

Investigating the role of neurotrophins in the development of pain responses in animal models of joint pain

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This thesis is dedicated to the loving memory of my mother, Sharon
S. Gowler.

Ithaka, C.P. Cavafy

*As you set out for Ithaka
hope the voyage is a long one,
full of adventure, full of discovery.*

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Abstract

Background: The chronic joint disease osteoarthritis (OA) represents a significant global problem, not only at the present time, but also for the future. Characterised by articular cartilage degeneration, inflammation of the synovium, and subchondral bone changes, it is the chronic pain associated with OA which presents the most serious consequences. There is a clear need to understand the mechanisms underlying chronic joint pain, and to identify novel therapeutic targets. Two targets which may have therapeutic potential are BDNF and cordycepin.

Objectives: The objectives of this thesis are two-fold; firstly to establish a slow progressing murine model of OA that is representative of post-traumatic OA, and secondly to investigate peripheral targets which may modulate chronic OA pain.

Methods: Surgical destabilisation of the medial meniscus (DMM) was carried out in adult C57BL/6 mice. Weight bearing asymmetry and hindpaw withdrawal threshold were measured up to 16 weeks post-surgery. Joint pathology was then assessed post-mortem at 16 weeks post-surgery. Another cohort of adult C57BL/6 mice underwent a modified surgical destabilization of the medial meniscus. Pain behaviour and joint pathology outcomes were measured 16 weeks and 20 weeks post-surgery.

Osteoarthritis was induced in adult male Sprague Dawley rats via intra-articular injection of monosodium iodoacetate (MIA) or vehicle (50ul 0.9% saline. A second cohort of male Sprague Dawley rats underwent either meniscal transection (MNX) or sham surgery. Rats then received intra-articular injections of either trkB-fc or human IgG. Pain behaviour was tested up to 3 hours post injection.

Adult C57BL/6 mice underwent DMM or sham surgery. Pain behaviour was measured up to 16 weeks post-surgery. From 14 weeks post-surgery mice were orally dosed with either cordycepin or vehicle every two days for two weeks. Joint pathology was then assessed post-mortem at 16 weeks post-surgery.

Results: There was a significant increase in weight bearing asymmetry from 13 weeks post DMM surgery in C57BL/6 mice, but no changes in hindpaw withdrawal thresholds. There were also significant increases in chondropathy and synovitis at 16 weeks post-surgery. When the surgical induction of the DMM model was modified there were still significant changes in joint pathology, but no significant changes in pain behaviour.

Intra-articular injection of TrkB/fc chimera in rats with established MIA induced joint pain was found to acutely reduce weight bearing asymmetry and increase ipsilateral hindpaw withdrawal thresholds. There was also a significant reduction in pain behaviour in rats with MNX established joint pain when TrkB/fc chimera was injected into the knee joint.

Following systemic administration of cordycepin in mice with DMM induced joint pain there was a significant reduction in weightbearing asymmetry when compared to vehicle treated mice. There was also a significant reduction in DMM induced chondropathy, subchondral bone thickening, and osteophytosis in mice treated with cordycepin compared to vehicle treated mice.

Conclusions: The changes in pain behaviour outcomes between the traditional and modified DMM, despite similar joint pathology outcomes, suggests a role for meniscal damage as a peripheral driver of OA pain.

Localised injection of TrkB/fc chimera into the knee joint of rats with both MIA and MNX induced joint pain was found to acutely reverse joint pain. This implies that peripheral BDNF may be involved in mediating OA joint pain.

Oral administration of cordycepin was found to reduce both pain behaviour and joint pathology changes in the DMM model in mice. These results suggest a role for local protein translation underlying both OA chronic pain and joint damage.

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GOWLER, P. R. W., WALSH, D., SUZUKI, R. and CHAPMAN, V., 2015. Nerve Growth Factor Mediated Sensitization in the Knee Joint in Mice. In. Cutting Edge Osteoarthritis, Oxford, UK, June 2015.

Abbreviations

α -CaMKII: Ca^{2+} -calmodulin-dependent protein kinase II

ACLT: Anterior cruciate ligament transection

ADAMTS: α -disintegrin and metalloprotease

ALP: Alkaline phosphatase

AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ASIC: Acid sensing ion channels

ATF-3: Activating transcription factor-3

ATP: Adenosine triphosphate

BDNF: Brain derived neurotrophic factor

BMI: Body mass index

BML: Bone marrow lesion

BMP-7: Bone morphogenetic protein 7

cAMP: Cyclic adenosine monophosphate

CBT: Cognitive behavioural therapy

CCI: Chronic constriction injury

CCL2: Chemokine (C-C motif) ligand 2

CD68: Cluster of differentiation 68

CFA: Complete Freund's adjuvant

CGRP: Calcitonin Gene Related Peptide

COX-2: Cyclooxygenase-2

CPEB: Cytoplasmic polyadenylation binding protein

DALY: Disability-adjusted life years

DCV: Dense core vesicles

DMM: Destabilisation of the medial meniscus

DMOAD: Disease modifying osteoarthritis drug

DNA: Deoxyribonucleic acid

DPX: Dibutyl phthalate xylene

DRASIC: Dorsal root acid sensing ion channel

DRG: Dorsal root ganglion

ECM: Extracellular matrix
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
EMLA: Eutectic mixture of local anaesthetics
EPSC: Excitatory postsynaptic current
ERK: Extracellular signal-related kinase
EtOH: Ethanol
FRAP: Fluoride-resistant acid phosphatase
GABA: gamma-Aminobutyric acid
GAG: Glycosaminoglycan
GFAP: Glial fibrillary acidic protein
GI: Gastrointestinal
GLAST: Glutamate aspartate transporter
GLT-1: Glutamate transporter-1
GPCR: G protein coupled receptor
HRP: Horseradish peroxidase
IB4: Isolectin B4
Iba1: Ionized calcium-binding adapter molecule 1
ICC: Intra class correlation
IgG: Immunoglobulin
iGluR: Ionotropic glutamate receptors
IL-1 β : Interleukin 1 β
IL-8: Interleukin-8
IPL: Infrapatellar ligament
IPSC: Inhibitory postsynaptic current
JNK: c-Jun N-terminal kinases
KCC2: Potassium-chloride co-transporter 2
LAN: Lateral articular nerve
LPS: Lipopolysaccharide
MAN: Medial articular nerve
MAPK: Mitogen-activated protein kinase

MCL: Medial collateral ligament
 MCLT: Medial collateral ligament transection
 mGluR: Metabotropic glutamate receptors
 MIA: Monosodium iodoacetate
 MIP-2: Macrophage inflammatory protein - 2
 MM: Medial meniscus
 MMP: Matrix metalloproteinases
 MMTL: Medial meniscotibial ligament
 MNX: Medial mensicus transection
 MPO: myeloperoxidase
 MRI: Magnetic resonance imaging
 mRNA: Messenger ribonucleic acid
 MTP: Medial tibial plateau
 NGF: Nerve growth factor
 NICE: National Institute for Health and Care Excellance
 NK1: Neurokinin 1
 NMDA: N-methyl-D-aspartate
 NO: Nitric oxide
 NSAID: Nonsteroidal anti-inflammatoy drug
 NT-3: Neurotrophin- 3
 NT-4/5: Neurotrophin - 4/5
 OA: Osteoarthritis
 PAG: Periaqueductal grey
 PAN: Posterior articular nerve
 PBS: Phopshate buffered saline
 PFA: Paraformaldehyde
 PGE2: Prostaglandin E2
 PKC: Protein kinase C
 PPAR γ : Peroxisome proliferator-acitvated receptor
 gamma
 PVP: Polyvinylpyrrolidone

qRT-PCR: quantitative real time polymerase chain reaction

ROS: Reactive oxygen species

RTX: Vanilloid resiniferatoxin

RVM: Rostral ventromedial medulla

SCB: Subchondral bone

SGC: Satellite glial cells

SP: Substance P

TGF β : Transforming growth factor β

TIMP: Tissue inhibitors of metalloproteinases

TNF- α : Tumour necrosis factor - α

TRAP: Tartrate-resistant acid phosphatase

TREK1: Potassium channel subfamily K member 2

TrkA: Tropomyosin receptor kinase A

TrkB/fc: Tropomyosin receptor kinase B/ full chain

TrkB: Tropomyosin receptor kinase B

TRPM8: Transient receptor potential cation channel subfamily M member 8

TRPV1: Transient receptor potential cation channel subfamily V member

TTBS: Trizma Triton x-100 buffered saline

TTXr: Tetrodotoxin resistant

VEGF: Vascular endothelial growth factor

VPL: Ventral posterolateral nuclei

WT: Wild type

Chapter 1: General Introduction

1.1 Pain

1.1.1 Peripheral Nociceptors

Nociception is the process through which noxious stimuli are detected, encoded, and then relayed to the central nervous system. This section will focus on the peripheral nociceptors which are the primary afferent fibres which first detect painful stimuli, and relay this information to the dorsal horn of the spinal cord.

1.1.1.1 Classifying primary afferent fibres

Charles Sherrington was one of the first researchers to propose the idea of a neuron which responds to noxious stimuli (Sherrington, 1906). He found that the scratch reflex can be induced by electric, thermal, and mechanical insult (Sherrington, 1906). These neurons are the nociceptors and their role is to detect potentially harmful environments and aid organisms in their survival. Primary afferent fibres can be subdivided into several different classes based on a variety of different metrics including; morphology, size, and chemical phenotype.

There is a broad distinction between those nociceptors which are myelinated, A δ fibres, and those which are not myelinated, C fibres. Both C fibres and A δ fibres have relatively small diameters, however due to their myelination A δ fibres have a much higher conductance velocity than C fibres. It has been thought for a long time that A δ fibres encode the 'first pain' due to their faster conduction velocity and that C fibres encode the later 'second pain' (McMahon *et al*, 2013). Cutaneous A fibres have a monotonically increasing response to an increasing mechanical stimulus, whereas the response from C fibres is found to plateau (Slugg *et al*, 2000). The magnitude of

response from A- fibres is also much greater than C fibres (Slugg *et al*, 2000).

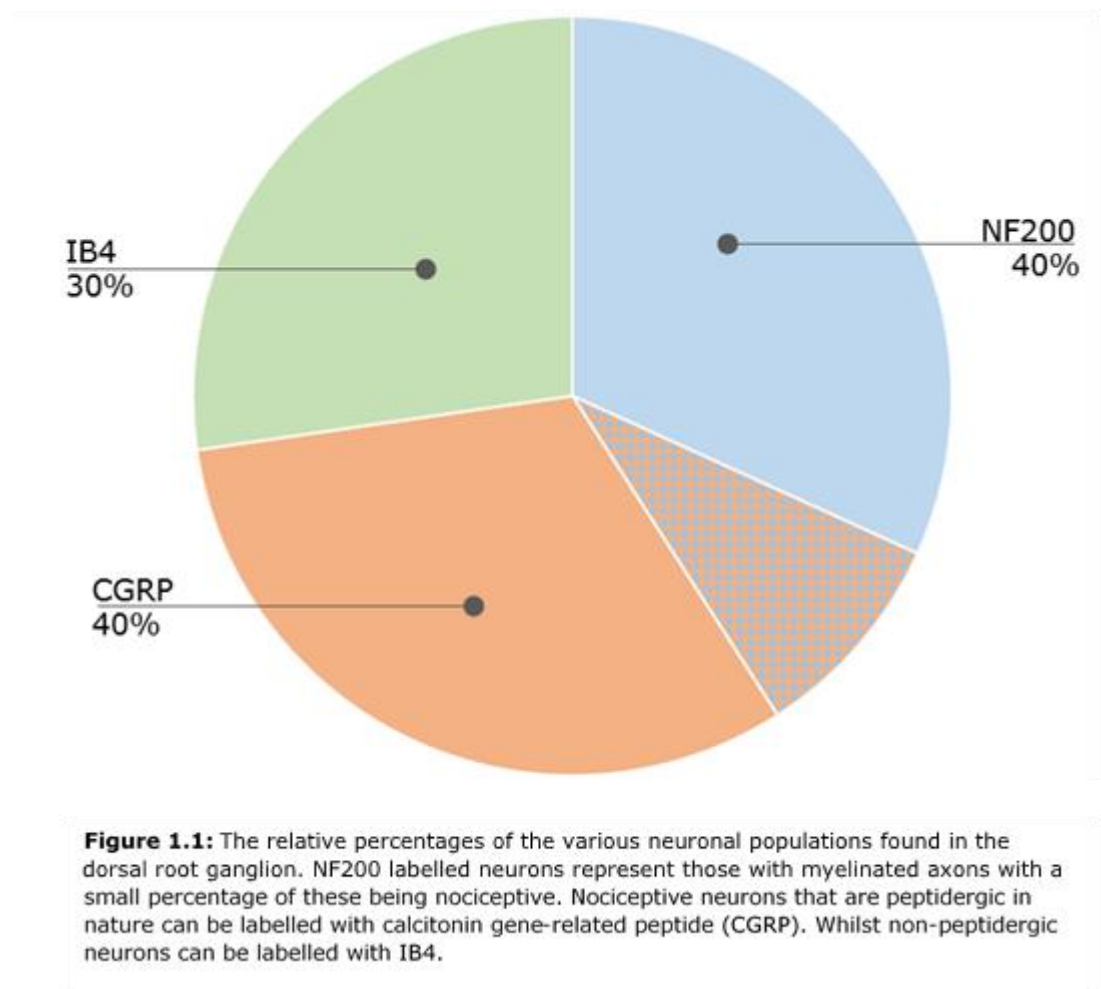
Another method for classifying nociceptors is to group them based on the sensory information they encode. There are multiple forms of adverse stimuli which need to be detected by nociceptors, such as thermal, chemical, mechanical, and electrical. In order to encode all of these sensory modalities there either needs to be a class of nociceptors for each noxious stimuli, or the nociceptors need to be able to respond to different sensory modalities. It is the latter strategy which evolution has decided to favour. In the primary afferent fibres found in the hairy skin of monkeys it was found that both A δ and C fibres respond to thermal stimuli, with A δ fibres being less sensitive than C fibres to heat (Davis *et al*, 1993). The same fibres were also sensitive to chemical insult, with C fibres being less sensitive than A δ fibres (Davis *et al*, 1993). Following chemical insult there was sensitization of both A δ and C fibres to other modalities of input with increased heat and mechanical sensitivity (Davis *et al*, 1993). Whilst mechanically sensitive C fibres are easily excited upon the application of a mechanical force it has been found that they stop responding to a tonic pressure after around 10s (Schmidt *et al*, 2000). However, after 20s of constant pressure the previously mechanically insensitive fibres started firing, suggesting a role for this subset of neurons in the encoding of tonic pressure (Schmidt *et al*, 2000).

The different types of nociceptors also have different thermal thresholds. The unmyelinated C fibres of the monkey have a thermal threshold of around 39-40°C, and this threshold is reliant on the temperature at the receptor level and not at the skin level (Tillman *et al*, 1995). A δ fibres can be separated into two different subtypes; type I A δ fibres and type II A δ fibres. This separation is based on the fibres response to thermal stimuli, type I A δ fibres have a slow response latency and have a thermal threshold of around 53 °C, whilst type II A δ have a much shorter response latency and have a thermal threshold of 46 °C (Treede *et al*, 1995).

Type II A δ fibres were found to stop discharging very soon after they initially responded (Treede *et al*, 1995).

Sensory neurons can also be described in terms of their neurochemical phenotype (**Figure 1.1**). The large light cells which are thought to be mechanically sensitive neurons and not involved with nociception have been found to bind the neurofilament antibody RT97 (Lawson *et al*, 1984). RT97 was found to bind almost exclusively with the large light cells and did not stain small dark nociceptive neurons, around 35% of ganglion neurons were found to be RT97 immunoreactive (Lawson *et al*, 1984). The nociceptive neurons can be divided into two different groups based on whether or not they store and release peptides. The most common peptide found in the vast majority of peptidergic neurons is calcitonin gene related peptide (CGRP), as such this protein is an ideal marker for peptidergic neurons. It was found that between 29 and 37% of DRG neurons express CGRP (Alvarez *et al*, 1991). These CGRP immunoreactive neurons were mostly small cells with an average diameter of around 23 μ m, and their processes terminated mainly in laminae I and II outer (IIo) (Alvarez *et al*, 1991). The antibody LA4 binds to cell surface oligosaccharides and is thought to exclusively bind to non-peptidergic neurons (Alvarez *et al*, 1991). Around 26-40% of neurons in the DRG were LA4 positive, again these neurons were small diameter neurons ($\sim$ 25 μ m), however unlike peptidergic neurons these cells terminated predominantly in lamina II inner (IIi) (Alvarez *et al*, 1991). There was very little co-staining between these two populations of neurons (Alvarez *et al*, 1991). Isolectin B4, a protein from *Griffonia simplicifolia*, also binds to cell surface oligosaccharides, and can also be used as a marker for non-peptidergic neurons (Silverman & Kruger, 1990). IB4 was strongly co-localised with fluoride -resistant acid phosphatase (FRAP), which is a sensory neuron specific enzyme, there was also very little colocalization between IB4 and CGRP (Silverman & Kruger, 1990). It is interesting to note as well that tropomyosin receptor kinase A (TrkA), the high affinity receptor for nerve growth factor (NGF), is

expressed predominantly by peptidergic neurons (Averill *et al*, 1995, Molliver *et al*, 1995). The high affinity receptor for NGF, TrkA, is expressed by around 55% of small diameter neurons and 90% of these neurons also co-stained with CGRP (Averill *et al*, 1995). There was very little cross staining between TrkA and IB4 (Averill *et al*, 1995, Molliver *et al*, 1995).



1.1.1.2 Peripheral Transduction

In order to properly encode adverse events it is necessary for there to be signal transduction, whereby the energy of the noxious stimuli is converted into action potentials and relayed by the sensory nerves to the spinal cord (**Figure 1.2**). This section will briefly

detail some of the more important receptors found in peripheral afferent fibres which play a role in signal transduction.

TRPV1

The transient receptor potential cation channel subfamily V member 1 (TRPV1) is formed of 6 transmembrane domains which make up a non-selective cation channel (Bolcskei *et al*, 2005). TRPV1 was first discovered in the rat as the receptor which was sensitive to capsaicin, the active molecule of chilli peppers (Caterina *et al*, 1997). The capsaicin receptor was found to be a vanilloid receptor, and as such was activated by both capsaicin and the vanilloid resiniferatoxin (RTX) (Caterina *et al*, 1997). TRPV1 was predominantly located in small diameter neurons (Caterina *et al*, 1997). *In vivo* experiments in mice genetically modified to not express TRPV1 have revealed a lot about the role of this protein in both acute and chronic nociception. In TRPV1^{-/-} mice there was no nocifensive response to intraplantar injection of capsaicin or RTX, however the behavioural response to another type of painful stimulus, formalin, was the same as the wild type mouse (Caterina *et al*, 2000). These results suggest that TRPV1 is involved in transducing some, but not all, chemical insults. Following exposure to pH 5 buffer there was an increase in cytoplasmic Ca²⁺ in WT neurons, however TRPV1^{-/-} neurons did not respond to changes in pH, suggesting that TRPV1 is also responsive to acidosis (Caterina *et al*, 2000). TRPV1^{-/-} mice had a normal response to both noxious punctate stimuli and a noxious tail pinch, suggesting that TRPV1 does not play a role in mechanical stimuli transduction (Caterina *et al*, 2000). TRPV1 expressing neurons in wild type mice responded vigorously to an increase in temperature past 43°C, whereas TRPV1^{-/-} neurons did not respond (Caterina *et al*, 2000).

ASIC

Acid sensing ion channels (ASICs) are thought to play a role in detecting changes in pH. When the extracellular pH was lowered below 6.9 a Na⁺ inward current was activated in *Xenopus laevis* oocytes with cloned ASIC (Waldmann *et al*, 1997). This channel could only be activated by extracellular changes in pH, decreasing pH intracellularly did not activate the channel (Waldmann *et al*, 1997). ASIC immunoreactivity was present in peripheral afferent fibres, notably the small and medium sized DRG cells (Olson *et al*, 1998). ASIC was also seen to co-localise with both substance P and CGRP in capsaicin sensitive cells (Olson *et al*, 1998). Both the localisation and the function of these channels suggest they have a role to play in nociception, especially for detecting noxious changes in pH. Indeed, in dorsal root acid sensing ion channel (DRASIC) null mice there was a reduced response of C fibres in skin nerve preparations to extracellular pH of 5, compared to wild type mice (Price *et al*, 2001). These results were mirrored *in vivo*, injection of pH 4 saline into the gastrocnemius muscle in DRASIC null mice was associated with a decreased hyperalgesic response compared to wild type mice (Price *et al*, 2001).

P2X

P2X is a family of ligand gated ion channels activated by extracellular adenosine triphosphate (ATP) (Valera *et al*, 1994). It is thought that P2X3 plays an important role in nociception. In both cultured tooth pulp cells and stretch receptors, 10µM ATP was enough to evoke a train of action potentials (Cook *et al*, 1997). There was significant immunoreactivity of P2X3 found in the nociceptors primarily in small diameter neurons (Cook *et al*, 1997). Behaviourally, ATP has been shown to cause mechanical allodynia following intraplantar injection (Tsuda *et al*, 2000). This mechanical allodynia could be blocked by the P2X antagonist PPADS, suggesting a role for P2X in establishing ATP induced nociception (Tsuda *et al*, 2000).

TREK1

TREK1 is a two pore K⁺ channel which is sensitive to a variety of modalities of stimuli. TREK1 is the first mammalian K⁺ channel identified to be mechanosensitive, the channel opens in response to both membrane stretch and cell swelling (Patel *et al*, 1998). It is thought that TREK1 may detect mechanical force which is directly applied to the lipid bilayer (Patel *et al*, 1998). TREK1 is found in 60% of sensory neurons, all of which have small-medium diameters (Alloui *et al*, 2006). Of the neurons which expressed TREK1, 41% were positive for substance P, 32% were positive for IB4, and 40% were positive for TRPV1 (Alloui *et al*, 2006). TREK1^{-/-} mice showed changes in nocifensive behaviour to a variety of different stimuli. There was a decrease in withdrawal latency to increasing tail temperature up to 50°C, however at more extreme temperatures there was no difference in withdrawal latency between TREK1^{-/-} and WT mice (Alloui *et al*, 2006). There was also a decrease in paw withdrawal threshold in response to von Frey hairs in TREK1^{-/-} mice compared to WT, however there was no change in their response to more extreme mechanical stimuli (Alloui *et al*, 2006). These results suggest that TREK1 may have an important role to play in transducing lower intensity mechanical or thermal stimuli.

TRPM8

A substantial population of sensory neurons are activated by exposure to menthol, or cold temperatures. It was found that these stimuli activated a calcium permeable channel which was a member of the TRP family of receptors (McKemy *et al*, 2002). Following a decrease in environmental temperature from 50°C to 35°C there was a rapidly activating inward current (McKemy *et al*, 2002). This receptor, TRPM8, was found to be expressed by a sub-population of C fibres (McKemy *et al*, 2002). The TRPM8 antagonist BCTC completely removed the Ca²⁺ increase induced by menthol in trigeminal neurons (Madrid *et al*, 2006). Application of BCTC also increased the temperature threshold in 92% of neurons, as well as eliminating the cooling induced inward current (Madrid *et al*, 2006). There is also behavioural data in TRPM8 null mice which shines a

light on the function of the receptor. Injection of icilin, a TRPM8 agonist, caused a robust jumping behaviour in wild type mice, however in TRPM8 null mice this behaviour was completely nullified (Colburn *et al*, 2007). TRPM8 null mice also showed no preference for chambers when one was 50°F and one was room temperature, unlike wildtype mice, who avoided the cold chamber (Colburn *et al*, 2007). These studies support a role of this receptor in transducing noxious cold stimuli.

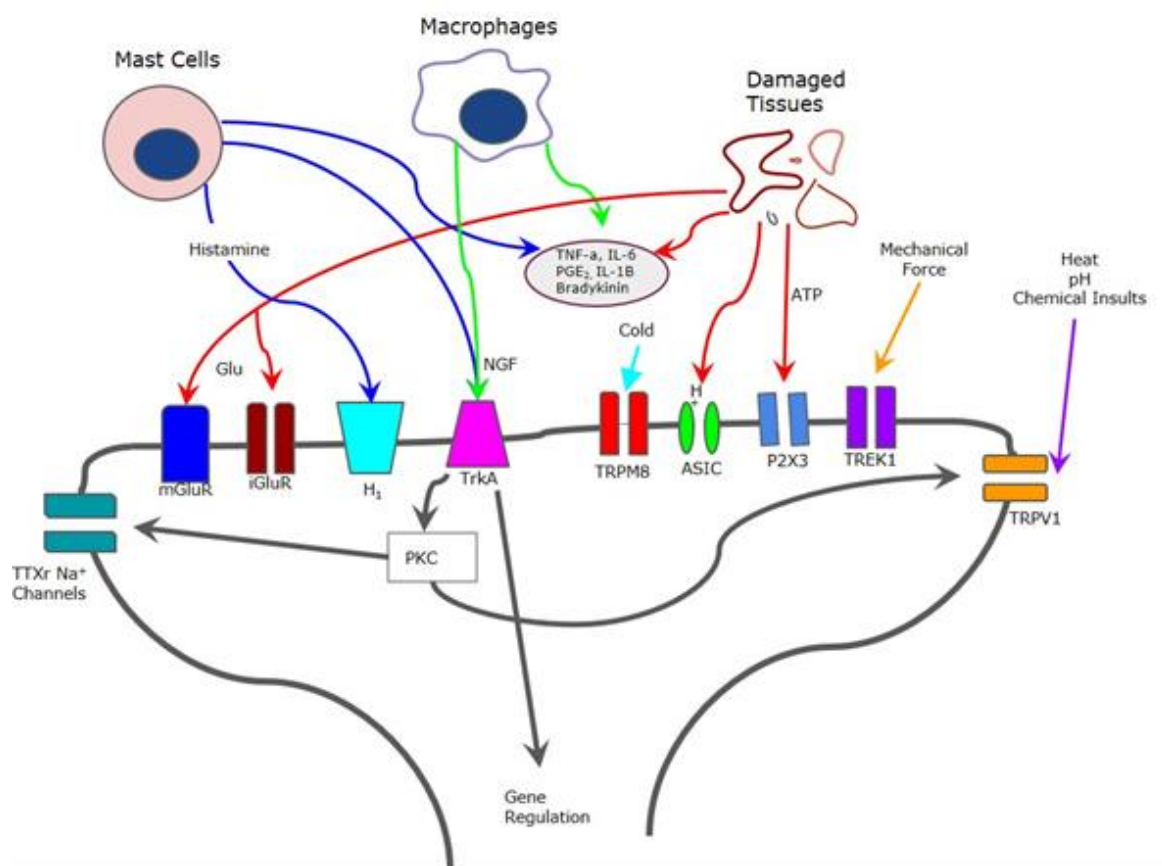


Figure 1.2: Schematic showing a selection of peripheral transducers and the noxious stimuli they detect. TTXr- tetrodotoxin resistant. mGluR- metabotropic glutamate receptors. iGluR- ionotropic glutamate receptors. TrkA- tropomyosin receptor kinase A. TRPM8- transient receptor potential cation channel subfamily M member 8. ASIC- Acid sensing ion channel. P2X3- P2X purinoreceptor 3. TREK1- Potassium channel subfamily K member 2. TRPV1- transient receptor potential cation channel subfamily V member 1.

1.1.2 The Dorsal Horn of the Spinal Cord

1.1.2.1 The Structure of the Dorsal Horn

Peripheral inputs enter the spinal cord via the dorsal horn. The primary afferent fibres enter the spinal cord from the periphery via the dorsal horn. The structure of the dorsal horn was first described in the cat (Rexed, 1952). It was found that the cells of the grey matter were organised into several layers of laminae which extend across the length of the spinal cord (Rexed, 1952). There were 10 laminae described in the spinal cord, with the dorsal horn comprising of laminae I to VI (Rexed, 1952). Staining of the rat spinal cord has found that the cytoarchitectonic structure is similar to that of the cat, with only very minor differences (Molander *et al*, 1984). A β fibres, which encode non-noxious information, such as light mechanical stimulation, terminate in laminae III-V in the cat dorsal horn (Brown & Fyffe, 1981). Mechanical nociceptors terminate primarily in laminae I & II, with a small number of afferents terminating in lamina V (Light & Perl, 1979). CGRP labelled neurons terminate in laminae I and IIo, whilst IB4 positive neurons were only found in the middle third of lamina II (Lorenzo *et al*, 2008). The TrkA immunoreactive processes also terminated predominantly in laminae I and IIo, much like the CGRP positive peptidergic neurons (Averill *et al*, 1995, Molliver *et al*, 1995).

When primary afferent fibres enter the dorsal horn they terminate in multi-synaptic structures known as glomeruli (Todd, 1996). There are two distinct types of glomeruli, type 1 are found in the middle and ventral part of lamina II and are thought to involve unmyelinated primary afferent neurons, whilst type 2 are found in the ventral part of lamina II and the dorsal part of lamina III which involve myelinated peripheral neurons (Todd, 1996a). The glomeruli contain synapses with inhibitory interneurons, type 1 glomeruli are inhibited by GABAergic neurons, whilst the inhibitory interneurons which synapse with type II glomeruli co-express both GABA and glycine (Todd, 1996a).

In lamina I there were two distinct groups of neurons based on morphology, projection neurons and neurons which did not project (Grudt & Perl, 2002). In lamina II there are 5 different morphological subtypes of neurons; islet neurons, radial neurons, central cells, medial-lateral neurons, and vertical neurons (Grudt & Perl, 2002). Islet cells extend mainly in the rostral and caudal directions, radial neurons have dendrites which radiate in all directions, and central cells were mostly located in lamina II and have a dense and short dendritic arbor (Grudt & Perl, 2002). Medial-lateral neurons have dendrites which extend in both the medial-lateral and dorso-ventral directions, and vertical neurons were named after the vertical orientation of their dendrites whose cell bodies are mostly found in lamina IIo (Grudt & Perl, 2002). The morphologically different cells in lamina II are also functionally different (Yasaka *et al*, 2010). Islet cells are GABAergic, whilst radial and vertical cells were found to be glutamatergic (Yasaka *et al*, 2010). There was diversity in the excitatory or inhibitory nature in the rest of the morphologically distinct groups of interneurons (Yasaka *et al*, 2010). Descending serotonergic neurons from the brainstem are found to synapse in all laminae of the dorsal horn, although the densest staining was seen in laminae I & IIo (Hoffert *et al*, 1983). These serotonergic neurons were found to synapse with both nociceptive and wide dynamic range neurons (Hoffert *et al*, 1983).

1.1.2.2 Spinal Processing of Pain

When A δ and C fibres synapse in the dorsal horn the major excitatory transmitter is glutamate (Yoshimura & Jessell, 1990). The majority of fast excitatory synaptic transmission is mediated by the ionotropic α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subclass of glutamate receptors (Li *et al*, 1999a). Fast excitatory transmission within the dorsal horn is also mediated by the kainate receptor (Li *et al*, 1999b). The other ionotropic glutamate receptor, the N-methyl-D-aspartate (NMDA) receptor, is also present in the dorsal horn, and is blocked in a voltage

dependent manner by Mg^{2+} (Mayer *et al*, 1984). Metabotropic glutamate receptors have also been found in the dorsal horn as well (Berthele *et al*, 1999).

GABA_A and glycine receptors are also found within the dorsal horn of the spinal cord (Todd *et al*, 1996a). Both these receptors have been found to be co-expressed in dorsal horn neurons (Todd *et al*, 1996b). These two ionotropic receptors mediate fast inhibitory transmission, whilst the metabotropic GABA_B receptor is thought to mediate a slower and longer lasting inhibition (Towers *et al*, 2000).

The substance P (SP) receptor neurokinin 1 (NK1) is also found within the dorsal horn of the spinal cord especially lamina I (Nakaya *et al*, 1994). Substance P is found in a population of nociceptors (Lawson *et al*, 1997) and as such is also thought to have a role in the processing of pain within the spinal cord.

1.1.3 Ascending Pathways

Pain is an emotive response to a noxious stimulus. For this to be the case the brain has to be involved in its processing. This section will briefly detail the pathways which transmit information from the dorsal horn of the spinal cord to the brain (**Figure 1.3**). Within the dorsal horn there are projection neurons which travel rostrally up the spinal cord to various areas of the brain. Retrograde labelling of the parabrachial area and thalamus of rats showed that the vast majority of projection neurons were localised in lamina I of the dorsal horn (Hylden *et al*, 1989). Similar results were found in the primate when horseradish peroxidase (HRP) was injected into the thalamus and midbrain (Willis *et al*, 1979). In both rats and primates there was some retrograde labelling of lamina V following injection of HRP into the thalamus and midbrain (Hylden *et al*, 1989, Willis *et al*, 1979). A large number of spino-parabrachial projection neurons ($\frac{1}{3}$) and spino-PAG projection neurons ($\frac{2}{3}$) receive monosynaptic input from primary afferent C fibres (Ruscheweyh *et al*, 2004).

1.1.3.1 The spinothalamic tract

The first major ascending pathway to discuss is the spinothalamic tract which projects from the dorsal horn of the spinal cord to the thalamus (Ruscheweyh *et al*, 2004). Two subsets of spinothalamic neurons which were insensitive to mechanical and thermal stimuli were found to be excited by histamine and mustard oil (Andrew & Craig, 2001). This suggests that the spinothalamic tract can transmit itch sensation as well as pain. Within the thalamus the majority of projection neurons terminate in the ventral posterolateral nuclei (VPL) (Applebaum *et al*, 1979). The neurons which terminate in the VPL represent numerous functional classes of projection neurons (Applebaum *et al*, 1979). Both anterograde and retrograde labelling of thalamocortical neurons found that the thalamus transmits information to the somatosensory cortex (Stevens *et al*, 1993). The majority of the projections to the somatosensory cortex from the thalamus originate from the ventroposterior inferior nucleus, the ventroposterior lateral nucleus, posterior nucleus, and the centrolateral nucleus (Stevens *et al*, 1993). The somatosensory cortex is where the location and intensity of pain is processed. The thalamus also projects to the insular cortex, where there is thermosensory activation by non-noxious cooling stimuli (Craig *et al*, 2000). It is clear to see that the thalamus plays a key role in transmitting nociceptive information across the brain.

1.1.3.2 The spinoparabrachial tract

The second of the major ascending pathways is the spinoparabrachial tract which projects from lamina I and V of the spinal cord to the parabrachial area of the midbrain (Hylden *et al*, 1989). There are projections from the parabrachial area to the central nucleus of the amygdala, with three distinct pathways to the medial, lateral, and lateral capsular portions of the central nucleus (Bernard *et al*, 1993). It is believed that the parabrachial area to lateral capsular portion of the central nucleus pathway is primarily nociceptive in nature (Bernard *et al*, 1993). The parabrachial area

projects heavily to the hypothalamus as well (Bester *et al*, 1997). It is thought that the projection from the parabrachial area to the dorsomedial part of the ventromedial hypothalamic nucleus is associated with the aversive reaction to noxious stimuli (Bester *et al*, 1997). There are also a significant number of projections from the parabrachial area to the thalamus (Krout & Loewy, 2000). This suggests that information from the spinoparabrachial tract can be integrated into the spinothalamic tract. Lastly, there are links between the parabrachial area and the periaqueductal grey (PAG) which is an area of the brain associated with the descending control of pain (Krout *et al*, 1998).

1.1.4 Descending Controls

The processing of pain is not a one way system from the periphery to the brain, there are also descending pathways from the brain to the spinal cord which have an inhibitory effect on spinal processing (**Figure 1.3**). The idea of a neuronal network which modulates spinal excitability was initially reviewed in the late 1970's (Basbaum & Fields, 1978). There are three tiers to the descending control of pain; the periaqueductal grey in the midbrain, the rostral ventromedial medulla and finally at the spinal cord (Basbaum & Fields, 1978). This section will briefly detail how nociception is mediated at each of these three levels.

1.1.4.1 The periaqueductal grey

The periaqueductal grey (PAG) is located in the midbrain and is connected with a high variety of regions in the brain. Results from double retrograde staining in the brains of rat found that there were projections from all over the medial frontal cortex to the PAG (Neafsy *et al*, 1986). It has also previously been shown that there are projections between the parabrachial area, which is a part of the spinobrachial tract, and the PAG (Krout *et al*, 1998). There are projections from the PAG to the rostral ventromedial medulla (RVM) (Morgan *et al*, 2008). Around 2/3rds of the neurons which project to the RVM were found to be immunoreactive for GAD67 a marker for

GABAergic neurons (Morgan et al, 2008). There were GABAergic neurons which projected to GABAergic ON cells in the RVM and non-GABAergic neurons which project to GABAergic OFF cells (Morgan et al, 2008). This suggests that neurons from the PAG may excite OFF cells and inhibit ON cells in the RVM. It is thought that mGluRs play an important role in the PAG's ability to modulate nociception. Application of agonists for group I and II mGluRs in the PAG were found to reduce the nociceptive response to formalin (Maione et al, 2000). These inputs to the PAG from across the brain and outputs to the RVM suggest that the PAG is involved in integrating cortical information before influencing descending modulation.

1.1.4.2 The rostral ventromedial medulla

The RVM receives inputs from several brain areas most notably the PAG (Morgan et al, 2008). The RVM also projects neurons down to the dorsal horn of the spinal cord (Basbaum & Fields, 1978). It therefore seems that the RVM plays a key role as the final integration point for descending control. The RVM is thought to modulate spinal activity through ON and OFF cells. OFF cells are believed to play a role in inhibiting spinal nociceptors, when they are forced to fire continuously OFF cells are capable of stopping a thermal induced tail flick reflex (Fields et al, 1983). Before a tail flick response in response to a heat stimulus the OFF cells were found to pause firing, however application of the opioid morphine was able to disinhibit the OFF cell (Fields et al, 1983). This suggests that this regulation of OFF cells in the RVM may be involved in opioid induced analgesia. Observing the latency of the tail flick response whilst measuring spontaneous ON cell firing has shown that the latencies were longer when the ON cells were silent than when the ON cells were active (Heinricher et al, 2009). This suggests that the ON cells are capable of actually facilitating spinal nociception.

1.1.4.3 The influence of descending control on the spinal cord

There are three major descending pathways from the brain which mediate nociception in the spinal cord. The first as previously mentioned are the GABAergic neurons which originate from the RVM (Morgan et al, 2008). Secondly, there are the serotonergic neurons which originate from the raphe nuclei (Bowker et al, 1983). Lastly, there are the noradrenergic neurons originating from the locus coeruleus (Westlund et al, 1983). There are also some peptidergic and cholinergic neurons which input into the dorsal horn from the raphe nuclei (Bowker et al, 1983). The GABAergic neurons are thought to play a direct role in causing inhibition in the substantia gelatinosa (Kato et al, 2006). Stimulating the RVM with both glutamate and electrical stimuli was found to increase both the frequency and amplitude of inhibitory postsynaptic currents (IPSCs) (Kato et al, 2006). Blocking GABA receptors in the substantia gelatinosa with bicuculline was found to completely block the RVC mediated increase in IPSCs (Kato et al, 2006)

1.1.5 Plasticity in Pain

1.1.5.1 Peripheral sensitization following inflammation

Inflammation occurs following an injury or insult to vascularised tissue. Its purpose is to protect the damaged area, to remove bacteria and necrotic tissues, and finally to initiate tissue repair. Inflammation is characterised by redness, swelling, heat, loss of function, and pain. This section will briefly detail peripheral changes in nociceptors following inflammation.

The first cells to migrate to the site of inflammation following injury are the neutrophils (Cassatella, 1995). They are responsible for clearing away debris and foreign objects from the site of inflammation. They achieve this by engulfing the objects via phagocytosis, before breaking them down with intracellular enzymes including myeloperoxidase (MPO), and lysozyme (Rausch & Moore, 1975). A secondary role for neutrophils is to attract other inflammatory cells, such as macrophages and mast cells to the site

of injury via the release of cytokines including TNF- α , IL-1 β , TGF β , & IL-8 (Cassatella, 1995). As neutrophils age they undergo apoptosis before being phagocytosed and destroyed by macrophages (Savill *et al*, 1989). However, despite playing a role in abolishing the threat of foreign bodies in the wound, when neutrophils are removed from the wound there was no change in the levels of wound debridement or monocyte infiltration (Simpson & Ross, 1972).

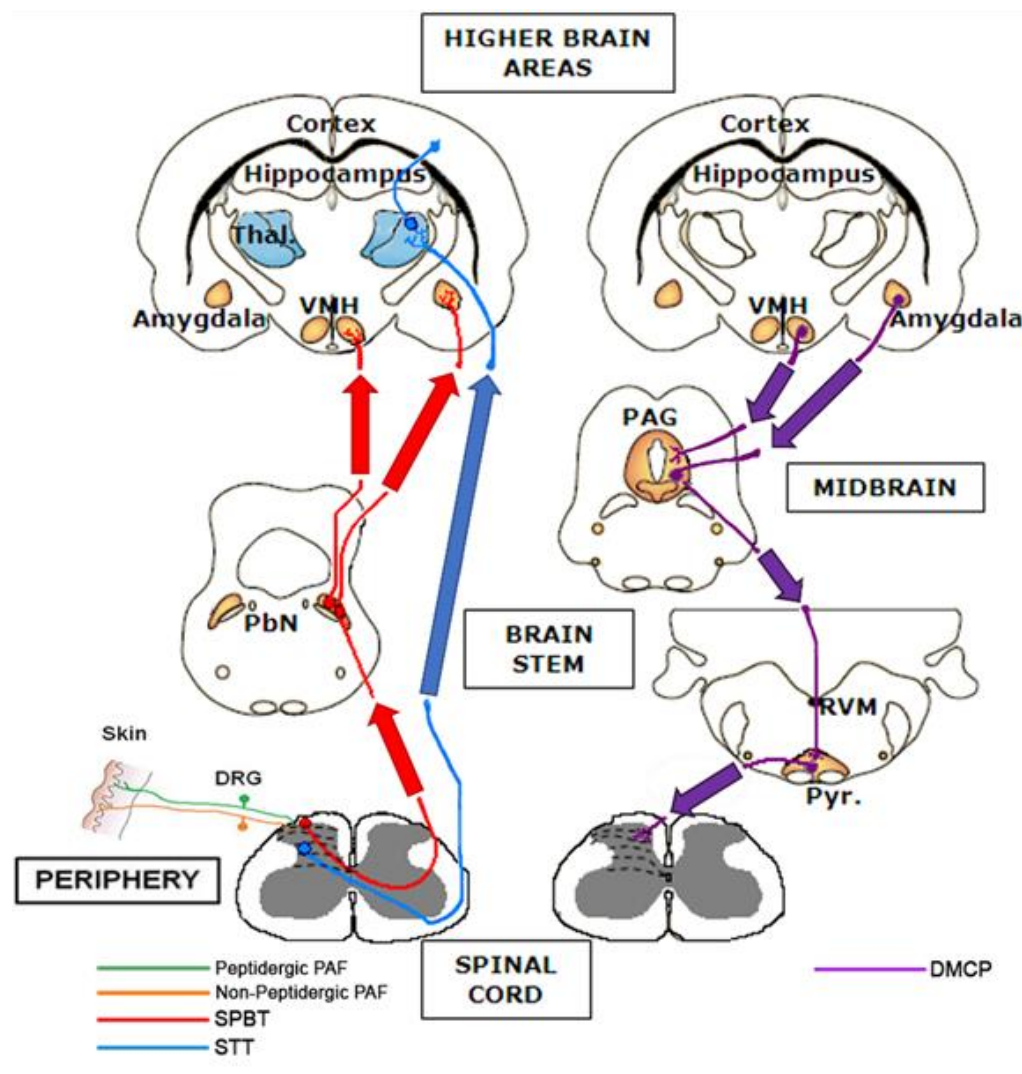


Figure 1.3: Schematic showing the ascending and descending pathways between the brain and the spinal cord. SPBT- Spinoparabrachial tract. STT- Spinothalamic tract. DMCP- descending modulatory control pathway. Adapted from Hunt & Mantyh, 2001.

Circulating monocytes then enter inflamed tissues and become macrophages. There are two subtypes of macrophage, the classical M1 macrophage and the M2 macrophage which plays a role in

resolving inflammation (Italiani *et al*, 2014). Distinct clusters of genes are regulated at different phases of inflammation, M1 macrophage gene clusters are seen in the inflammatory stage, whilst M2 macrophage gene clusters are found during the resolution phase (Italiani *et al*, 2014). M1 macrophages are regulated by a host of chemotactic factors and cytokines including, IL-1 β , TNF- α , and CCL2 (Beuscher *et al*, 1990, Wetzler *et al*, 2000). Keratinocytes are the major source for CCL2, as well as the neutrophil chemoattractant MIP-2 (Wetzler *et al*, 2000). However, macrophages themselves can also produce various cytokines and chemotactic factors including IL-1 α , IL-1 β , and TNF- α (Hubner *et al*, 1996). The maximal levels for these inflammatory mediators were found at 15 to 24 hours after injury (Hubner *et al*, 1996). Peak macrophage infiltration has been observed three days post injury, by which point they are the primary phagocytic cell in the wound (Leibovich & Ross, 1975). Macrophages are essential for correct wound healing, when macrophages were removed from the wound there was a clear reduction in debridement as well as a significant delay in the onset of fibrosis and the rate at which fibroblasts proliferated (Leibovich & Ross, 1975).

During injury there is a change in the chemistry of the wound site from damaged cells, degranulation of mast cells, and secretions from inflammatory cells. The resultant 'inflammatory soup' of kinins, prostanoids, growth factors, cytokines, chemokines, protons, and ATP sensitizes nociceptors (Woolf & Ma, 2007). These inflammatory sensitizers bind to nociceptor receptors and activate a multitude of intracellular pathways. One such signal transduction pathway, the CAMP-Epac-PKC pathway, has been found in primarily IB4+ neurons (Hucho *et al*, 2005). In fact administration of the Epac specific activator CPTOMe induced mechanical hyperalgesia which could be inhibited with the PKC inhibitor ϵ V1-2 (Hucho *et al*, 2005). There is also evidence of the p38-MAPK pathway being important in inflammatory pain, with the inflammatory mediator TNF- α enhancing the TTX-resistant Na⁺ current of nociceptors (Jin &

Gereau, 2006). The p38 inhibitor SB202190 caused a significant decrease in baseline TTX-resistant Na⁺ currents and blocked the TNF- α induced increase (Jin & Gereau, 2006). Another intracellular signalling pathway that has been linked to inflammatory pain is the JNK pathway, there was an increase in JNK activation in rat DRGs following intraplantar injection of CFA and NGF (Doya *et al*, 2005). The thermal hyperalgesia seen in response to CFA and NGF insult was also blocked with the JNK inhibitor SB600125 (Doya *et al*, 2005).

Nerve growth factor has been shown to sensitize TRPV1 channels through Src kinase dependent phosphorylation (Zhang *et al*, 2005). As well as this increased TRPV1 sensitivity there was also an increase in the expression of TRPV1 in the surface membrane following exposure to NGF (Zhang *et al*, 2005). Changes in the phosphorylation of voltage gated sodium channels are also thought to play a role in peripheral sensitization. In Na(V)1.9^{-/-} mice there was a significant reduction in the hypersensitivity induced by intraplantar injection of a variety of inflammatory mediators including bradykinin, capsaicin, PGE2, and IL-1 β (Amaya *et al*, 2006). Alongside the changes seen in the peripheral terminals following inflammation there are also transcriptional changes in the DRG soma. One key example of this is brain derived neurotrophic factor (BDNF). Following intraplantar injection of CFA there was a significant increase in BDNF mRNA in the DRGs (Mannion *et al*, 1999). This is thought to be linked to neuronal activity as there was a significant increase in BDNF mRNA expression in response to electrical stimulus (Mannion *et al*, 1999).

1.1.5.2 The role of glial cells in peripheral nociception

Neurons are not the only cells which are found in the peripheral nervous system. It is important to briefly touch upon the glial cells which are also present in the periphery and the potential role they play in nociception. Glial cells provide support for neuronal cells, and in the periphery these cells take the shape of Schwann cells, which make up the insulating myelin sheath, and satellite glial cells

(SGCs). SGCs surround the soma of DRG neurons, and are connected to each other via gap junctions. Recent studies have proposed an active role for both Schwann cells and SGCs in the processing of noxious stimuli. Following denervation it was found that Schwann cells upregulated 5 different growth factors including NGF and BDNF (Hoke *et al*, 2006). Increases in NGF and BDNF have been linked to chronic pain conditions previously, so glial induced upregulation of these neurotrophins could contribute to pain states. Following insult with lipopolysaccharide (LPS) there was an upregulation in TNF α mRNA in Schwann cells, which was reduced with erythropoietin exposure (Campana, 2006). There is also evidence for increases in SGC expression following neuronal injury. In the sciatic nerve transection model there was a significant increase in the numbers of perineuronal macrophages 2-4 days after the injury (Lu & Richardson, 1993). These increases were only seen in the corresponding DRG to the peripheral transection (Lu & Richardson, 1993). There is also evidence that the number of gap junctions between SGCs changes in response to a changing physiological environment. In the mouse, rat, and guinea pig it was found that in basal conditions dye coupling was evident in approximately 30% of cases, however when the pH was increased to 8 there was a significant increase in both the amount of dye coupling and the number of cells coupled to dye injected neurons (Huang *et al*, 2005). There was also a significant increase in the amount of dye coupling when the extracellular K⁺ concentration was increased, and this effect of a high K⁺ concentration was nullified by decreasing extracellular Ca²⁺ levels (Huang *et al*, 2005). Expression of the glial glutamate transporters glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1) is altered in a neuropathic pain state. GLAST and GLT-1 were shown to be downregulated 7 days following chronic constriction injury (CCI) of the sciatic nerve, which was linked to a 30% decrease in glutamate uptake activity (Sung *et al*, 2003). This decrease in glutamate uptake from surrounding glial cells may increase neuronal

excitability. It is clear to see that the role of peripheral glial cells in modulating nociception is not insignificant.

1.1.5.3 Central Sensitisation

Following intense peripheral injury there is an increased excitability and sensitivity in the spinal cord (Woolf 1983). This central sensitization can outlast past the point of injury resolution and is important in the induction and maintenance of chronic pain (Woolf 1983). Central sensitisation is a result of continual excitement of spinal neurons by peripheral input leading to long lasting changes in their sensitivity.

Glutamate represents the main excitatory neurotransmitter released by nociceptors which causes the depolarisation of post-synaptic neurons (Di Biasi & Rustioni, 1988). When nociceptors fire at intense frequency, either following peripheral insult or nerve injury, there is a significant increase in the release of glutamate (Inquimbert *et al*, 2012). The expression of the ionotropic glutamate receptor AMPA in the superficial laminae of the dorsal horn is high (Polgar *et al*, 2008), and therefore increased release of glutamate will lead to an increased depolarisation in the dorsal horn. As well as the increased glutamate release there is also an upregulation of AMPA receptor following peripheral nerve injury (Harris *et al*, 1996). AMPA receptors therefore are likely to play a role in the induction of central sensitization. Similarly, in the hippocampus AMPA receptor binding is increased following the induction of long term potentiation (Maren *et al*, 1993).

In the spinal cord there is a network of glutamate transporters which maintain the levels of extracellular glutamate during normal physiological conditions (Robinson & Dowd, 1996). However, following chronic constriction injury a down regulation of both glial and neuronal glutamate transporters (Sung *et al*, 2003) hinders removal of extracellular glutamate. This coupled with the injury induced increase in neuronal glutamate release means there is higher levels of synaptic glutamate following intense neuronal

stimulation, which can bind to extrasynaptic metabotropic glutamate receptors (mGLURs) present throughout the superficial laminae (Azkue *et al*, 2000, Tolle *et al*, 1993). These events further depolarizes the postsynaptic neuron, removing the voltage dependent Mg^{2+} block of NMDA receptors, and allowing further Ca^{2+} influx (Mayer *et al*, 1984). In neuropathic pain models there is also evidence for an increased phosphorylation of the NR1 subunit of the NMDA receptor which coincides with increased mechanical allodynia (Gao *et al*, 2005).

Two forms of potentiation which contribute to central sensitization have been described; homosynaptic potentiation and heterosynaptic potentiation. Homosynaptic potentiation involves the activity dependent strengthening of a synaptic connection localised to the site of activity. An example of homosynaptic potentiation is wind up, whereby a tetanus of low frequency stimuli causes an increased electrical response from the innervated postsynaptic neuron (Mendell & Wall, 1965). Heterosynaptic potentiation involves the activity dependent strengthening of not only the activated synapse but also nearby synapses which are not being directly activated. Heterosynaptic potentiation may underlie secondary hyperalgesia, whereby areas outside of nociceptor receptive fields exhibit altered responses leading to allodynia and hyperalgesia (LaMotte *et al*, 1992).

As well as an increase in the excitatory systems within the dorsal horn following peripheral stimulation there are also changes in the inhibitory systems. There is evidence for a PKC mediated reduction in GABAergic and glycinergic inhibition of spinothalamic tract neurons following central sensitization (Lin *et al*, 1996)

1.1.5.4 Glial Cells of the Dorsal Horn

Microglia represent the tissue resident macrophages of the central nervous system, and are derived from primitive macrophages in the yolk sac (Ginhoux *et al*, 2010). Microglia in the dorsal horn have

different morphologies based on their functionality; a resting state with long fine processes and an activated state with fewer and shorter processes (Svensson *et al*, 1993a). When microglia are activated there is an upregulation of microglial markers, including ionised calcium-binding adapter 1 (Iba1) (Ito *et al*, 1998). Microglia have been heavily implicated in the central processing of pain. Following both spinal nerve cryoneurolysis and chronic constriction injury there was a significant increase in microglia activation in the rat (Colburn *et al*, 1997). Indeed, administration of minocycline, a potent inhibitor of microglia, is capable of preventing the onset of neuropathic pain (Raghavendra *et al*, 2003).

Microglia widely express the purinoceptors P2X and P2Y, which respond to extracellular ATP (James & Butt, 2002). Indeed, ATP activates ERK, JNK and p38 MAP kinase activity in microglia (Suzuki *et al*, 2004). All three of these kinases are involved in ATP induced TNF α release from microglia (Suzuki *et al*, 2004). Specifically, it has been shown that P2X7 is responsible for JNK and p38 induced TNF α release (Suzuki *et al*, 2004). As well as TNF α , microglia also release the cytokines IL-6 and IL-1 β (Ferrari *et al*, 1997). Overall there is strong evidence supporting a role of spinal microglia in the central processing of pain (Milligan & Watkins, 2009).

The second type of glial cell present in the dorsal horn of the spinal cord are the astrocytes. Astrocytes are vital for the survival and normal function of spinal neurons, primarily through the uptake of extracellular glutamate (Anderson & Swanson, 2000). Astrocyte activity can be measured by an increase in the immunofluorescence of glial fibrillary acidic protein (GFAP) (Svensson *et al*, 1993a). Following injury astrocytes appear to be activated following the activation of microglia. After a chronic constriction injury there was increased ipsilateral immunofluorescence for GFAP (Garrison *et al*, 1991). Following spinal nerve ligation there are changes in ERK activation in neuronal cells immediately after injury, and then ERK activation in microglia a few days post injury, and finally there was increased ERK activation in astrocytes by 10 days post injury

(Zhuang *et al*, 2005). There is evidence that microglial activation plays a role in the subsequent astrocyte activity, for example eliminating activated microglia reduced the increase in GFAP expression following spinal nerve transection (Svensson *et al*, 1993b). Like microglia, astrocytes are thought to have a more active role in nociception, with evidence of NMDAR mediated glutamatergic astrocyte-neuron signalling contributing to the central processing of pain (Bardoni *et al*, 2010).

1.2 Osteoarthritis

1.2.1 Epidemiology of Osteoarthritis

The chronic joint disease osteoarthritis (OA) presents a serious public health problem for both the present and the future. OA is the most common form of arthritis (Lawrence *et al*, 2008) and is hugely prevalent, in 1990 an estimated 15% of the adult US population reported having arthritis (Lawrence *et al*, 1998). A study which took radiographs of 1,420 participants found evidence of knee OA in 33% of cases (Felson *et al*, 1987). A more recent estimate of OA prevalence estimated that there were 26.9 million adults aged 25 or above in the US with clinical OA (Lawrence *et al*, 2008). A study of OA diagnosed in general practise in the UK found that an estimated 12.5% of adults aged 45 or older had documented OA of the knee joint (Bedson *et al*, 2005). It is thought that the numbers of people afflicted with OA could increase significantly in the future. In the US it is estimated that by the year 2020 an estimated 18.2% of adults will have arthritis (Lawrence *et al*, 1998). Indeed, it is predicted that by 2020 it will be one of the top ten causes of burden in the developed world as measured by disability-adjusted life years (DALY) (Murray & Lopez, 1997).

A major contributing factor to the increasing prevalence of OA is age, since the chances of developing OA are positively correlated with age. Radiographic evidence of OA in the knee was found in 27.4% of under 70 year olds, compared to 34.1% in those aged 70-79, and 43.7% in those aged 80 and over (Felson *et al*, 1987). This relationship between age and OA is not isolated to the US, studies in both the Netherlands and the UK have shown that the prevalence of OA increased strongly with age (Bedson *et al*, 2005, Van Saase *et al*, 1989). Sex is also a risk factor for OA, with women more likely to have OA than men. Radiographic knee OA was evident in 52.6% of women aged 80 or over compared to 32.6% of men (Felson *et al*, 1987). As well as this the majority of people diagnosed with knee OA in general practice in the UK were women (Bedson *et al*, 2004). However, apart from OA in the hips and knees, it is suggested that the differences between men and women aren't that large (Van Saase *et al*, 1989). There is some evidence that ethnicity may also be a risk factor for OA. It was found that African Americans had slightly greater prevalence of both symptomatic and radiographic OA of the knee (Jordan *et al*, 2007). There was also a significantly greater prevalence of more severe radiographic knee OA in African Americans when compared to Caucasians (Jordan *et al*, 2007).

Prevalence of OA is also heavily linked to obesity, which is another reason why this disease represents a very serious issue for the future. In both males and females the incidence of knee OA was positively correlated with both body mass index (BMI) and skinfold thickness (Anderson & Felson, 1988). Subjects who had BMIs above 30 had a much higher incidence of OA than those participants with BMIs between 25 and 30 (Anderson & Felson, 1988). Similar results were found in a separate population where there was a significantly higher average BMI in men and women with definite OA of the knee compared to healthy controls (Hochberg *et al*, 1995). Previous injury to the knee is also a risk factor for developing knee OA later in life. A study of American footballers 20 years after they last played found that there was a significant increase in OA prevalence

in player who had a prior knee injury (Moretz *et al*, 1984). Soccer players also had a greater prevalence of knee OA when compared to a control group, this difference was linked to the greater number of knee injuries in the footballers (Kujala *et al*, 1995). Amongst the other exogenous risk factors for OA, there is also evidence that your occupation can influence the prevalence of knee OA (Anderson & Felson, 1988).

Alongside idiopathic OA there are also examples of secondary OA developing as a result of different disorders. Notably, around half of those who suffer from diabetes are predicted to suffer from arthritis of some form (King & Rosenthal, 2015). However, this link may be a result of increased obesity in diabetes and arthritis patients.

Hereditary hemochromatosis is a disease whereby there is tissue specific iron overload. Patients with this disorder have a high risk of developing severe secondary OA, and thus when compared to the general population they undergo joint replacement surgery at an earlier age (Sahinbegovic *et al*, 2010). Ehlers-Danlos syndrome is a group of hereditary connective tissue disorders, individuals with this syndrome develop premature osteoarthritis due to joint hypermobility (Callewaert *et al*, 2008). Another systemic connective tissue disorder, Marfan's syndrome, also shows signs of premature arthrosis, again as a result of joint hypermobility (Callewaert *et al*, 2008).

1.2.2 Pathophysiology of Osteoarthritis

1.2.2.1 The structure of the synovial joint

Joints are complex structures within the body which are essential for movement. Synovial joints represent the most common type of joint within the body and are made up of a variety of different types of tissue including synovium, bone, cartilage, ligaments and menisci. The knee joint is a classic example of a synovial joint, and is the point where the distal epiphysis of the femur meets the proximal epiphysis of the tibia. This section will detail the structure of synovial joints using the knee joint as an example.

The bones and ligaments of the knee joint

As mentioned earlier, the knee joint is a hinge joint between the femur and the tibia allowing the movement of the legs in mammals. The epiphysis of the femur has two protrusions which are known as the medial and lateral femoral condyles. These condyles articulate with the tibia. Between the two condyles is the femoral groove. The epiphysis of the tibia has two flattened plateaus which articulate with the femoral condyles, these are the medial and lateral tibial plateaus. The smaller bone of the lower leg, the fibula is not directly involved in movement and as such does not connect to the femur directly. The patella sits on the anterior surface of the femur and slides up and down during movement providing much needed leverage for movement. There are four major ligaments within the knee joint which support and connect the bones of the joint. The medial and lateral collateral ligaments, as the names suggest, run along the medial and lateral sides of the joint respectively, and prevent the knee joint from moving too far medially or laterally. The other two major ligaments within the joint are strong ligaments which connect the tibia and femur known as the cruciate ligaments. The anterior cruciate ligament is linked to the anterior portion of the tibia, and the posterior cruciate ligament is linked to the posterior portion of the tibia, the two ligaments cross over each other hence the name cruciate.

The menisci of the knee joint

There are two menisci in the knee joint, the lateral and medial menisci, named after their position on the tibia. They are semi-lunar in shape and are attached to the tibial plateaus through a meniscotibial ligament. The cells of the menisci are known as fibrochondrocytes, they have a round morphology and are situated within lacunae, like chondrocytes, however they produce a fibrocartilage matrix (McDevitt & Webber, 1990). The extracellular matrix (ECM) of the menisci are primarily comprised of type 1 collagen, with smaller amounts of type II, III, and V collagen

(McDevitt & Webber, 1990). The meniscal ECM also contains proteoglycan, matrix glycoproteins, and elastins (McDevitt & Webber, 1990). The menisci are thought to have numerous functions within the joint including; shock absorption, improving joint stability, and improving weight distribution (Seedhom et al, 1974). An experiment which applied stresses to the knee joints of cadavers estimated that the lateral meniscus carried 70% of the load, whilst the medial meniscus carried 50% of the load (Seedhom et al, 1974).

Cartilage

A thin layer of hyaline cartilage lines the two femoral condyles and the tibial plateaus and provides a reduced friction surface to aid the movement of the joint. The only cell type in the cartilage are the chondrocytes, their role is the synthesis and remodelling of the ECM (Gray et al, 1988). The ECM of cartilage is again mostly comprised of collagens, with specifically type II, IX, and XI collagens forming the striated fibrils within the matrix (Bruckner & van der Rest, 1994). It has been found that the biosynthesis undertaken by articular chondrocytes changes under mechanical stress, including reduced GAG production (Gray et al, 1988). There are four main layers within hyaline cartilage which are determined by the orientation of the collagen within them. The four layers starting from the articular surface are the uncalcified superficial layer (layer 1), the intermediate layer (layer 2), the deep layer (layer 3), and the calcified cartilage (layer 4) (Imhof et al, 1997). Layer 1 is comprised of densely packed fibrils which align parallel to the surface, layer 2 is filled with randomly distributed fibrils, layer 3 is the thickest layer and its fibrils are perpendicular to the surface, whilst layer 4 is a thin layer of calcified cartilage (Imhof et al, 1997).

The synovium

Synovial joints are characterised by the presence of a synovium, which is a fibrous capsule which encapsulates the joint. The synovial

membrane is comprised of two layers, the first layer is comprised of mostly fibroblast-like synoviocytes (Barland et al, 1962). The second layer is mostly comprised of macrophages. A healthy synovial lining is usually 2-3 cells deep and heavily vascularised. The synovial membrane is responsible for synthesising and releasing the hyaluronic acid of the synovial fluid (Barland et al, 1962). The synovial fluid inside the joint plays an important role in both providing nourishment for chondrocytes in the non-vascularised cartilage and providing joint lubrication (Lorenz & Richter, 2006).

1.2.2.2 Pathophysiology of Osteoarthritis

All structures of the joint are affected by the onset of osteoarthritis (**Figure 1.4**). This next section will briefly detail the pathophysiology of osteoarthritis.

Cartilage degradation in osteoarthritis

In osteoarthritis, the cartilage loses its smooth appearance and becomes fibrillated and degraded. Over time the loss of cartilage becomes severe and the underlying subchondral bone can be exposed. In healthy cartilage there is a fine balance between cartilage degradation and synthesis, this balance is lost in OA (Sandell & Aigner, 2001). During OA there are several changes seen within the cartilage. It has been suggested that there is apoptosis of chondrocytes within the cartilage during OA (Hashimoto et al, 1998). However, the percentage of chondrocytes undergoing apoptosis and its subsequent role in driving OA pathophysiology is debated (Sandell & Aigner, 2001). There is increased metabolic degradation of the extracellular matrix of the cartilage in OA. Cells in the synovium, as well as chondrocytes, produce cytokines which have been found to increase the synthesis of matrix metalloproteinases (MMPs) in OA (Sandell & Aigner, 2001). There is also a reduction in the expression of tissue inhibitors of metalloproteinases (TIMPs) in human OA synovial fluid (Yoshihara et al, 2000). The α -disintegrin and metalloprotease with thrombospondin motifs (ADAMTS) family of proteins are thought to

be involved in cartilage degradation (Tortorella et al, 2000). Cathepsins, which are cysteine proteases, are also been found in OA cartilage and subchondral bone (Lang et al, 2000). In healthy tissue chondrocytes express bone morphogenetic protein 7 (BMP-7), which plays a role in cartilage and bone synthesis (Chubinskaya et al, 2000). In OA there is a significant decrease in BMP-7 protein (Chubinskaya et al, 2000). Another key characteristic of OA is the development of osteophytes, which are new bone spurs growing from the joint (Sandell & Aigner, 2001). There is evidence that the cytokine TGF- β is involved in the formation of osteophytes, as repeated injection of the growth factor was enough to induce osteophyte development in the mouse (van Beuningen et al, 1994).

Synovitis in osteoarthritis

Unlike rheumatoid arthritis, OA is not considered as primarily an inflammatory disorder, however synovitis is still an important change seen in the disease. There are significant structural changes in the synovium in OA including thickening of the synovial lining, increased vascularisation, and infiltration of inflammatory cells (Smith et al, 1997). As a result of the inflammatory cell migration there was also an increase in IL-1 α , IL- β , and TNF- α within the synovium (Smith et al, 1997). As already mentioned these inflammatory mediators are thought to increase MMP expression and decrease MMP enzyme inhibitor expression (Sandell & Aigner, 2001). Changes in cell infiltration and vascularisation are more prominent in early OA rather than the later stage disease (Benito et al, 2005).

Meniscal damage in osteoarthritis

Meniscal damage is commonly seen in OA patients. Evidence of meniscal tears or destruction was seen in 35% of MRI scans of patients with OA, with greater prevalence of damage in the medial meniscus (Englund et al, 2008). There is also evidence for collagen degradation and increased vascularisation within the outer portion of the meniscus in knee joints with high chondropathy (Ashraf et al,

2010). Cartilage loss was significantly increased in patients with damaged or malpositioned menisci (Hunter et al, 2006). This highlights the importance of the meniscus in maintaining the structure of the joint.

Subchondral bone changes in osteoarthritis

There are also concomitant changes in the subchondral bone in OA. As in the cartilage, there is a delicate balance of turnover in healthy bones mediated by osteoblasts and osteoclasts (Tombran-Tink & Barnstable, 2004). In OA there is evidence of subchondral bone sclerosis during the development of the disease (Moskowitz 1999). This suggests an imbalance in remodeling within the subchondral bone. Bone marrow lesions are also prevalent in patients with OA, an MRI study found that there were bone marrow lesions in 56% of knee joints (Sowers et al, 2003). It is still not clear whether the subchondral bone changes seen in OA are the cause of cartilage loss in OA, or whether cartilage loss leads to subchondral sclerosis.

1.2.3 Knee Pain

1.2.3.1. Innervation of the knee joint

To understand where knee pain in OA originates it is important to consider which of the different joint structures are innervated. One of the first analyses of the pattern of innervation within the knee joint was carried out in the cat (Freeman & Wyke, 1967). There are two main groups of nerves which innervate the knee joint, the primary nerves (posterior, medial, and lateral articular nerves), and the accessory nerves (which originate from muscular nerves) (Freeman & Wyke, 1967). The posterior articular nerve (PAN) originates from the posterior tibial nerve and terminates at the lateral and medial regions of the posterior capsule, the posterior cruciate ligament, and the posterior fat pads (Freeman & Wyke, 1967). The PAN only innervates tissues within the tibiofemoral joint (Freeman & Wyke, 1967). The medial articular nerve (MAN) branches off the saphenous nerve and splits into two branches at

the medial femoral condyle (Freeman & Wyke, 1967). Like the PAN, the MAN only innervates articular tissues such as the medial collateral ligament, the medial ligaments of the medial meniscus, and the infrapatellar ligament (Freeman & Wyke, 1967). The other primary nerve to innervate the knee joint is the lateral articular nerve (LAN), which originates from the lateral popliteal nerve and innervates the lateral aspects of the tibiofemoral joint (Freeman & Wyke, 1967). However, unlike the PAN and the MAN, the LAN innervates tissues outside of the knee joint, primarily the tibiofibular joint and the peroneal muscles (Freeman & Wyke, 1967). The position of the accessory articular nerves were found to be much more variable than the primary articular nerves, the majority of these nerves originate from the muscles above the knee joint and from extensor muscles (Freeman & Wyke, 1967).

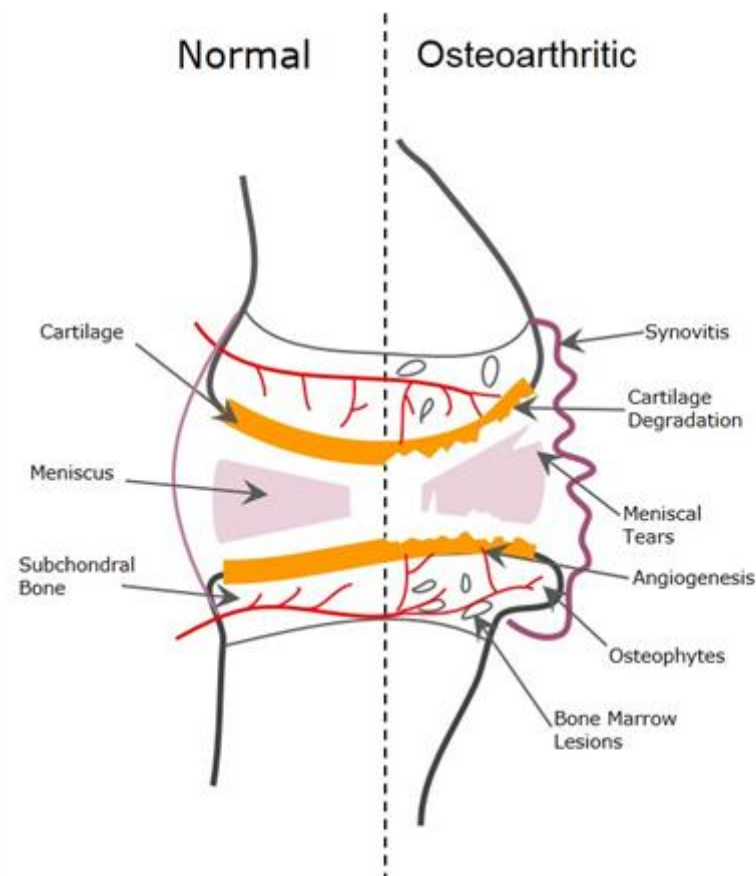


Figure 1.4: Diagram highlighting some of the characteristic structural changes seen in osteoarthritic knee joints when compared to normal knee joints.

There are four distinct morphological types of nerve endings which innervate the knee joint (Freeman & Wyke, 1967). Type I nerve endings are characterised by globular corpuscles, these nerve endings are found primarily in the posterior and anterior fibrous capsule of the knee (Freeman & Wyke, 1967). Type II nerve endings are characterised by a conical capsule and are primarily located in the medial and lateral fibrous capsule of the knee (Freeman & Wyke, 1967). Type III nerve endings have a fusiform capsule, and unlike type I and II nerve endings, are only found in the ligaments and tendons of the knee joint (Freeman & Wyke, 1967). Type I, II, and III nerve endings are thought to be the knee joints mechanoreceptors (Freeman & Wyke, 1967). The fourth class of nerve endings, type IV, are characterised by free nerve endings and represent the pain sensitive system of the knee joint (Freeman & Wyke, 1967). Type IV nerve endings are found in two forms, firstly they are found as a mesh-like network which innervates the fibrous capsule and the periosteum of the tibia, femur, and patella, secondly they are found as free nerve terminals which are found in the ligaments and tendons of the knee joint (Freeman & Wyke, 1967).

Retrograde labelling experiments in the rat have further elucidated the innervation of the knee joint. Firstly, it was noted that the afferents of the knee joint were found primarily in the L3 and L4 dorsal root ganglia (Salo & Theriault, 1997). Secondly, it was found that there are peptidergic neurons located in the joint, with 10% of neurons expressing substance P, and 33% of neurons expressing CGRP (Salo & Theriault, 1997). Interestingly, there appears to be no innervation of the knee joint by IB4 positively labelled neurons (Ivanavicius *et al*, 2004).

1.2.3.2. Normal nociception of the knee

Based on the pattern of innervation within the knee joint it is possible to predict which joint structures will detect normal nociceptive pain. Previously, the various tissues within the human knee joint have been probed without intra-articular anaesthesia, the

conscious experience of pain provided key information about nociception of the knee joint (Dye *et al*, 1998). The most severe pain was experienced when the fibrous capsule was cut open, there was also intense pain experienced when the fat pad, ligaments, and tendons were probed (Dye *et al*, 1998). However, there was no pain experienced when the cartilage was probed, even at the highest force of 500g (Dye *et al*, 1998). These findings are as expected with the most pain being found in the areas most highly innervated by the free nerve endings, whilst in the aneural cartilage there was no nociception.

It is also important to briefly mention the receptors present in the peripheral terminals of the primary articular nociceptors. Intra-articular injection of either $\alpha\beta$ methylene ATP or ATP in the rat causes a short-lasting excitation of C and A δ fibres, this excitation was blocked by a P2X antagonist, suggesting the presence of functional P2X receptors in articular nerve terminals (Dowd *et al*, 1998a). Adenosine A1 receptor agonists also excited rat knee joint afferents, and an A1 antagonist reduced ATP induced excitation indicating the presence of A1 receptors in nociceptive afferents (Dowd *et al*, 1998b). Immunohistochemical and retrograde labelling experiments have shown that around 40% of knee joint afferents in the mouse express the vanilloid receptor TRPV1 (Cho & Valtschanoff, 2008). There was also ASIC3 immunoreactivity in 31% of knee joint afferents in naive mice (Ikeuchi *et al*, 2008).

1.2.3.3 Osteoarthritic Pain

Of all the symptoms of OA, pain is the most significant, and is often the reason patients seek medical attention. During chronic OA pain there is abnormal functioning at all levels of the nociceptive pathway. The pathological changes observed in the joint sensitize the peripheral afferents suggesting a peripheral origin for chronic OA pain; however a substantial number of total knee joint arthroplasty patients still experience chronic pain suggesting there is also a central component (Beswick *et al*, 2012, Bourne *et al*, 2010).

There is evidence that subchondral bone lesions play an important role in chronic OA pain. Towards the late stages of OA, after severe cartilage breakdown, subchondral bone is exposed to microfractures and eventually lesioning (Madry *et al*, 2010). Indeed, there was a significant correlation between cartilage loss and the extent of bone marrow lesioning (Hunter *et al*, 2006b). The subchondral bone is highly vascularised, suggesting that it is also highly innervated (Madry *et al*, 2010). A MRI study of patients with OA found that there was strong positive correlation between reported pain and the extent to which the subchondral bone was exposed (Moisio *et al*, 2009). An experiment of patients with radiographic evidence of osteoarthritis investigated the link between BMLs and chronic joint pain (Felson *et al*, 2001). There was evidence of BMLs in 77.5% of patients that reported joint pain compared to just 30% in patients who did not report chronic joint pain (Felson *et al*, 2001). Patients that reported pain also had significantly larger BMLs (30%) than those patients that did not report pain (2%) (Felson *et al*, 2001).

As previously mentioned, there is significant inflammation of the synovial fluid in OA affected joints (Smith *et al*, 1997). There is increased vascularisation and infiltration of immune cells in the synovium of OA patients (Smith *et al*, 1997). Synovial inflammation could therefore contribute to OA pain. Indeed, there is a significant correlation between the extent of synovitis and patient reported pain (Hill *et al*, 2007). With the infiltration of inflammatory cells into the synovium there is an increase in the expression of the cytokines IL-1 α , IL-1 β , and TNF- α (Smith *et al*, 1997). As previously discussed in section **1.1.5** these inflammatory cytokines play an important role in peripheral sensitization. There is also evidence of the nociceptive neuropeptides CGRP and substance P being found in the joint capsule following inflammation (Ralphs & Benjamin, 1994). It is clear that synovitis is important in OA induced joint pain.

Articular cartilage under normal circumstances is not innervated or vascularised, and therefore is not involved in normal nociception. However, following OA there is increased vascularisation and

innervation of the articular cartilage (Walsh *et al*, 2010). Therefore it is possible that the articular cartilage is important as a peripheral driver of joint pain. Indeed, in a murine model of OA there was upregulation of several proteins in the articular cartilage implicated in nociception, most notably NGF (Driscoll *et al*, 2016). An MRI study of patients with both painful and non-painful OA found that full thickness defects in the cartilage were most commonly found in patients that experienced chronic pain (Sowers *et al*, 2003).

There are other tissues within the knee joint that are innervated, and therefore may contribute to chronic OA pain. These include the ligaments and tendons within the knee. An MRI study investigated the relationship between damage in different structures within the knee and patient perceived pain (Torres *et al*, 2006). There was relationship between pain and several joint structure defects including meniscal tears (Torres *et al*, 2006). However, there were not enough examples of ligament tears to analyse any potential correlation with pain outcomes (Torres *et al*, 2006).

As well as peripheral sensitization, there is evidence for a centrally mediated phenotype of OA. Patients with OA experienced a greater temporal summation to repeated painful stimuli and had a reduced descending inhibition, which are both indicators for central sensitization (Arendt-Nielson *et al*, 2010). There was also a negative correlation between reported knee joint pain and the pressure pain threshold at distal sites, suggesting that the spread of sensitivity is greater in patients experiencing more joint pain (Arendt-Nielson *et al*, 2010). In fact, in patients who experience high pain but have a low radiographic grade of OA had greater pain catastrophizing, sleep deprivation and increased anxiety (Finan *et al*, 2013). This high pain but low radiographic OA group also had a significantly increased response to painful stimuli at distal sites from the joint compared to the high pain and high OA group (Finan *et al*, 2013). These data suggest that in a certain subgroup of people the peripheral input from the OA affected joint may not be as important as central sensitization in driving chronic joint pain.

Functional imaging studies have provided information about the role of supraspinal areas in the maintenance of chronic joint pain. In patients who display signs of central sensitization there was a significantly greater response in the brainstem to punctate stimuli when compared to healthy controls (Gwilym *et al*, 2009). There is also evidence that there is differential activation of the dorsolateral prefrontal cortex in response to electrical stimulation of the knee in OA patients compared to healthy controls (Hiramatsu *et al*, 2014).

There is also evidence for a psychological component to chronic OA pain. Patients with co-morbid insomnia and OA reported improved sleep and a reduction in joint pain following cognitive behavioural therapy (CBT) (Vitiello *et al*, 2009). This improvement in both sleep and pain was found to be maintained up to a year post CBT (Vitiello *et al*, 2009). Other psychological interventions, including hypnosis and relaxation therapy, have been reported to have long lasting effects on the perception of pain in OA patients (Gay *et al*, 2002).

1.2.4 Treating Osteoarthritis Pain

The National Institute for Health and Care Excellence (NICE) guidelines recommend exercise as a core non-pharmacological treatment for osteoarthritic pain regardless of pain severity or age of patient (NICE guidelines, 2014). In terms of pharmacological treatments for OA pain NICE recommends paracetamol and topical NSAIDS as an initial intervention, if the pain persists then NICE recommends the use of opioids, NSAIDS, and COX-2 inhibitors (NICE guidelines, 2014). Total joint replacement surgery is only recommended when all other core treatment options have been considered (NICE guidelines, 2014).

Paracetamol tends to only have moderate clinical effects on joint pain when taken as a monotherapy, however there were significant improvements in pain relief, joint function, and quality of life when

paracetamol was given as a combined therapy with ibuprofen (Doherty *et al*, 2011). A clinical trial for a topical NSAID diclofenac solution over 12 weeks showed moderate improvements in reported pain as well as joint function and stiffness, topical application of the NSAID showed no changes in the number of GI tract related adverse effects, compared to placebo (Roth & Shainhouse, 2004).

The COX-2 inhibitor rofecoxib and the NSAID diclofenac have been shown to have a comparable effects in terms of reducing walking induced pain and improving physician's assessments of the diseases status (Cannon *et al*, 2000). Twice daily doses of opioids over 26 weeks decreased reported pain and improved patients' sleep (Caldwell *et al*, 2002). However, a meta-analysis of opioid effectiveness in the treatment of knee or hip OA pain found that there were only 4% more responders to opioids than to the placebo treatment (Nuesch *et al*, 2009). There was only a 15% difference in the average improvement in pain relief and only a 13% difference in the average improvement in joint function; however experimental groups were 55% more likely to develop adverse effects than placebo groups (Nuesch *et al*, 2009).

These results suggest that current drug treatments are only partially effective OA pain and therefore the development of novel therapeutics is a priority. Of current interest as a current therapeutic target is nerve growth factor (NGF). One clinical trial for the NGF monoclonal antibody tanezumab reported reduced pain on walking by 62% as well as improving joint stiffness and function (Lane *et al*, 2010). However, in 16 patients severe worsening of OA pathology was seen which required total joint replacement (Lane *et al*, 2010). A more thorough understanding of the roles of NGF on joint structure and pain is required to understand the mechanistic basis for these events. Knowledge of the potential role(s) of the other neurotrophins, including brain derived neurotrophic factor (BDNF), in OA related joint pain is currently limited.

1.3 Brain derived neurotrophic factor

1.3.1 Brain derived neurotrophic factor

Brain derived neurotrophic factor (BDNF) was the second neurotrophin to be discovered and was first purified from pig brain in 1982 (Barde et al, 1982). It has a molecular weight of 12.3 kDa and was differentiated from the first neurotrophic factor NGF based on lack of response to NGF antibodies (Barde et al, 1982). The rat gene for mature BDNF is composed of four 5' exons and one 3' exon, 8 separate mRNAs are transcribed from this gene (Timmusk et al, 1993). There was differential expression of these BDNF mRNAs in central and peripheral tissues (Timmusk et al, 1993). BDNF is synthesised in a precursor form with a molecular weight of 32 kDa before being cleaved to make the mature form of the protein (Mowla et al, 2001). ProBDNF is still however biologically active and can stimulate TrkB autophosphorylation similar to mature BDNF (Mowla et al, 2001). BDNF has a vital role in development with the majority of BDNF null mice not surviving beyond 48 hours (Jones et al, 1994). In BDNF null mice which survived had significant deficits in the number of cells in sensory ganglia and in the numbers of dorsal root neurons, but no deficits in muscle innervation or motor neuron expression (Jones et al, 1994).

1.3.2.1 Expression of BDNF

In the adult levels of BDNF in the CNS are high, suggesting a role of BDNF outside of development (Altar et al, 1997, Hofer et al, 1990). Indeed, application of BDNF to the adult hippocampus enhanced synaptic strength for up to 3 hours, which was reversed by the tyrosine kinase receptor inhibitor K252a (Kang & Schuman, 1995).

Significant BDNF protein immunoreactivity has been demonstrated in the striatum as well as the frontal and cingulate areas of the cortex (Altar et al, 1997). Levels of BDNF mRNA are high in the

cortex and the hippocampus, however there are much lower levels in the striatum (Hofer et al, 1990). Due to this disconnect between expression of BDNF protein and mRNA it has been suggested that there is anterograde transport of BDNF from the cell bodies to the neuropil. Inhibiting axonal transport with colchicine increased BDNF immunoreactivity in the cell body whilst decreasing immunoreactivity in the neuropil (Altar et al, 1997). Ablation of extrinsic afferents also reduced the levels of BDNF protein quantified by ELISA (Altar et al, 1997).

BDNF is stored in dense core vesicles (DCV) in both spinal and amygdala neurons (Salio et al, 2007). Interestingly there is co-storage of BDNF with both CGRP and substance P within these DCVs (Salio et al, 2007). BDNF has been found to be released in the dorsal horn following high frequency burst stimulation, mimicking the physiological firing of C fibres (Lever et al, 2001). Dorsal horn BDNF release was also induced through application of capsaicin (Lever et al, 2001).

BDNF is also found in the peripheral nervous system, with BDNF immunoreactivity in DRG neurons and the superficial laminae of the dorsal horn (Michael et al, 1997, Zhou et al, 2004). Significant colocalization between CGRP and BDNF immunoreactivity has been reported (Michael et al, 1997). BDNF has also been found to be expressed in microglia in laminae I & II of the dorsal horn of the spinal cord (Merighi et al, 2008). Outside of the nervous system there is significant BDNF immunoreactivity in keratinocytes and the myenteric plexus of the small intestine (Zhou et al, 2004).

1.3.2.2 Expression of the TrkB Receptor

Like NGF and the other neurotrophins, BDNF binds to two receptors, a tyrosine receptor kinase and p75 (Chao & Hempstead, 1995). BDNF binds with high affinity to the tyrosine receptor kinase TrkB. Indeed, co-expression of TrkB and BDNF was found to induce morphological changes in transfected cells (Klein et al, 1991). BDNF was also found to phosphorylate TrkB in transfected cells (Klein et

al, 1991). Neurotrophin- 3 (NT-3) also phosphorylates TrkB (Klein et al, 1991). Alternative splicing of TrkB mRNA results in 3 separate isoforms of the protein, one full length and the other two are truncated (Middlemas et al, 1991). The truncated isoforms of TrkB share the same extracellular and transmembrane domains as the full length protein, but have shortened cytoplasmic tails (Middlemas et al, 1991). Full length TrkB and truncated TrkB are found in neuronal cells, whilst the truncated version appears to be localised to non-neuronal cells, like those found in the pia mater (Yan, et al, 1997).

Like BDNF, there is significant immunoreactivity for TrkB across the nervous system, in both the brain and the spinal cord (Zhou et al, 1993). Within the brain TrkB is highly expressed in several areas including; the frontal cortex, the olfactory bulb, the thalamus, the hypothalamus, purkinje cells in the cerebellum, and both sensory and motor nuclei of the brainstem (Yan et al, 1997). There is high colocalisation of TrkB and BDNF within the brain (Yan et al, 1997). Within the spinal cord full length TrkB immunoreactive cells were found predominantly in laminae I & II in the rat and mouse (Salio et al, 2005). In 10% of dorsal root neurons there was colocalisation of both BDNF and TrkB, compared to around 35% of neurons expressing both BDNF and TrkA (Salio et al, 2005). Terminals which are immunoreactive for TrkB and BDNF make up the C bouton of peptidergic glomeruli in lamina II (Salio et al, 2005).

1.3.2.3 p75 receptor

All neurotrophic factors also bind with low affinity to the p75 receptor (Chao & Hempstead, 1995). The p75 receptor is a member of the TNF super family of receptors, it is made up of four cysteine rich extracellular domains, which provides the binding site for the neurotrophins (Chao & Hempstead, 1995). The p75 receptor is found in a wide variety of different cell types including schwann cells, motor neurons, and purkinje cells (Chao & Hempstead, 1995).

p75 neurotrophin receptor was originally thought to play an important role in cell apoptosis (Chao & Hempstead, 1995). However, there is also evidence that p75 NTR plays a more complex role in Trk signalling. For example it can influence ligand specificity when it is co-expressed with Trk receptors. In fact, NT-4/5 and NT-3 mediated phosphorylation of TrkB was found to be reduced when p75 and TrkB were co-expressed, whilst BDNF specificity was increased (Bibel *et al*, 1999).

1.3.2 BDNF and Pain

1.3.2.3 Regulation of BDNF in inflammatory and neuropathic pain states.

In the adult nervous system BDNF is thought to be involved in nociceptive processing. Indeed there is plenty of evidence that BDNF expression is regulated by both inflammatory and neuropathic pain states. Following intraplantar injection of CFA there was an increase in the number of BDNF immunoreactive neurons in the DRG and the number of BDNF immunoreactive terminals in the dorsal horn (Cho *et al*, 1997a). In the same model of inflammation there was also an increase in the number of BDNF mRNA positive neurons, this inflammation induced increase was prevented by treatment with an NGF antibody (Cho *et al*, 1997b). Treatment with NGF also increased the release of BDNF in dorsal root neurons (Lever *et al*, 2001). Peripheral inflammation induced by CFA was also found to up-regulate full length TrkB in the dorsal horn of the spinal cord (Mannion *et al*, 1999).

BDNF expression changes following nerve injury, following axotomy there is an increase in BDNF expression in lumbar DRG cells, in particular in medium to large sized cells (Michael *et al*, 1999). There is also an increase in the numbers of cells co-labelled with BDNF and TrkB following axotomy (Michael *et al*, 1999). Following the chronic constriction injury model of neuropathic pain there was an increase in the numbers of BDNF immunoreactive neurons in the DRG and an increase in the numbers of BDNF labelled axons in the

deeper laminae of the spinal cord (Ha et al, 2001). In the murine sciatic nerve ligation model there were increased levels of the full length TrkB protein in the dorsal horn of the spinal cord (Yajima et al, 2002). These findings in rodent models appear to have translational relevance as levels of BDNF are increased in chronic pain patients. For example, there are increased levels of BDNF in the cerebrospinal fluid of both chronic migraine and primary fibromyalgia patients (Sarchielli et al, 2007). Interestingly, there was a strong positive correlation between the duration of chronic pain and the expression of BDNF (Sarchielli et al, 2007). BDNF expression is also significantly increased in several tissues of patients with chronic pancreatitis (Zhu et al, 2001). Again, it was found that there was a strong positive correlation between the levels of BDNF expression and reported pain severity and frequency in patients with chronic pancreatitis (Zhu et al, 2001). As discussed below there is also evidence for a link between BDNF and chronic OA joint pain (**1.3.3**).

1.3.2.4 The role of BDNF in mediating normal nociception

Unlike NGF, the extent to which BDNF affects normal nociception is still debated. There is some evidence that BDNF plays a role in nociception under normal conditions. For example, there was an increase in the phosphorylation of TrkB receptors in the spinal cord following chemical, mechanical, and heat stimulation in naive animals (Pezet et al, 2002). As previously mentioned BDNF is also found to be released following high frequency short burst stimulation (Lever et al, 2001). However, others have found that baseline pain behaviour is not altered by BDNF. Indeed, mopping up endogenous BDNF with TrkB/fc chimera did not alter the baseline behavioural sensitivity (Mannion et al, 1999). Removal of endogenous BDNF had no effect on A and C fiber evoked responses (Kerr et al, 1999). While it is not clear whether or not endogenous BDNF is involved in normal nociception, exogenous application of the neurotrophin can dramatically alter nociception. Intrathecal application of BDNF enhanced the C fiber mediated nociceptive

reflex (Kerr et al, 1999). Exogenous BDNF also increased the release of several neurotransmitters including glutamate and substance P (Merighi et al, 2008). Behaviourally, intrathecal administration of BDNF was associated with thermal hyperalgesia in naive mice (Groth & Aanonsen, 2002).

1.3.2.5 Interactions between BDNF and NMDA

There has been a lot of research into the interaction between BDNF and NMDA in modulating nociceptive processing. Application of BDNF enhances NMDA induced depolarisation in the spinal cord of naive rats (Kerr et al, 1999, Garraway et al, 2003). Blocking NMDA receptors was also shown to prevent BDNF evoked synaptic facilitation (Garraway et al, 2003). Co-administration of BDNF and the NMDA antagonist, d-APV, inhibited BDNF induced thermal hyperalgesia (Groth & Aanonsen, 2002). One of the mechanisms through which BDNF may modulate NMDA activity is through phosphorylation of NMDA subunits. Application of BDNF to isolated spinal cords increased levels of phosphorylated NR1, a NMDA subunit in laminae I, II, and V of the spinal cord (Slack & Thompson, 2002). Sequestering BDNF with TrkB-IgG prevented BDNF induced phosphorylation of NR1 (Slack & Thompson, 2002). The BDNF induced phosphorylation of NR1 is thought to be mediated by the TrkB receptor, as the Trk inhibitor K252a blocked NR1 phosphorylation (Slack et al, 2004). In cortical and hippocampal neurons BDNF was found to enhance the tyrosine phosphorylation of the NR2B subunit of NMDA (Lin et al, 1998). The phosphorylation of the NR2B subunit was found to be specific to BDNF, as administration of NGF did not affect phosphorylation of NR2B (Lin et al, 1998).

There is substantial information available regarding the intracellular mechanisms through which BDNF modulates NMDA activity. It is thought that the MEK/ERK, and PKC/PLC pathways are necessary for the BDNF induced NR1 subunit phosphorylation. Administration of the MEK inhibitor, U0126, and the PKC inhibitor, chelerythrine chloride, reduced the levels of NR1 subunit phosphorylation induced

by BDNF (Slack et al, 2004). The PLC inhibitor U73122 has also been shown to stop BDNF induced synaptic facilitation (Garraway et al, 2003). The augmented hyperalgesia and allodynia in mice with neuropathic pain following intra-theal injection of BDNF was reversed with a PKC inhibitor (Yajima et al, 2005). Inhibition of PKC was also found to abolish the BDNF induced Ca^{2+} concentration increase in cultured mouse spinal neurons (Yajima et al, 2005). As described earlier, phospho-ERK is a key signalling molecule implicated in central sensitization and levels of phospho-ERK in the dorsal horn of the spinal cord are increased following administration of BDNF (Pezet et al, 2002). Conversely, removal of endogenous BDNF with TrkB-IgG decreased the number of phospho-ERK labelled neurons following capsaicin stimulation (Pezet et al, 2002).

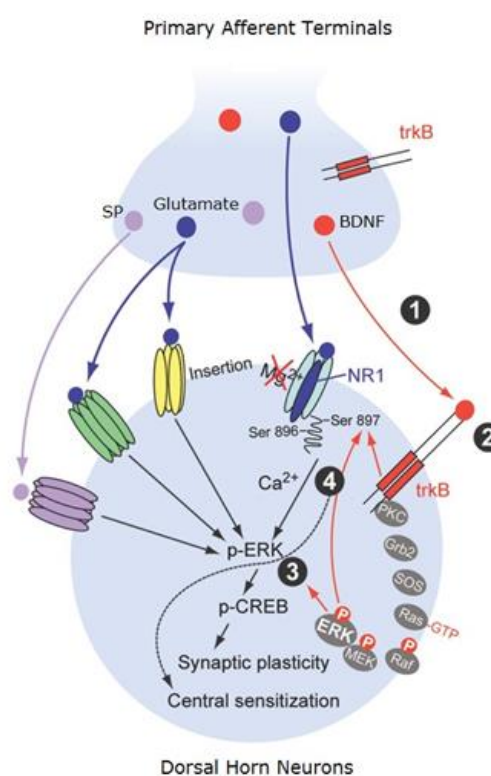


Figure 1.5: Diagram highlighting some of the mechanisms through which BDNF is thought to contribute to central sensitization. 1) BDNF is released from primary afferent terminals. 2) BDNF binds to its high affinity receptor trkB. 3) Increased levels of phospho-ERK. 4) Phosphorylation of the NR1 subunit of NMDA receptors. Adapted from Pezet & McMahon, 2006.

1.3.3 BDNF and Osteoarthritis pain

As previously mentioned (1.2.4) monoclonal antibodies for NGF have shown real promise as therapeutics for OA pain (Lane *et al*, 2010, Sanga *et al*, 2013, Tiseo *et al*, 2014). This section of the thesis will detail the current evidence concerning the contributions of BDNF to chronic OA pain. The literature regarding BDNF's involvement with OA and OA induced joint pain is far from complete. Nevertheless, the information that is available does indicate a potential role for BDNF in arthritis. Firstly, in patients with longstanding rheumatoid arthritis levels of BDNF in the synovial fluid were higher than healthy controls. Following anti-TNF treatment there was a reduction in these levels of BDNF (Grimsholm *et al*, 2008b). In mice with inflammatory arthritis immunoreactivity for BDNF was present in the synovium and there was also staining for BDNF and TrkB in articular chondrocytes (Grimsholm *et al*, 2008a). In OA patients there was a significant increase in plasma levels of BDNF, compared to healthy controls (Simao *et al*, 2014). There was also BDNF detected in the synovial fluid of patients with OA, however there was no data available on the levels of BDNF in the synovial fluid of a healthy population (Simao *et al*, 2014). Interestingly, there was a correlation between patient's perception of pain on the WOMAC scale and the plasma levels of BDNF (Simao *et al*, 2014). BDNF levels also appear to be linked to activity, in a study of elderly women with knee OA there was both an acute and chronic increase in plasma concentration of BDNF following exercise (Gomes *et al*, 2014). BDNF is produced by synovial fibroblasts in humans with OA (Klein *et al*, 2012). When these fibroblasts were stimulated with ATP there was an increase in the release of BDNF, and this ATP induced increase was thought to be mediated by the purinergic receptor P2X4 (Klein *et al*, 2012). Due to the localisation and expression of BDNF in OA patients as well as the documented involvement of BDNF in the processing of pain it is conceivable that BDNF is involved in chronic OA pain. However, the literature

regarding BDNF and OA is sparse, therefore further preclinical work is required to investigate the potential role of BDNF and the therapeutic potential of this target.

1.4 Polyadenylation as a Potential Therapeutic Target

In the search for therapeutic agents which can provide pain relief to OA patients, researchers are increasingly interested in the potential of disease modifying osteoarthritis drugs (DMOADs) (Hunter, 2011). These compounds which aim to reverse or halt the progression of OA pathology could provide analgesia to patients with peripherally driven OA pain. One biological mechanism which could be a target for DMOADs could be cytoplasmic polyadenylation.

1.4.1 Polyadenylation

Polyadenylation is a part of the process of protein translation and is thought to play an important role in the metabolism of mRNA. Polyadenylation involves the addition of a long tail of adenine nucleotides (poly(A) tail) to the 3'- terminus of post-transcriptional mRNA (Darnell *et al*, 1971, Edmonds *et al*, 1971). A complex of proteins (cleavage/polyadenylation specificity factor [CPSF], cleavage stimulation factor [CstF], cleavage factors I and II [CFI, CFII], polyadenylate polymerase [PAP], and polyadenylate binding protein 2 [PAB II]) recognizes the polyadenylation signal sequence (AAUAAA) and effects the cleavage of the 3' terminus of newly transcribed mRNA and subsequent polyadenylation (Colgan & Manley, 1997). The poly(A) tail is then bound by poly(A) binding protein (Pab1p) which stabilizes newly transcribed mRNA (Coller *et al*, 1998). If mRNA undergoes sufficient deadenylation it is subsequently decapped and degraded before it can be translated (Muhlrad *et al*, 1994). This process typically takes place in the nucleus, however some mRNAs

undergo polyadenylation in the cytoplasm and this mechanism allows for local translation away from the nucleus.

1.4.2 Cytoplasmic Polyadenylation

The length of the poly(A) tail of mRNA plays an important role in regulating translation. In *Xenopus* oocytes research has shown that mRNAs with shortened poly(A) tails are stored in the cytoplasm until later polyadenylation and subsequent translation during maturation (Reviewed in de Moor *et al*, 2005). Mammalian cells have also been shown to store mRNAs with shortened poly(A) tails suggesting a role for cytoplasmic polyadenylation (Meijer *et al*, 2007). The process of polyadenylation in the cytoplasm is regulated by cytoplasmic polyadenylation element binding protein (CPEB) (Hake & Richter, 1994). Once the poly(A) tail is elongated the mRNA is no longer inactive and can begin the process of translation (Reviewed in Radford *et al*, 2008).

1.4.3 Cordycepin and Cytoplasmic Polyadenylation

Cordycepin (3' deoxyadenosine) is a structural analogue for adenosine, and is produced by the fungus *Cordyceps militaris* (Cunningham *et al*, 1950). Cordycepin's similarity to adenosine means it is incorporated into the poly(A) tail and can inhibit polyadenylation (Beach & Ross, 1978). Cordycepin is therefore a useful tool for investigating the contribution of cytoplasmic polyadenylation to various biological processes.

There is evidence that local translation, mediated by cytoplasmic polyadenylation, may be involved in the chronification of pain. Hyperalgesic priming is a model for the transition from acute to chronic pain, whereby a second acute insult causes an increased and persistent hyperalgesia compared to the initial insult (Ferrari *et al*, 2013a). Injection

of cordycepin at the site of the noxious insult was found to both prevent and reverse hyperalgesic priming in rats (Ferrari *et al*, 2013a). Alpha calmodulin-dependent protein kinase II (α -CaMKII) is thought to be involved in the onset of hyperalgesic priming (Ferrari *et al*, 2013b). Interruption of CPEB mediated polyadenylation of α -CaMKII by cordycepin prevented hyperalgesic priming (Ferrari *et al*, 2013b).

Cordycepin has also been shown to impact inflammation. In lipopolysaccharide stimulated macrophages cordycepin was found to inhibit NO production through the down regulation of iNOS and COX-2 (Kim *et al*, 2006). In a model of inflammation induced osteoporosis oral administration of cordycepin was found to reduce the expression of IL-6 and TNF- α (Zhang *et al*, 2014). In airway smooth muscle cells exposed to TNF- α cordycepin was also found to reduce the expression of other pro-inflammatory mediators including IL-8 and chemokine (C-X-C motif) ligand 1 (CXCL1) (Kondrashov *et al*, 2012).

Alongside these effects on the chronification of pain and inflammation there is also evidence that cordycepin can prevent bone loss in models of osteoporosis. *In vitro* data showed that cordycepin was able to attenuate osteoclastogenesis as well as the bone resorbing activity of osteoclasts (Dou *et al*, 2016). In the ovariectomized model of osteoporosis, cordycepin administration has been found to have osteoprotective effects in both rats and mice (Dou *et al*, 2016, Zhang *et al*, 2015).

The effects of cordycepin on hyperalgesic priming and inflammation suggest that it may have therapeutic potential for OA pain. However, the effects of cordycepin on bone loss

in models of osteoporosis suggest that it may also affect bone turnover in OA. If this is the case then cordycepin may be able to prevent or reverse some aspects of OA pathology.

1.5 Aims

The use of a wide range of animal models is important to fully understand the underlying mechanisms behind human disease, especially with a heterogeneous disease like osteoarthritis. The first aim of this thesis is to establish a slow progressing model of murine OA which will complement the other models used by the group.

The potential role of BDNF in mediating chronic OA joint pain is not known, to address this gap in knowledge the expression of BDNF and its receptor TrkB will be assessed in human synovium taken post-arthroplasty. The contribution of this neurotrophin to peripherally driven joint pain will be investigated in two models of OA pain in the rat.

Lastly, the importance of polyadenylation regulated translation in regulating both pain and joint pathology will be investigated in the DMM model of OA through the use of cordycepin as a novel therapeutic intervention.

Chapter 2: General Methods

All experiments involving animals conformed to the regulations set out by the UK Home Office in the Animal (Scientific Procedures) Act 1986. Experiments involved either male C57BL/6 mice (7-8 weeks at time of arrival, Charles River, Kent, UK) or male Sprague Dawley rats (175-199g at time of arrival, Charles River, Kent, UK). Mice were housed in individually ventilated cages, whilst rats were housed in open top cages. All animals had access to food and water *ad libitum*.

2.1 Behavioural Assessments of Pain

Pain behaviour was measured through two different methods: weight bearing asymmetry and paw withdrawal threshold. Prior to baseline data being recorded animals were habituated to the behavioural tests for either two days (rats) or one week (mice). The experimenter was blinded to the experimental status of the animals (model/sham, treatment/vehicle) for the duration of the behavioural testing.

2.1.1 Weight bearing asymmetry

An incapacitance tester (Linton Instrumentation, UK) was used to detect changes in weight distribution across the hindpaws in both rats and mice (Sagar et al, 2015).

Before each testing session, the incapacitance tester was calibrated with brass weights, the weight of which was selected to be as close as possible to the approximate weight the animals will place on each pad. The incapacitance tester was set to measure the average weight placed on each pad over a 3 second period of time. Animals were placed in a

Perspex chamber above the split sensors. Animals were positioned in such a manner that their hindpaws were placed in the centre of each sensor, their forepaws were located on the sloped front wall of the chamber, and their tails were rested outside the back of the chamber. When animals were positioned correctly the average weight distribution across the two sensors was measured. Weight bearing changes were expressed as the weight placed on the ipsilateral sensor as a percentage of the total weight across both sensors. Between testing each animal the equipment was tared. After all testing had been completed the incapacitance tester and Perspex chamber were cleaned with 20% EtOH, and any detritus between the sensors was removed.

2.1.2 Hindpaw withdrawal threshold

Von Frey monofilaments (Touch Test, forces 0.07-26g) were used to measure allodynia through the application of a known force to the plantar aspect of the hindpaw. A modified version of the up-down method described by Chaplan was used (Chaplan *et al*, 1994). Animals were placed in a raised cage and given time to acclimatize to the testing chamber. Starting with either the 4g hair (for rats) or the 0.6g hair (for mice), von Frey hairs were applied to the plantar side of the hindpaw for 3 seconds. If the animal withdrew its paw from the stimulus the next lowest hair was then applied. This was repeated until a response was no longer observed. At which point the weight of the hairs was increased again until a response was evoked, this weight was then taken as the threshold. Conversely, if the starting hair did not evoke a response the weight of the hairs was increased until a response was observed. Once there was a withdrawal, descending weights of filaments were applied until there was

no evoked withdrawal. The weight of hairs applied to the hindpaw were then increased again until withdrawal was evoked; this weight was then taken as the threshold. In rats, the 26g hair was the maximum weight of hair used, whilst in mice it was the 2g filament. The thresholds for both the ipsilateral and contralateral paws were recorded. Following testing the testing chambers were cleaned thoroughly with 20% EtOH.

2.2 Surgical Models of Osteoarthritis

Two different surgical techniques were used to induce osteoarthritis in 8-9 week old male C57BL/6 mice (Charles River, Kent, UK). These two models were the traditional destabilisation of the medial meniscus (tDMM) (Glasson et al, 2007) and the modified destabilisation of the medial meniscus (mDMM). In 175-199g male Sprague Dawley rats (Charles River, Kent, UK) osteoarthritis was induced through transection of the medial meniscus (MNX) (Bove et al, 2006).

2.2.1 Induction and maintenance of general anaesthesia

An isoflurane vaporiser (Medicalia Isotec Mk3) was used to administer a gaseous anaesthetic to induce and maintain general anaesthesia. Animals were transferred to a Perspex box for induction, and the lid was then secured. Oxygen (O₂) was used to carry the isoflurane, and the O₂ flow rate was set to 1L/min. The isoflurane vaporiser was set to 3% for induction. Waste gases were collected by an activated charcoal scavenger (Harvard Apparatus). Animals were judged to be anaesthetised when they were areflexive, and there was a change in breathing rate and depth. Once anaesthesia was confirmed the animals were removed from the induction box and transferred to a nose cone. During the procedure the

isoflurane vaporiser was turned down to 2% for maintenance of anaesthesia.

2.2.2 Preparing the animal for surgery

Following the induction of anaesthesia, animals were transferred to a nose cone and prepared for surgery. Animals were placed in a supine position on top of a heat mat. Both the ipsilateral and contralateral hind legs were then shaved from the ankle to the groin. All loose hair was then removed from the preparation area. The diameters of both the ipsilateral and contralateral knees were then measured using electronic callipers, to enable us to monitor knee swelling post-surgery. The ipsilateral knee was then swabbed with the disinfectant chlorhexidine (Ecolab, Cheshire). Finally, eutectic mixture of local anaesthetics (EMLA) cream was applied to the surgical site. Animals were then transferred to the surgical table ready for the procedure.

2.2.3 Surgical destabilisation of the medial meniscus

All surgeries were carried out in aseptic conditions. The surgeon wore a sterile surgical gown, sterile gloves, a hair net and a face mask. The surgical area was disinfected, and all drapes were autoclaved prior to surgery. All tools were sterilised before use, and a heat bead sterilizer was used to sterilise the micro scalpel blade between surgeries. A surgical assistant ensured that the surgeon did not have contact with anything that was not previously sterilised.

Surgeries were completed with the aid of a surgical camera and HD monitor (**Fig. 2.1**).

Mice were then tested to ensure they were fully areflexic before any incisions were made. The initial incision was made approximately 5mm to the medial side of the infrapatellar

ligament using a number 11 scalpel blade. A pair of sharp scissors was then used to open a surgical window which ran parallel to the infrapatellar ligament. The window was large enough to be able to see the meniscus, the heads of both the femur and the tibia and the medial collateral ligament (MCL). It was possible at this point to observe the meniscus below layers of muscle and fat. Using a pair of fine curved forceps the muscle and fat on top of the meniscus was blunt dissected away. Care was taken not to damage any of the supporting ligaments in the knee capsule especially the MCL. Following this dissection the meniscus was exposed. This was the end point for the sham surgeries.

In the traditional destabilisation of the medial meniscus (Glasson *et al*, 2007) the medial meniscotibial ligament (MMTL) was transected. A pair of fine forceps was used to hold the infrapatellar ligament out of the way, with particular care taken not to damage this tissue. A 5mm micro-scalpel blade was then used to fully transect the MMTL. The forceps were then used to ensure that the meniscus was loose in the joint. In the modified destabilisation of the medial meniscus the MMTL was left intact, and the supporting coronary ligaments were transected. The 5mm micro-scalpel was used to cut the coronary ligaments between the tibial plateau and the meniscus. Great care was taken not to damage the articular cartilage during this part of the procedure. Once the coronary ligaments were transected, forceps were used to ensure the meniscus was destabilised.

Following surgery the incision was closed up using surgical staples. If necessary, a small amount of topical tissue adhesive (GLUture, World Precision Instruments) was applied to the wound.

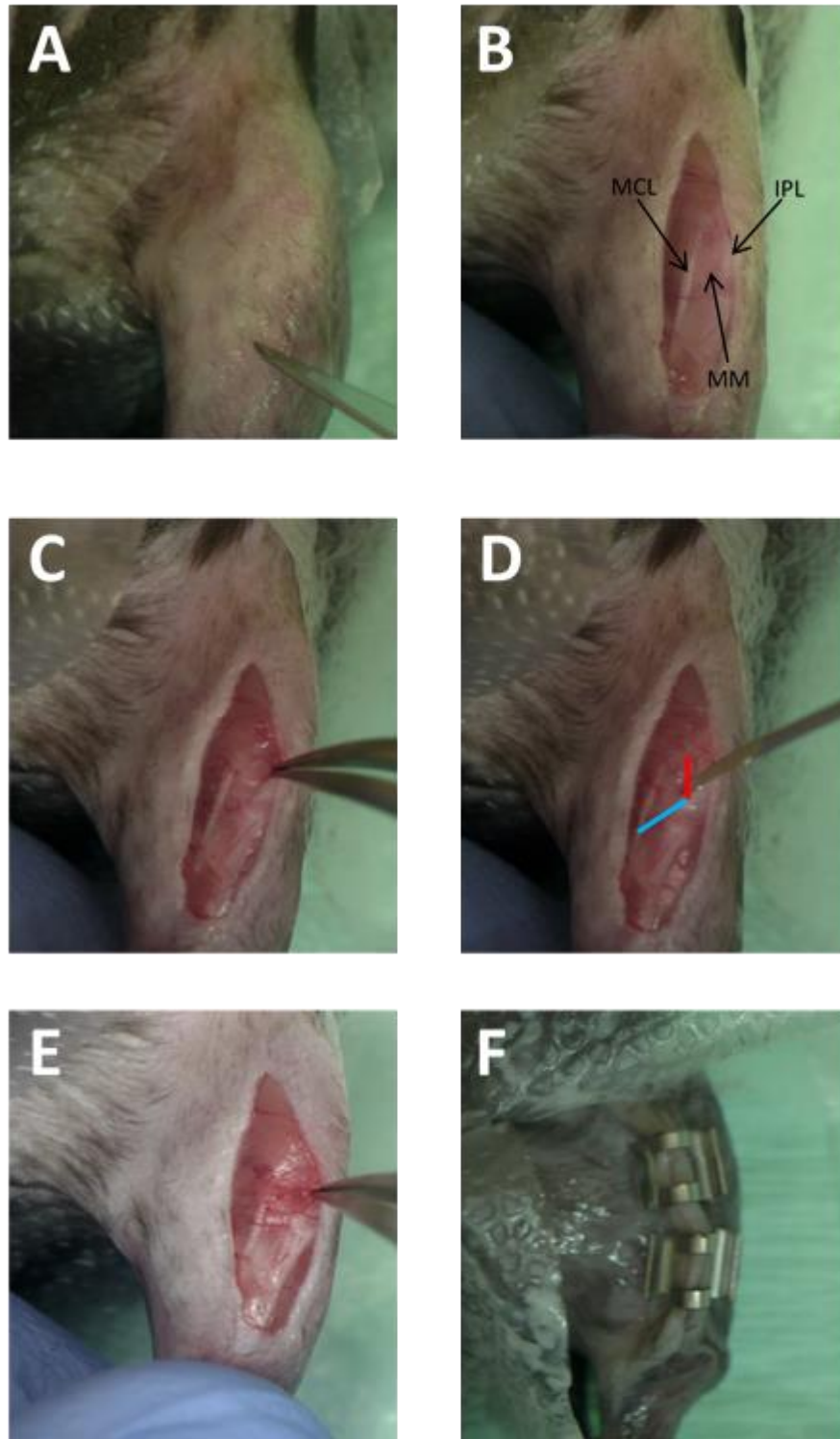


Figure 2.1: Destabilisation of the Medial Meniscus (DMM) in the mouse: This series of images shows the surgical procedure for the DMM. **A:** The knee prepared for surgery. **B:** The location of the medial collateral ligament (MCL), medial meniscus (MM), and infrapatellar ligament (IPL). **C:** The blunt dissection of the connective tissues on top of the medial meniscus, after this step the sham surgery is complete. **D:** The transection of the ligaments attaching the MM to the tibial plateau (the red line indicates the cut made during the traditional DMM, whilst the blue line indicates the cut made during the modified DMM). **E:** The end point of the surgery whereby the medial meniscus is destabilised and there is a space seen between the tibia and femur. **F:** The end of surgeries the wound was sealed with wound clips.

2.2.4 Surgical transection of the medial meniscus

All MNX surgeries were performed under aseptic technique as described in **2.2.3**. A surgical camera and HD monitor were used to perform the procedures (**Fig. 2.2**).

A toe pinch was used to assess whether or not rats were areflexive. Once the animal was suitably anaesthetised, a pair of scissors was used to open a surgical window approximately 10mm to the medial side of the infrapatellar ligament. The window was large enough to view the tibial and femoral heads, the medial meniscus, and the medial collateral ligament. A cauterising iron was used to cauterise the supporting tissue which lies on top of the MCL. Once this tissue was removed, a pair of vanna scissors was used remove a small section of the MCL allowing access to the medial meniscus. This point in the procedure was considered the end point for the sham surgery.

The thickest part of the medial meniscus was then raised up with the use of rat toothed forceps, and the meniscus was transected with the use of vanna scissors. The surgery was considered complete when the medial meniscus was fully transected, and there was a visible gap between the tibial and femoral heads.

Sterile 8-0 suture was used to close up the connective tissue underlying the skin with a series of 3-4 mattress sutures. The skin was then closed up with sterile 4-0 suture with a continuous stitch. Topical tissue adhesive was used if necessary.

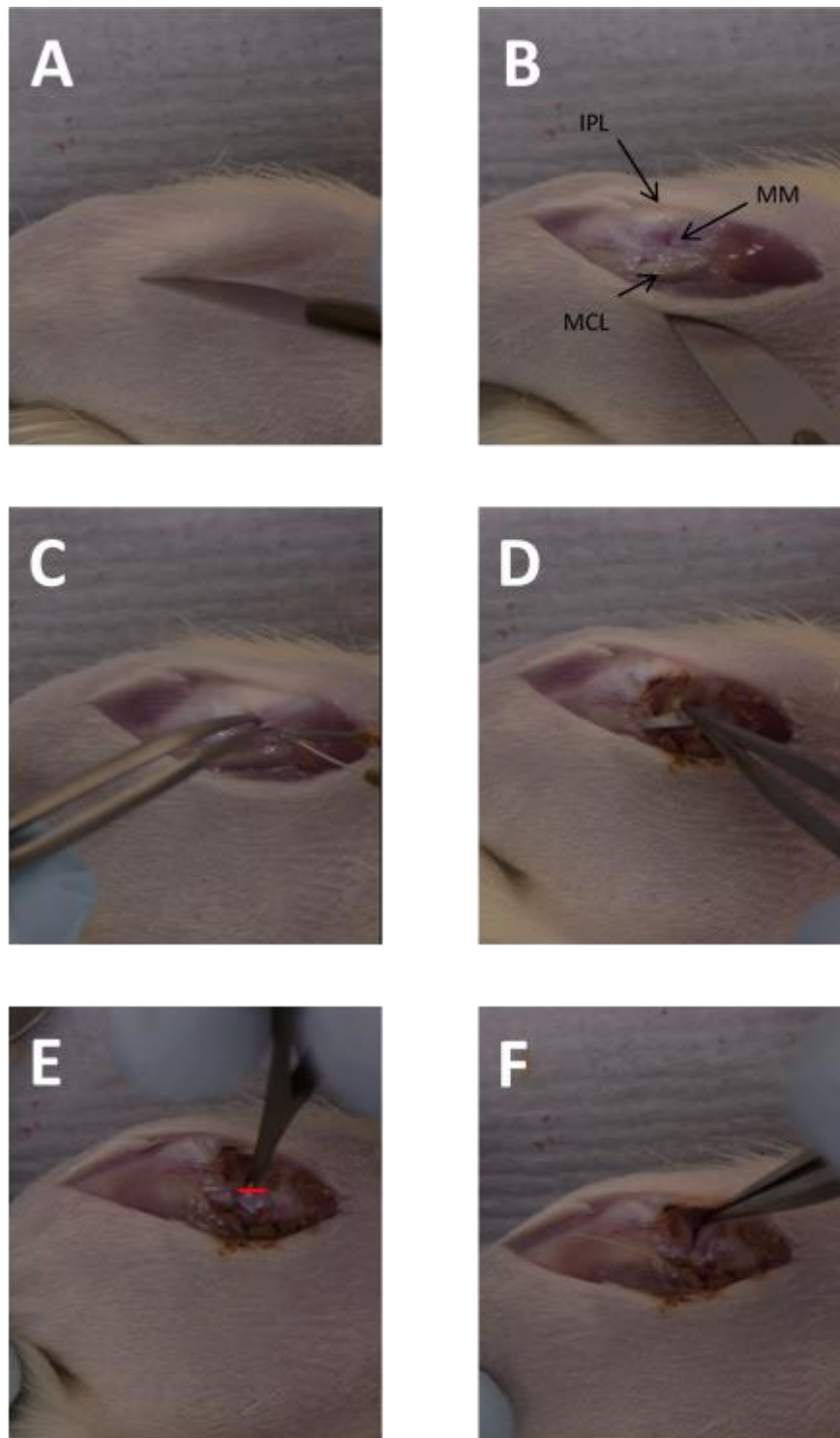


Figure 2.2: Medial meniscus transection (MNX) in the rat: This series of images shows the surgical procedure for the MNX. **A:** The knee prepared for surgery. **B:** The location of the medial collateral ligament (MCL), medial meniscus (MM), and infrapatellar ligament (IPL). **C:** The dissection of the connective tissues on top of the medial meniscus with the use of a cautery torch. **D:** The removal of the portion of the MCL covering the MM, after this step the sham surgery is complete. **E:** The transection of the MM (the red line indicates the cut made through the meniscus). **F:** The end point of the surgery whereby the medial meniscus is transected and there is a space seen between the tibia and femur.

2.2.5 Recovery from surgery

Following the conclusion of surgery, animals were removed from the nose cone and weighed before being taken to the recovery room. The recovery room was lit only by a red light. Animals were placed in a recovery cage which was on top of an electronic heat blanket. The recovery cage had vet bedding, mash, and aqua gel. The animal's recovery from anaesthesia was carefully monitored. Upon recovery, animals were monitored closely to ensure that their locomotion was not impeded as a consequence of the surgery. Animal welfare was monitored daily for the first three days post operation, and then weekly for the duration of the study. Surgical clips were removed one week post-surgery.

2.3 Intra-articular Injection of Substances

Animals were anaesthetised as previously described (**2.2.1**). Once areflexic, animals were transferred to a nose cone and anaesthesia was maintained at 2%. Animals were placed in a supine position. An electric shaver was used to remove hair from both the ipsilateral and contralateral hind legs, from the toes to the groin. All loose hair was then removed from the procedure site. Electronic callipers were then used to measure the diameters of both the ipsilateral and contralateral knee joints. A toe pinch was used to ascertain whether or not the animal was areflexive. The injection site was then disinfected through the application of chlorhexidine. The ipsilateral joint was then bent to 90°, and the infrapatellar ligament was identified. A 0.3ml insulin syringe was used to administer substances to mice and a 0.5ml insulin syringe was used for rats. Animals were injected through the infrapatellar ligament

into the intra-articular space. If any bleeding was observed an autoclaved swab was used to stem the bleeding. Following injection animals were carefully monitored as they recovered.

2.4 Intra-plantar Injection of Substances

All intra-plantar injections were carried out in 7-8 week old C57BL/6 mice (Charles River, Kent, UK).

Mice were anaesthetised as previously described (**2.2.1**), once the animals were anaesthetised, they were transferred to a nose cone and anaesthesia was maintained at 2% isoflurane. Animals were placed in a prone position, with the plantar aspect of both the ipsilateral and contralateral hind-paws facing upwards. The injection site was disinfected with chlorhexidine. A 29 gauge needle was used to administer substances. Animals were injected in the area just behind the walking pads of the ipsilateral hind-paw. Following injection, animals were removed from the nose cone and their recovery was carefully monitored.

2.5 Transcardial Perfusion

Both mice and rats were perfused at the conclusions of their studies.

Animals were initially injected with a suitable dose of sodium pentobarbital (0.4ml in mice, 1.5ml in rats) (Merial, Harlow) into the peritoneal space. Animals were prepared for transcardial perfusion when they were fully areflexive. The initial incision was made just below the rib cage with a pair of sharp scissors, with care taken not to cut the liver. A pair of forceps was used to move the liver away from the diaphragm. Using scissors, the diaphragm was carefully cut away exposing

the chest cavity. Incisions were made along the length of the ribcage on both the ipsilateral and contralateral sides. A pair of haemostats was then used to lift the sternum up and the connective tissue holding the heart in place was transected. A small incision was made close to the apex of the heart, in the left ventricle. A gavage needle was inserted into the ventricle and secured in place with haemostats. A large incision was then made in the aorta.

The animal was then exsanguinated by passing saline (0.9% v/v) through the perfusion needle, and around the animal's circulatory system. The animal was judged to be exsanguinated when the fluids ran clear and when the liver had lost its colour. At this point paraformaldehyde (PFA) (4%) was run through the system. Substantial muscle contractions should be seen as PFA circulates around the animal. The stiffness of the limbs and the changing texture of the liver were used as a guide to ensure that the animal was perfused correctly.

2.6 Histopathology

2.6.1 Tissue preparation for histopathology

At the end point of studies animals were euthanised with an appropriate technique as described in schedule 1 of the Animal (Scientific Procedures) Act 1986. Both the ipsilateral and contralateral knees were removed post-mortem and kept in neutral buffered formalin for 48 hours. The knee joints were then decalcified in ethylenediaminetetraacetic acid (EDTA) buffer (In 2.5L dH₂O; 22g NaOH, 3.4g Trizma base, 250g EDTA, 187.5g polyvinylpyrrolidone [PVP], pH 7) for 10 days for mice knee joints, and 5 weeks for rat knee joints. During decalcification the knee joints were agitated at room

temperature on a shaker (Scientific Laboratory Supplies). Following decalcification the knee joints were processed at Kings Mill Hospital by Roger Hill. The samples were dehydrated and then immersed in paraffin wax. Once processed, the knee joints were embedded in paraffin wax blocks ready for cutting. Using a microtome the tissue was sectioned into 5µm slices and mounted on slides.

2.6.2 Haematoxylin and eosin Staining

Under a fume hood the samples were firstly de-waxed in xylene, before being rehydrated in progressively less concentrated ethanol solutions. The samples were then placed in Mayer's haematoxylin for 15 minutes and then rinsed in water. The samples were then exposed to acid alcohol for 20 seconds before being dipped in 1% eosin solution. The tissue was then placed into increasingly more concentrated ethanol solutions and then xylene. Slides were then mounted with dibutyl phthalate xylene (DPX) and left to dry.

2.6.3 Safranin-O staining

Under a fume hood the slides were initially de-waxed in xylene, and then rehydrated in progressively less concentrated ethanol solutions. Slides were then placed in Weigert's haematoxylin for 2 minutes and then rinsed in water. Samples were then submerged in acid alcohol for 20 seconds and again rinsed in water. Slides were then placed into fast green for 5 minutes, before being dipped into glacial acetic acid. Slides were then placed into safranin-O for 5 minutes before being rinsed in water. Slides were then dehydrated in increasing

concentrations of ethanol before being placed in xylene. Slides were then mounted with DPX and left to dry.

2.6.4 Microscopic scoring

Microscopic assessment of joint pathology remains the gold standard in pre-clinical studies for investigating OA development. Two scoring systems were used in this thesis; one for coronal sections and one for sagittal sections. For coronal sections a modified version of the OARSI recommended cartilage damage scoring system was used (Glasson *et al*, 2007). We also measured synovitis and osteophyte on a 0-3 score (**Table 2.1**). For sagittal sections we used the same chondropathy scoring system as for the coronal sections (0-6). We also measured synovitis (0-3), subchondral bone density (0-5), osteophyte size (0-3), osteophyte maturity (0-3), proteoglycan loss (0-1), and chondrocyte apoptosis (0-1) (**Table 2.2**). Sections were scored on a light microscope. To ensure that the scorer did not know the surgical status of the sections all slides were blinded and not unblinded until the end of scoring.

2.6.5 Macroscopic scoring

Macroscopic scoring enables researchers to quickly and cost-effectively assess changes in chondropathy across the entire knee joint. The scoring system used in this thesis was initially published by Guincamp *et al* (Guincamp *et al*, 1997) in the MIA model of osteoarthritis (**Table 2.3**). It has also been previously used in studies from our group for assessing

Table 2.1: Scoring criteria for joint pathology in the mouse (coronal). Scoring system was adapted from Glasson *et al*, 2010.

Cartilage Damage	Scoring Criteria
0	Normal
1	Small fibrillations without the loss of surface cartilage
2	Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina
3	Vertical clefts to the calcified cartilage extending to <25% of the articular surface
4	Vertical clefts to the calcified cartilage extending to 25-50% of the articular surface
5	Vertical clefts to the calcified cartilage extending to >50-75% of the articular surface
6	Vertical clefts to the calcified cartilage extending to >75% of the articular surface
Synovitis	Scoring Criteria
0	Synovial lining 1 cell thick
1	Mild: Synovial lining 2-3 cells thick
2	Moderate: Synovial lining 4-5 cells thick
3	Severe: Synovial lining >6 cells thick
Osteophyte Score	Scoring Criteria
0	None
1	Small, same thickness as the adjacent cartilage
2	Medium, 1-3x the thickness as the adjacent cartilage
3	Large, >3x the thickness as the adjacent cartilage

Table 2.2: Scoring criteria for joint pathology in the mouse (sagittal). Scoring system based on Jackson *et al* (2014).

Cartilage Damage	Scoring Criteria
0	Normal
1	Small fibrillations without the loss of surface cartilage
2	Vertical clefts down to the layer immediately below the superficial layer and some loss of surface lamina
3	Vertical clefts to the calcified cartilage extending to <25% of the articular surface
4	Vertical clefts to the calcified cartilage extending to 25-50% of the articular surface
5	Vertical clefts to the calcified cartilage extending to >50-75% of the articular surface
6	Vertical clefts to the calcified cartilage extending to >75% of the articular surface
Synovitis	Scoring Criteria
0	Synovial lining 1 cell thick
1	Mild: Synovial lining 2-3 cells thick
2	Moderate: Synovial lining 4-5 cells thick
3	Severe: Synovial lining >6 cells thick
Chondrocyte Hypertrophy	Scoring Criteria
0	No chondrocyte hypertrophy in the non-calcified cartilage
1	Enlarged chondrocyte lacunae with lack of stain around a collapsed cell in the non-calcified cartilage

Osteophyte Score	Scoring Criteria
0	None
1	Small, same thickness as the adjacent cartilage
2	Medium, 1-3x the thickness as the adjacent cartilage
3	Large, >3x the thickness as the adjacent cartilage
Osteophyte Maturity	Scoring Criteria
0	None
1	Predominantly cartilaginous
2	Mixed cartilage and bone with active vascular invasion and endochondral ossification
3	Predominantly bone
Proteoglycan Loss	Scoring Criteria
0	Normal staining of non-calcified cartilage
1	Decreased but not complete loss of Saf-O staining over 1-100% of articular surface
2	Complete loss of Saf-O staining in the non-calcified cartilage extending to <25% of the articular surface
3	Complete loss of Saf-O staining in the non-calcified cartilage extending to >25-50% of the articular surface
4	Complete loss of Saf-O staining in the non-calcified cartilage extending to >50-75% of the articular surface

5	Complete loss of Saf-O staining in the non-calcified cartilage extending to >75% of the articular surface
Subchondral Bone Thickening Scoring Criteria	
0	Normal trabecular bone with >50% marrow space
1	2 or more 'wide' vertical trabecular struts of bone that extend from the cartilage to the growth plate or 'solid' bone spanning up to 1/3 rd of the width of the epiphysis
2	'solid' bone spanning up to >1/3 rd - <2/3 rd of the width of the epiphysis
3	'solid' bone spanning up to >2/3 rd of the width of the epiphysis

cartilage damage and loss, following intra-articular injection of MIA. (Nwosu *et al*, 2015).

Prior to scoring the knee joints had to be separated. Knee joints were removed post-mortem from the animal, with the use of bone cutters to cut the bone mid-femur and mid-tibia. Scissors were then used to trim away excess muscle to expose the underlying joint. A scalpel was then used to carefully cut both the lateral and medial collateral ligaments. The anterior and posterior cruciate ligaments were then carefully transected with great care being taken not to damage the articular cartilage. The joint was then slowly twisted and the scalpel was used to cut through the meniscus effectively disarticulating the tibia from the femur. Vana scissors were then used to cut away any excess tissue covering the tibial

plateaus or femoral chondyles. The knee joints were then scored using a dissecting microscope.

The scoring system published by Guincamp (Guincamp *et al*, 1997) scored 6 separate compartments; the patella, the femoral groove, the lateral femoral chondyle, the medial femoral chondyle, the lateral tibial plateau, and the medial tibial plateau. Each of these compartments was scored on a scale of 0-4 (**Table 2.3**). The sums of these scores were then taken to provide a total value between 0-24. For our studies, the patella was not scored as it was taken along with the synovium for microscopic histopathological and immunohistochemical work. Therefore our joints were scored on a scale of 0-20.

Table 2.3: Macroscopic scoring criteria for chondropathy in the rat. Scoring system based on Guincamp *et al* (1997).

Cartilage Damage Score	Scoring Criteria
0	Normal appearance
1	Slight yellow discolouration of the chondral surface
2	Little cartilage erosions in load-bearing areas
3	Large erosions extending down to the subchondral bone
4	Large erosions with large areas of subchondral bone exposure

2.7 Immunohistochemistry

2.7.1 Tissue processing for immunohistochemistry

At the end of experiments mice were injected with sodium pentobarbital and then transcardially perfused (**2.5**). Using a pair of scissors the skin from the back of the animal was removed. A scalpel was then used to make two incisions either side of the spinal column before the muscle lying on top of the spinal column was removed with the use of rangeur scissors. Once the muscle was removed from the top of spinal column a strong pair of vana scissors were then used to carefully cut along the dorsal aspect of the column on both the ipsilateral and contralateral sides until the top of the spinal column could be removed. Following this the spinal cord is exposed, using a pair of fine forceps the cauda equine was lifted up and a pair of fine vana scissors were used to cut through the processes in order to remove the spinal cord from the column. Once the spinal cord was removed the lumber enlargement was isolated from the cord, before the menisci surrounding the cord were then carefully removed. The lumber enlargement was then post-fixed in 4% PFA for 48 hours. Tissue was then placed in 30% sucrose in 0.1M phosphate buffered saline (PBS)/0.02% sodium azide for cryoprotection, before being stored in a +4°C refrigerator until the tissue was sectioned.

2.7.2 Staining for glial cells

The spinal cords were sectioned on a sledge microtome in preparation for immunohistochemistry. We stained cryosections of L3/L4/L5 spinal cords (n=6-8 mice per group, 5-6 sections per mouse). Iba-1 was used to stain for microglia, whilst GFAP was used to stain for astrocytes.

Immuohistochemistry was carried out on free floating spinal sections. Following washes in 0.1M PBS, sections were blocked with 3% goat serum and 0.3% Triton X-100 in 0.1M PBS for 1 hour at room temperature. Sections were incubated with either rabbit anti-Iba-1 (Wako: 019-19741) diluted 1:1000 in Trizma Triton X-100 buffered saline (TTBS) for 72 hours at 4°C or rabbit anti-GFAP (Abcam: ab48050) diluted 1:100 in TTBS for 18 hours at room temperature. Sections were then washed again in 0.1M PBS. The sections were then incubated for 2 hours with either Alexafluor 488 conjugated goat anti-rabbit secondary antibody (Iba-1) diluted 1:300 in TTBS or Alexfluor 568 conjugated goat anti-rabbit secondary antibody (GFAP) diluted 1:300 in TTBS at room temperature. Sections were mounted on gelatinized slides, before being air-dried overnight at room temperature and coverslipped using Fluoromount.

2.7.3 Quantification

Both Iba-1 and GFAP immunostaining was imaged using a 20 x 0.4 NA objective lens on a Leica DMIRE2 fluorescence microscope, running micromanager equipped with a Hamamatsu Orca C4642-95 camera. Images were acquired using an exposure time of 150ms (Iba-1) or 50ms (GFAP). Each image was taken from approximately the same area covering the superficial laminae, the area covered by each of the quadrants was 403.2 x 307.2µm. The numbers of activated microglia were counted manually in each quadrant. Microglia were considered activated if the diameter of the cell body was twice the size of the processes, whilst non-activated microglia had long processes extending out from a non-swollen cell body. The experimenter was blinded to the

conditions the sections were from whilst counting microglia numbers.

For GFAP analysis the mean fluorescence grey intensity was measured with Velocity 5.5 for each image.

2.8 Molecular quantification

2.8.1 ELISA analysis

BDNF protein levels were quantified using a BDNF ELISA kit (ThermoScientific: ERBDNF). Following the end of experiments rats were euthanised according to Schedule 1 of the A(SP)A 1986. Plasma and synovial fluid were then collected from each rat. Synovial fluid was collected on protein saver card and eluted into 200µl of ddH₂O before being loaded into the ELISA wells, whilst 100µl of plasma from each animal was loaded into the wells without dilution. The ELISA was then run according to the manufacturer's guidelines. The results were analysed using the software found at elisaanalysis.com.

2.8.2 RNA extraction and cDNA synthesis

Vascularised sections (50µg) from human synovium samples were homogenised in 1ml of ice cold TriReagent before 0.2ml of 1-bromo-3-chloro-propane were added. Following centrifugation the upper aqueous phase was removed and RNA was precipitated with 0.5ml isopropanol. The RNA was then purified with the addition of 2.5µl of DNase I and 10µl RDD buffer. RNA was then extracted again through the addition of 400µl of phenol/chloroform/isoamyl alcohol. RNA was then precipitated through the addition of 40µl 3M sodium acetate (pH 5.2) and 800µl of absolute ethanol. RNA was

finally stored in 40µl of DEPC-treated water. For cDNA synthesis, 150ng of RNA was reverse transcribed using M-MLV reverse transcriptase in a total volume of 20µl. Reactions were incubated at 25°C for 10 minutes, 37°C for 50 minutes, and terminated after 70°C for 15 minutes.

2.8.3 Taqman quantitative real time polymerase chain reaction

Gene expression as measured by Taqman quantitative real time polymerase chain reaction (qRT-PCR) was quantified using the relative standard curve method as previously reported (Erhuma *et al*, 2007). Primers and probes (**Table 2.4**) were designed using PrimerExpress software. Primers and probes were then synthesised by Eurofins MWG Synthesis. Data are presented as a ratio of gene expression with reference to β -actin.

2.9 Statistical analysis

All data in this thesis were analysed by GraphPad Prism V7.1. Outliers were detected through the use of Grubb's test for outliers. Distribution of data was assessed through the use of D'agostino-Pearson omnibus test. More specific information regarding the tests used to assess data can be found in each chapter.

Table 2.4: The sequences for forward primers, reverse primers, and probes used for RT-PCR. NTRK2 is the gene for trkB.

Gene of Interest	Sequence (5' -> 3')
huB-actin	Fwd: CCTGGCACCCAGCACAAT Rev: GCCGATCCACACACGGAGTACT Pro: ATCAAGATCATTGCTCCTCCTGAGCGC
huIL-6	Fwd: CGGGAACGAAAGAGAAGCTCTA Rev: AGGCGCTTGTGGAGAAGGA Pro: CTCCCCTCCAGGAGCCCAGCTATG
huTNF-a	Fwd: CCCAGGGACCTCTCTCTATACA Rev: GGT TTGCTACAACATGGGCTACA Pro: CTCTGGCCCAGGCAGTCAGATCATCT
huBDNF	Fwd: CGTGATAGAAGAGCTGTTGGATGA Rev: GACGTGTACAAGTCTGCGTCCTT Pro: ACCAGAAAGTTCGGCCCAATGAATGAAGAAAAC
huNTRK2	Fwd: ACGATGGTGCAAACCCAAAT Rev: CCGGTTTTATCAGTGACGTCTGT Pro: CACCACGAACAGAAGTA

Chapter 3: Establishing a slow progressing model of murine osteoarthritis.

3.1 Introduction

3.1.1 General Background

The use of animal models of human diseases remains vital in enabling researchers to investigate novel therapeutic targets. For animal models to remain the gold standard for *in vivo* experimentation it is important that the model should accurately portray the symptoms and pathological characteristics of the human disease. Currently there is a lack of consensus over any single animal model of osteoarthritis representing the entirety of the disease in the human. This is perhaps most salient for researchers investigating OA pain. A meta-analysis of papers with the keywords 'animal model/models', 'OA', and 'pain' reported finding 18 different methods for inducing OA in 9 different species (Little & Zaki, 2012). These papers also varied greatly in the pain outcomes they measured with several behavioural tests being reported. The models of OA currently used can be sorted into roughly three categories; chemically induced, spontaneously occurring, and surgically induced. The monosodium iodoacetate (MIA) model of OA is the most prevalent in OA pain literature, being used in approximately 50% of cases (Little & Zaki, 2012). However, due to the nature of OA it is possible that the use of multiple models is required to investigate its multiple facets. It

is therefore important when planning experiments to choose the most appropriate model for the questions you are asking.

Intra-articular injection of MIA remains the most prevalent model of osteoarthritis for researching chronic joint pain. MIA has been shown to decrease the viability of chondrocytes through apoptosis, with evidence of increased cytochrome c release and decreased mitochondrial membrane potential following exposure to MIA (Jiang *et al*, 2013). Previous studies have shown that there is a rapid change in joint pathology following intra-articular injection of MIA. Fifteen days after doses of 0.3mg and 3mg MIA in the rat there were several changes noted including; significant losses of articular cartilage in both the tibial plateaus and the femoral condyles, evidence of osteophytes, and subchondral bone remodelling (Guincamp *et al*, 1997). Following a dose of 1mg MIA in the rat there was a development of histopathology over time; 8 days post injection there was chondrocyte death, synovial infiltration, and proteoglycan loss in the cartilage, 14 days post-injection there were lesions found in the articular cartilage, at days 21, 28, and 35 this cartilage loss became more pronounced and significant bone remodelling was also observed (Ivanavicius *et al*, 2007). Likewise, following a dose of 1mg MIA in the mouse there was significant cartilage loss observed at day 28 (Ogbonna *et al*, 2013).

The MIA model has been used extensively in pain research. In the rat it is seen to produce a robust increase in weight bearing asymmetry and decrease in paw withdrawal threshold from 7 days post injection (Orita *et al*, 2011. Sagar *et al*, 2011). In the mouse, there was a dose dependent decrease in paw withdrawal threshold from day 7 following injection of

0.5mg, and 0.75mg, and from day 3 following injection of 1mg of MIA (Ogbonna *et al*, 2013). The same study found that there were no changes in weight-bearing asymmetry at the lower doses, however there was a significant decrease in the weight borne on the ipsilateral paw from day 3 following injection of 1mg MIA (Ogbonna *et al*, 2013). These behavioural differences appear to be age related as 22 month old mice showed reduced weight bearing asymmetry when compared to 3 month old mice injected with the same dose of MIA, these differences are seen despite the joint pathology being the same (Ogbonna *et al*, 2015).

There have been numerous studies which have looked at the source of pain in the MIA model of OA. During the first 4 days following intra-articular injection of MIA in rat knees there was an increase in IL-6 and TNF α before a significant increase in the levels of NGF at day 7 in the peripheral tissue (Orita *et al*, 2011). There is increased sensitivity in primary afferent fibres days after intra-articular injection of MIA. Fourteen days post MIA there was a dose dependent increase in firing frequency in afferents following noxious torque when compared to control rats (Schuelert & McDougall, 2009). There were also neuronal changes observed with increases in CGRP immunoreactivity in the ipsilateral DRGs at days 7 and 14 post injection, as well as an increase in Iba1 positive cells in the ipsilateral dorsal horn (Orita *et al*, 2011). Previous work from our own group has supported these findings, showing increased levels of microglia activation in the spinal cord at days 7, 14, and 28 post injection (Sagar *et al*, 2011). There was also significantly increased GFAP fluorescence at day 28 (Sagar *et al*, 2011). In the mouse there was also a significant increase in the numbers of microglia 28 days post MIA

injection, however there were no changes in GFAP positive cells (Ogbonna *et al*, 2013). These changes in spinal glial cell activation have been linked to pain processing. Indeed, attenuating microglia activity through the COX inhibitor nimesulide led to increased paw withdrawal thresholds and a decreased weight-bearing asymmetry in rats (Sagar *et al*, 2011). There is also evidence for neuropathic pain mechanisms playing a role in the pain outcomes of the MIA model. Activating transcription factor- 3 (ATF-3) is a marker of neuronal damage, in rats injected with MIA there was ATF-3 immunoreactivity observed in L4 and L5 DRGs (Ivanavicius *et al*, 2007). However, in the mouse there was no difference in ATF-3 immunoreactivity 28 days post MIA injection (Ogbonna *et al*, 2013).

The MIA model is the most prevalent model of OA induction in research investigating the underlying mechanisms behind chronic joint pain (Little & Zaki, 2013). Not only is it a versatile model due to the dose dependent changes in joint pathology and pain outcomes, it is also considered fairly robust. The time course of this model with changes in joint pathology seen as early as 8 days post injection (Ivanavicius *et al*, 2007) also make this model highly attractive to researchers. However, the severe and rapid changes brought about by injection of MIA may not be useful for investigating early OA. There are also questions about how representative cartilage gene changes induced by MIA are when compared to the human disease (Barve *et al*, 2007).

Some animal models of OA rely on the spontaneous development of the disease over time, which is evident in multiple species including guinea pigs, dogs, and genetically

modified mice (Little & Zaki, 2012). The Dunkin Hartley strain of guinea pig develops spontaneous OA pathology from around 3 months of age, with a progression to moderate to severe OA at 12 months (Huebner *et al*, 2002). This strain of guinea pig exhibits increased serum levels of osteocalcin, a biomarker of osteoblast activity, and synovial fluid levels of keratan sulphate, a biomarker of proteoglycan turnover at 12 months of age (Huebner *et al*, 2002). Behavioural testing of guinea pigs is notoriously difficult, however there is evidence for peripheral changes in nociception in the Dunkin Hartley strain of guinea pigs. In older Dunkin Hartley guinea pigs there was spontaneous firing of knee joint afferents in 33% of recorded fibres compared to 0% in younger animals (McDougall *et al*, 2009). There was also a greater afferent firing rate observed in older animals in response to noxious joint rotation (McDougall *et al*, 2009). However, whilst there was a high correlation between OA severity and both age and weight, there was a lack of significant correlation between OA severity and any of the electrophysiological outcomes (McDougall *et al*, 2009). There is also evidence of spontaneously occurring OA pathology in genetically modified mice. Peroxisome proliferator-activated receptor gamma (PPAR γ) is a transcription factor which when activated reduces synthesis of OA catabolic and inflammatory factors (Vasheghani *et al*, 2013). In PPAR γ knockout mice the joint pathology changes gradually with 6 month old mice showing roughening of the articular surface, and increased proteoglycan loss, whilst at 14 months of age the mice show severe cartilage degradation, loss of chondrocytes, and synovial inflammation (Vasheghani *et al*, 2013). PPAR γ knockout mice also showed increased expression of several catabolic and inflammatory factors at 14

months including; MMP-13, ADAMTS-5, syndecan-4, HIF2a, and COX-2 (Vasheghani *et al*, 2013).

Due to the slow progressing nature of the pathological changes, spontaneous models may best represent the age-related nature of the human disease. However, there are several limitations to the use of spontaneous models for investigating the underlying mechanisms of OA pain. Firstly, unlike chemically or surgically induced OA it is very difficult to find the appropriate age-matched controls for spontaneous models. Secondly, the costs involved with housing and maintaining these experimental animals for such long times may be prohibitive. Thirdly, the variability in disease severity and onset offers an impracticality not seen in alternative methods of OA induction.

In the human condition, trauma to the joint increases prevalence and severity of OA pathology. Animal models with surgically induced OA pathologies aim to recreate this joint instability. In the mouse, the destabilisation of the medial meniscus (DMM) model has been used extensively in the literature. In this model the medial meniscotibial ligament is transected, this leads to greater movement in the anterior-posterior axis, with the greatest bone remodelling and loss found in the posterior portion of the medial tibial plateau (Moodie *et al*, 2011). Mild to moderate OA like pathology was seen at 4 weeks post model induction, with the severity of the pathology increasing to moderate to severe lesioning by 8 weeks (Glasson *et al*, 2007). Levels of tissue inhibitors of metalloproteinase 2 (TIMP2) are increased in C57BL/6 mice just 1 month after DMM surgery, after 4 months there is increased expression of MMPs, VEGF-A, and VEGF-B (Mi *et al*,

2012). In TIMP2 knockout mice the development of OA pathology was significantly faster when compared to wild type mice with cartilage degradation seen just 1 month after surgery (Mi *et al*, 2012). Calcitonin is a natural peptide hormone which has anti-resorptive effects when bound to calcitonin receptors on osteoclasts (Sondergaard *et al*, 2012). When mice genetically over-expressed salmon calcitonin there was a reduction in proteoglycan and histopathological damage when compared to wild type mice 7 weeks after DMM (Sondergaard *et al*, 2012).

Pain behaviour in the DMM model has been shown to be biphasic, with decreases in paw withdrawal threshold and increases in weight bearing asymmetry during the first 3 days post operation, the postoperative pain resolves and returns at 12 weeks post-surgery (Driscoll *et al*, 2016). Others have found changes in paw withdrawal threshold from as early as 4 weeks post-surgery (Miller *et al*, 2012). Following DMM surgery there was also a decrease in cold plate latency at the 12 week timepoint (Driscoll *et al*, 2016). Mice also show significantly decreased mobility 8 weeks post DMM surgery when compared to shams (Miller *et al*, 2012). Blockade of TNF α can alleviate postoperative pain behaviour but has no impact on pain behaviour at later timepoints, whereas application of TrkAd5 to reduce NGF levels attenuated pain behaviour both immediately postoperatively and at 16 weeks post-surgery (McNamee *et al*, 2010). NGF was found to be upregulated in articular cartilage following DMM surgery, and cartilage injury was enough to induce upregulation of NGF *in vitro* (Driscoll *et al*, 2016). In terms of the central changes, there was a bilateral increase in activated microglia in the

DRGs 8 weeks post-surgery, these changes in microgliosis were not seen in ADAMTS5 null mice (Tran *et al*, 2016).

Another surgical model commonly used in OA pain research is the meniscal transection (MNX) model in the rat. In the MNX model the medial meniscus is transected following removal of the medial collateral ligament. Histopathological changes 21 days post MNX surgery include cartilage loss, presence of osteophytes, loss of proteoglycans and chondrocytes (Bove *et al*, 2006). There were also increases seen in synovial inflammation, angiogenesis, and the number of vascular channels crossing the osteochondral junction at day 35 (Ashraf *et al*, 2011). Increases in weight bearing asymmetry and decreases in paw withdrawal threshold were seen 3 days post-surgery and were maintained until day 21 (Bove *et al*, 2006), other studies have shown pain behaviour being maintained until day 35 (Ashraf *et al*, 2011). There were no changes in paw withdrawal latency to the Hargreaves test, or to mechanical hyperalgesia as measured by the Randall-Selitto test (Bove *et al*, 2006). Changes in pain behaviour were reversed by application of the NSAID indomethacin, the corticosteroid dexamethasone, and the antiangiogenic PP1-2458 (Ashraf *et al*, 2011). Like the other models, there is evidence for peripheral sensitization following the MNX surgery with changes reported in the firing patterns of A β high threshold mechanoreceptors 2 months after the onset of the model (Wu & Henry, 2009).

Surgical models attempt to accurately portray the pathology and pain outcomes of trauma induced osteoarthritis in the human. The DMM model in mice may be especially useful for investigating the early stages of OA because of its slow

progressing pathology and pain behaviour. However, there are questions about the reliability of surgical models of OA especially compared to the high reproducibility of chemical induction, indeed it has been shown that not all animals develop mechanical allodynia following MNX (Bove *et al*, 2006).

3.1.2 Rationale

Previously our group has primarily used the MIA and MNX models of OA to investigate the underlying mechanisms behind chronic joint pain in the rat (Ashraf *et al*, 2014). As well as the slow progressing nature of the DMM model, it also has the benefit of being a mouse model allowing researchers to utilize transgenic mice to further probe mechanisms. One of the goals of my thesis was to establish the DMM model and to confirm that I could replicate the published joint pathology and pain behaviour as previously described in this model (Glasson *et al*, 2007, Driscoll *et al*, 2016).

Since our group has primarily used rats in the past, in order to validate the pain behaviour outcomes in mice I needed to measure weight bearing asymmetry and paw withdrawal threshold in a reliable model of mouse pain. Carrageenans are a group of large polysaccharides which causes significant inflammation when injected (Ghosh *et al*, 2013, Hargreaves *et al*, 1988, Lu *et al*, 2013). This has led to carrageenan being used as a robust model for inflammatory pain and was an ideal positive control for the pain behaviour measurements in mice.

One of the major advantages of chemical models of OA, such as the MIA model, is that the administered dose can be

altered to change the time course and severity of the disease (Guincamp *et al*, 1997). One of the goals of this chapter was to investigate whether modifying the method of surgical destabilisation of the medial meniscus will alter the progression of both pain behaviour and joint pathology in mice. In the published traditional DMM model the medial meniscotibial ligament (MMTL) is transected to free the meniscus (**2.2.3**). It was hypothesised that leaving the MMTL intact, and only transecting the coronary ligaments that attach the meniscus to the tibial plateau would destabilise the medial meniscus to a lesser degree than transection of the MMTL. This modified model would allow an investigation of how varying the surgical induction of the model can potentially impact upon the development of both pain and pathology. Additionally, this modified DMM may represent a milder alternative to the traditional DMM, which may allow more thorough investigation of early mechanisms of OA pain.

3.1.3 Aims

The aim of this chapter was to characterise the pain behaviour and the presence of spinal markers of central sensitization in the traditional DMM model. The second aim was to identify the potential importance of the transection of the medial meniscotibial ligament in the development of joint pathology and pain.

Objectives

To validate pain behavioural testing in the mouse in a reliable model of pain.

To replicate the pain behaviour and joint pathology of the DMM model reported in the wider literature.

To investigate the effects of modifying the DMM surgery on pain behaviour and joint pathology, and to determine the impact of duration of the model.

To investigate potential central mechanisms underlying the pain behaviour associated with the traditional and modified DMM model of osteoarthritis.

3.2 Material and Methods

General methods are detailed more thoroughly in **Chapter 2**. All experiments in this chapter were performed in male C57BL/6 mice (Charles River, Kent, UK).

3.2.1 Intraplantar injection of carrageenan

Eight male C57BL/6 mice aged approximately 7-8 weeks were used. Mice were anaesthetised with isoflurane (**2.2.1**) and then injected with either 10 μ L 1% carrageenan (n=4) or 10 μ L saline (n=4) in the plantar aspect of the left hindpaw (**2.4**). Both weight bearing asymmetry and paw withdrawal threshold were then measured at 24, 48, and 72 hours post injection (**2.1**). At the end of the study the mice were euthanised by a rising concentration of CO₂.

3.2.2 Destabilisation of the medial meniscus

18 male C57BL/6 mice were used in this experiment aged approximately 8-9 weeks. Following anaesthesia with

isoflurane (**2.2.1**) mice underwent either destabilisation of the medial meniscus (n=10) or sham surgery (n=8) of the left knee joint (**2.2.3**). Weight bearing asymmetry and paw withdrawal threshold were then measured weekly for 16 weeks (**2.1**). Note one mouse was euthanised at one week post-surgery due to a breakdown of the wound. The remaining mice were euthanised at 16 weeks post-surgery by an intra-peritoneal injection of pentobarbital, before being transcardially perfused (**2.5**).

Once death was confirmed, knee joints were dissected by carefully cutting through the femur and tibia with a large pair of scissors. Excess muscle tissue was trimmed from the joint before the tissue was processed as detailed in **2.6.1**. Once the knee joints were sectioned, six sections taken from different depths of the knee were stained with haematoxylin and eosin (**2.6.2**). These slides were then scored for chondropathy, synovitis, and osteophytosis (**2.6.3**).

Due to the subjective nature of scoring joint pathology in OA models it is vitally important to ensure that the scorer shares good inter-observer validity with other researchers. The scorer for the subsequent studies was PG who had no prior histopathological experience. To ensure that the training of PG was sufficient for the valid scoring of joint pathology, sections from a previous DMM study from King's College London were scored by **PG** and the two experienced histologists **PM** and **SS**. Inter-observer reliability was assessed by intra class correlation (ICC), whereby an ICC of above 0.75 indicated an excellent agreement between scorers, whilst an ICC of above 0.4 indicates a good agreement between scorers. An ICC of below 0.4 would indicate poor or no agreement between scorers.

3.2.3 Modified Destabilisation of the Medial Meniscus

Thirty-four male C57BL/6 mice (Charles River, Kent, UK) aged approximately 8-9 weeks were used. Mice were anaesthetised with isoflurane (**2.2.1**), and then either had the modified destabilisation of the medial meniscus surgery (n=16) or sham surgery (n=18) (**2.2.3**). Following the respective surgeries, pain behaviour (weight bearing asymmetry and paw withdrawal threshold) was measured weekly for 16 weeks (**2.1**). At the end of the experiment, mice were euthanised by an intra-peritoneal injection of pentobarbital and perfusion fixation (**2.5**). Knee joints were harvested and processed as previously described in **2.6.1**. Six sections per joint were stained for H & E (**2.6.2**) before being microscopically scored (**2.6.3**).

Thirty-five male C57BL/6 mice (Charles River, Kent, UK) aged approximately 8-9 weeks were used. Surgeries were carried out under isoflurane induced general anaesthesia (**2.2.1**) and mice underwent either modified DMM (n=22) or sham surgery (n=13) in their left knee joint (**2.2.3**). Following surgery both weight bearing asymmetry and paw withdrawal threshold were measured for **20 weeks** (**2.1**). At the end of the experiment the mice were euthanised by intra-peritoneal injection of sodium pentobarbital and transcardially perfused (**2.5**). The knee joints were then dissected out from the animal and processed as previously described (**2.6.1**). Six sections from throughout each joint were then stained with H & E, and subsequently microscopically scored (**2.6.2**, **2.6.3**).

3.2.4 Spinal immunohistochemistry

Immunohistochemistry was performed using spinal cord sections from both the traditional and modified DMM model at 16 weeks post-surgery. Following dissection, spinal cords were processed for IHC as described in **2.7.1**. Spinal sections (n=6 sections per mouse) from both traditional and modified DMM and sham operated mice were stained for the microglia marker Iba1 and the astrocyte marker GFAP (**2.7.2**). Images of Iba1 and GFAP stained sections of the superficial laminae of the dorsal horn were captured using a DMIRE2 microscope (**2.7.3**). These images were then analysed and the numbers of activated microglia were counted, and GFAP immunofluorescence quantified in the dorsal horn as previously described (**2.7.3**).

3.2.5 Data Analysis

Data were analysed and then graphically presented using Prism V.7 (Graphpad, San Diego, California, US). Data were tested for normal distribution using the D'agostino and Pearson normality test, if data were found to be normally distributed then parametric analyses were carried out and if data were found to be not normally distributed then non-parametric analyses were used. Weight bearing data are presented as the change in weight bearing asymmetry from baseline (mean $\pm$ SEM). Paw withdrawal threshold is presented as the mean threshold $\pm$ SEM. The differences in both weight bearing asymmetry and paw withdrawal threshold between DMM and sham operated animals over time was assessed by

2-way ANOVA, with the surgical condition as the between-subjects factor and time post surgery as the within-subjects factor. The histological parameters were presented as the mean score $\pm$ SEM. Differences between DMM and sham operated joints were assessed by Mann-Whitney U test. Spearman's ρ was used to determine if there was a statistical dependence between weight bearing asymmetry and joint pathology. The percentage change in activated microglia in the ipsilateral dorsal horn from the contralateral dorsal horn was presented as mean $\pm$ SEM. Differences in the percentage change in activated microglia between DMM and sham operated animals was assessed by unpaired t-test. GFAP immunofluorescence was presented as the average sum grey intensity value (mean $\pm$ SEM). Differences in the GFAP immunofluorescence between DMM and sham operated animals was assessed by unpaired t-test.

3.3 Results

3.3.1 Carrageenan model evokes pain behaviour in mouse.

Two-way ANOVA with repeated measures showed significant difference over time ($F_{(3,24)} = 3.099$, $p = 0.0457$), and between groups ($F_{(1, 24)} = 11.22$, $p = 0.0027$) in weight bearing asymmetry following carrageenan injection, however the interaction between the two was found to be non-significant ($F_{(3,24)} = 2.46$, $p=0.087$). Looking at the data it appears that there is an increase in weightbearing asymmetry 24 hours post injection of carrageenan when compared to saline injected animals (**Fig. 3.1.A**). Two-way ANOVA with repeated

measures showed no significant difference over time ($F_{(3,24)} = 1.596$, $p = 0.2165$), but did show a significant difference between groups ($F_{(1, 24)} = 9.94$, $p = 0.0043$) for the ipsilateral hind-paw withdrawal thresholds. However, there was no significant interaction between these variables ($F_{(3, 24)} = 2.27$, $p = 0.1$). Again, looking at the data suggests that at the 24 hour timepoint there is a decreased ipsilateral paw withdrawal threshold when compared to saline controls. There was no difference in the contralateral hind-paw withdrawal thresholds between carrageenan and saline injected mice at any timepoint (**Fig. 3.1.C**).

3.3.2 Destabilisation of medial meniscus induces weight-bearing asymmetry.

Two-way ANOVA with repeated measures showed significant difference over time ($F_{(15,240)} = 2.286$, $p = 0.0048$), and between groups ($F_{(1, 240)} = 56.87$, $p = <0.0001$) for weight bearing asymmetry following traditional DMM surgery. The interaction between these two variables was found to also be significant ($F_{(15, 240)} = 2.87$, $p = 0.0004$). These results suggest that there was a significant difference between DMM operated mice and sham operated mice over time. It appears that the weight bearing asymmetry of DMM operated mice starts to diverge from that of the sham operated mice from 10 weeks post surgery. This difference in weight bearing asymmetry slowly increases up until 16 weeks post surgery. However, hindpaw withdrawal thresholds were not significantly altered following DMM surgery even at 16 weeks post-surgery (**Fig. 3.2.B**). Two-way ANOVA with repeated measures showed significant difference over time ($F_{(7,105)} = 2.567$, $p =$

0.0176), but not between groups ($F_{(1, 15)} = 0.04999$, $p = 0.8261$). There was also no significant interaction between these variables either ($F_{(7,105)} = 1.081$, $p = 0.381$). Similarly, there were no significant differences in the contralateral hindpaw withdrawal thresholds (**Fig. 3.2.C**) at any time point.

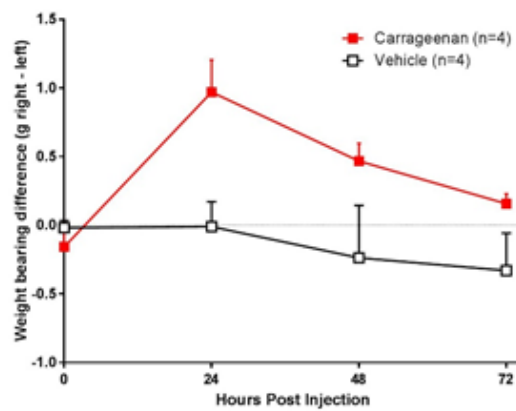
3.3.3 Inter-observer validation for the scoring of chondropathy, synovitis, and osteophytosis.

Table 3.1 reports the intraclass correlations for the average scores given for each animal for chondropathy, synovitis, and osteophytes. An ICC of above 0.75 indicates an excellent agreement between scorers, whilst an ICC of above 0.4 indicates a good agreement between scorers. As can be seen there was excellent agreement for several measures including; MTP chondropathy, synovitis, and osteophyte presence.

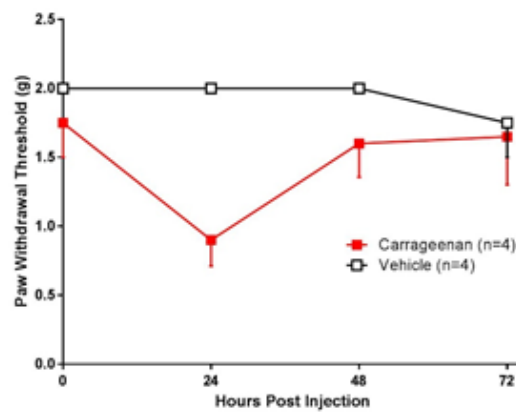
Table 3.1: ICCs for inter-observer reliability. The intraclass correlation for the average score of chondropathy in the four compartments in the knee joint, synovitis, and osteophytes between three scorers (**PG**, **PM**, & **SS**).

Average Measures	ICC (95% CI)	P value
Medial Tibial Plateau	0.91 (0.78-0.97)	<0.001
Lateral Tibial Plateau	0.73 (0.37-0.91)	0.001
Medial Femoral Condyle	0.81 (0.54-0.93)	<0.001
Lateral Femoral Condyle	0.78 (0.45-0.93)	<0.001
Synovitis	0.94 (0.87-0.97)	<0.001
Osteophytes	0.99 (0.98-1.00)	<0.001

A



B



C

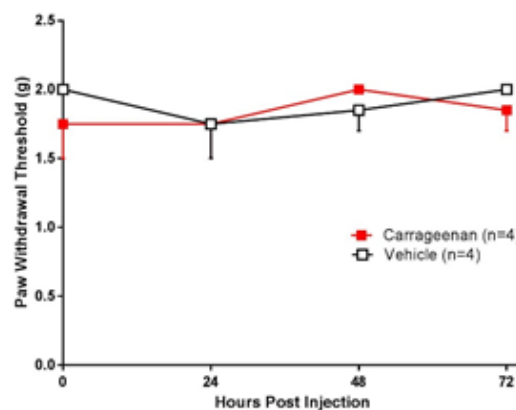
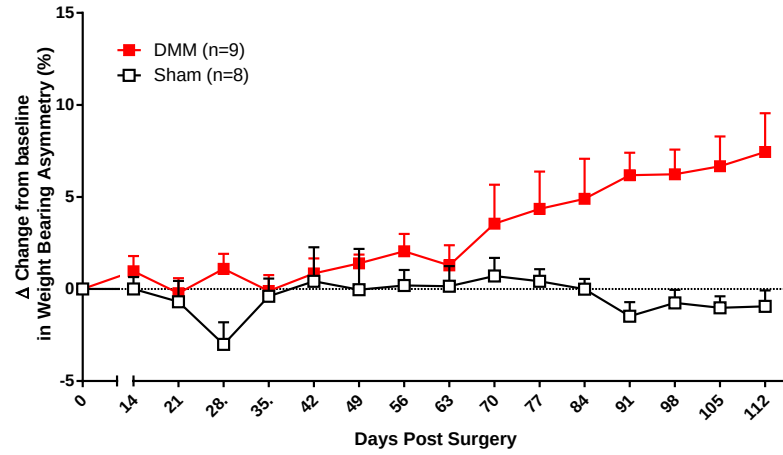
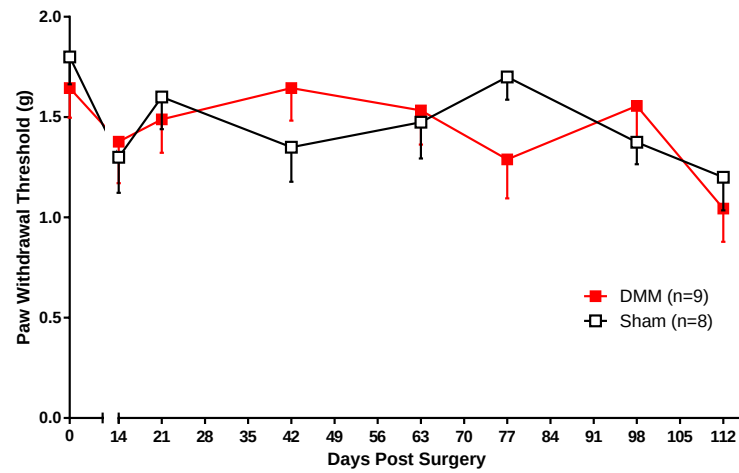


Figure 3.1: Behavioural response to intra-plantar injection of 1% carrageenan in the mouse. Male C57BL/6 mice were injected with either 10µl 1% carrageenan (n=4) or 10µl saline (n=4) in the ipsilateral hindpaw. Pain behaviour was measured up to 3 days post injection. **A:** Weight-bearing difference following intra-plantar injection of carrageenan. **B:** Ipsilateral hindpaw withdrawal threshold the following carrageenan injection. **C:** Contralateral hindpaw withdrawal thresholds following carrageenan insult. Data analysed by 2-way ANOVA

A



B



C

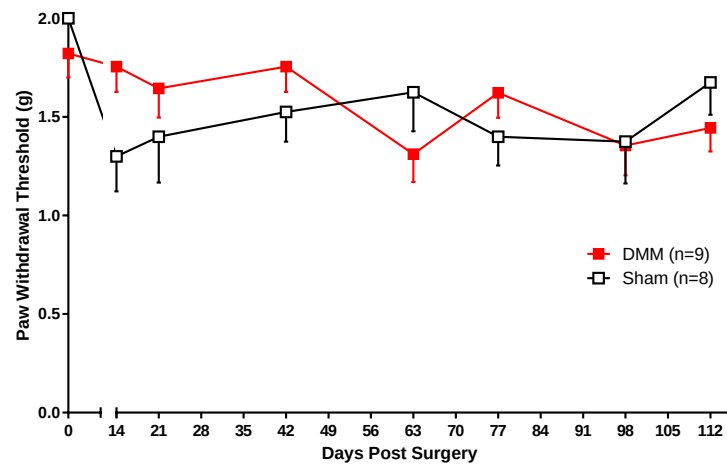


Figure 3.2: Changes in pain behaviour in mice following traditional destabilisation of the medial meniscus. Adult male C57BL/6 mice underwent either destabilisation of the medial meniscus (DMM) (n=9) or sham (n=8) surgery. Weight-bearing asymmetry and paw withdrawal threshold were measured up to 16 weeks post model induction. **A:** Weight-bearing asymmetry following DMM surgery. **B:** Ipsilateral hindpaw withdrawal threshold following surgeries. **C:** Contralateral hindpaw withdrawal thresholds following surgeries. Data analysed by 2 way ANOVA.

3.3.4 Destabilisation of the medial meniscus causes increases in chondropathy and synovitis 16 weeks post-surgery.

With the significant increase in weight bearing asymmetry reported in **3.3.2** it was important to confirm that the DMM surgery was actually modelling the pathophysiological changes to the joint observed in the human disease. In order to achieve this, chondropathy in the medial tibial plateau, synovitis, and osteophytosis were scored.

There was a significant difference between the cartilage score in the medial tibial plateau of the ipsilateral joints of DMM operated mice compared to sham operated mice (**Fig 3.3.A**). DMM operated mice had an average cartilage damage score of 2.82 ± 0.24 , whilst sham operated mice had an average score of 0.21 ± 0.1 ($p < 0.01$). There was also a significant difference in synovial inflammation between the ipsilateral joints of DMM and sham operated mice (**Fig 3.3.B**). DMM operated mice had an average synovitis score of 2.11 ± 0.27 , whilst sham operated mice had an average score of 0.54 ± 0.13 ($p < 0.01$). There was a small but significant difference between the levels of osteophytosis between the ipsilateral joints of DMM and sham operated mice (**Appendix 7.1**). There was almost no presence of osteophytes in DMM mice, and there were no osteophytes in the sham operated mice.

3.3.5 Modified destabilisation of the medial meniscus surgery at 16 weeks.

To understand better the relationship between the key aspects of the DMM surgery and pain behaviour and joint pathology,

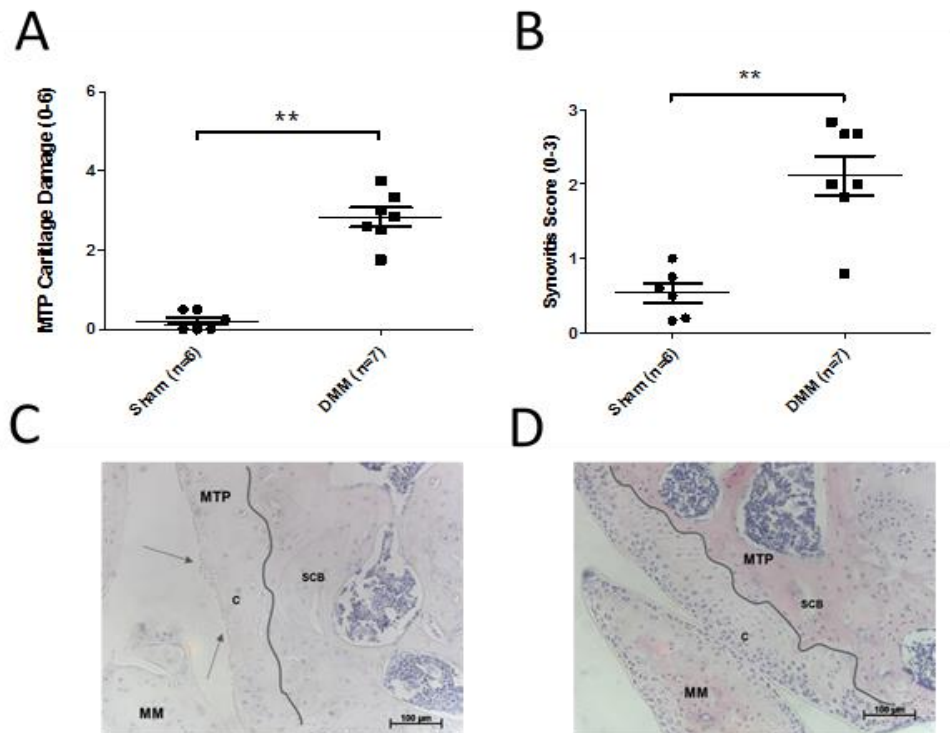


Figure 3.3: Changes in joint pathology in mice following destabilisation of the medial meniscus. Adult male C57BL/6 mice underwent either destabilisation of the medial meniscus (DMM) (n=7) or sham (n=6) surgery. Damage to the cartilage and synovial inflammation were scored 16 weeks post model induction. **A:** Compares medial tibial plateau cartilage damage between DMM and sham mice. **B:** Compares synovitis between DMM and sham mice. Data analysed by Mann Whitney U test. ** = $p < 0.01$ between DMM & Sham. **C:** Representative image of the cartilage damage at 16 weeks post DMM. **D:** Representative image of the cartilage damage at 16 weeks post sham surgery. The grey line represents the osteochondral junction. Arrows indicate areas of cartilage damage. MTP = medial tibial plateau, MFC = medial femoral condyle, MM = medial meniscus, C = cartilage, SCB = subchondral bone

the model was replicated using a modified surgical procedure as outlined in section **2.2.3**.

Following the modified DMM surgery, there was no evidence of weight bearing asymmetry or changes in hindpaw withdrawal thresholds between mice that underwent the modified DMM surgery versus sham surgery. There was a modest non-significant increase in weight bearing asymmetry, in the modified DMM mice from baseline, of $3.8 \pm 1.8\%$ at 16 weeks post-model induction. In the sham operated mice, weight bearing asymmetry had increased by $1.5 \pm 0.78\%$ at 16 weeks. There was no significant difference in weight bearing between sham and DMM operated mice at any time point over 16 week period (**Fig. 3.4.A**). Two-way ANOVA with repeated measures showed significant difference over time ($F_{(14, 434)} = 3.042$, $p = 0.0002$), but not between groups ($F_{(1, 32)} = 1.782$,

$p = 0.153$). There was no significant interaction between these variables ($F_{(14, 434)} = 0.7757$, $p = 0.696$). Similarly, there was no significant difference between the withdrawal thresholds of the ipsilateral hindpaws between sham and modified DMM mice at any time point across the 16 weeks following model induction (**Fig. 3.4.B**). Two-way ANOVA with repeated measures showed significant difference over time ($F_{(7, 252)} = 4.067$, $p = 0.0003$), but not between groups ($F_{(1, 32)} = 3.6$, $p = 0.067$). There was no significant difference between the surgical condition over time ($F_{(7, 252)} = 0.8542$, $p = 0.5435$). There was no significant difference in the contralateral hindpaw withdrawal thresholds of sham and modified DMM mice at any time point studied (data not shown).

3.3.6 Modified DMM causes chondropathy and synovitis 16 weeks post-surgery.

Despite the lack of effect of the modified DMM surgery on pain behaviour in mice, analysis of the knee joints revealed the presence of joint pathology. Indeed, there was significant chondropathy in the modified DMM mice, compared with sham mice. DMM mice had an average cartilage damage score of 2.79 ± 0.26 ($p < 0.01$) for the medial tibial plateau at 16 weeks post-surgery. Sham operated mice had an average cartilage damage score of 0.6 ± 0.37 for the medial tibial plateau at the same timepoint. In addition, there was a significantly higher synovitis score in the modified DMM mice compared to sham mice. The average synovitis score for the modified DMM mice was 1.98 ± 0.24 ($p < 0.01$) compared with the average score of 0.36 ± 0.11 in sham mice.

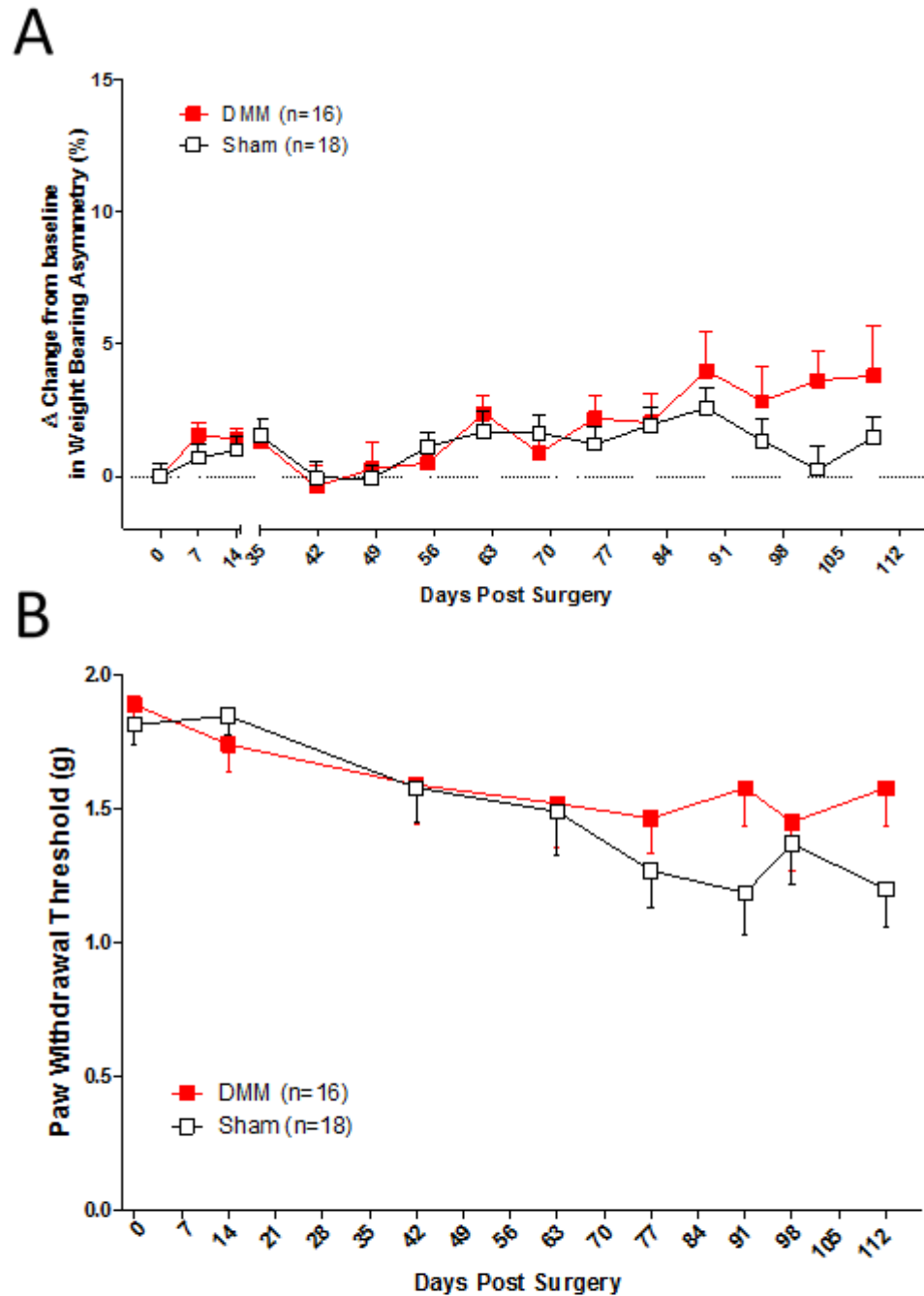


Figure 3.4: Changes in pain behaviour in mice following the modified destabilisation of the medial meniscus. Adult male C57BL/6 mice underwent either destabilisation of the medial meniscus (DMM) (n=16) or sham (n=18) surgery. Weight-bearing asymmetry and paw withdrawal threshold were measured up to 16 weeks post model induction. **A:** Shows the changes in weight-bearing asymmetry following DMM surgery. **B:** Shows changes in the paw withdrawal threshold of the ipsilateral hindpaw following surgeries. Data analysed by 2 way ANOVA.

There was no evidence for the presence of osteophytes in either the DMM mice or the sham mice (**Appendix 7.1**). It is interesting that there were still significant differences in chondropathy and synovitis between modified DMM mice and sham operated mice, despite there being no difference in either pain behaviour measurement.

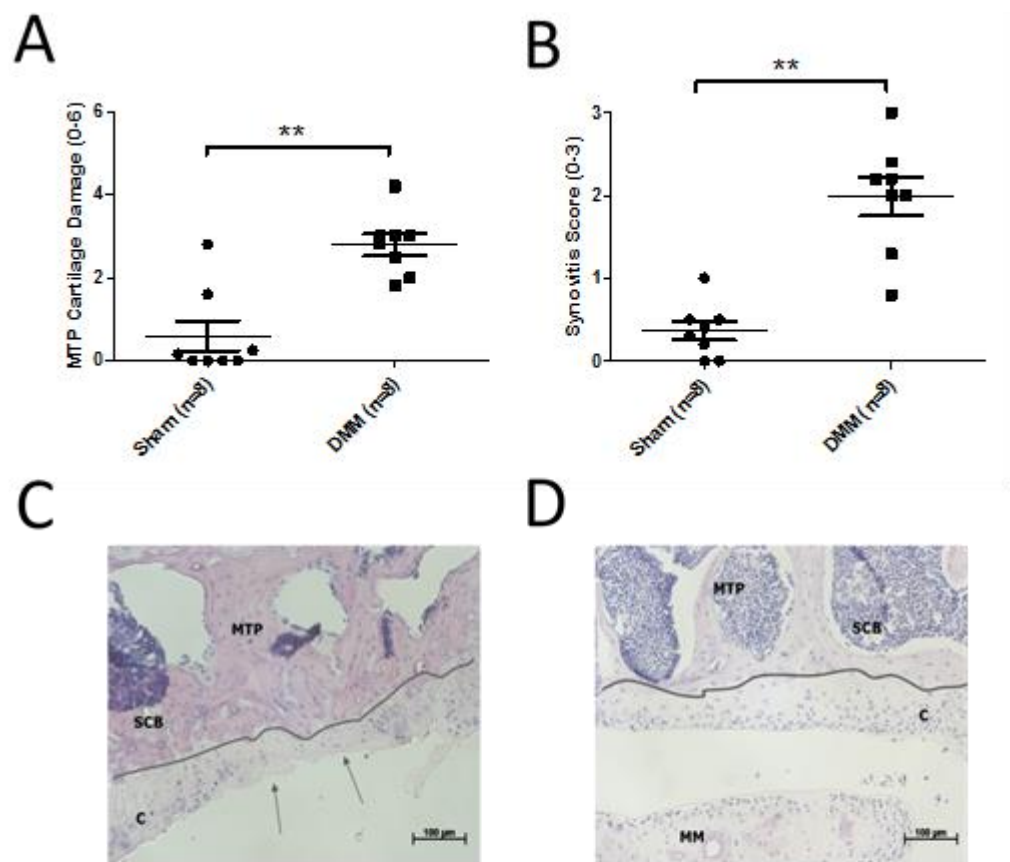


Figure 3.5: Changes in joint pathology in mice following the modified destabilisation of the medial meniscus. Adult male C57BL/6 mice underwent either modified destabilisation of the medial meniscus (DMM) (n=8) or sham (n=8) surgery. Damage to the cartilage and synovial inflammation were scored 16 weeks post model induction. **A:** Compares medial tibial plateau cartilage damage between DMM and sham mice. **B:** Compares synovitis between DMM and sham mice. Data analysed by Mann Whitney U test. **= p<0.01 between DMM & Sham. **C:** is a representative image of the cartilage damage found 16 weeks post DMM. **D:** is a representative image of the cartilage damage found 16 weeks post sham surgery. The grey line represents the osteochondral junction. Arrows indicate areas of cartilage damage. MTP = medial tibial plateau, MFC = medial femoral condyle, MM = medial meniscus, C = cartilage, SCB = subchondral bone

3.3.7 Modified destabilisation of the medial meniscus at 20 weeks post-surgery.

To gauge whether the modified DMM model is potentially more slowly developing in terms of pain behaviour, an additional study was performed to assess potential changes in pain behaviour up to 20 weeks post-surgery.

In this separate cohort of mice, modified DMM surgery did not result in any evidence of weight bearing asymmetry compared to sham surgery up to 20 weeks post-model induction (**Fig. 3.6.A**). Two-way ANOVA with repeated measures showed significant difference over time ($F_{(9, 316)} = 3.919$, $p = <0.0001$), but not between groups ($F_{(1, 33)} = 0.0722$, $p = 0.7883$), and there was no significant difference between the two variables ($F_{(16, 528)} = 0.5589$, $p = 0.8303$). As before, there was no significant difference between the ipsilateral hindpaw withdrawal thresholds of modified DMM versus sham mice at any time point (**Fig. 3.6.B**). Two-way ANOVA with repeated measures showed significant difference over time ($F_{(8, 282)} = 9.586$, $p = <0.0001$), but not between groups ($F_{(1, 33)} = 0.007$, $p = 0.9322$). There was no significant difference in the ipsilateral hindpaw withdrawal thresholds between groups over time ($F_{(8, 282)} = 0.8367$, $p = 0.57$). Contralateral hindpaw withdrawal thresholds of modified DMM and sham operated mice were not statistically different from each other (data not shown).

3.3.8 Chondropathy and significant synovitis at 20 weeks following modified DMM surgery.

At 20 weeks following modified DMM surgery, the average chondropathy score (**Fig. 3.7.A**) was 2.24 ± 0.55 compared to

0.38± 0.14 for sham operated mice. However, this trend did not reach statistical significance. There was however a significant synovitis score (**Fig. 3.7.B**) for the modified DMM mice (1.65± 0.25) compared to sham operated mice (0.32± 0.14). As in previous studies there was very little evidence for the presence of osteophytes in either the DMM or sham operated mice (**Appendix 7.1**).

3.3.9 No correlation between pain behaviour and joint pathology at 16 weeks post DMM.

In the traditional DMM model the severity of MTP cartilage damage (**Fig. 3.8.A**) was not significantly correlated (Spearman ρ : 0.036, $p = \text{n.s.}$) with the change in weight bearing asymmetry from baseline. Likewise, synovitis (**Fig. 3.8.B**) was also not correlated with pain behaviour at 16 weeks post DMM (Spearman ρ : -0.11, $p = \text{n.s.}$).

Similarly, in the modified DMM model neither MTP cartilage damage (**Fig. 3.8.C**) (Spearman ρ : 0.073, $p = \text{n.s.}$) nor synovitis (**Fig. 3.8.D**) (Spearman ρ : 0.14, $p = \text{n.s.}$) were significantly correlated with weight bearing asymmetry at 16 weeks post-surgery.

3.3.10 No evidence of markers of central sensitization in the DMM model of OA.

As previously discussed in **1.1.5.4** spinal microglia activation has been strongly linked to the induction of central sensitization. It was therefore important to determine whether the DMM model of OA also leads to activation of microglia in the spinal cord.

There was no significant difference in the percentage change in the number of activated microglia in the ipsilateral dorsal

horn of mice that underwent traditional DMM surgery, compared to the ipsilateral dorsal horn of sham operated mice (**Fig 3.9.A**). This was also the case for the modified DMM surgery compared to sham control (**Fig 3.9.B**). Like spinal microglia, there is also a significant increase in GFAP immunofluorescence (a marker for astrocytes) in the ipsilateral dorsal horn at day 28 in the MIA model of OA pain in rats (Sagar *et al*, 2011). In the present studies, there was no significant change at 16 weeks post-surgery in GFAP immunofluorescence as measured by the sum grey intensity values between the ipsilateral dorsal horns of mice that underwent traditional DMM versus sham surgery (**Fig 3.10.A**), or modified DMM mice and sham operated mice (**Fig 3.10.B**).

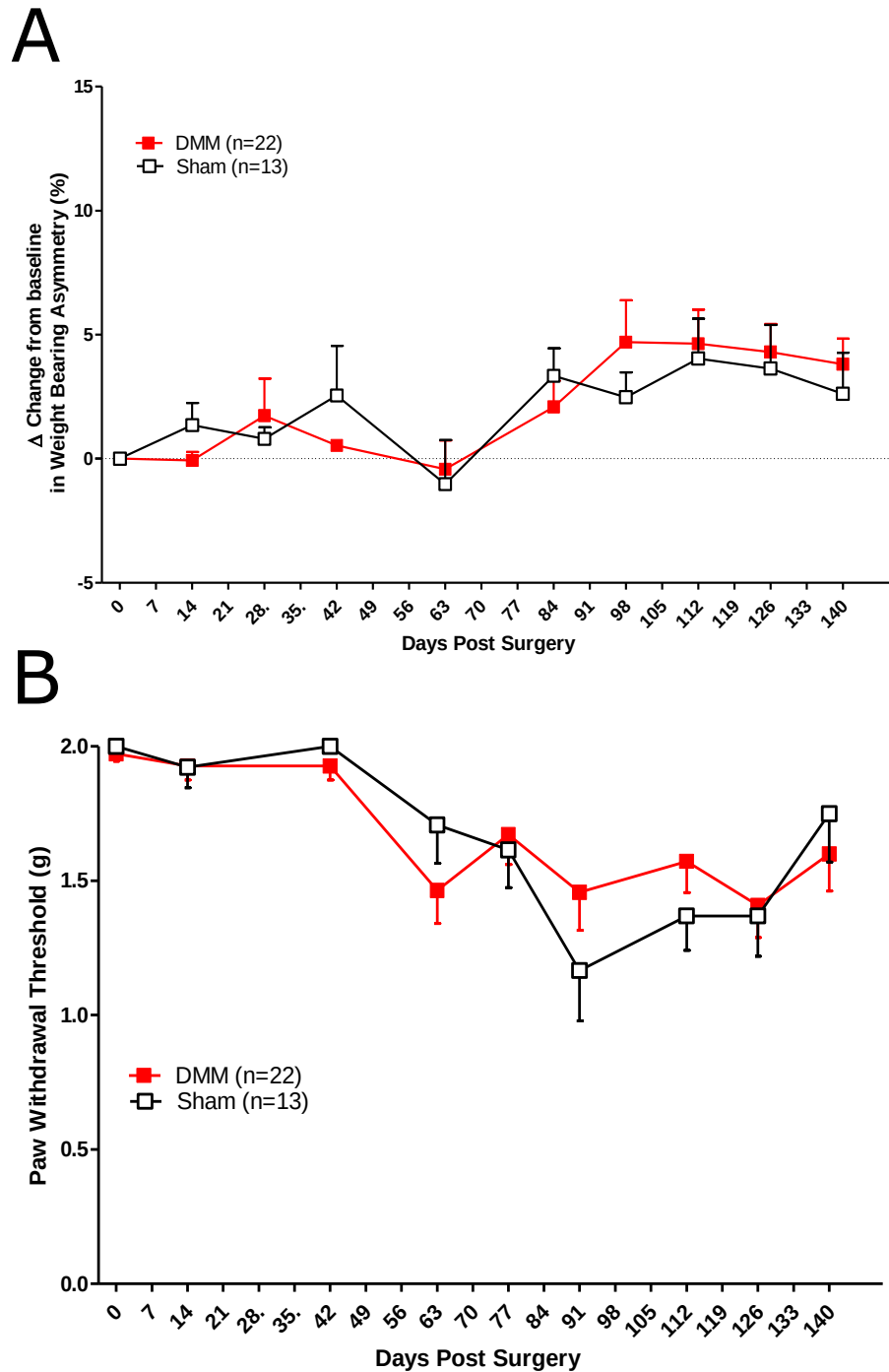


Figure 3.6: Changes in pain behaviour in mice 20 weeks after the modified destabilisation of the medial meniscus. Adult male C57BL/6 mice underwent either destabilisation of the medial meniscus (DMM) (n=22) or sham (n=13) surgery. Weight-bearing asymmetry and paw withdrawal threshold were measured up to 20 weeks post model induction. **A:** Changes in weight-bearing asymmetry following DMM surgery. **B:** shows changes in the paw withdrawal threshold of the ipsilateral hindpaw following surgeries. Data analysed by 2 way ANOVA.

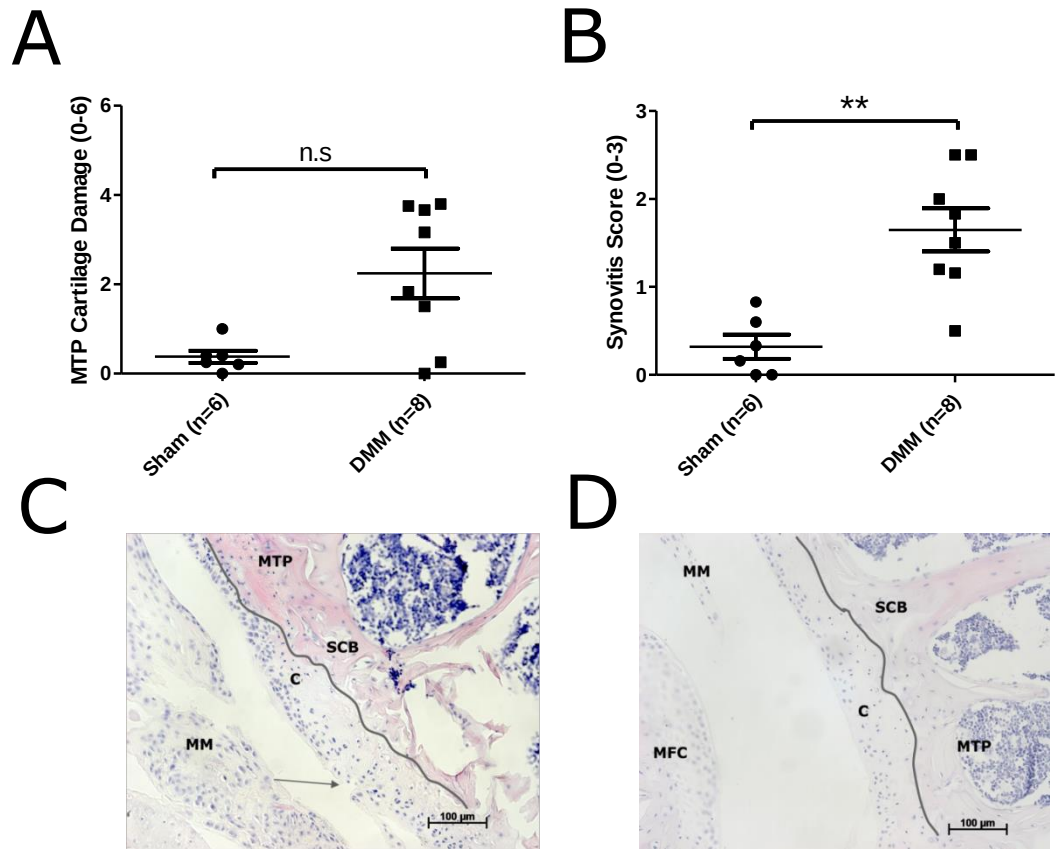


Figure 3.7: Changes in joint pathology in mice 20 weeks after the modified destabilisation of the medial meniscus. Adult male C57BL/6 mice underwent either modified destabilisation of the medial meniscus (DMM) (n=8) or sham (n=6) surgery. Damage to the cartilage and synovial inflammation were scored 20 weeks post model induction. **A:** Compares medial tibial plateau cartilage damage between DMM and sham mice. **B:** Compares synovitis between DMM and sham mice. Data analysed by Mann Whitney U test. ** = $p < 0.01$ between DMM & Sham. **C:** Representative image of the cartilage damage found 20 weeks post DMM. **D:** Representative image of the cartilage damage found 20 weeks post sham surgery. The grey line represents the osteochondral junction. Arrows indicate areas of cartilage damage. MTP = medial tibial plateau, MFC = medial femoral condyle, MM = medial meniscus, C = cartilage, SCB = subchondral bone

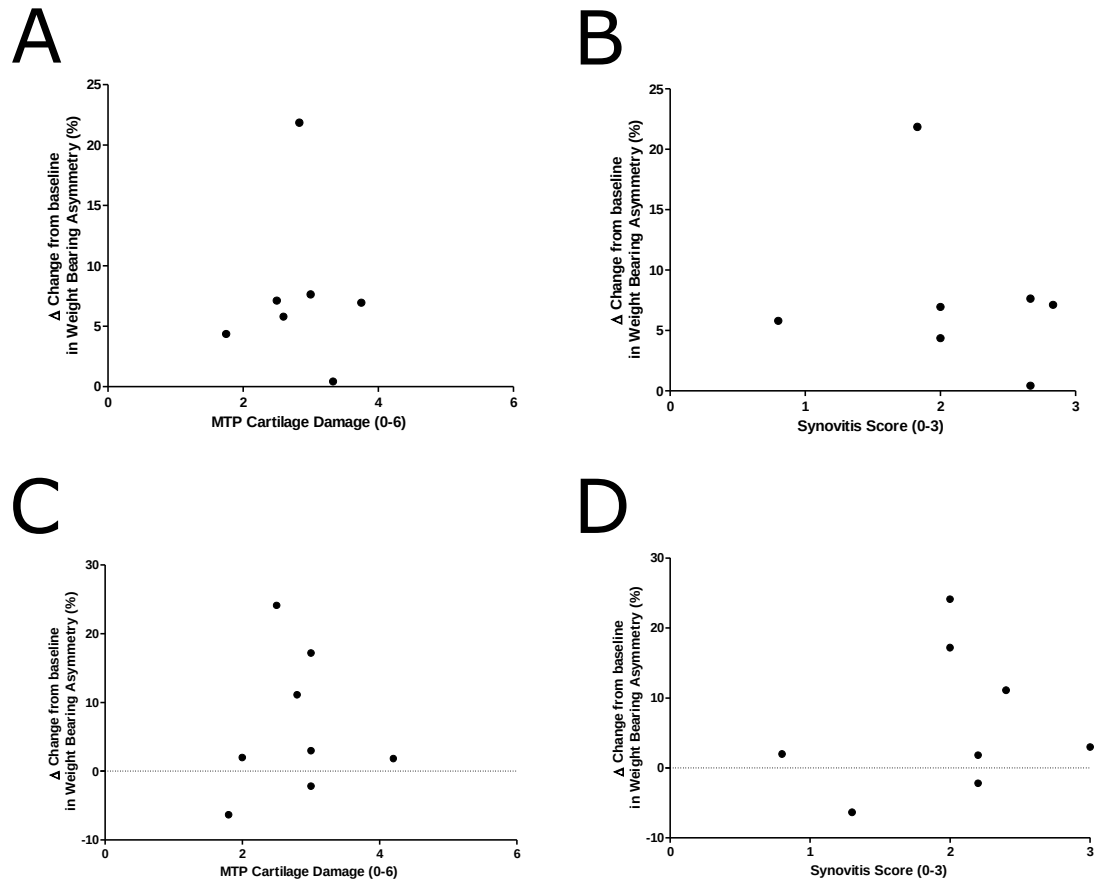


Figure 3.8: Correlation between joint pathology (MTP cartilage damage and synovitis) and pain behaviour (weight bearing asymmetry) 16 weeks after either traditional or modified DMM surgery. Adult male C57BL/6 mice underwent either modified DMM (n=8) or traditional DMM (n=7) surgery. Damage to the cartilage and synovial inflammation were scored 16 weeks post model induction as was weight bearing asymmetry. **A:** Correlation between medial tibial plateau cartilage damage and weight bearing asymmetry following traditional DMM. Spearman $\rho = 0.036$, $p = \text{n.s.}$ **B:** Correlation between synovitis and weight bearing asymmetry following traditional DMM. Spearman $\rho = -0.11$, $p = \text{n.s.}$ **C:** Correlation between medial tibial plateau cartilage damage and weight bearing asymmetry following modified DMM. Spearman $\rho = 0.07$, $p = \text{n.s.}$ **D:** Correlation between synovitis and weight bearing asymmetry following modified DMM. Spearman $\rho = 0.14$, $p = \text{n.s.}$

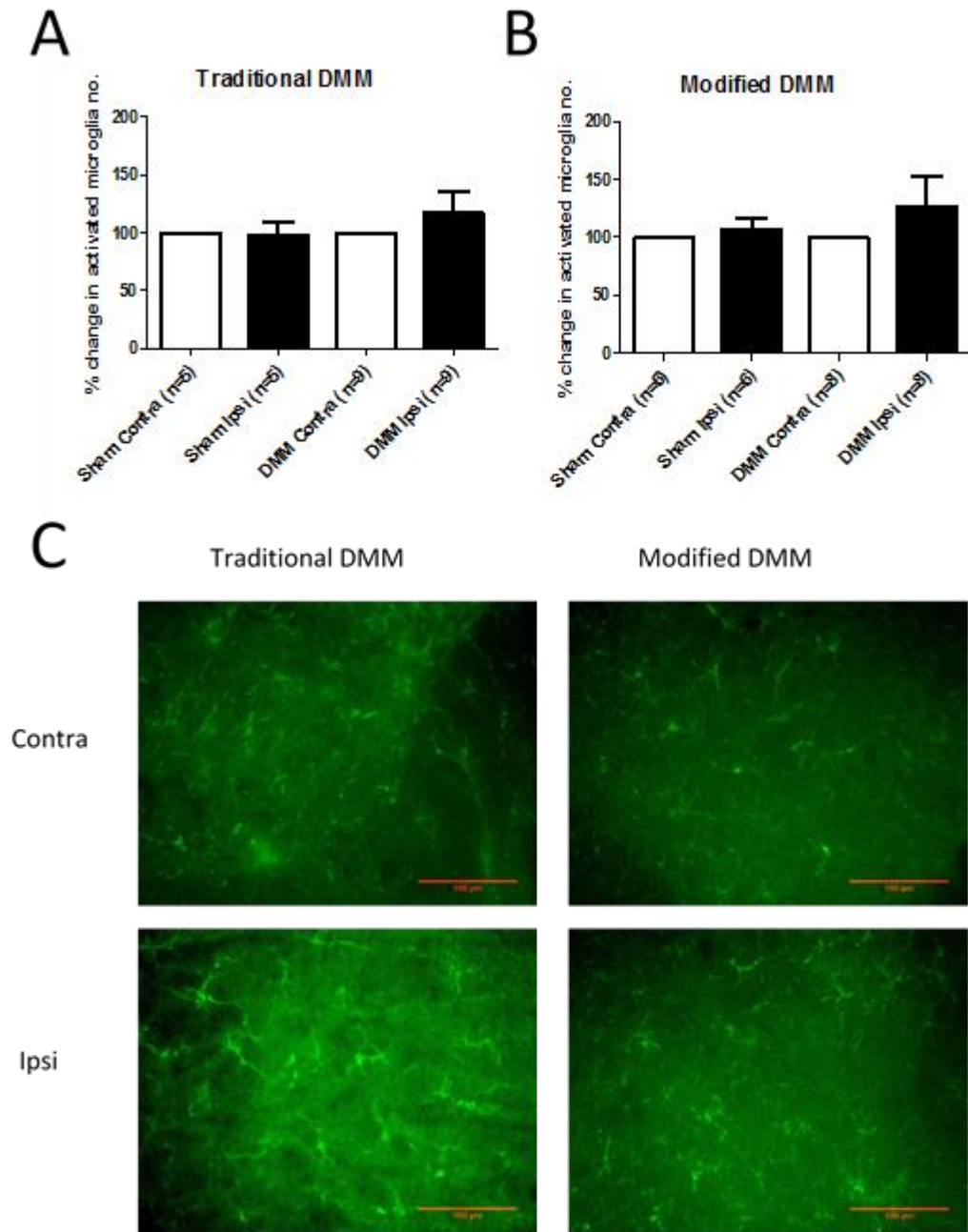


Figure 3.9: Activation of microglia in two murine models of OA. Adult male C57BL/6 mice underwent traditional DMM, modified DMM, or sham surgery. IBA1 immunostaining was investigated 16 weeks after surgery. Data are shown as a percentage change in activated microglia when compared to the contralateral dorsal horn. **A:** Quantification of IBA1 immunostaining in mice that underwent traditional DMM. **B:** Quantification of IBA1 immunostaining in mice that underwent modified DMM. **C:** Representative images of IBA1 immunostaining in the superficial dorsal horn of mice that underwent traditional or modified DMM surgery. Data were analysed by unpaired t-test $p=n.s.$

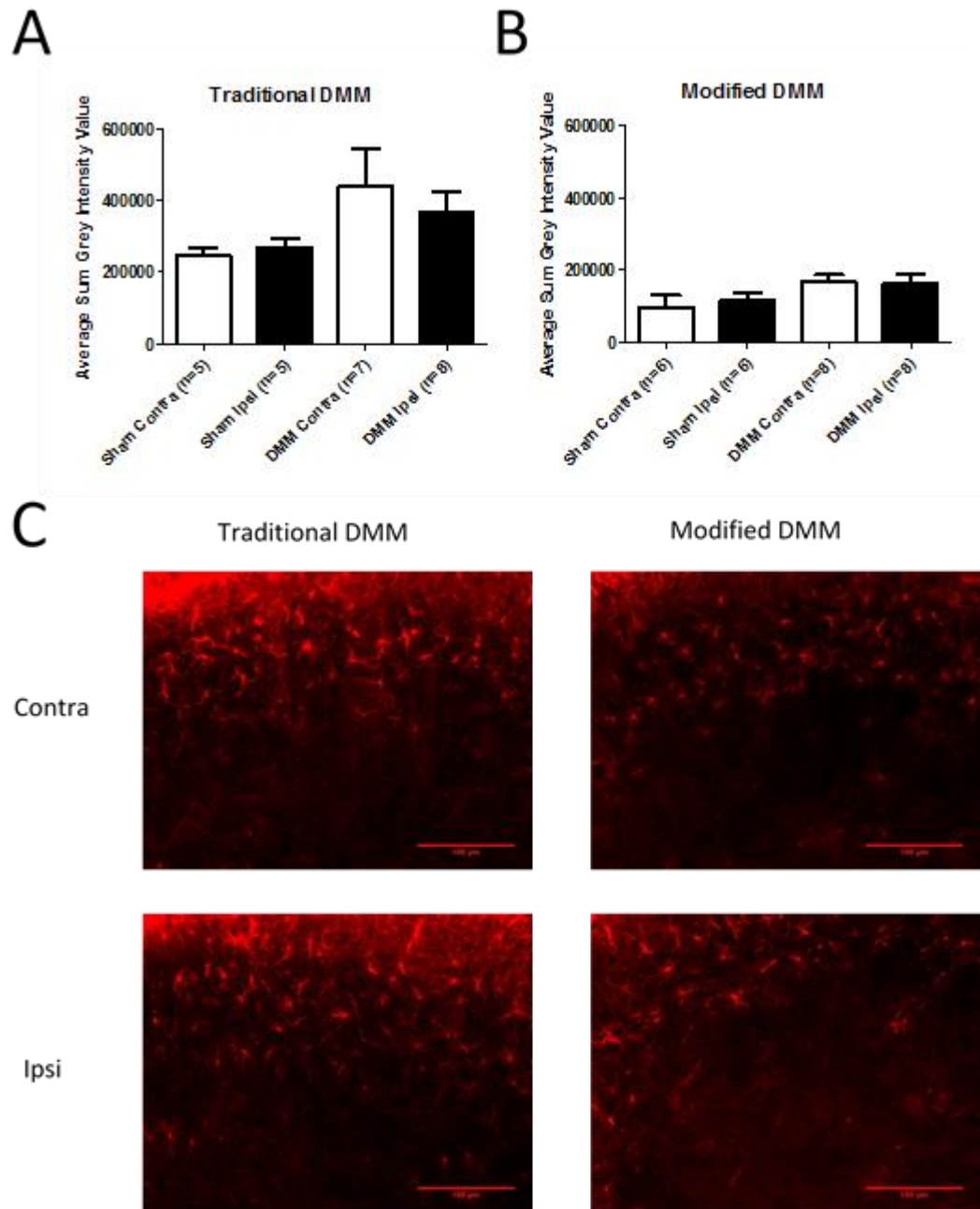


Figure 3.10: GFAP immunostaining in two murine models of OA. Adult male C57BL/6 mice underwent traditional DMM, modified DMM, or sham surgery. GFAP immunostaining was investigated 16 weeks after surgery. Data are shown as the average sum grey intensity value as measured by the Velocity software. **A:** Quantification of GFAP immunostaining in mice that underwent traditional DMM. **B:** Quantification of GFAP immunostaining in mice that underwent modified DMM. **C:** Representative images of GFAP immunostaining in the superficial dorsal horn of mice that underwent traditional or modified DMM surgery. Data were analysed by unpaired t-test, $p=n.s.$

3.4 Discussion

3.4.1 Summary of major findings

As expected, intra-plantar injection of carrageenan was found to produce an acute pain response in naïve C57BL/6 mice. There were changes in both weight bearing asymmetry and paw withdrawal within the first 24 hours. This study of acute inflammatory pain confirmed that the two behavioural measures of pain had utility and reproducibility in this strain of mice.

Following traditional DMM surgery in C57BL/6 mice, there was an increase in weight bearing asymmetry from 10 weeks post model induction. This increase in weight bearing asymmetry was significantly different from the sham mice up to 16 weeks post-surgery. There was no difference in the hindpaw withdrawal thresholds between DMM and sham mice at any time point across the 16 weeks. At 16 weeks post DMM surgery there was significant cartilage damage and synovitis in DMM knees, compared to sham mice.

Following the modified DMM surgery where the MMTL is left intact, weight bearing between the two hindlimbs was comparable to sham operated animals. Nevertheless, this modified DMM surgery did result in significant joint pathology at 16 weeks post-surgery, both in terms of chondropathy and synovitis score. In both the traditional and modified DMM models, there was no correlation between the severity of pain behaviour and joint pathology at the 16 week timepoint.

Increasing the duration of the modified DMM model to 20 weeks did not reveal any change in weight bearing, suggesting no change in pain response. At 20 weeks post-surgery there was a trend towards cartilage damage in the modified DMM

model, compared to sham mice. At this timepoint, there was a significant difference in the synovitis score between the modified DMM mice and sham controls.

There was no significant difference in the levels of activated microglia or astrocytes in the ipsilateral spinal cord of either the traditional or modified DMM surgery, or their respective sham operated controls.

3.4.2 Measuring pain responses in mice

The significant pain behaviour seen after intra-plantar injection of carrageenan was to be expected based on previous studies (Ghosh *et al*, 2013, Hargreaves *et al*, 1988, Lu *et al*, 2013). Carrageenan is thought to induce a robust inflammatory response through mediators such as interleukin-1 β (IL-1 β) and tumour necrosis factor- α (TNF- α) (Albyrak *et al*, 2013). Previous studies have shown pain behaviour is present up to 24 hours post injection before starting to resolve (Hargreaves *et al*, 1988), and these findings mirror the results of my study. One of the aims of this study was to confirm the methods for measuring pain behaviour in the mouse were reliable. The results from this study indicate that I could accurately measure pain behaviour through two behavioural tests.

3.4.3 Surgical models of OA

Surgical models of OA mimic traumatic OA in humans by changing the weight bearing upon the joint (Fang & Beier, 2014). Here I have shown that at 16 weeks post-DMM there is significant chondropathy and synovitis present in the

ipsilateral knee joints. There was also an increase in weight bearing asymmetry from 10 weeks post-surgery. These results replicate previous findings (Driscoll *et al*, 2016). However, we could not replicate the decrease in paw withdrawal threshold that others have observed. Behavioural testing is a subjective practise; there is the possibility that the experimenter (**PG**) was too conservative in the assessment of paw withdrawal from the mice. However, in other studies of acute pain there were significant differences in paw withdrawal observed in mice by the same experimenter (**3.3.1** & **Appendix 7.2**). Weight bearing asymmetry is thought to measure the pain on loading localised to the injured knee joint, whilst the paw withdrawal threshold represents distal pain, which may indicate central sensitization. The fact that we found no significant changes in paw withdrawal threshold may imply that in the DMM model at the 16 week time point there are less central changes compared to other models. This finding is consistent with the lack of changes in microglia and astrocyte immunostaining in the dorsal horn of the spinal cord in the DMM model. It was perhaps surprising that we did not see any changes considering a recent study which reported a significant increase in microglia activation in the spinal cord between DMM and sham operated animals (Tran *et al*, 2016). However, the increase in activated microglia was only seen when they considered both the ipsilateral and contralateral dorsal horns together. Overall, in my study a similar number of activated microglia were evident in the dorsal horn as reported by Tran *et al.*, however we did not see a significant difference from the sham. Currently, there is no information in the literature regarding changes in astrocyte immunoreactivity in the DMM model. Here I show that whilst there is an increase in the intensity of GFAP immunoreactivity in the dorsal horn of

the spinal cord in the DMM model, compared to the sham, significance was not reached.

3.4.4 Relationship between pain and pathology

One of the aims of this chapter was to investigate whether similar joint pathology and pain behaviour was achievable with a less invasive approach to destabilise the medial meniscus, which did not require the transection of the medial meniscotibial ligament. Interestingly this modified model did not result in significant pain behaviour at 16 or 20 weeks post-surgery, compared to sham controls. However, chondropathy and synovitis were evident at both timepoints in the modified DMM.

In the human disease whilst radiographic evidence of OA was linked to a higher prevalence of joint pain, there is still a subset of people that show structural changes within the knee joint but lack chronic joint pain (Davis *et al*, 1992). It has also been shown that a change in the radiographic evidence for joint damage is not necessarily indicative of a change in pain severity, or other clinical outcomes (Dieppe *et al*, 1997). It therefore appears that there is a dissociation between pain and pathology in the human condition.

The modified DMM is interesting in that it has a similar severity of joint pathology compared to the traditional DMM at 16 weeks post-surgery, but does not show a significant change in pain behaviour. Further investigation of the differences between the traditional and modified models of DMM might help to understand what leads to joint pathology becoming chronically painful. The correlation analyses within this chapter showed that in both the traditional and modified

DMM models there was no significant correlation between joint pathology (cartilage damage and synovitis) and pain behaviour. Therefore there is the possibility that other structural changes within the joint may be responsible for OA pain. One structure which may be of particular interest is the meniscus itself. Indeed, there is a correlation between meniscal tears and patient reported pain (Torres *et al*, 2006). Menisci from patients with osteoarthritis are more highly vascularised and innervated than those without chondropathy (Ashraf *et al*, 2010). Therefore meniscal damage may play an important role as a peripheral driver of OA pain.

3.4.5 The influence of age on joint pathology

Previous studies have reported changes in articular cartilage with age. There is evidence that in naïve C57BL/6 mice at 40 weeks of age there is thickening of collagen fibrils, increased nanostiffness and a reduced glycosaminoglycan/DNA ratio (Stolz *et al*, 2009). In naïve BALB/c mice mild increases in cartilage degradation and synovitis have been reported at 36 weeks of age, and by 52 weeks this has progressed to moderate OA comparable with a $\alpha 1$ integrin knockout model of OA (Zemmyo *et al*, 2003). In naïve C57 black mice there was an increase in both the incidence and severity of osteoarthritis as mice aged up to 16 months (Yamamoto *et al*, 2005). These OA related changes occurred concomitantly with an age related reduction in both protein and saccharide synthesis (Yamamoto *et al*, 2005). Both naïve C57BL/6 mice and BALB/c mice display severe OA at 2 years and 1 year of age respectively (Stoop *et al*, 1999). This spontaneous

development of OA was linked to a localised degradation of type II collagen in both strains (Stoop *et al*, 1999).

There are also several other factors to consider when establishing a murine model of OA. Previous work has shown that older animals develop a more severe form of OA when compared to younger animals; therefore we might be able to modify the onset and progression of OA by inducing the model in animals of different ages (Loeser *et al*, 2012).

The strain of mouse used in these studies may have affected the results observed. In studies using the 129/SvEv mouse, increases in cartilage damage are seen from 4 weeks post-surgery, and the severity of damage increase up to 26 weeks post-surgery (Botter *et al*, 2009, Glasson *et al*, 2007). A similar disease progression was seen in C57BL/6 mice following DMM surgery, however there was mild OA like pathology seen just 2 weeks post-surgery (Inglis *et al*, 2009). In terms of measuring pain outcomes the C57BL/6 mouse is the primary strain of mouse used in previously published DMM experiments (Driscoll *et al*, 2016, Inglis *et al*, 2009, Malfait *et al*, 2010). Therefore it would appear the decision to use C57BL/6 mice for these studies was appropriate for our needs. It may also be worth considering novel methods for assessing pain behaviour; other possible behavioural tests include monitoring changes in activity levels, gait analysis, or utilising a grimace scale. Indeed, other groups have observed how activity levels are significantly affected by arthritis (Costello *et al*, 2010). Other groups have already used alternative pain outcomes in the DMM model of OA. The use of a system to detect spontaneous activity in mice found significant decreases in locomotion and grooming from 8 weeks post-surgery (Inglis *et al*, 2009). These systems however can be

prohibitively expensive for the information they provide. Changes in the ethological behaviour of burrowing may also represent a potential pain outcome for preclinical studies. This behavioural test has been used previously in rats with CFA-induced inflammatory pain (Wodarksi *et al*, 2016) However, burrowing will provide information regarding systemic changes in pain, but does not tell us anything about the unilateral changes likely to be seen in the DMM model of OA. Grimace scales have been shown to provide reliable information with acute pain states (up to 24 hrs), however, for chronic pain models, such as CCI, the grimace scale is not sensitive enough to show changes in pain (Langford *et al*, 2010). For a model such as DMM induced OA, the chosen pain outcomes obviously need to be suitable for long-term use.

3.4.6 Limitations

The knee joints dissected from the mice for these studies were sectioned in the coronal plane, which I found led to some inconsistencies in orientation. It was identified that some knee joints were becoming miss-orientated during the embedding process. To overcome this problem future experiments with the DMM model will use sagittal sectioning of the knee joints for histological assessment. We predict that embedding the knee joints ready for sagittal sectioning will minimize the potential for misorientation which will maximise the numbers of useable sections.

There is also the possibility that the sham surgery, which involves blunt dissection of the connective tissues over the joint, of these animals may destabilise the joint over such a long time frame exacerbating the age related changes in the joint.

3.4.7 Conclusions

A surgical model of murine OA with both significant changes in pain behaviour and joint pathology was established. When the meniscus was destabilised in a less aggressive manner there was no change in pain behaviour outcomes despite the presence of significant joint pathology. These results suggest that meniscal damage may be an important peripheral driver behind DMM induced joint pain.

Chapter 4: Investigating the role of BDNF in rodent models of osteoarthritis.

4.1 Introduction

4.1.1 General Background

In the search for novel therapeutics for chronic OA joint pain, monoclonal antibodies for nerve growth factor (NGF) have been shown to be highly efficacious. Clinical trials of a range of different monoclonal antibodies which act to sequester NGF have reported a reduction in pain in patients with established OA (Lane *et al*, 2010, Sanga *et al*, 2013, Tiseo *et al*, 2014). Indeed pre-clinical work has highlighted the role of this neurotrophin in OA. Immunohistochemical analysis revealed the presence of NGF in the chondrocytes of human articular cartilage, there was also staining of both TrkA and p75 in these samples (Gigante *et al*, 2003). In human meniscal cartilage staining for NGF, p75, and TrkA was evident in chondrocyte like cells, but there was no staining in meniscal fibroblasts (Gigante *et al*, 2003). A significant proportion of the dorsal root ganglia neurons (65%) which innervate the subchondral bone express TrkA (Aso *et al*, 2014).

In osteoarthritic chondrocytes there is increased expression of both NGF and TrkA mRNAs when compared to healthy chondrocytes (Iannone *et al*, 2002). Interestingly, NGF expression in chondrocytes was correlated to the extent of cartilage damage, as was TrkA expression (Iannone *et al*, 2002). In cultured chondrocytes NGF was released following

mechanical stimulation, as well as in response to IL-1 β (Pecchi *et al*, 2014). In the rat MIA model of OA there was also an increase in NGF mRNA in the cartilage 35 days post model induction (Im *et al*, 2010). Chondrocytes do not appear to be the sole source of increased NGF in OA. In arthritic mice knees there was significant NGF expression in the inflammatory infiltrates of the synovium, compared to healthy controls (Grimsholm *et al*, 2008a). Increased NGF expression has also been linked to the increased osteochondral angiogenesis seen in OA (Walsh *et al*, 2010).

Preclinical experiments in animal models of OA have investigated the role of NGF in chronic joint pain. Intra-articular injection of NGF into naïve rats caused acute changes in pain behaviour with increased weight bearing asymmetry and lowered paw withdrawal thresholds (Ashraf *et al*, 2014). In the rat MIA model of OA intra-articular injection of NGF increased spinal response to knee extension, and caused an increase in the receptive fields of the neurons which innervate the knee (Sagar *et al*, 2015). In rats with MIA and MNX induced joint pain there was an augmented increase in weight bearing asymmetry following intra-articular injection of NGF (Ashraf *et al*, 2014). Pretreatment with the NSAID indomethacin blocked the NGF-induced augmentation in weight bearing asymmetry (Ashraf *et al*, 2014). These results suggest that NGF is involved in the peripheral inflammatory pain of OA. As previously discussed, NGF's effects are mediated through the TrkA receptor, and there is preclinical evidence that it is this receptor that plays a key role in mediating OA pain. Therapeutic treatment with AR786, a selective inhibitor of TrkA activity, in both the MIA and MNX models of OA reversed OA induced joint pain (Nwosu *et al*,

2014). AR786 was also able to prevent the development of MNX induced joint pain when rats were pretreated with the compound (Nwosu *et al*, 2014). Pretreatment with a NGF monoclonal antibody, muMab911, also prevented MIA induced joint pain, and when given as a therapeutic treatment muMab911 reversed established joint pain (Xu *et al*, 2016).

Monoclonal antibodies for NGF were found to be effective in reducing OA related pain in patients (Lane *et al*, 2010, Sanga *et al*, 2013, Tiseo *et al*, 2014). However, there were reports of adverse effects in the joint in some patients (Hochberg *et al*, 2015). There was a noticeable increase in the number of these adverse effects during concomitant dosing with NSAIDs (Hochberg *et al*, 2015). Therefore, investigating the potential roles of other neurotrophins in mediating chronic OA pain may lead to the development of treatments which reduce pain without the occurrence of rapid joint degradation.

Like NGF, brain derived neurotrophic factor (BDNF) and its high affinity receptor, tropomyosin receptor kinase B (TrkB) have also been linked to pain. Spinal administration of BDNF in naïve animals is associated with an enhanced C fiber nociceptive reflex as well as thermal hyperalgesia (Kerr *et al*, 1999, Groth & Aanonsen, 2002). Whether BDNF may play a role in mediating pain at the peripheral level has yet to be explored. Nevertheless BDNF and TrkB are present in the knee joint of mice with inflammatory arthritis (Grimsholm *et al*, 2008a). BDNF was present in synovial fluid and plasma of OA patients, and plasma levels of BDNF were correlated with patient experienced pain (Simao *et al*, 2014). Despite this evidence of clinical associations, there is little further available information regarding BDNFs potential role in OA pain.

4.1.2 Rationale

Due to the lack of previous research into the potential role of BDNF in mediating chronic joint pain, there are a lot of questions which need answering.

The starting point for these studies was to investigate the effect, if any, of exogenous application of BDNF to the knee joint of naïve rats. This would help us understand the role of BDNF in normal nociception in the knee joint.

As previously discussed BDNF and its high affinity receptor TrkB are upregulated in both inflammatory and neuropathic pain states (**Chapter 1.3**). It is therefore possible that TrkB is upregulated in the knee joints of OA rats and sensitivity to BDNF maybe increased in models of OA.

Although answering the above questions will provide new evidence that BDNF can cause pain responses in models of OA, this does not provide evidence that this molecule contributes to OA pain. To answer this question the effects of decreasing or removing endogenous BDNF from the knee joint of rats with established OA is required. The chimeric compound TrkB/fc is formed of the extracellular domain of the high affinity BDNF receptor, TrkB, and the fc region of human IgG1. TrkB/fc chimera has been shown to sequester endogenous BDNF (Endo *et al*, 2009, Yajima *et al*, 2005). Administration of TrkB/fc chimera in both the hippocampus and the spinal cord blocks BDNFs effects *in vivo* on synaptic plasticity (Chen *et al*, 2012, Vaynman *et al*, 2006, Ying *et al*, 2008). Furthermore spinal application of TrkB/fc chimera reversed pain in neuropathic pain models in both rats and mice (Endo *et al*, 2009, Yajima *et al*, 2005). The TrkB/fc chimera appears to be an ideal experimental tool to

investigate the potential roles of endogenous BDNF in peripheral pain sensitization in models of OA.

It was important to choose the most appropriate animal model of OA to investigate the potential role of BDNF in chronic joint pain. Given the lack of knowledge in this area, it was important to use a robust and well-understood model known to display chronic joint pain at a reliable time point. The monosodium iodoacetate (MIA) model is a chemical model of OA, in which differing doses of MIA injected into the intra-articular space lead to varying amounts of joint damage and pain depending upon the dose administered (Guincamp *et al*, 1997). Our lab has previously used a dose of 1mg of MIA, injected into the joint space of adult Sprague Dawley rats (Sagar *et al*, 2011). This dose of MIA produced changes in weight bearing asymmetry and paw withdrawal threshold from 7 days post injection, as well as significant cartilage loss, bone remodelling, and synovial inflammation at 21 days post injection (Ivanavicius *et al*, 2007, Sagar *et al*, 2011). In order to ensure we had joint pathology that best represents the end stages of the human disease we chose 21 days post MIA injection as our time point for therapeutic intervention. However, because there is such a severe loss of chondrocytes in the MIA model there are some questions as to the extent with which this model represents the human disease (Barve *et al*, 2007). It is therefore good practice to replicate any effects of an intervention in the MIA model in a surgical model of OA, to improve the translational relevance. In the rat, transection of the medial meniscus (MNx) is known to lead to joint pathology reminiscent of the human disease by 21 days post-surgery (Bove *et al*, 2006). Previous work by our lab has shown that there are significant changes in both weight

bearing asymmetry and paw withdrawal thresholds which are maintained until 35 days post-surgery (Ashraf *et al*, 2011). It was therefore decided that the MNX model would be used to compare our intervention results in the MIA model.

4.1.2 Aims

The aim of this chapter is to investigate the peripheral contribution of the neurotrophin BDNF to established OA joint pain in the rat.

Objectives

To assess the effects of intra-articular injection of BDNF in naïve rats on pain behaviour.

To determine the effects of intra-articular injection of BDNF on pain behaviour in two models of OA pain.

To explore the impact of sequestration of endogenous BDNF in the knee joint of rats on pain behaviour in the MIA and MNX models.

To determine whether levels of BDNF and/or TrkB are altered in human OA samples.

4.2 Materials and Methods

General methods are provided in more detail in **Chapter 2**. All the experiments in this chapter utilised male Sprague Dawley rats (Charles River, Kent, UK).

4.2.1 Intra-articular injection of BDNF in naïve rats.

Twenty-eight male Sprague Dawley rats weighing 175-199g were used. Rats were anaesthetised with isoflurane (**2.1**) before undergoing intra-articular injection (**2.3**) of either 10µg/50µl BDNF (n=7), 1µg/50µl BDNF (n=7), 100ng/50µl BDNF (n=6), or 50µl 0.9% saline (n=8). Weight bearing asymmetry and paw withdrawal threshold were measured 1, 3, and 6 hours after injection, and then subsequently measured 1 day, 4 days, and 7 days post-injection.

4.2.2 Intra-articular injection of BDNF in rats with MIA induced joint pain.

Thirty-two male Sprague Dawley rats weighing 175-199g were used. Rats were anaesthetised with isoflurane (**2.1**) before receiving intra-articular injections (**2.3**) of either 1mg/50µl MIA (n=16) or 50µl 0.9% saline (n=16). Weight bearing asymmetry and paw withdrawal thresholds were measured for 21 days post injection. At 21 days post MIA model induction, rats were anaesthetised again with isoflurane (**2.1**) before receiving intra-articular injections (**2.3**) of either 1µg/50µl BDNF (n=15), 1µg/50µl NGF (n=3), or 50µl 0.9% saline (n=14). Weight bearing asymmetry and paw withdrawal thresholds were then measured for 14 days post injection.

4.2.3 Intra-articular injection of TrkB/fc chimera in rats with MIA induced joint pain.

Thirty male Sprague Dawley rats underwent injection of either 1mg/50µl MIA (n=20) or 50µl 0.9% saline (n=10). When the model was established at 21 days post injection, rats were once again anaesthetised by isoflurane (**2.1**) and received

intra-articular injection of either 100ng/50µl TrkB/fc chimera (n=15) or 100ng/50µl human IgG (n=15). The final group sizes were therefore; MIA + TrkB/fc (n=10), MIA + IgG (n=5), saline + TrkB/fc (n=10), and saline + IgG (n=5). Weight bearing asymmetry and paw withdrawal thresholds were measured 1 hour and 3 hours post injection. Rats were then euthanised and tissues were collected.

To investigate the long term effects of a single intra-articular dose of TrkB/fc thirty male Sprague Dawley rats weighing 175-199g were used. Rats were anaesthetised with isoflurane (2.1) before receiving intra-articular injections (2.3) of either 1mg/50µl MIA (n=20) or 50µl 0.9% saline (n=10). Weight bearing asymmetry and paw withdrawal thresholds were then measured for 21 days post injection. At 21 days post MIA model induction, rats were anaesthetised again with isoflurane (2.1) before receiving intra-articular injections (2.3) of either 100ng/50µl TrkB/fc chimera (n=15) or 100ng/50µl human IgG (n=15). Weight bearing asymmetry and paw withdrawal thresholds were measured at 1 hour, 3 hours, 24 hours, 1 week and 2 weeks post injection.

4.2.4 Intra-articular injection of TrkB/fc chimera in rats with MNX induced joint pain.

Thirty male Sprague Dawley rats weighing 175-199g were used for this experiment. Rats were anaesthetised with isoflurane (2.1) before undergoing either transection of the medial meniscal (MNX) surgery (n=20) or sham surgery (n=10) (2.2.4). Weight bearing asymmetry and paw withdrawal thresholds were measured for 21 days post injection. Rats were then anaesthetised again before

undergoing intra-articular injection of either 100ng/50µl TrkB/fc chimera (n=15) or 100ng/50µl human IgG (n=15). The final group sizes were; MNX + TrkB/fc (n=10), MNX + IgG (n=5), sham + TrkB/fc (n=10), and sham + IgG (n=5). Weight bearing asymmetry and paw withdrawal thresholds were then measured for 3 hours post injection. Rats were then euthanised before tissues were collected (**2.5**).

4.2.5 Macroscopic scoring of joint pathology

Knee joints were collected post-mortem from rats 21 days following either injection of 1mg MIA (n=20), injection of 50µl saline (n=10), meniscal transection surgery (n=20), or sham surgery (n=10). Knee joints were disarticulated as described in **2.6.4**. The following compartments were then scored for signs of chondropathy; medial tibial plateau, lateral tibial plateau, medial femoral condyle, lateral femoral condyle, and the femoral groove (**2.6.4**). The sums of these individual scores were taken to give each a total chondropathy score for each knee joint on a scale of 0-20.

4.2.6 ELISA analysis

It was important to make sure that the ELISA did not come up with false positives by detecting the TrkB/fc and BDNF complex. In order to investigate this increasing concentrations (0.01x, 0.1x, 1x, 10x, & 100x of the concentration of BDNF) of TrkB/fc chimera were added to a standard curve of BDNF before being measured with the ELISA.

BDNF expression in the plasma was also measured to compare the MIA model in the rat to previous clinical findings (Simao *et*

al, 2014). Plasma was collected from rats immediately following euthanasia by intra-peritoneal injection of sodium pentobarbital. These samples were diluted 1:2 before BDNF concentration was measured using an ELISA kit.

Synovial fluid was collected by joint wicking, whereby discs of protein saver card were placed in the knee joint post mortem to absorb the synovial fluid. These discs were then placed in 100ul of distilled water in order to elute out the proteins stored in the discs for 24 hours. The eluted fluid was then diluted 1:2 before BDNF concentration was measured using an ELISA kit.

4.2.7 Gene Expression

Gene expression as measured by qRT-PCR was used to investigate a potential relationship between inflammation and BDNF in human synovium. Synovial tissue samples from 16 patients (10 men, 6 women, median age 72 [interquartile range 64-74 year]) with OA of the knee were taken during total knee replacement (**Appendix 7.3**). Sections 4µm in depth were taken from each of the samples and then stained with H&E. Synovitis was measured according to the following scale: 0 = normal (synovial lining <4 cells thick, sparse, cellular distribution, with few or no inflammatory cells), 1 = mild inflammation (synovial lining 4 or 5 cells thick, increased cellularity with some inflammatory cells), 2 = moderate inflammation (synovial lining 6 or 7 cells thick, dense cellularity with inflammatory cells but not lymphoid aggregates), 3 = severe inflammation (synovial lining > 7 cells thick, dense cellularity and inflammatory cell infiltration, may contain perivascular lymphoid aggregates) (Haywood *et*

al, 2003). The two groups investigated were a low inflammation group (a score of 1) and a high inflammation group (a score of 3). Samples were gender matched and the ages between each pair of samples was ± 10 years. RNA was extracted and cDNA synthesis was completed as previously described (**2.8.2**). The target genes of interest were IL-6 and TNF- α as positive controls, and BDNF and the TrkB gene, NTRK2. Taqman qRT-PCR was performed as previously described (**2.8.3**). Data are presented as a ratio of gene expression with reference to β -actin.

4.2.8 Data Analysis

Data were analysed and then graphically presented using Prism V.7 (Graphpad, San Diego, California, US). Data were tested for normal distribution using the D'agostino and Pearson normality test, if data were normally distributed then parametric analyses were used and if data were found to be not normally distributed then non-parametric analyses were used. Weight bearing data are presented as the weight borne on the contralateral limb minus the weight borne on the ipsilateral limb divided by the total weight borne and multiplied by 100 (mean $\pm$ SEM). Paw withdrawal threshold is presented as the mean threshold $\pm$ SEM. The differences in both weight bearing asymmetry and paw withdrawal threshold between MIA/MNX and sham animals over time was assessed by 2-way ANOVA, with the model induction condition as the between-subjects factor and time post model induction as the within-subjects factor. Multiple comparisons between different treatment groups at different time points was done using Bonferroni's multiple comparisons test. Differences between the macroscopic scores of cartilage damage between

MIA/MNX and sham rats were tested by unpaired t-test. An unpaired t-test was also used to test differences in the levels of BDNF in plasma and synovial fluid between MIA and saline injected animals. Differences in the expression of IL-6 and NTRK2 mRNA between the low inflammation and high inflammation groups of human synovia were also analysed by unpaired t-test.

4.3 Results

4.3.1 Intra-articular injection of BDNF in naïve rats.

Intra-articular injection of BDNF (100ng/50µl, 1µg/50µl, 10µg/50µl) in naïve rats did not significantly alter weight bearing asymmetry **Fig. 4.1.A** or hindpaw withdrawal thresholds (**Fig. 4.1.B**). For weightbearing asymmetry two-way ANOVA with repeated measures showed significant difference over time ($F_{(6, 144)} = 3.281, p = 0.0047$), but not between groups ($F_{(3, 24)} = 1.644, p = 0.2057$). There was no significant interaction between these two variables ($F_{(18, 144)} = 0.7586, p = 0.7448$). For the ipsilateral hindpaw withdrawal thresholds two-way ANOVA with repeated measures showed no significant difference over time ($F_{(6, 144)} = 1.623, p = 0.14$), or between groups ($F_{(3, 24)} = 1.309, p = 0.29$). There was also no significant difference between groups over time ($F_{(18, 144)} = 1.141, p = 0.319$). There was a modest increase in weight bearing asymmetry at 1 hour post injection following injection of 1µg BDNF ($10.6 \pm 3.2\%$ increase), however this was not significant when compared to injection of 0.9% saline ($4.27 \pm 2.2\%$ increase). There was also a modest increase in weight bearing asymmetry 24 hours following injection of 10µg of BDNF ($8.7 \pm 4.6\%$ increase), but this was not significant when compared to the saline control ($2.2 \pm 1.1\%$

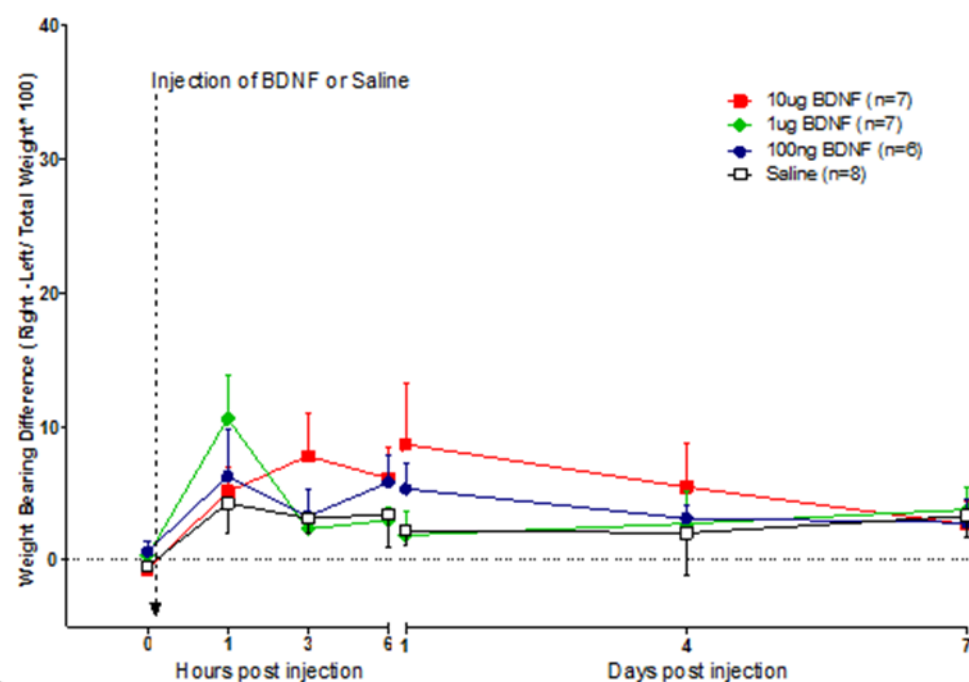
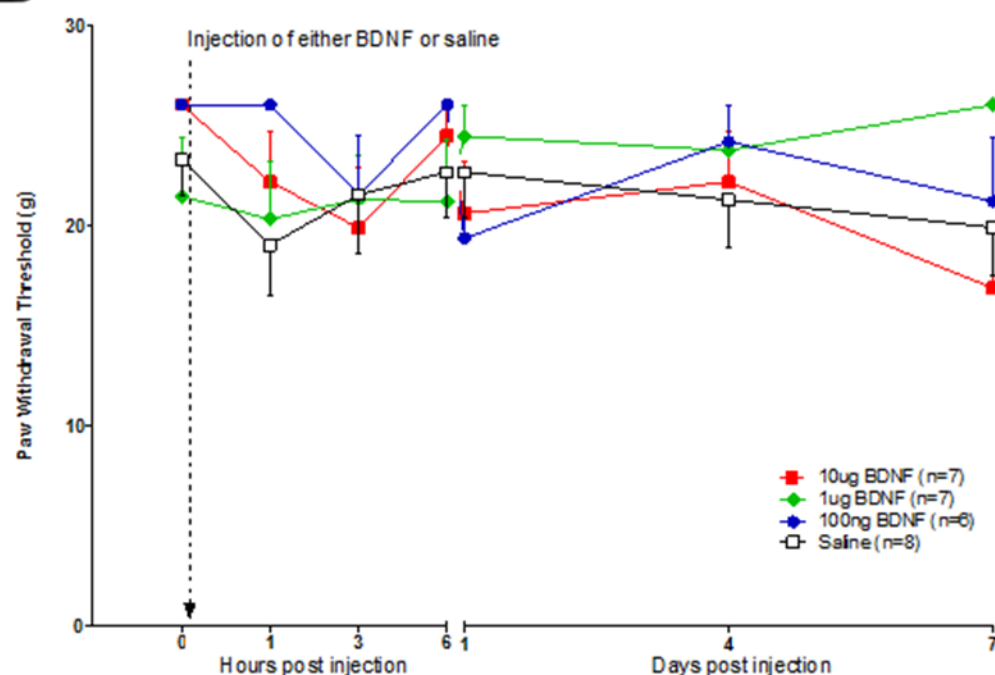
A**B**

Figure 4.1: Intra-articular injection of BDNF in naïve rats: Adult male Sprague Dawley rats were injected with 10 μ g BDNF (n=7), 1 μ g BDNF (n=7), 100ng BDNF (n=6) or 0.9% saline (n=8) into the knee joint capsule. **Fig. 4.1.A** shows the weight-bearing asymmetry following intra-articular injection. **Fig. 4.1.B** shows changes in the paw withdrawal threshold of the ipsilateral hindpaw following injections. Data analysed by 2 way ANOVA.

increase). Unsurprisingly, there were no significant changes in the contralateral hindpaws of rats following intra-articular injection of BDNF or saline into the ipsilateral knee (data not shown).

4.3.2 Augmented pain behaviour in response to intra-articular BDNF in rats with MIA induced OA.

Following intra-articular administration of 1µg BDNF in rats 21 days post MIA injection there appears to be a further increase in weight bearing asymmetry and a further decrease in paw withdrawal threshold. Two-way ANOVA with repeated measures showed no significant difference over time ($F_{(3, 81)} = 0.04579$, $p = 0.7124$), but did show a significant difference between groups ($F_{(4, 27)} = 7.894$, $p = 0.0002$) following intra-articular injection of either BDNF, NGF, or saline. There was no significant interaction between weight bearing asymmetry in each group over time. Looking at the data it appears that there is an increase in weight bearing asymmetry in MIA rats injected with BDNF when compared to saline injected controls. Multiple comparisons between each of the groups at the timepoints after injection show some significant difference between groups at different timepoints. Five days following BDNF administration weight bearing asymmetry (**Fig 4.2.A**) was significantly augmented ($30.6 \pm 5.3\%$, $p = 0.0163$) in rats with MIA induced OA, compared to injection of saline in rats with MIA induced OA ($10.9 \pm 6.3\%$). Weight bearing asymmetry was still increased 14 days post injection of BDNF, compared to injection of saline in rats with MIA induced OA. Ipsilateral paw withdrawal thresholds (**Fig. 4.2.B**) appeared lower 24 hours post injection of BDNF, compared to saline injected animals. Paw withdrawal thresholds remained lower in

BDNF injected rats for 14 days, compared with saline injected rats. Two-way ANOVA with repeated measures showed no significant difference over time ($F_{(3, 81)} = 1.943$, $p = 0.129$), but did show differences between groups ($F_{(4, 27)} = 8.777$, $p < 0.0001$) following the intra-articular injection of either BDNF, NGF, or saline. The interaction between these two variables was not found to be significant ($F_{(12, 81)} = 0.5186$, $p = 0.8969$). Area under the curve analysis (**Fig. 4.2.C**) of paw withdrawal thresholds over the 14 days post injection revealed that BDNF injected rats and NGF injected rats had significantly lower paw withdrawal thresholds

compared to saline injected animals. As a positive control intra-articular injection of NGF in rats with MIA induced OA also appeared to augment weight bearing asymmetry, compared to saline injected rats (**Fig 4.2.A**).

There was no change in contralateral paw withdrawal thresholds following intra-articular injection of BDNF or saline at any time point (data not shown).

4.3.3 Intra-articular administration of TrkB/fc acutely reversed MIA induced joint pain.

Intra-articular injection of TrkB/fc chimera acutely reversed weight bearing asymmetry and paw withdrawal thresholds in animals with MIA induced joint pain (**Fig. 4.3.A, B**). Two-way ANOVA with repeated measures showed no significant difference over time ($F_{(2, 52)} = 1.278$, $p = 0.29$) but did show a significant difference between groups ($F_{(3, 26)} = 10.67$, $p = < 0.0001$) following injection of either TrkB/fc or IgG control. However, there was a non-significant interaction between these variables ($F_{(6, 52)} = 1.551$, $p = 0.1805$). A multiple comparisons test analysed differences between the MIA + TrkB/fc group and the MIA + IgG group post injection. At one

hour post TrkB/fc injection weight bearing asymmetry was significantly decreased ($3.7 \pm 1.8\%$, $p = 0.0161$), compared to IgG injected rats ($15.8 \pm 3.8\%$). This significant effect was maintained at 3 hours post injection (**Fig. 4.3.A**). Ipsilateral hindpaw withdrawal thresholds (**Fig. 4.3.B**) were increased at 1 hour and 3 hours post-injection of TrkB/fc. Two-way ANOVA with repeated measures showed no significant difference over time ($F_{(2, 52)} = 0.8523$, $p = 0.43$), but did show a significant difference between groups ($F_{(3, 26)} = 8.546$, $p = 0.0004$). And there was a significant difference between groups over time ($F_{(6, 52)} = 3.576$, $p = 0.0292$). Area under the curve analysis showed that over the behavioural timecourse there was a significant decrease in ipsilateral paw withdrawal thresholds following intra-articular administration of TrkB/fc (**Fig. 4.6.A**). Contralateral paw withdrawal thresholds were not significantly altered by TrkB/fc at any time point (data not shown).

The next study investigated potential prolonged effects of a single intra-articular injection of TrkB/fc. Two-way ANOVA with repeated measures showed no significant difference in weight bearing difference over time ($F_{(6, 156)} = 0.5434$, $p = 0.77$), but did show a significant difference between groups ($F_{(3, 26)} = 33.43$, $p = <0.0001$). However the interaction between these two variables was not significant ($F_{(18, 156)} = 1.169$, $p = 0.2935$)(**Fig 4.4.A**). One hour after injection of TrkB/fc chimera there looks like there is a reduction in the weight bearing asymmetry in rats. Three hours post TrkB/fc injection there appeared to be a difference between the MIA + IgG injected rats and the MIA + TrkB/fc injected rats. There appears to be little difference between TrkB/fc treated MIA rats and IgG treated MIA rats from 6 hours post injection to two weeks post injection.

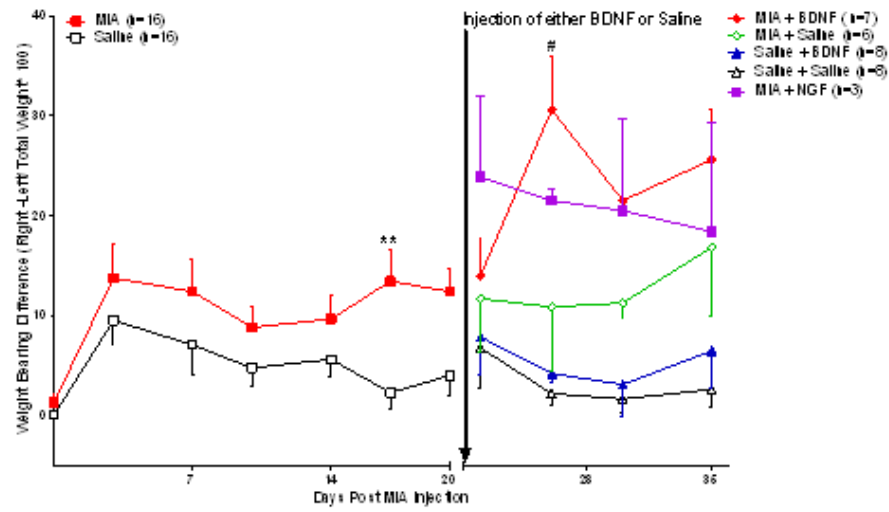
The MIA-induced decrease in the ipsilateral hindpaw withdrawal thresholds were reversed following intra-articular injection of TrkB/fc (**Fig. 4.4.B**). Two-way ANOVA with repeated measures showed significant difference over time ($F_{(6, 156)} = 4.556$, $p = 0.0003$), and between groups ($F_{(3, 26)} = 20.35$, $p = <0.0001$). The interaction between these variables was also significant ($F_{(18, 156)} = 1.752$, $p = 0.0359$). Multiple comparisons between groups at individual timepoints suggest that there was a significantly increased ipsilateral paw withdrawal threshold in MIA rats injected with TrkB/fc (16.7 ± 2.3 g, $p = 0.0092$), compared to rats injected with IgG (8.5 ± 0.9 g) at one hour post injection. Again, this effect was transient and by 24 hours the ipsilateral paw withdrawal thresholds had returned to lower levels consistent with the MIA IgG treated rats (7 ± 0.6 g).

4.3.4 Intra-articular administration of TrkB/fc acutely reversed MNX induced joint pain.

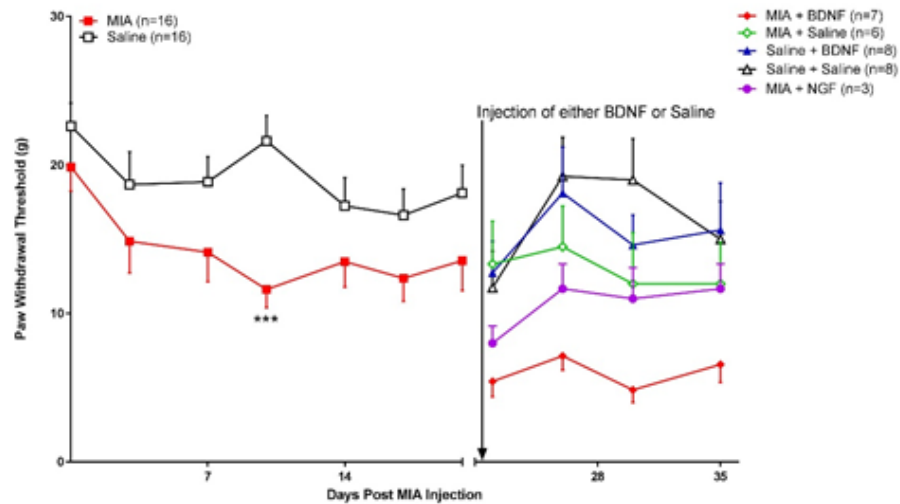
Weight bearing asymmetry was increased in rats that underwent meniscal transection (MNX) from 14 days post-surgery compared to sham operated animals (**Fig. 4.5.A**). The ipsilateral hindpaw withdrawal thresholds were also lowered in the MNX rats 14 days post-surgery (**Fig. 4.5.B**).

Intra-articular injection of TrkB/fc chimera 21 days post MNX surgery decreased weight bearing asymmetry. Two-way ANOVA with repeated measures showed no significant difference over time ($F_{(2, 52)} = 0.1588$, $p = 0.85$), but did show a significant difference between groups ($F_{(3, 26)} = 15.76$, $p = <0.0001$) following intra-articular injection of TrkB/fc or IgG control. However, there was no significant interaction between these variables ($F_{(6, 52)} = 1.653$, $p = 0.1515$). It appears that there is a slight decrease in weight bearing asymmetry in rats

A



B



C

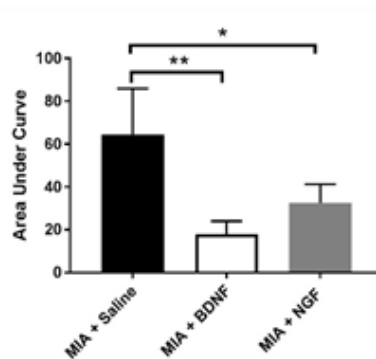


Figure 4.2: Intra-articular injection of BDNF in rats with MIA induced OA: Adult male Sprague Dawley rats were injected with 1µg/50µl BDNF, 1µg/50µl NGF, or 50µl saline 21 days after induction of the MIA model. **Fig. 4.2.A** shows the weight-bearing asymmetry following BDNF or NGF administration. 2 way ANOVA w/ Bonferroni multiple comparisons test. **= $p < 0.01$ MIA vs Sal, #= $p < 0.05$ MIA + BDNF vs MIA + Sal. **Fig. 4.2.B** shows changes in the paw withdrawal threshold of the ipsilateral hindpaw following BDNF or NGF administration. 2 way ANOVA w/ Bonferroni correction. *= $p < 0.05$ MIA vs Sal. **Fig. 4.2.C** shows the area under the curve for the paw withdrawal thresholds post BDNF and NGF administration. Unpaired t-test, **= $p < 0.01$ MIA+BDNF vs MIA+Sal, *= $p < 0.05$ MIA+NGF vs MIA+Sal.

with MNX induced OA one hour post TrkB/fc injection compared to IgG injection (**Fig. 4.5.A**). Three hours post TrkB/fc injection weight bearing asymmetry was significantly decreased ($4.6 \pm 2.8\%$, $p = 0.0038$) when compared to IgG injection ($17.6 \pm 4.4\%$) in MNX rats.

Two-way ANOVA with repeated measures showed significant difference in ipsilateral hindpaw withdrawal thresholds over time ($F_{(2, 52)} = 3.204$, $p = 0.0487$), but not between groups ($F_{(3, 26)} = 2.747$, $p = 0.063$). (**Fig. 4.5.B**). There was no significant interaction between these two variables ($F_{(6, 52)} = 1.585$, $p = 0.1703$). Area under the curve analysis of ipsilateral paw withdrawal threshold over the behavioural timecourse showed there was no significant difference in rats treated with IgG and TrkB/fc (**Fig. 4.6.B**).

Contralateral paw withdrawal thresholds in MNX rats were once again not changed following any intervention at any time point (data not shown).

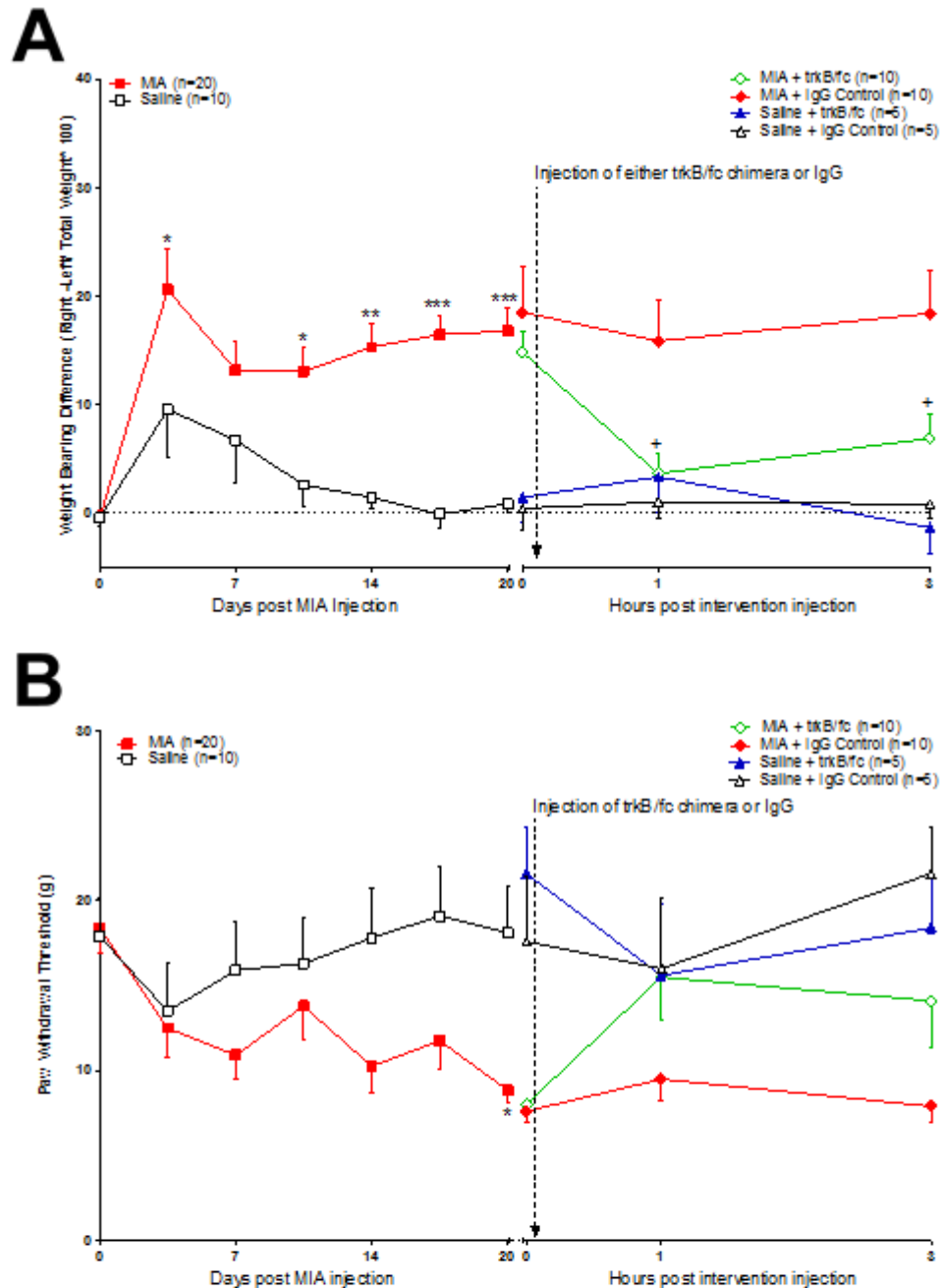
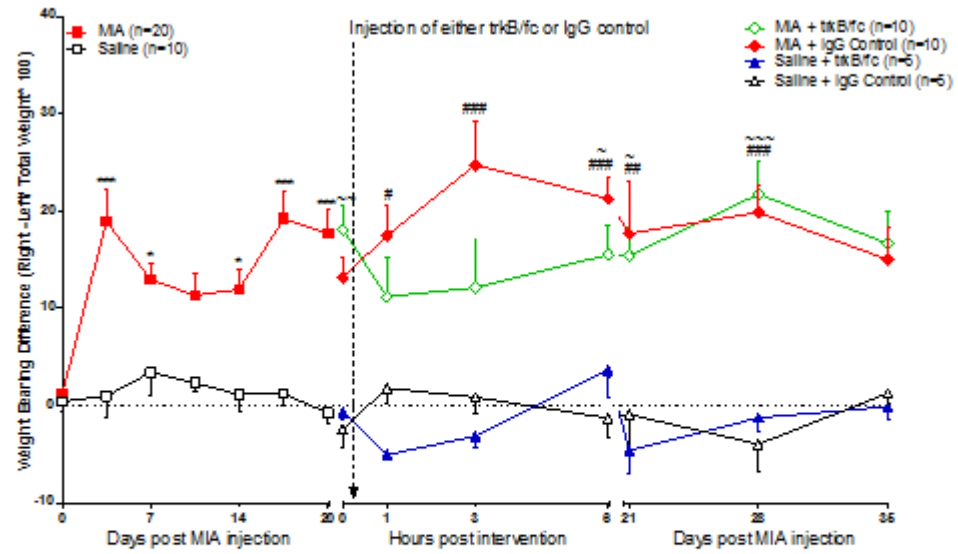


Figure 4.3: Acute effects of intra-articular injection of TrkB/fc in rats with MIA induced OA: Adult male Sprague Dawley rats were injected with 100ng/50µl TrkB/fc or 100ng/50µl human IgG 21 days after induction of the MIA model. **Fig. 4.3.A** shows the weight-bearing asymmetry following TrkB/fc administration. 2 way ANOVA w/ Bonferroni correction. *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$ MIA vs Sal, += $p < 0.05$, MIA + TrkB/fc vs MIA + IgG. **Fig. 4.3.B** shows changes in the paw withdrawal threshold of the ipsilateral hindpaw following TrkB/fc administration. 2 way ANOVA w/ Bonferroni correction. *= $p < 0.05$ MIA vs Sal

A



B

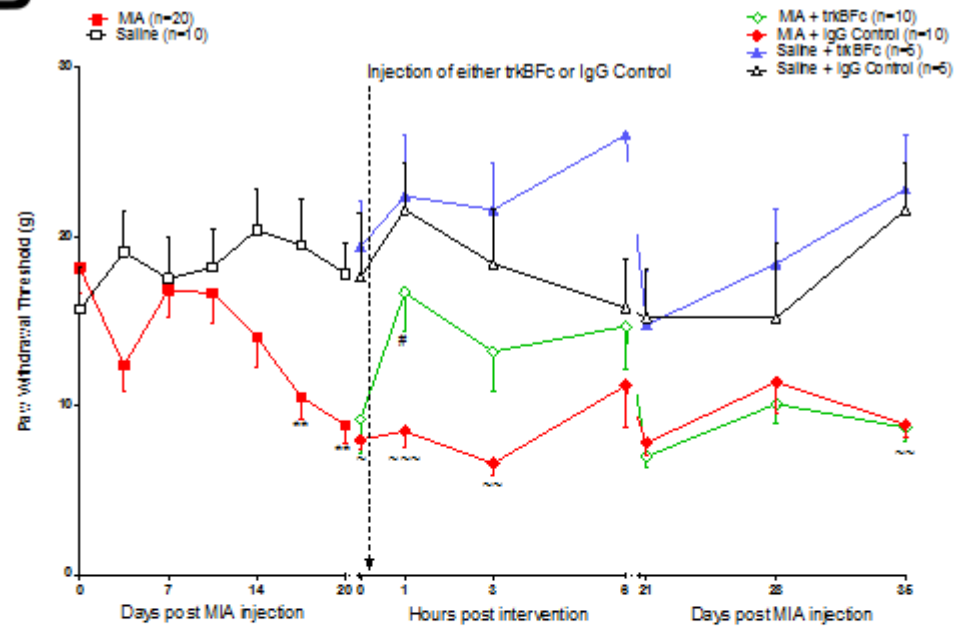


Figure 4.4: Intra-articular injection of TrkB/fc in rats with MIA induced OA: Adult male Sprague Dawley rats were injected with 100ng/50µl TrkB/fc or 100ng/50µl human IgG 21 days after induction of the MIA model. Pain behaviour was measured for 2 weeks post injection. **Fig. 4.4.A** shows the weight-bearing asymmetry following TrkB/fc administration. 2 way ANOVA w/ Bonferroni correction. *= $p<0.05$, **= $p<0.001$ MIA vs Sal, #= $p<0.05$, ##= $p<0.01$, ###= $p<0.001$, MIA + IgG vs Sal + IgG, ~= $p<0.05$, ~~~= $p<0.001$, MIA + TrkB/fc vs Sal + IgG. **Fig. 4.4.B** shows changes in the paw withdrawal threshold of the ipsilateral hindpaw following TrkB/fc administration. 2 way ANOVA w/ Bonferroni correction. **= $p<0.01$ MIA vs Sal, #= $p<0.05$ MIA + TrkB/fc vs MIA + IgG, ~= $p<0.05$, ~~~= $p<0.001$, MIA + IgG vs Sal + IgG.

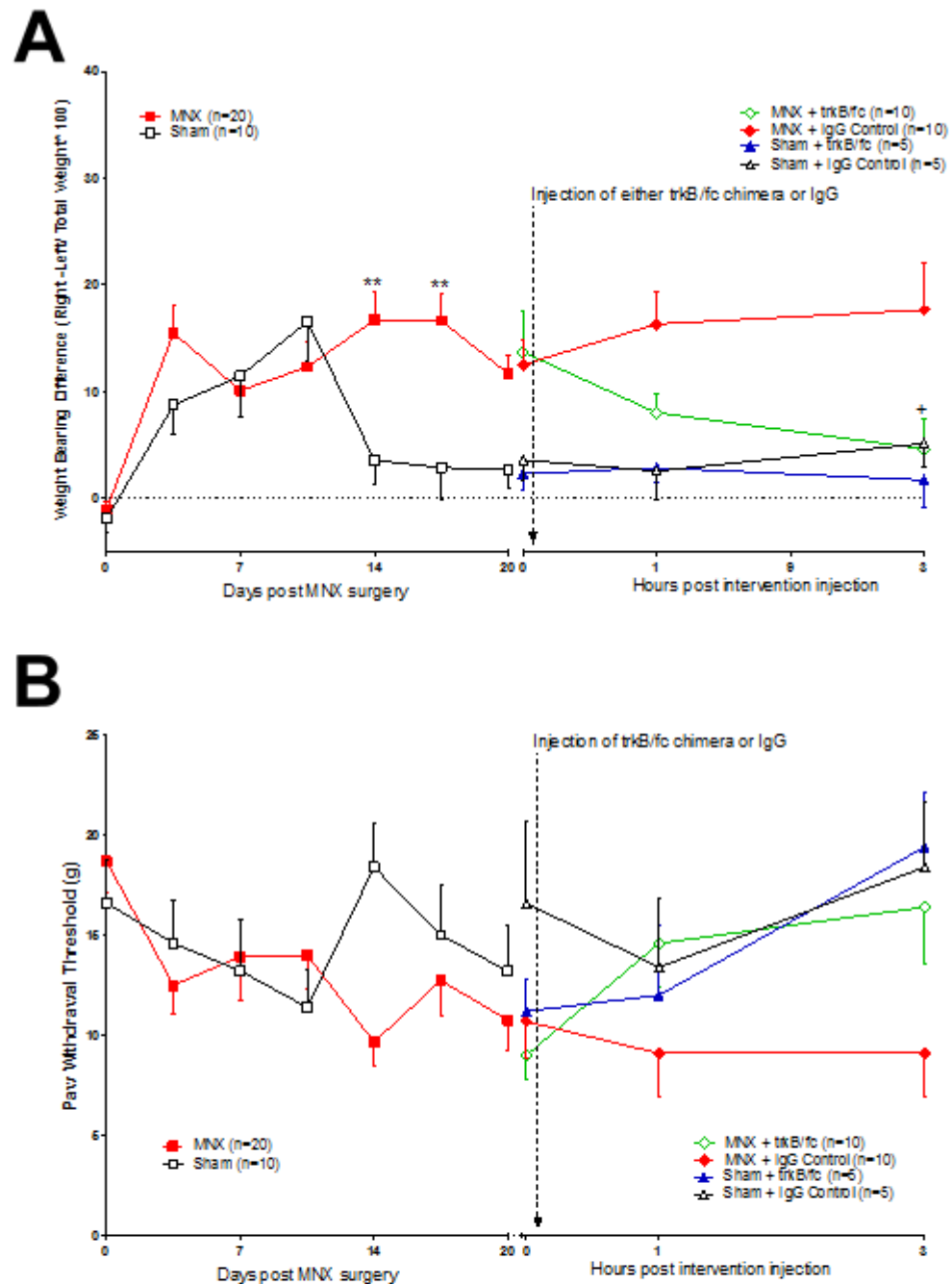


Figure 4.5: Acute effects of intra-articular injection of TrkB/fc in rats with MNX induced OA: Adult male Sprague Dawley rats were injected with 100ng/50 μ l TrkB/fc or 100ng/50 μ l human IgG 21 days after induction of the MNX model. **Fig. 4.5.A** shows the weight-bearing asymmetry following TrkB/fc administration. 2 way ANOVA w/ Bonferroni correction, **= $p < 0.01$ MIA vs Sal, += $p < 0.05$, MIA + TrkB/fc vs MIA + IgG. **Fig. 4.5.B** shows changes in the paw withdrawal threshold of the ipsilateral hindpaw following TrkB/fc administration. 2 way ANOVA w/ Bonferroni correction. $P = n.s$

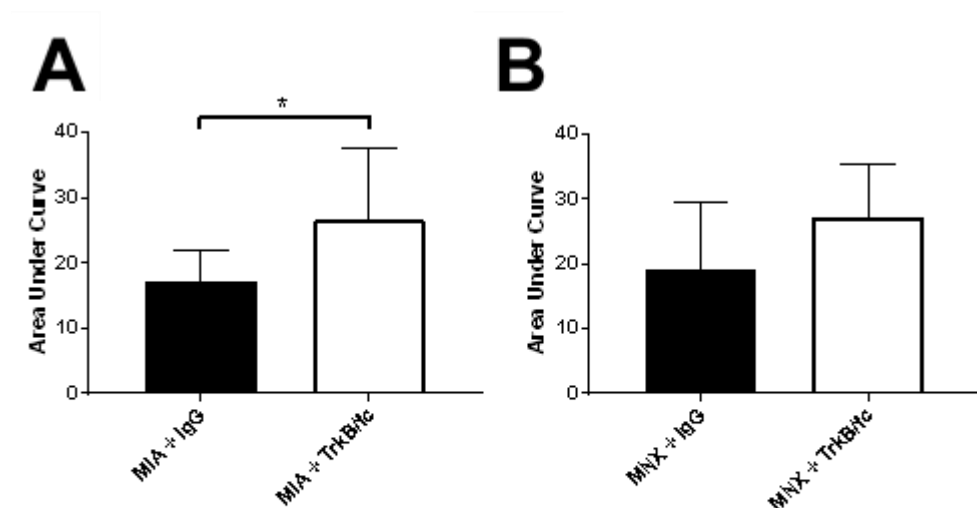


Figure 4.6: Area under the curve analysis of changes in ipsilateral hindpaw withdrawal threshold following intra-articular injection of 100ng/50 μ l TrkB/fc chimera in rats with MIA (**Fig. 4.6.A**) or MNX (**Fig. 4.6.B**) induced joint pain. * = $p < 0.05$ TrkB/fc vs IgG following unpaired t-test

4.3.5 Significant chondropathy in MIA and MNX rats

Twenty-one days following intra-articular injection of MIA significant chondropathy of the ipsilateral knee joint was evident, compared to saline injected rats (**Fig. 4.7.A**). There was a similarly significant chondropathy in the ipsilateral knee joint of rats 21 days following MNX surgery when compared to sham operated rats (**Fig. 4.7.C**.)

4.3.6 ELISA measurement of BDNF and effect of TrkB/fc

An experiment to ensure the ELISA kit would not return false positives by detecting a TrkB/fc BDNF complex was carried out. When increasing concentrations of TrkB/fc were added to a known quantity of BDNF and analysed by ELISA, there was a TrkB/fc dependent decrease in the quantity of BDNF measured by the kit (**Fig. 4.8**). When 100x TrkB/fc was added to 192pg/ μ l of BDNF, BDNF was not detectable by the ELISA. At

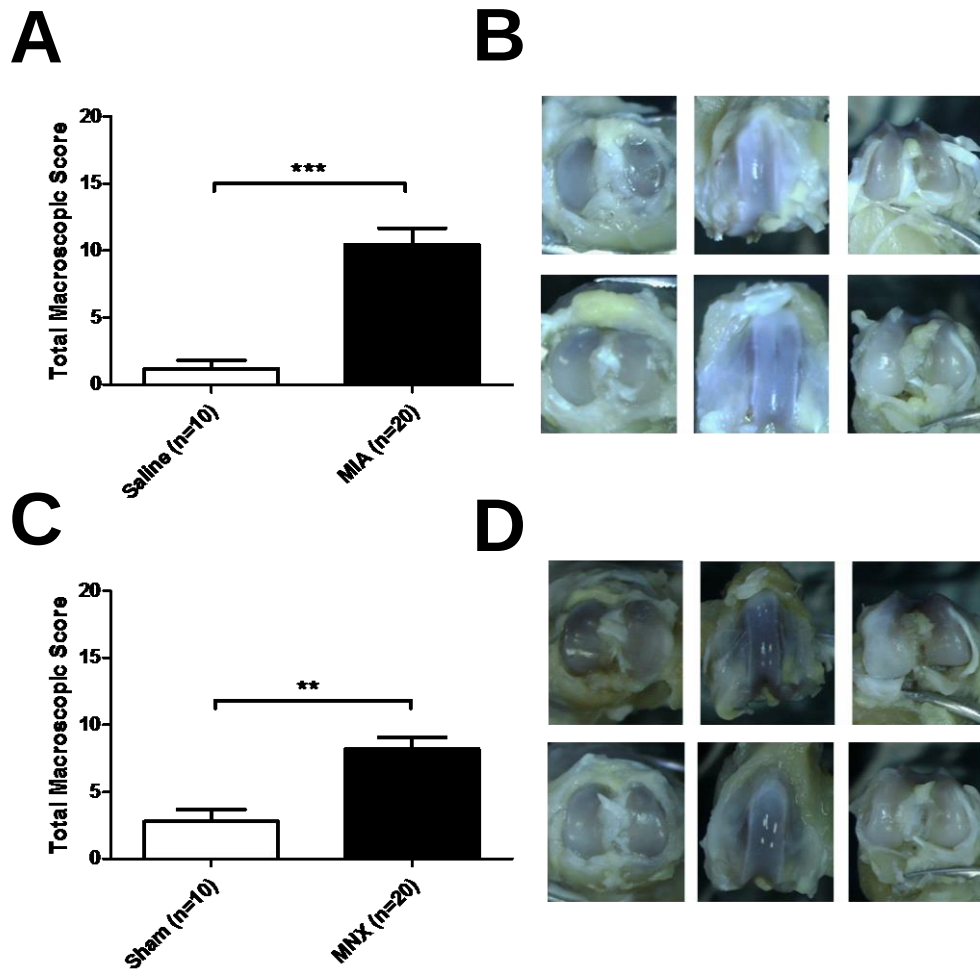


Figure 4.7: Chondropathy in rats with MIA and MNX induced OA: Ipsilateral knee joints from adult male Sprague Dawley rats 21 days following MIA injection (n=20), saline injection (n=10), MNX surgery (n=20), and sham surgery (n=10) were macroscopically scored for cartilage damage. **Fig. 4.7.A** shows the difference in total chondropathy score between MIA and saline treated rats. Data analysed by unpaired t-test, ***=p<0.001 MIA vs saline. **Fig. 4.7.B** shows representative images of the different compartments of the knee following MIA injection (Top row from L-R; tibial plateaus, femoral groove, and femoral condyles) or saline injection (Bottom row from L-R; tibial plateaus, femoral groove, and femoral condyles). **Fig. 4.7.C** shows the difference in total chondropathy score between MNX and sham treated rats. Data analysed by unpaired t-test, **=p<0.01 MIA vs saline. **Fig. 4.7.D** shows representative images of the different compartments of the knee following MNX surgery (Top row from L-R; tibial plateaus, femoral groove, and femoral condyles) or sham surgery (Bottom row from L-R; tibial plateaus, femoral groove, and femoral condyles).

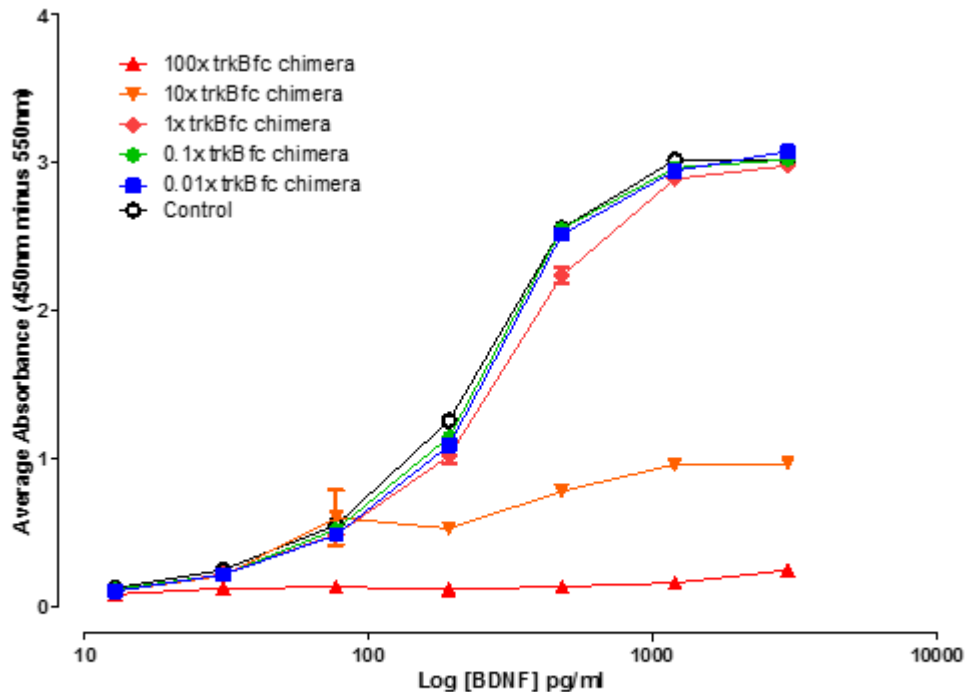


Figure 4.8: The effect of adding different concentrations (100x, 10x, 1x, 0.1x, 0.01x) of TrkB/fc chimera to a standard curve of BDNF as measured by ELISA.

10x TrkB/fc the measured concentration of BDNF was 96.5pg/ μ l, compared to the standard of 192pg/ μ l. At 1x TrkB/fc chimera the measured concentration of BDNF was 152.1pg/ μ l compared to the standard of 192pg/ μ l.

ELISA measurement of BDNF in rat plasma indicated no difference in the concentration of BDNF in MIA versus control rat plasma (**Fig. 4.9.B**). There was also no change in plasma BDNF concentration in rats treated with intra-articular injection of TrkB/fc chimera, compared to rats treated with vehicle.

ELISA analysis of BDNF in synovial was also performed. In control (non-arthritis) rats BDNF was detected in 5/ 10 synovial fluid samples for both groups (vehicle and TrkB/fc). BDNF was detected in 3/10 samples collected from MIA rats treated with intra-articular vehicle. Likewise BDNF was

detected in 3/10 samples taken from MIA rats treated with TrkB/fc. This means that any analysis of actual levels can only be considered pilot data. However, when the samples with detectable levels of BDNF were analysed there was a significantly higher concentration of BDNF in the MIA + IgG group, compared to the saline + IgG group (**Fig. 4.10.B**). There was no effect of the TrkB/fc treatment.

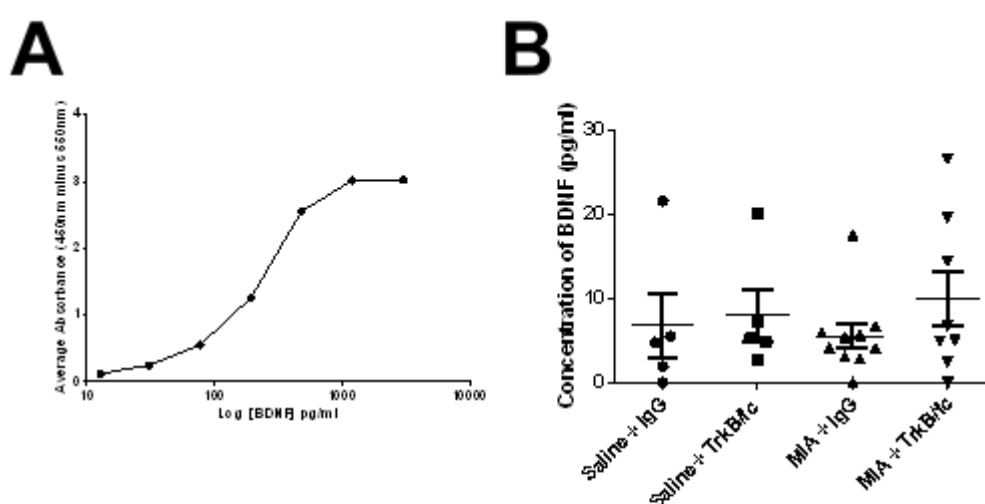


Figure 4.9: BDNF concentration in plasma following intra-articular injection of TrkB/fc chimera. **Fig. 4.9.A :** standard curve of absorbance values for known concentrations of BDNF. **Fig. 4.9.B.:** the plasma concentration of BDNF in MIA and saline treated rats following intra-articular injection of either IgG control or TrkB/fc chimera. There was no significant difference between any groups following unpaired t-test.

4.3.7 Gene Expression

To start the forward-translation of these data, the expression of four genes in human OA synovium was studied.

Unfortunately the gene expression of both BDNF and TNF- α were below the detection limit in the clinical samples obtained. However, both IL-6 and NTRK2 mRNA were quantified in the human synovial samples. IL-6 mRNA was detected in 4/8 samples in both the low inflammation group and the high inflammation group. There was a non-significant increase in IL-6 mRNA expression in the high inflammation group (**Fig.**

4.11.A). NTRK2 was detected in all of the high inflammation samples and 6/8 low inflammation samples. Again there was a non-significant increase in NTRK2 mRNA expression in the high inflammation group, compared to the low inflammation group (**Fig. 4.11.B**).

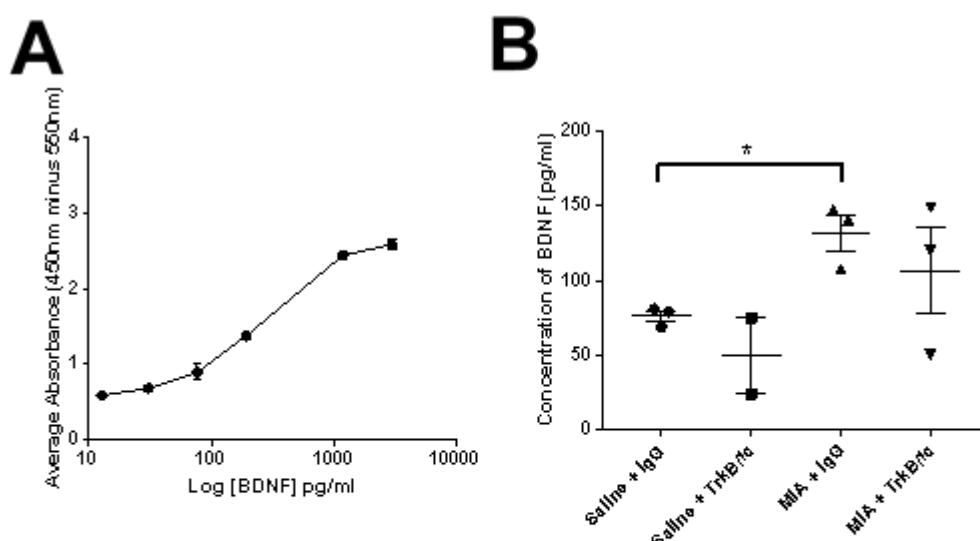


Figure 4.10: BDNF concentration in synovial fluid following intra-articular injection of TrkB/fc chimera. **Fig. 4.10.A** : the standard curve of absorbance values for known concentrations of BDNF. **Fig. 4.10.B.**: the concentration of BDNF in the synovial fluid of MIA and saline treated rats following intra-articular injection of either IgG control or TrkB/fc chimera. * = $P < 0.05$ between MIA + IgG and saline + IgG following unpaired t-test.

4.4 Discussion

4.4.1 Summary of major findings

There was no significant change in weight bearing asymmetry or ipsilateral hindpaw withdrawal threshold following intra-articular injection of BDNF in naive rats. Intra-articular injection of 1 μ g BDNF at 21 days post MIA induction resulted in a significant increase in weight bearing asymmetry and a decrease in the ipsilateral hindpaw withdrawal threshold. Intra-articular injection of TrkB/fc chimera, which is designed

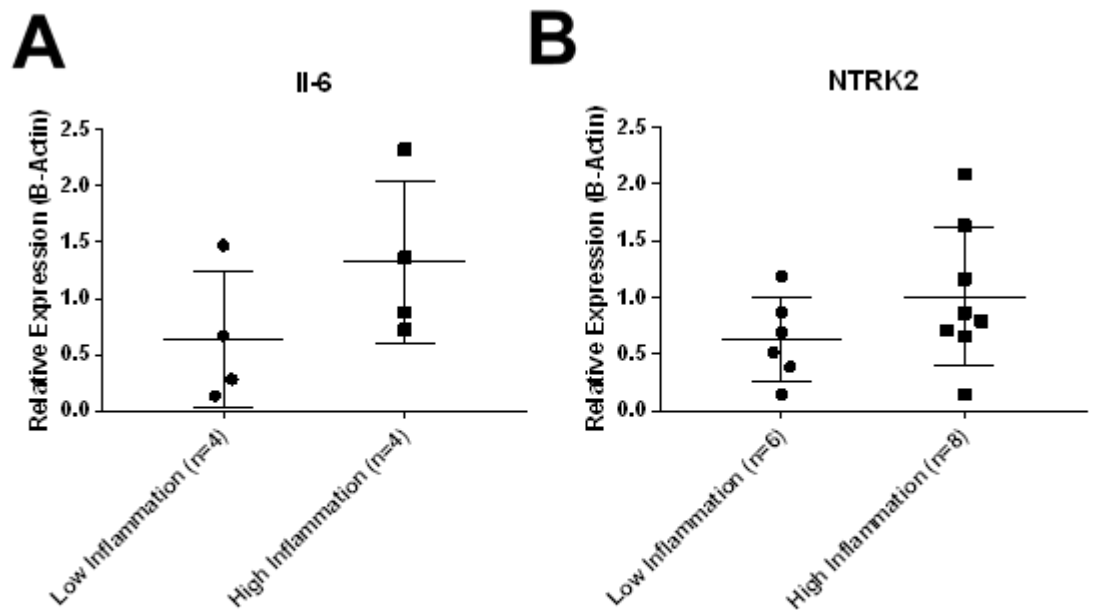


Figure 4.11: Expression of IL-6 (Fig. 4.11.A) and NTRK2 (Fig. 4.11.B) mRNA in the synovial tissue of human patients with either low synovial inflammation or high synovial inflammation. Data are presented as the ratio of gene expression with reference to β -actin.

to sequester endogenous BDNF, acutely reversed both MIA and MNX induced joint pain. Intra-articular injection of 100ng Trkb/fc chimera 21 days post MIA decreased weight bearing asymmetry and increased hindpaw withdrawal threshold within 3 hours of administration. By 24 hours post TrkB/fc chimera injection weight bearing asymmetry and paw withdrawal thresholds returned to pre-treatment levels. There was no further change in pain behaviour observed up to 2 weeks post-treatment. These findings were replicated in a surgical model of OA in the rat as well. The results of ELISA analysis of the synovial fluid were inconclusive. The levels of BDNF in the synovial fluid were too low to be consistently detected by ELISA. However, in the samples where BDNF was detected, there was a significant increase in the levels of BDNF in MIA injected rats compared to saline controls.

4.4.2 Expression of BDNF and TrkB in the osteoarthritic knee

BDNF has been shown to induce acute increases in pain behaviour in naïve mice when administered intrathecally (Groth & Aanonsen *et al*, 2002). However, we found there was no change in either weight bearing asymmetry or hind paw withdrawal threshold following intra-articular injection of BDNF in naïve rats. When BDNF was injected into the knee joints of rats with established chronic joint pain there was an augmented pain response. This suggests that there may be a change in the expression of TrkB in the osteoarthritic joint.

Expression of BDNF mRNA was found within synovial fluid taken from patients with OA (Barthel *et al*, 2009, Simao *et al*, 2014). A potential source for this BDNF could be synovial fibroblasts. Fibroblasts taken from the synovial fluid of patients with OA were found to release BDNF in the presence of ATP (Klein *et al*, 2012). In the synovial tissue of OA patients there is a substantial infiltration of CD68+ macrophages (Benito *et al*, 2005). There is also a significant increase in the numbers of activated macrophages in the knee joint of rats from 2 weeks post MIA injection (Piscaer *et al*, 2011). Both macrophages and monocytes have previously been found to express TrkB (Shibayama & Koizumi, 1996). BDNF has also been shown to be released by monocytes (Schulte-Herbruggen *et al*, 2005). This could therefore represent one source of increased TrkB in the osteoarthritic knee joint.

BDNF and TrkB could also be located in the neurons within the knee joint. BDNF has been found to be co-localised with CGRP in dorsal root neurons (Michaels *et al*, 1997). There is also co-

localisation of both BDNF and TrkB in dorsal root neurons (Salio *et al*, 2005). Around 33% of the neurons which innervate the knee joint express CGRP (Salo & Theriault, 1997). There is increased angiogenesis and subsequently increased innervation in the knee joints of patients with OA (Ashraf *et al*, 2010). The increased number of neurons within the knee joint during OA may also be a potential source of BDNF and TrkB.

4.4.3 The role of BDNF in mediating peripheral sensitivity

The contributions of endogenous BDNF to chronic joint pain remain unknown. Therefore, the final part of this chapter was to investigate the effects of removing endogenous BDNF from the knee joints of rats with both MIA and MNX induced OA. The chimeric compound TrkB/fc was chosen as the intervention as it has been shown to reduce levels of endogenous BDNF in models of neuropathic pain (Endo *et al*, 2009, Yajima *et al*, 2005). In both the MIA and MNX models of OA intra-articular injection of 100ng TrkB/fc chimera acutely reversed pain behaviour to a similar extent, suggesting that BDNF's contributions to joint pain are generalizable to the disease itself and not the method of induction.

The mechanism through which BDNF may mediate peripheral sensitization of the joint is still not known. In the central nervous system BDNF plays an important role in modulating and regulating neurotransmission (Merighi *et al*, 2008). It is thought that presynaptic TrkB in the spinal cord can regulate the release of glutamate, substance P, and CGRP (Merighi *et*

al, 2008). It is possible that the presence of BDNF in the periphery changes the excitability of primary afferent neurons, and therefore has a direct effect on pain processing and eventual pain behaviour.

There could be a link between peripheral inflammation and BDNF in OA. BDNF and TrkB are both expressed by macrophages (Schulte-Herbruggen *et al*, 2005, Shibayama & Koizumi, 1996). The release of BDNF from macrophages is enhanced by both IL-6 and TNF- α (Schulte-Herbruggen *et al*, 2005), cytokines which are known to be increased in OA. The acute effects of TrkB/fc in the models of OA may be attributed to a change in the low grade inflammatory response within the joint.

Another way in which BDNF has been shown to effect spinal nociception has been through the sensitization of NMDA receptors. Human articular chondrocytes express functional NMDA receptors, with OA chondrocytes expressing an increased variety of subunits when compared to normal chondrocytes (Ramage *et al*, 2008). Thus, an increase in BDNF in the joint may play a role in sensitizing chondrocyte NMDAR which could lead to further chondrocyte apoptosis. Interestingly, when NMDAR in the joint space was inhibited by intra-articular injection of magnesium sulfate there was a decrease in joint pain and pathology progression (Lee *et al*, 2009). By reducing the local levels of BDNF in the knee joint, TrkB/fc could potentially be reducing the sensitization of NMDAR in the joint. However, whether these mechanisms would lead to an acute effect on pain behaviour as seen in this chapter is unclear.

4.4.4 The role of BDNF in mediating central sensitivity

Although the effects of intra-articular injection of BDNF are reminiscent of the effects of intra-articular injection of NGF in MIA rats (Ashraf *et al*, 2014). There were differences in the effects of the two neurotrophins. NGF treatment resulted in an increase in weight bearing asymmetry in rats with MIA induced OA, but no change in hindpaw withdrawal threshold (Ashraf *et al*, 2014). However, following intra-articular injection of BDNF in MIA rats both weight bearing asymmetry was increased and hindpaw withdrawal thresholds were lowered. Since lowered hindpaw withdrawal threshold is a measure of central sensitization these results suggest that localised injection of BDNF into the knee joint may also modulate the central processing of pain. The mechanisms underlying these effects are unknown and it may be insightful to consider the known effects of BDNF on excitability in the spinal cord.

Under inflammatory conditions BDNF induced enhancement of the excitatory postsynaptic current (EPSC) in substantia gelatinosa neurones in the spinal cord is blocked by the Trk inhibitor K252a (Matayoshi *et al*, 2005). The BDNF induced enhancement of the EPSC was not affected by TTX but was blocked by lidocaine (Matayoshi *et al*, 2005). This suggests that BDNF increases glutamatergic transmission in the spinal cord via TrkB activation of lidocaine sensitive, TTX resistant Na⁺ channels. Following inflammation there is down-regulation of potassium-chloride co-transporter 2 (KCC2) (Zhang *et al*, 2008). The Trk inhibitor K252a reduced the inflammation induced decrease in KCC2 (Zhang *et al*, 2008). This suggests that BDNF can also modulate synaptic transmission through altering intracellular calcium levels.

Clearly both of these mechanisms may occur at the peripheral terminals of the sensory nerves in the models of joint pain, but further studies are required to demonstrate this.

4.4.5 The potential role of p75 NTR

Although my data with the TrkBfc chimera point to a role for TrkB in mediating the effects of BDNF, there is the potential that BDNF is exerting its effects through the less specific but lower affinity receptor p75 (Chao & Hempstead, 1995). The p75 receptor is also thought to be implicated in the processing of pain. In patients with the painful neuropathies Churg-Strauss syndrome and polyarteritis nodosa there were increased levels of p75 mRNA (Yamamoto *et al*, 2005). There was also a strong positive correlation between p75 mRNA and NGF mRNA in these disorders (Yamamoto *et al*, 2005). In p75 knockout mice there were increased thresholds to both thermal and mechanical noxious stimuli when compared to the wild type mice (Bergmann *et al*, 1998). However, administration of NGF still evoked both thermal and mechanical hyperalgesia, indicating that p75 is not solely responsible for NGF mediated nociception (Bergmann *et al*, 1998). In the nerve crush model of neuropathic pain, anti-p75 intervention was seen to partially reduce mechanical allodynia (Fukai *et al*, 2010). In another model of neuropathic pain, the spinal nerve ligation model, anti-p75 reduced both heat and cold hyperalgesia (Obata *et al*, 2006). There was also an increase in p75 immunoreactivity in the intact spinal nerve following the SNL procedure (Obata *et al*, 2006).

One mechanism through which p75 NTR might mediate hyperalgesia is through the regulation of bradykinin binding sites. In p75^{-/-} mice there was a reduction in NGF induced bradykinin upregulation when compared to wild type (Petersen *et al*, 1998). The administration of anti-p75 in wild type mice also had the same effect in reducing NGF induced bradykinin upregulation (Petersen *et al*, 1998). There is a lack of information regarding the expression of p75 in both healthy and osteoarthritic knee joints. However, there is the potential that NGF or BDNF exert their effects through this non-specific receptor in the knee joint.

BDNF is also not the only neurotrophin which binds to TrkB, the neurotrophins NT3 and NT4 also bind to the receptor (Chao & Hempstead, 1995). Therefore, there is the possibility that the intra-articular injection of TrkB/fc chimera was actually sequestering these alternative neurotrophic factors, rather than BDNF. There is a lack of information about the expression and localisation of these neurotrophins, NT3 and NT4, in the OA knee joint. Unfortunately, the results of the ELISA analysis of synovial fluid did not provide strong enough evidence that TrkB/fc lowered synovial levels of BDNF. However, this is likely to be because the method of collecting synovial fluid was diluting the sample too much to be detected by the ELISA kit.

4.4.6 Future Directions

Considering the augmented effect of BDNF administration in the MIA model of OA, priority should be given to experiments which investigate this apparent increased sensitivity to the

neurotrophin in established OA. It would be interesting to see if there was increased expression of BDNF's high affinity receptor TrkB in the joint, the DRG, and the superficial dorsal horn in rat models of OA. The results of the ELISA tests for BDNF in the synovial fluid were inconclusive; this may be due to the method used to collect the synovial fluid. During the extraction of the synovial fluid from the protein saver card the sample may have been diluted too much for use in the ELISA. Current research has shown that the magnetic capture of biomarkers in the synovium of rats is possible with the use of magnetic nano-particles bound to antibodies (Yarmola *et al*, 2016). This technique may provide a more accurate way to assess the quantity of BDNF within the knee joints of experimental rats. Whilst the previous research with this technique has been exclusively used post-mortem, the possibility of using magnetic capture to assess changing levels of BDNF in a slow progressing model of OA would be hugely interesting.

Data in this chapter has shown how a single injection of TrkB/fc chimera can reverse OA induced joint pain acutely. However, TrkB/fc chimera has a very short half-life; therefore future work should investigate longer duration interventions. It would be highly interesting to see the results of repeated dosing of TrkB/fc chimera in both the MNX and MIA models of OA, and whether this results in a longer duration reversal of pain behaviour. Administration of an NGF monoclonal antibody has shown long lasting therapeutic effects in the MIA model of OA (Xu *et al*, 2016). The effects of a BDNF monoclonal antibody in a model of OA have yet to be investigated, but could provide a lot of interesting information.

The effects of TrkB/fc on pain behaviour in rats with MIA and MNX induced OA suggest a role for BDNF in established OA pain. However, it remains to be seen what role, if any, BDNF plays in the origins of joint pain. A preventative study whereby an anti-BDNF treatment is given before the induction of a model of OA would provide information as to whether BDNF is involved with the development of joint pain, or is just involved in the later stages of the disease.

Whilst the studies in this chapter investigate the contributions of BDNF directly in the knee joint, in the clinic chronic intra-articular drug administration is not feasible. Future experiments should investigate systemic BDNF interventions, to see if the same analgesic effects are observed.

Future studies should also utilize specific antagonists for the TrkB and p75 receptors in order to establish the mechanisms through which BDNF exerts its effects in the joint.

4.4.7 Conclusions

The results in this chapter provide the first direct evidence for a peripheral contribution of BDNF to chronic joint pain. Due to the rapid effect of TrkB/fc chimera administration, it appears that BDNF may play a role in mediating peripheral sensitivity in OA.

Chapter 5: Therapeutic administration of cordycepin reverses DMM induced joint pain and pathology

5.1 Introduction

5.1.1 Rationale

As previously discussed (**1.2.4**) current therapeutic treatments for OA pain provide only partial relief. Reversing or slowing the rate of osteoarthritis progression may provide a long term solution to patients with peripherally driven chronic joint pain. Therefore research into disease modifying osteoarthritis drugs (DMOADs) represents an important area of investigation. The ultimate goal of a successful DMOAD is to slow the natural history of the disease, which in the case of osteoarthritis would be to lower the number of patients that experience joint failure (Manno *et al*, 2012). In pre-clinical studies, histopathological analysis of the knee joint post-mortem can provide substantial information regarding the ability of DMOADs to halt or reverse the progression of osteoarthritis.

One compound that might have the potential of being a DMOAD in osteoarthritis is cordycepin (3' deoxyadenosine). Cordycepin is produced by *Cordyceps militaris*, and was first isolated from the fungus in 1950 (Cunningham *et al*, 1950). Due to the structural similarity cordycepin has with adenosine, it can interfere with molecular events that require adenosine,

and has been shown to inhibit the poly-adenylation of newly transcribed mRNA (Beach & Ross, 1978, Kondrashov *et al*, 2012). One of the physiological effects cordycepin has been associated with is an attenuation of the production of reactive oxygen species (ROS) (Won *et al*, 2009). ROS have been seen to be able to stimulate osteoclastogenesis, thus increasing bone resorption (Garrett *et al*, 1990). It has therefore been previously hypothesised that cordycepin may be able to inhibit osteoclast generation, and therefore have an osteoprotective effect (Dou *et al*, 2016). Indeed, cordycepin has been shown to reduce osteoclast numbers in primary bone marrow monocyte cultures in a dose dependent manner (Dou *et al*, 2016). Cordycepin also dose-dependently reduced the extent of bone resorption seen with cultured osteoclasts (Dou *et al*, 2016). This cordycepin induced reduction in osteoclastogenesis, and subsequent reduction in bone resorption was linked to effects on ROS (Dou *et al*, 2016). In the ovariectomized model of osteoporosis in both mice and rats cordycepin has been shown to reduce bone loss (Dou *et al*, 2016, Zhang *et al*, 2015). As previously discussed (**1.2.2.**) subchondral bone changes, specifically increased osteoclast activity, are also considered to be an integral component of osteoarthritis. Therefore cordycepin could have the potential to reduce or reverse the progression of joint pathology seen in OA.

Interestingly, cordycepin has also previously been shown to have anti-inflammatory properties. In LPS stimulated macrophages cordycepin was shown to reduce both NO and iNOS release in a dose dependent manner (Kim *et al*, 2006). Cordycepin also significantly decreased LPS induced both the release of TNF- α , and COX-2 expression (Kim *et al*, 2006). In

an inflammatory model of osteoporosis in the rat cordycepin was also found to reduce IL-1 β and TNF- α serum levels (Zhang *et al*, 2014). Since there is also an inflammatory component to osteoarthritis (**1.2.2.**), cordycepin may also directly affect inflammatory pain arising from the knee joint.

It is important when investigating novel therapeutics to use the most appropriate animal model for the questions being asked. Since cordycepin has the potential of acting as a DMOAD, it was decided that the DMM model of OA in the mouse would be an appropriate model. Because the ultimate goal of a DMOAD is to slow the natural progression of disease, the earlier it can be given to the patient the better. Therefore, a model that is slowly progressing is of huge benefit. The DMM model is a slow progressing model of OA, this means that cordycepin can be administered at a variety of different time-points, before, during, and after the establishment of OA. The surgical induction of the model also reproduces trauma induced OA in the human, giving it high clinical relevance.

5.1.2 Aims

The aim of this chapter was to assess the therapeutic potential of cordycepin on joint pain and pathology in a murine model of osteoarthritis.

Objectives

1. To investigate the effects on orally administered cordycepin on DMM induced joint pain

2. To establish a scoring system for joint pathology for sagittal sections in the mouse
3. To investigate the effects of orally administered cordycepin on joint pathology in mice with DMM induced OA

5.2 Materials and Methods

General methods are provided in more detail in **Chapter 2**. All the experiments in this chapter utilised male C57BL/6 mice (Charles River, Kent, UK).

5.2.1 Induction of the DMM model

Fifty male C57BL/6 mice were used in this experiment aged approximately 8-9 weeks. Following anaesthesia with isoflurane (**2.2.1**), mice underwent either destabilisation of the medial meniscus (n=30) or sham surgery (n=20) of the left knee joint (**2.2.3**). Weight bearing asymmetry and paw withdrawal threshold were then measured weekly for 14 weeks post-surgery (**2.1**).

5.2.2 Oral Dosing of Cordycepin

At 14 weeks after DMM or sham surgery mice were dosed via oral gavage once every two days for two weeks. During this time both weight bearing asymmetry and paw withdrawal thresholds were measured twice weekly. Mice were dosed with either 8mg/kg of cordycepin prepared in 23% propylene glycol (PPG) in distilled water (n=30) or the vehicle (n=20). The final groups were: DMM plus cordycepin (n=15); DMM plus vehicle (n=15); sham plus cordycepin (n=10); sham plus vehicle (n=10). One mouse from the sham plus vehicle group was

euthanized due to oesophageal damage following oral dosing. Sixteen weeks after DMM or sham surgery mice were euthanized by intraperitoneal injection of sodium pentobarbital and tissues were either collected fresh (n=22) or following transcardial perfusion (**2.5**) (n=27).

5.2.3 Joint Histology

In order to investigate the ability of cordyepin to modify the progression of pathology, it is important to robustly quantify the changes in joint structure. Previously, mice knee joints were sectioned along the coronal plane. Due to technical difficulties with the embedding process leading to sections with the wrong orientation it was decided that future studies would section the joints along the sagittal plane. It was important to therefore establish a scoring system that allowed for the quantification of joint pathology in sagittal sections.

Both ipsilateral and contralateral knee joints were dissected from mice by cutting through the femur and tibia, before excess muscle was removed. The knee joints were then processed as described in **2.6.1**.

Once the knee joints were embedded in wax blocks 60 sagittal sections 5µm in thickness were taken from the medial side of the joint. Two sections (sections 20-21) were stained for H&E and Saf-O respectively to assess joint pathology. These sections were used for scoring, selected on the basis of the most consistent joint landmarks between all the blocks. Sections taken from the most medial aspect of the joint (sections 1-10) had variable amount of medial tibial plateau exposed. The last sections taken per block (sections 40-60) were occasionally found to have progressed past the anterior

cruciate ligament suggesting that they were in the lateral side of the joint. The spare middle sections were kept for future experiments.

The scoring system used to assess joint pathology was based primarily on the criteria defined by Jackson *et al* (2014). Whilst cartilage damage was assessed based on the scoring criteria from Glasson *et al*, (2010). The scoring criteria used for this study is described in **Table 2.2**. It was important that there was good inter- and intra- observer reliability with this scoring system. To this end a selection of samples were blinded and scored by two scorers (**PG** and **SA**), one week later the same selection of samples were re-blinded and scored again by **PG** and **SA**.

5.2.4 Data Analysis

Data were analysed and then graphically presented using Prism V.7 (Graphpad, San Diego, California, US). Normal distribution of data was tested with a D'Agostino and Pearson normality test. If data were normally distributed parametric analyses were used, and if data were not normally distributed then non-parametric analyses were used. Weight bearing asymmetry was presented as the average weight bearing difference between the left and right hindpaws as a percentage of the total weight (mean $\pm$ SEM). The differences in both weight bearing asymmetry and paw withdrawal threshold between DMM and sham operated animals over time was assessed by a 2-way ANOVA, with the surgical intervention (DMM vs Sham) condition as the between-subjects factor and time post surgery as the within-subjects factor. Differences in weight bearing asymmetry and paw

withdrawal thresholds following oral dosing with either cordycepin or vehicle were also assessed by 2-way ANOVA, with the treatment conditions as the between-subjects factor (DMM + cordycepin, DMM + vehicle, sham + cordycepin, and sham + vehicle), whilst time post treatment was used as the within-subjects factor. Multiple comparisons between-subjects at individual timepoints underwent Bonferroni correction. The differences between the area under the curve for cordycepin and vehicle treated animals were assessed by unpaired t-test. Paw withdrawal threshold is presented as the mean threshold $\pm$ SEM. The differences in both weight bearing asymmetry and paw withdrawal threshold between DMM and sham operated animals over time was assessed by 2-way ANOVA with Bonferroni correction. Changes in the joint pathology were presented as the mean $\pm$ SEM. Differences in each of the outcome measures between groups was assessed by Mann Whitney U test.

5.3 Results

5.3.1 Oral administration of cordycepin reverses DMM induced joint pain.

Two-way ANOVA with repeated measures showed significant difference in weight bearing asymmetry over time ($F_{(13, 624)} = 2.037$, $p = 0.0162$), and between DMM and sham operated animals ($F_{(1, 48)} = 22.92$, $p = <0.0001$). However, there was no significant interaction between these variables ($F_{(13, 624)} = 1.063$, $p = 0.3893$). Multiple comparisons between DMM and sham operated animals suggest there was a significant increase in weight bearing asymmetry (**Fig. 5.1.A**) in mice that underwent DMM surgery at 12 weeks post-surgery

($7.5 \pm 1.1\%$, $p = 0.0002$), compared to sham operated animals ($0.1 \pm 1.9\%$). Two-way ANOVA with repeated measures showed no significant difference in weight bearing over time ($F_{(4, 180)} = 0.88$, $p = 0.48$), but did show a significant difference between groups ($F_{(3, 45)} = 8.27$, $p = 0.0002$) following cordycepin administration. However, there was no significant interaction between these variables ($F_{(12, 180)} = 0.297$, $p = 0.9903$). From 2 hours post-cordycepin administration it looks like there was a lowered weight bearing asymmetry in DMM mice, compared with DMM mice treated with vehicle. This lowered weight bearing asymmetry in DMM mice was maintained following the two weeks of dosing with cordycepin. An area under the curve analysis (**Fig. 5.1.B**) showed that there was a significantly lower weight bearing asymmetry in cordycepin treated DMM mice ($12.98 \pm 1.7\%$) compared with vehicle treated DMM mice ($21 \pm 2.98\%$, $p = 0.0251$) over the two weeks of dosing. The DMM model per se did not lead to a change in ipsilateral hindpaw withdrawal threshold compared to the sham surgery at any timepoint (**Fig. 5.1.C**). Two-way ANOVA with repeated measures showed significant difference over time ($F_{(13, 624)} = 13.14$, $p = <0.0001$), but not between groups ($F_{(1, 48)} = 0.005$, $p = 0.94$). There was no significant interaction between these variables ($F_{(13, 624)} = 1.032$, $p = 0.4181$). Neither cordycepin nor vehicle treatment altered ipsilateral hindpaw withdrawal thresholds. Two-way ANOVA with repeated measures showed significant difference over time ($F_{(4, 112)} = 6.409$, $p = 0.0001$), but not between groups ($F_{(1, 28)} = 2.763$, $p = 0.108$). There was also no significant difference between groups over time ($F_{(4, 112)} = 1.009$, $p = 0.4057$). Similarly, there was no change in contralateral hindpaw withdrawal thresholds at any time during the experiment between any groups (Data not shown).

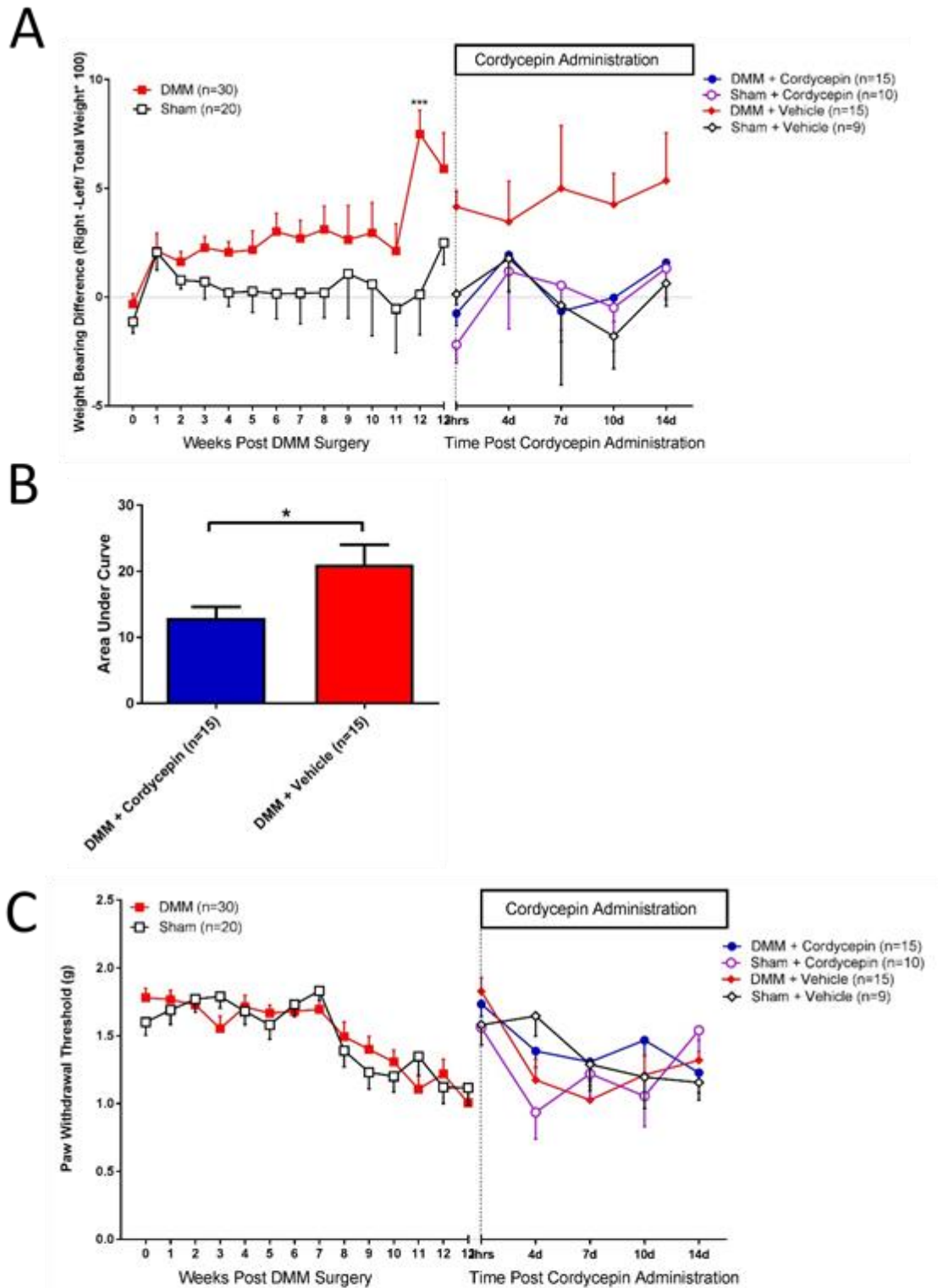


Figure 5.1: Effects of cordycepin on pain behaviour in mice with DMM induced OA. Adult male C57BL/6 mice underwent either DMM (n=30) or sham (n=20) surgery. Pain behaviour was measured up to week 16. Mice were orally dosed with either cordycepin (n=25) or vehicle (n=24). **A:** Weight bearing asymmetry in mice following cordycepin administration. Data analysed by 2 way ANOVA with Bonferroni correction. DMM vs Sham, *** = $P < 0.001$ **B:** Area under the curve analysis comparing weight bearing asymmetry in DMM mice dosed with either cordycepin or vehicle. Data analysed by t-test, DMM + cordycepin vs DMM + vehicle, * = $p < 0.05$. **C** Ipsilateral paw withdrawal threshold in mice following cordycepin administration. Data analysed by 2 way ANOVA with Bonferroni correction.

5.3.2 Inter- and intra-observer reliability for the scoring of joint pathology in sagittal sections.

Table 5.1 shows the intra-class correlations for the average scores given for the different criteria for the same sections scored on two separate occasions for the scorer **PG**. **Table 5.2** shows the ICCs for the average scores for the different criteria scored on two separate occasions for the scorer **SA**. The results of these analyses show that there was excellent intra-observer reliability for each of the scorers for the scoring system used.

Table 5.3 shows the weighted kappa for the scoring criteria between the two scorers **PG** and **SA**. The results of this analysis show that there was on the whole high agreement between the two scorers when scoring the same sections. There was excellent agreement between the two scorers for cartilage damage, osteophyte size, proteoglycan loss, and chondrocyte hypertrophy. There was intermediate/good agreement between the two scorers for osteophyte maturity and subchondral bone thickening. On this basis this scoring system was employed to evaluate any potential effects of cordycepin on DMM joint pathology

5.3.3 Oral administration of cordycepin reduced DMM induced joint pathology.

Sixteen weeks following DMM surgery there was a significant increase in cartilage damage in the medial tibial plateau (**Fig. 5.2.A, Fig. 5.3.A-C**) (3 [2.5-4.5], $p < 0.01$), compared to time-matched sham surgery (0 [0-0.25]). The magnitude of cartilage damage in DMM mice treated with cordycepin there was

Table 5.1: Intra-observer reliability for the scorer PG, scoring the same sections one week apart.

Measurement	ICC (95% CI)	p
Cartilage	0.99 (0.98-1)	<0.001
Osteophyte Size	1	No disagreement
Osteophyte Maturity	0.79 (0.3-0.94)	0.006
Proteoglycan	0.96 (0.86-0.99)	<0.001
Subchondral Bone	0.99 (0.96-1)	<0.001

Table 5.2: Intra-observer reliability for the scorer SA, scoring the same sections one week apart.

Measurement	ICC (95% CI)	p
Cartilage	0.92 (0.72-0.98)	<0.001
Osteophyte Size	0.97 (0.89-0.99)	<0.001
Osteophyte Maturity	0.97 (0.91-0.99)	<0.001
Proteoglycan	0.89 (0.92-0.97)	<0.001
Subchondral Bone	0.97 (0.89-0.99)	<0.001

Table 5.3: Inter-observer reliability between the scorers SA vs PG, scoring the same sections. Agreement thresholds: >0.75 = excellent. 0.4-0.75 = intermediate to good, <0.4 = poor.

Measurement	Weighted Kappa	Agreement
Cartilage	0.83	Excellent
Osteophyte Size	0.93	Excellent
Osteophyte Maturity	0.55	Intermediate/Good
Proteoglycan	0.9	Excellent
Subchondral Bone	0.67	Intermediate/Good
Chondrocytes	0.98	Excellent

significantly less (0.25 [0-1.25], $p<0.05$) than that seen in DMM mice treated with vehicle (**Fig. 5.2.A**).

Consistent with the cartilage damage score, chondrocyte hypertrophy was significantly greater (**Fig. 5.2.B, Fig. 5.3.D-F**) in DMM mice (1 [1-1], $p<0.01$), compared with sham control mice (0 [0-1]) at 16 weeks post-surgery. Again in DMM mice treated with cordycepin there was significantly less chondrocyte hypertrophy (0 [0-0.25], $p<0.05$) compared to DMM mice treated with vehicle (**Fig. 5.2.B**).

Other features of joint pathology were also evaluated, proteoglycan loss (**Fig. 5.2.E, Fig. 5.3.J-L**) as measured by loss of safranin-O staining was also significantly higher in DMM mice (1.5 [0.5-1.5], $p<0.01$) than sham mice (0 [0-0.3]).

DMM mice treated with cordycepin had significantly less proteoglycan loss (0 [0-0.25], $p<0.01$), when compared to DMM mice treated with vehicle.

To evaluate potential effects of cordycepin on bone features of OA, both size (**Fig. 5.2.C, Fig. 5.3.G-I**) and maturity (**Fig. 5.2.D**) of osteophytes were scored. Both osteophyte size (0.5 [0.5-1], $p<0.05$) and maturity (1 [0.5-1], $p<0.01$) were significantly increased in DMM mice compared with sham operated mice (size: 0 [0-0.1], maturity: 0 [0-0]). Treatment of DMM mice with cordycepin resulted in a significant reduction in both the size (0 [0-0.1], $p<0.05$) and maturity (0 [0-0.1], $p<0.05$) of osteophytes, compared to DMM mice treated with vehicle. A second bone feature studied was subchondral bone thickening (**5.2.F**), which had no significant between DMM mice (1 [0.5-3], $p=n.s.$) and sham mice (0 [0-0.6]). However, Cordycepin treatment resulted in a significant reduction in subchondral bone thickening (0 [0-0.375], $p<0.05$) compared to vehicle treated controls (1 [0.5-3]).

Finally, there was a significant increase in synovitis (**Fig. 5.2.G, Fig. 5.3.M-O**) in DMM mice (2 [1-3], $p < 0.05$) compared to sham operated mice (0.5 [0.1-1]). Cordycepin treatment did not significantly alter synovitis in DMM mice, compared to vehicle treated DMM mice.

5.4 Discussion

5.4.1 Summary of main findings

There was a significant increase in weight bearing asymmetry over time following DMM surgery in mice, compared to sham operated mice. There was no significant difference in the ipsilateral hindpaw withdrawal thresholds at any time point. This was accompanied by significant changes in joint pathology including cartilage damage, osteophyte size and maturity, proteoglycan loss, chondrocyte hypertrophy, and synovitis. The features of the DMM model described herein are consistent with those reported in Chapter 3 and indicate that this model is reproducible.

The scoring system for joint pathology in the sagittal sections was found to have mostly excellent intra- and inter-observer reliability.

Cordycepin treatment in DMM mice from 14 weeks following establishment of the model reversed the DMM induced increase in weight bearing asymmetry, a surrogate marker of pain. At the end of the treatment period the magnitude of joint pathology can be compared between DMM mice treated with vehicle versus cordycepin. Following two weeks treatment, cordycepin significantly reduced cartilage damage,

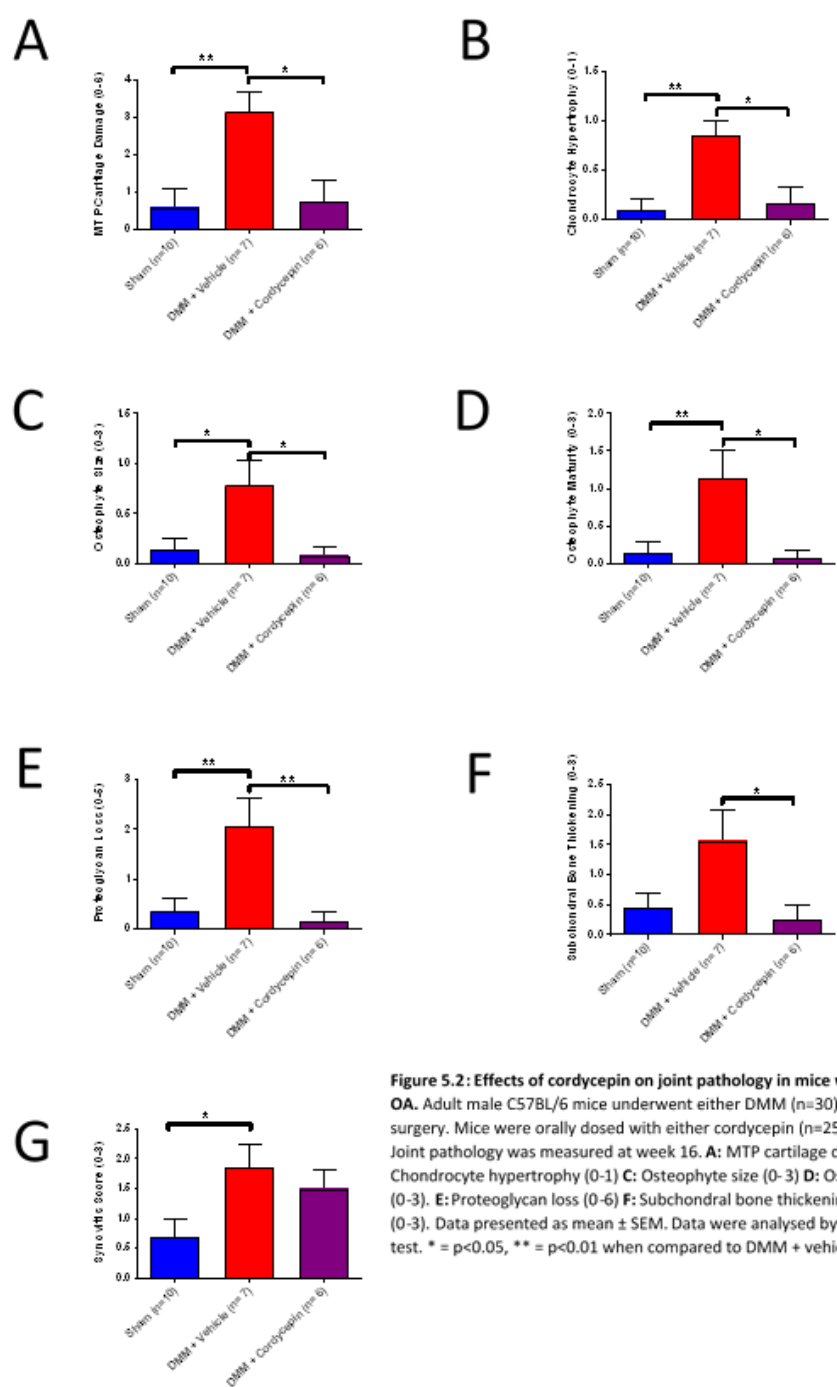


Figure 5.2: Effects of cordycepin on joint pathology in mice with DMM induced OA. Adult male C57BL/6 mice underwent either DMM (n=30) or sham (n=20) surgery. Mice were orally dosed with either cordycepin (n=25) or vehicle (n=24). Joint pathology was measured at week 16. **A:** MTP cartilage damage (0-6) **B:** Chondrocyte hypertrophy (0-1) **C:** Osteophyte size (0-3) **D:** Osteophyte maturity (0-3). **E:** Proteoglycan loss (0-6) **F:** Subchondral bone thickening (0-3) **G:** Synovitis (0-3). Data presented as mean $\pm$ SEM. Data were analysed by Mann Whitney U test. * = $p < 0.05$, ** = $p < 0.01$ when compared to DMM + vehicle group.

proteoglycan loss, subchondral bone thickening, osteophyte size, osteophyte maturity, and chondrocyte hypertrophy in DMM mice. Surprisingly there was no significant difference in synovitis score between DMM mice administered cordycepin or vehicle.

5.4.2 Cordycepin and inflammatory pain

Cordycepin had rapid effects on pain behaviour, which are unlikely to be accounted for by direct effects on structural damage within the joint. These inhibitory effects on pain behaviour were sustained following longer-term treatment, and it is feasible that the mechanisms underlying the analgesic effects of cordycepin may differ at early versus late stages of treatment.

Cordycepin has previously been shown to reduce the expression of several inflammatory mediators, including COX-2, IL-1 β , and TNF- α (Kim *et al*, 2006, Zhang *et al*, 2014). It is therefore feasible that cordycepin rapidly acts to reduce the levels of inflammatory mediators within the knee joint, which may account for the early reduction in weight bearing asymmetry in the DMM model. A limitation of this study was that separate experiments were not terminated at an early timepoint following treatment, and therefore any potential acute effects of treatment on inflammation were not studied. It is noteworthy however that two week cordycepin treatment did not alter the synovitis scores, measured as cellular infiltration, in DMM mice. In order to understand the potential mechanisms of cordycepin on pain mechanisms and inflammation, future studies could use a more specific marker for macrophages, such as CD68 (Haringman *et al*, 2005)

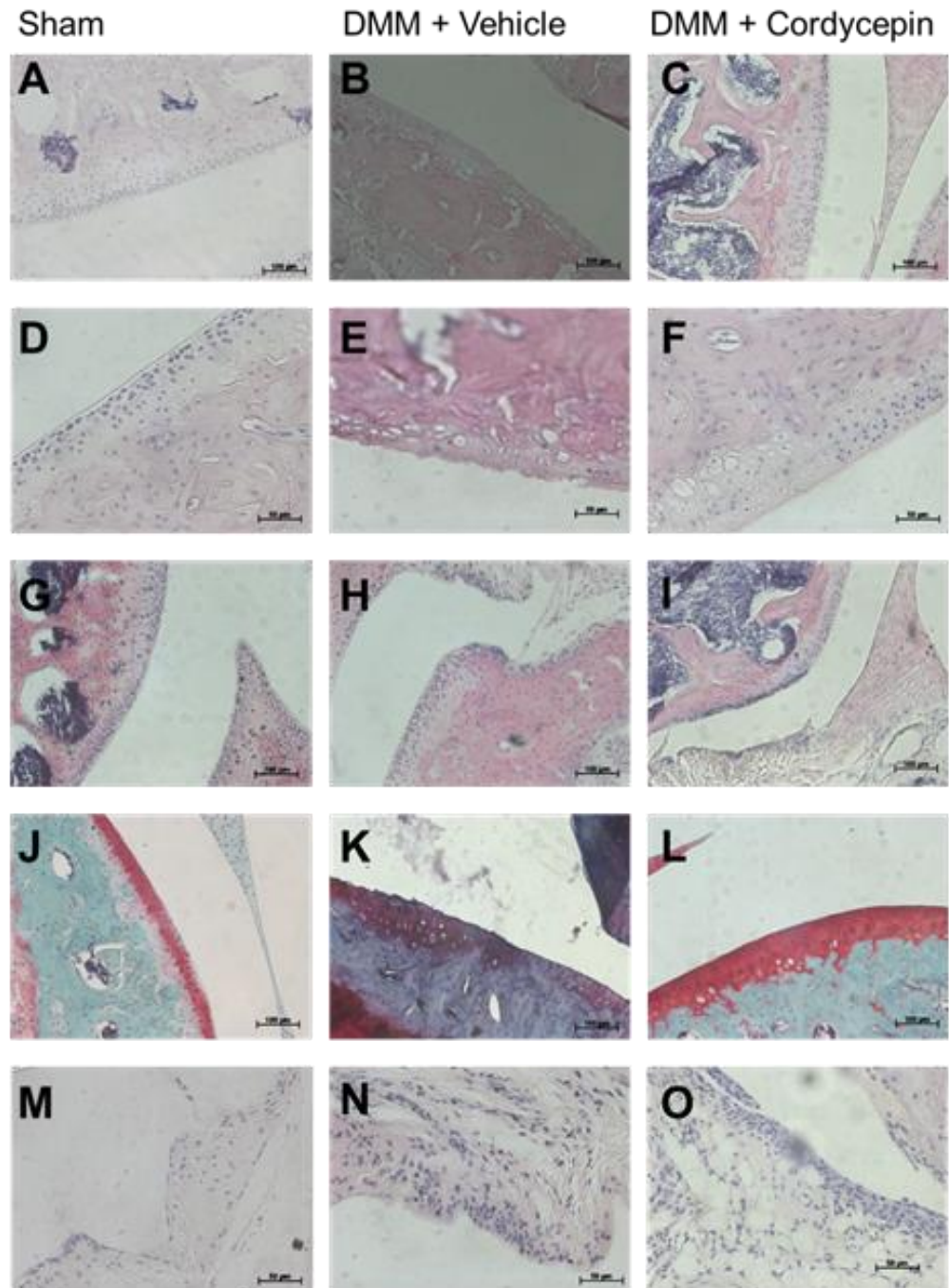


Figure 5.3: Representative images showing the effects of cordycepin on joint pathology in mice with DMM induced OA. Adult male C57BL/6 mice underwent either DMM (n=30) or sham (n=20) surgery. Mice were orally dosed with either cordycepin (n=25) or vehicle (n=24). Joint pathology was measured at week 16. Figures **A, D, G, J, & M** are from sham operated animals. Figures **B, E, H, K, & N** are from DMM operated animals treated with vehicle. Figures **C, F, I, L, & O** are from DMM operated animals treated with cordycepin. **Fig. 5.3.A-C:** Cartilage damage. **Fig. 5.3.D-F:** Chondrocyte hypertrophy. **Fig. 5.3.G-I:** Osteophytosis. **Fig. 5.3.J-L:** Proteoglycan loss. **Fig. 5.3.M-O:** Synovitis.

versus alternatively activated macrophages. Nevertheless, the lack of effect of cordycepin on synovitis is hard to reconcile with the proposal that direct inhibitory effects of acute cordycepin on inflammation may explain the rapid analgesic effects 2 hours following treatment in the DMM model.

The lack of effect of cordycepin on established synovitis, whilst still providing pain relief, provides insight into the relationship between these two events in models of OA. The 'inflammatory soup' comprises of kinins, growth factors, cytokines, chemokines, and numerous other factors sensitize nociceptors (Woolf & Ma, 2007). In patients with early OA there is evidence of synovial inflammation with increases in the levels of IL-1 α , IL-1 β , and TNF- α (Smith *et al*, 1997). Indeed, there is a correlation between the severity of synovitis and patient reported pain (Hill *et al*, 2007), supporting an important role in chronic joint pain. Previous work in the DMM model reported an increase in TNF α in the joint in the first 3 days post DMM surgery, but not at 16 weeks post-surgery (McNamee *et al*, 2010). In line with this finding, anti-TNF therapy reversed surgical pain at 3 days post model induction, but had no effect on established week 16 joint pain (McNamee *et al*, 2010). Collectively these findings support my data that the reduction of inflammation is not required for the reduction of pain behaviour at the later timepoints in the DMM model.

5.4.3 Cordycepin and local protein translation

The mechanism underlying the rapid effects of cordycepin on pain behaviour remains to be determined. The most obvious mechanism of effect is a direct action of cordycepin on sensory nerve function. Cordycepin has known effects on local protein

translation, which plays an important role in peripheral sensitization of sensory nerve terminals (Ferrari *et al*, 2013a).

Protein translation can be regulated through the elongation of cytoplasmic polyadenosine, this process is mediated by the cytoplasmic polyadenylation binding protein (CPEB) (Hake & Richter, 1994). This protein is not only found in cell bodies, but has also been localised to dendrites, suggesting local translation regulation (Wu *et al*, 1998). CPEB has been found to mediate polyadenylation, and regulate the translation of Ca^{2+} -calmodulin-dependent protein kinase II (α -CaMKII) (Wu *et al*, 1998). Cordycepin has been shown to prevent light-induced increase in α -CaMKII protein in the visual cortex by stopping CPEB mediated translation (Wells *et al*, 2001). Cordycepin has been shown to both prevent and reverse carrageenan induced hyperalgesic priming through the inhibition of protein translation (Ferrari *et al*, 2013a). These findings suggest that local protein translation in nerve terminals may be involved in pain processing and the transition from acute to chronic pain mechanisms. Local intra-plantar injection of cordycepin also reversed α -CaMKII induced priming, showing that α -CaMKII priming is dependent on local protein translation (Ferrari *et al*, 2013b). In separate experiments, systemic cordycepin reversed $\Psi\epsilon$ RACK induced priming (Ferrari *et al*, 2015). Interestingly, when cordycepin was given once priming was established, it could reverse priming at 4 hours post-injection, a similar time course to the change in pain behaviour reported in this chapter (Ferrari *et al*, 2013a, Ferrari *et al*, 2013b, & Ferrari *et al*, 2015).

Centrally, α -CaMKII has also previously been found to be critical for synaptic plasticity (Mayford *et al*, 1996). In the

dorsal horn of the spinal cord α -CaMKII is also involved in the maintenance of mechanical allodynia present in the SNI model of neuropathic pain (Katano *et al*, 2011). It is thought that one of the mechanisms through which α -CaMKII regulates synaptic plasticity is through the phosphorylation of AMPA receptors (Barria *et al*, 1997). There is also evidence that the interaction between α -CaMKII and the NR2B subunit of NMDAR are crucial for long term potentiation (Barria & Malinow, 2005). When the NR2B subunit was replaced by the NR2A subunit (which has lowered affinity for α -CaMKII) long term potentiation was attenuated (Barria & Malinow, 2005). Future studies could investigate the early molecular effects of cordycepin in the DMM model. To achieve this tissues could be collected a few hours after the initial administration of cordycepin.

5.4.4 Cordycepin: effects on joint pathology

The second objective of this chapter was to establish a scoring method for the sagittal knee joint sections in the mouse. The scoring system chosen has previously been used by other groups to assess joint damage (Jackson *et al*, 2014). The system was found to have both good inter-observer and intra-observer reliability. One of the most positive aspects of the scoring system is that it quantifies a wide range of OA hallmarks. Specifically, changes in cartilage damage, subchondral bone thickening, synovitis, chondrocyte hypertrophy, and osteophyte size and maturity were quantified between DMM injured mice and sham operated mice.

The final aim of this chapter was to assess the effects of cordycepin on joint pathology in the DMM model. DMM mice displayed significant joint damage across all measurements, compared to sham operated mice at 16 weeks post-surgery. After 14 days of cordycepin administration, from week 14, scores for all measures of joint damage, apart from synovitis, were significantly lower when compared to DMM mice treated with vehicle. Although the study design could not identify whether cordycepin can reverse DMM induced joint pathology, they do suggest that it can prevent further progression of DMM-induced joint damage.

In other conditions associated with bone remodelling, cordycepin can reverse unbalanced bone metabolism. For example in an ovariectomized model of osteoporosis cordycepin reversed the decrease in mechanical strength of the femur and reduced trabecular bone loss (Zhang *et al*, 2015). In addition, markers of osteoblast and osteoclast activity, alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activity respectively, were decreased by cordycepin in this model of osteoporosis (Zhang *et al*, 2015). Cordycepin has also been shown to reduce RANKL-induced osteoclast formation in bone marrow monocyte cultures via inhibition of ROSs (Dou *et al*, 2016), which act as intra-cellular mediators for osteoclast differentiation (Kanno *et al*, 2004, Lee *et al*, 2005). Since an imbalance in bone metabolism is a key player in the subchondral bone changes seen in OA (Tombran-Tink & Barnstable, 2004), inhibition of ROSs by cordycepin may be integral to restoring normal osteoblast and osteoclast activity in the model of OA.

ROSs do not just play a key role in osteoclastogenesis, they also have a crucial role in several other joint tissues. Indeed, in human patients with OA there is a significant increase in the serum levels of lipid hydroperoxide, suggesting increased systemic oxidative stress (Ertuk *et al*, 2012). Despite being located in avascular cartilage, chondrocytes still have mitochondria and still respire using diffused oxygen, and therefore do produce ROSs (Lee & Urban, 1997). This increase in ROS from OA chondrocytes has been found to change intracellular signalling and promote chondrocyte apoptosis (Henrotin *et al*, 2003). In fact this is the principle behind the MIA model of OA in the rat, whereby MIA induces apoptosis through increased ROS production (Jiang *et al*, 2013). Therefore, cordycepin administration may be reducing the ROS induced apoptosis of chondrocytes within the cartilage, and this in turn may explain the decreased chondropathy seen following cordycepin treatment.

Synovitis is also a key phenotype of osteoarthritis, with patients showing thickening of the synovial lining and inflammatory cell infiltration (Smith *et al*, 1997). As previously mentioned cordycepin has been shown to reduce the expression of numerous inflammatory mediators including COX-2, IL-1 β , and TNF- α (Kim *et al*, 2006, Zhang *et al*, 2014). Therefore it would be expected that cordycepin would reverse or reduce the extent of synovitis within the knee joint. However, our data did not show any differences in cellular infiltration following cordycepin administration. Another limitation of the work in this chapter is that the only measurement of synovitis taken was a semi-quantitative measure of synovial thickness. There remains the possibility that cordycepin is maybe altering the phenotype of

macrophages within the synovium. Future studies should address these concerns.

5.4.5 Future Directions

It is not known whether or not the changes in joint pathology seen post cordycepin administration are halting or reversing DMM induced joint pathology. It would be interesting to assess the joint pathology of the DMM model at a variety of different time-points. This way it would be possible to assess properly whether or not cordycepin has the potential to reverse osteoarthritic changes in joint pathology.

The advantage of using the DMM model of OA is that it changes in joint pathology are more slowly progressing than other models. Therefore it would be possible to investigate the effects of administering cordycepin before there is onset of OA pathology. If cordycepin is able to prevent or even reverse joint pathology, and then subsequently the development of joint pain, it could represent a future candidate as a DMOAD for the clinic.

The mechanism through which cordycepin alters joint pathology is still not known. Future work should focus on understanding the underlying mechanics behind these changes. Firstly, it is a priority to quantify the numbers of osteoclasts in mice with DMM induced OA who were given vehicle, compared to those mice who were dosed with cordycepin. This could be achieved through the use of tartrate-resistant acid phosphatase (TRAP) staining on the spare sections from this study.

As well as changes in the subchondral bone thickening following dosing of cordycepin, there were also changes in cartilage degradation observed. Therefore, future experiments

should also investigate the potential differential expression of cartilage degradation markers. Interesting targets would include ADAMTS-5, which is an aggrecanase, and has been linked to the degradation of articular cartilage (Song *et al*, 2007). Another key catabolic enzyme which may be interesting to investigate is matrix metalloprotease 13 (MMP13). This enzyme has been found to be involved with the breakdown of articular cartilage in a mouse model of OA (Wang *et al*, 2013).

This experiment used male mice; however future pre-clinical studies should also investigate cordycepins effects in female mice. This is due to the protective effects of oestrogen on bone metabolism. Experiments in mice following ovariectomy have found increased bone loss and bone resorption (Lean *et al*, 2003). There was also a reduction in the antioxidants glutathione and thioredoxin in the ovariectomised mice, suggesting a role for oestrogen in suppressing the effect of ROSs (Lean *et al*, 2003). Therefore it would be interesting to see if there was a differential response to cordycepin treatment in female mice.

5.4.5 Conclusions

Administration of the polyadenylation inhibitor, cordycepin, in a murine model of OA was found to significantly reduce weight bearing asymmetry and joint pathology when compared to vehicle.

Chapter 6: General Discussion

This thesis had two main aims:

To establish a slow progressing model of murine OA to complement the other established models used by researchers in the ARUK Pain Centre.

To investigate novel peripheral targets which modulate OA pain, specifically BDNF/ TrkB and cordycepin which modulates polyadenylation regulated translation and thereby inhibits inflammatory responses.

6.1 Establishing a Slow Progressing Murine Model of OA.

The DMM model is a slow progressing surgical model of OA in the mouse (see **3.1.1** for review). In order to establish this model it was important to confirm that destabilising the medial meniscus resulted in pain behaviour and changes in joint structure. To achieve this a scoring system for sagittal sections of mouse knee joints was replicated (based on Jackson *et al*, 2014) and validated as part of this thesis.

Using these approaches it was evident that pain behaviour, as indicated by weight bearing asymmetry, occurs from 12 weeks following DMM in the mouse. At 16 weeks following DMM injury pain behaviour was maintained, and there was significant chondropathy and synovitis within the injured joint. In addition to these classical features of OA, there was significant osteophytosis, proteoglycan loss, and chondrocyte hypertrophy at this timepoint in the DMM model. These data confirm that the DMM model of OA in the mouse results in both significant pain behaviour and joint pathology over a long

time course and therefore provides a useful model to investigate the mechanisms that precede the manifestation of overt pain behaviour associated with OA.

Investigation of the relationship between the type of joint injury and pain behaviour in the DMM model was also undertaken. Modifying the DMM surgery to avoid transecting the medial meniscotibial ligament still resulted in significant chondropathy and synovitis at 16 weeks post-surgery.

However, this modified model induction did not result in any evidence of pain behaviour using the tests employed. This suggests an important role of the meniscus in mediating joint pain in the DMM model. Understanding why there are marked differences in pain behaviour, but not joint pathology, between these two models will help us to better understand the mechanisms underlying chronic joint pain. These data suggest that the extent of damage to the meniscus, or the ligament, is likely to be important in mediating joint pain in this model. It is possible that transecting the MMTL causes greater damage to the meniscus, which may account for the difference in pain behaviour between the two surgical variations.

Previous work has shown differences in the progression of joint pathology and pain behaviour following either meniscectomy or meniscal transection. Following removal of the medial meniscus there was significantly less pain, compared to mice who underwent meniscal transection (de Melo Leite *et al*, 2014). There was also a significant increase in the numbers of cells in joint aspirates in mice that underwent meniscal transection compared to meniscectomy (de Melo Leite *et al*, 2014). However, meniscal transection also led to significantly increased joint pathology at day 49 post surgery when compared to meniscectomy, which may account for

these differences in pain behaviour (de Melo Leite *et al*, 2014). It should be noted that in this study the meniscal transection/ meniscectomy was performed after anterior cruciate ligament transection/ medial collateral ligament transection (ACLT/MCLT) (de Melo Leite *et al*, 2014). It has been suggested that the presence of the remnants of the meniscus within the knee joint may result in increased pain responses in mice. Alternatively the difference may be related to differences in the joint mechanics following meniscectomy versus meniscal transection. Indeed, differences in pain behaviour between animals following the transection of different ligaments to destabilise the medial meniscus, may reflect a change in the mechanics of the knee joint which impacts upon weight distribution in the knee joint. The meniscus is a highly innervated tissue within the knee joint, it is therefore not surprising that it may contribute to chronic joint pain in patients (Gronblad *et al*, 1985). In patients with radiographic evidence of knee OA there is a significant association between meniscal tears and pain severity (Torres *et al*, 2006). Menisci removed from patients with OA were found to produce both NO and PGE₂ in response to IL-1, TNF- α , and IL-17 (LeGrand *et al*, 2001), suggesting that the meniscus may be a source of inflammatory mediators, which contribute to pain responses. Destabilising or removing the medial meniscus is not the only method for inducing surgical OA in mice. Other models include those where the anterior cruciate ligament (ACLT), the medial collateral ligament (MCLT), or the cartilage itself are damaged (Glasson *et al*, 2007, Wright *et al*, 2003, Fitzgerald *et al*, 2008). Interestingly these models all show different outcomes from each other. The ACLT model exhibits severe cartilage damage from 4 weeks of induction compared to the milder

damage seen in the DMM model (Glasson *et al*, 2007). Whilst there is no evidence of cartilage damage in the MCLT model, there is a reduction in joint strength and an increase in inflammatory cells (Wright *et al*, 2003). Pain outcomes in these models have not been investigated in the mouse. The differences in these models, alongside the differences observed when the surgical induction of the DMM model was modified, highlights the diversity of the various models of OA.

6.2 Measuring Pain Behaviour in Rodents.

When clinical researchers investigate chronic pain they have access to several tools, such as the visual analogue scale (VAS) and the McGill pain questionnaire for assessing the patients experience (Hawker *et al*, 2011). These tools can provide a lot of information regarding both the severity and location of the pain. However, in animal models it is not possible to assess pain through the use of a questionnaire, so behavioural correlates for pain are needed. In this thesis both weight bearing asymmetry and paw withdrawal thresholds have been measured in mice and rats.

Weight bearing asymmetry aims to investigate changes in static weight bearing differences between arthritic and non-arthritic joints. This method is based upon the notion that pain originating from a joint results in the animal changing its posture to reduce the load on the injured limb. It is important to realise that pain may not be the only factor which may impact the way the animal distributes its weight, there is the possibility that changes to joint biomechanics will alter the animal's stance. This behavioural test should have good

translational value as a large proportion of individuals with OA experience weight-bearing related pain (Lo *et al*, 2009). There were significant weight bearing changes in mice 12 weeks post DMM surgery. Others have also shown changes in weight bearing asymmetry at this time point and onwards as well (Driscoll *et al*, 2016, McNamee *et al*, 2010). Previously, weight bearing changes have been shown within the first 3 days post-surgery (McNamee *et al*, 2010), although not confirmed in this thesis. Weight bearing asymmetry has been assessed in both surgical and chemical models of OA, in both rats and mice (Bove *et al*, 2006, Driscoll *et al*, 2016, Ogbonna *et al*, 2013, Orita *et al*, 2011).

A subset of OA patients also experience referred pain (Arendt-Nielsen *et al*, 2010). In order to investigate central sensitization in animal models of OA it's important to measure changes in mechanical sensitivity in areas which are distal to the site of injury. The lowering of the hindpaw withdrawal threshold is a measure of mechanical allodynia which can be used as a behavioural correlate of central sensitization in models of knee joint OA. This mechanical threshold is obtained through the use of von Frey hairs of a known weight. A decrease in the mechanical threshold is indicative of increases in mechanical sensitivity. In order to assess paw withdrawal threshold the experimenter needs to make a judgement as to whether responses are indeed a pain response or are just a by-product of the animal being startled. Therefore measuring paw withdrawal threshold does involve some subjectivity on the experimenter's behalf. It is therefore important for the experimenter to be correctly blinded and to ensure they assess each response in a reliable manner. Paw withdrawal thresholds have also been measured in surgical and chemical

models of OA, in rats and mice (Bove *et al*, 2006, Miller *et al*, 2012, Ogbonna *et al*, 2013, Sagar *et al*, 2011). However, there were no changes in paw withdrawal threshold over the 16 weeks of the DMM model, unlike previous reports in this model. Indeed, previous studies have reported a difference in the hindpaw withdrawal threshold (calculated as 50% paw withdrawal threshold rather than absolute paw withdrawal threshold) as early as 4 weeks post-surgery (Malfait *et al*, 2009, Miller *et al*, 2016). It is feasible that measuring the absolute mechanical threshold may not be as sensitive as measuring the 50% withdrawal threshold, and this requires further investigation.

The absence of a change in hindpaw-evoked pain response in the DMM model suggests a lack of central sensitization in the model, at least in our hands. Indeed, there was no significant change in the labelling of Iba1 or GFAP in the dorsal horn of the spinal cord 16 weeks post-surgery. In the MIA model there is increased activation of microglia and increased astrocyte expression (Sagar *et al*, 2011). Changes in glial activity in the dorsal horn are indicative of central sensitization, and in the MIA model there is a significant correlation between hindpaw withdrawal thresholds and IBA1 and GFAP labelling (Sagar *et al*, 2011).

The pain measures employed in this thesis measure evoked pain responses, however it is pertinent to investigate spontaneous incidents of pain in rodent models of OA. One potential correlate for spontaneous pain in rodents could be a change in their activity levels. Indeed, the laboratory animal behaviour observation registration and analysis system (LABORAS) has been used to show decreased activity in mice following DMM surgery (Miller *et al*, 2012). Whilst there are

many potential benefits to measuring spontaneous behaviour in animals the cost of these systems can be prohibitive. The use of a mouse grimace scale has also been used to assess pain in mice following vasectomy surgery, and could also provide information about the extent to which small rodents experience acute post-operative pain (Leach *et al*, 2012), but these scales have only been shown to be effective in short-term pain models (Matsumiya *et al*, 2012).

There are a few considerations which are vital when choosing which behavioural tests are used to assess pain. Firstly, it is important that the behaviours measured have some translational value when compared to the human disorder. Secondly, the behaviours measured should represent pain and not other behavioural states such as stress or fatigue. The behavioural tests should also be sensitive enough to be able to detect changes between animals that are experiencing pain and those that are not. Taking these considerations into account weight bearing asymmetry and hindpaw withdrawal threshold appear to be appropriate behavioural tests to investigate chronic joint pain in small rodents.

6.3 Novel Therapeutic Targets for the Treatment of OA Pain

The ultimate goal of pre-clinical research is to identify therapeutic targets which may have robust clinical effects. In this thesis two distinct mechanisms systems, BDNF/ TrkB and cordycepin, were investigated and their potential ability to modulate OA joint pain was assessed.

6.3.1 BDNF as a novel therapeutic target for the treatment of OA pain.

In order to properly understand the role of BDNF in joint pain it was important to understand how BDNF affected the naïve joint. Intra-articular injection of a range of doses of the neurotrophin had no effect on pain behaviour in naïve animals. However, when BDNF was injected into the knee joint of animals that had established MIA induced joint damage there was an augmented pain response. This suggests that BDNF may have a role in mediating chronic joint pain. The next step was to explore the role of endogenous BDNF by acutely decreasing levels in the knee joint. To achieve this a TrkB/fc chimera, a compound which has previously been shown to reduce BDNF levels *in vivo*, was injected into the joint space. Intra-articular injection of TrkB/fc reversed established MIA induced pain behaviour. To ensure the effects of TrkB/fc were common to osteoarthritis and not just to MIA induced chondrocyte loss the experiment was repeated in the surgical MNX model. Intra-articular injection of TrkB/fc also reversed MNX induced joint pain behaviour. The levels of BDNF in both plasma and synovial fluid from TrkB/fc treated rats were analysed by ELISA. Maybe not surprisingly there was no significant difference in the plasma levels of BDNF between groups.

Overall data presented in this thesis suggest that targeting BDNF and its receptor TrkB may have therapeutic potential. However, the mechanism underlying BDNF's effect on chronic OA joint pain is unknown. It is possible that BDNF's role in mediating OA joint pain is linked to inflammation within the joint. We know that TrkB is expressed by macrophages and

monocytes which are found in increased numbers in the joints of OA patients (Shibayama & Koizumi, 1996, Bondeson *et al*, 2010). In addition there is a high expression of BDNF mRNA within the synovial fluid of OA patients, and ATP induces the release of BDNF from synovial fibroblasts (Barthel *et al*, 2009, Klein *et al*, 2012). Additionally, BDNF is released from monocytes, and this release is enhanced by both TNF α and IL-6 (Schulte-Herbruggen *et al*, 2005). It is feasible that treatments which target the BDNF-TrkB pathway could have benefit for OA patients with an increased inflammatory response. BDNF may therefore also be an ideal therapeutic candidate for the more inflammatory rheumatoid arthritis. There are still a lot of questions which need to be answered before BDNF can be considered a clinically relevant target, however the initial findings presented in this thesis are promising.

6.3.2 Cordycepin as a potential therapeutic treatment for OA joint pain.

Another compound which may have future therapeutic potential is cordycepin. Cordycepin, due to its structural similarity to adenosine, is known to inhibit poly-adenylation of newly transcribed mRNA (Kondrashov *et al*, 2012). Oral administration of cordycepin in mice 14 weeks post DMM induction was associated with a significant reduction in weight bearing asymmetry, compared to mice dosed with vehicle. In addition, mice that had been orally dosed with cordycepin also had significantly reduced joint pathology compared to vehicle treated mice. Previously, cordycepin has been shown to have osteoprotective effects in models of osteoporosis (Dou *et al*,

2016, Zhang *et al*, 2015). Data in this thesis also suggests that cordycepin has chondroprotective effects.

Cordycepin has previously been shown to prevent and reverse hyperalgesic priming, the increased neuronal sensitivity following prior noxious insult, by inhibiting local protein translation (Ferrari *et al*, 2013a, Ferrari *et al*, 2013b, & Ferrari *et al*, 2015). Local protein translation can be mediated through the cytoplasmic polyadenylation binding protein (CPEB) which regulates the elongation of cytoplasmic polyadenosine (Hake & Richter, 1994). CPEB is thought to mediate the local translation of α -CaMKII through the regulation of polyadenylation (Wu *et al*, 1998). Cordycepin is able to stop the CPEB mediated synthesis of α -CaMKII (Wells *et al*, 2001). It appears that α -CaMKII is important in the transition from acute to chronic pain in the hyperalgesic priming model of pain (Ferrari *et al*, 2013b).

Cordycepin has also been shown to have anti-inflammatory properties, including reduction of LPS induced release of NO and PGE2 from macrophages and inhibiting the expression of various inflammatory mediators including IL-1 β , TNF- α , and COX-2 (Choi *et al*, 2014, Jeong *et al*, 2010). Cordycepin inhibits the activation of NF- κ B as well as inhibiting the MAPK and Akt pathways (Jeong *et al*, 2010). Cordycepin has also been found to interfere with TLR4 receptor activity in macrophages (Choi *et al*, 2014). Thus it appears that cordycepin works at various different levels of signalling pathways to reduce inflammatory signalling in macrophages. These effects on inflammation may also underlie the analgesic effects of cordycepin on mice with DMM induced joint pain.

6.3.3 The potential link between BDNF and local protein translation.

There is evidence that local translation plays an important role in regulating synaptic strength which may be mediated by BDNF. In the hippocampus BDNF induced enhancement of synaptic transmission was found to be reliant on local dendritic protein synthesis (Kang & Schuman, 1996). BDNF has also been shown to stimulate the local synthesis of α -CaMKII in hippocampal neurons (Aakulu *et al*, 2001). α -CaMKII is a critically important protein for synaptic plasticity (Mayford *et al*, 1996).

The link between BDNF and α -CaMKII may be important for mediating synaptic plasticity. In a model of inhibitory avoidance memory formation BDNF was found to be critical for the learning-dependent increase in α -CaMKII (Chen *et al*, 2012). The formation of inhibitory avoidance memory is also dependent on BDNF-TrkB signalling (Chen *et al*, 2012). BDNF and α -CaMKII are also important in mediating spinal plasticity. In the absence of supraspinal input there was an increase in both spinal BDNF and α -CaMKII mRNA in rats that had learned to avoid electrical stimulation (Gomez-Pinilla *et al*, 2007). Inhibiting BDNF and α -CaMKII was also found to inhibit facilitated learning in this model of operant conditioning (Gomez-Pinilla *et al*, 2007). Both BDNF and α -CaMKII are known to interact with NMDAR to regulate synaptic plasticity. The interaction between α -CaMKII and the NR2B sub-unit of NMDAR is important for long term potentiation (Barria & Malinow, 2005). The α -CaMKII/NMDAR complex is also important in maintaining synaptic strength, disrupting this complex was found to reduce the basal strength of the synapse (Sanhueza *et al*, 2011). BDNF has been found to

induce phosphorylation of both the NR1 and NR2B subunits of NMDAR (Slack & Thompson, 2002, Lin *et al*, 1998).

Cordycepin by interrupting the polyadenylation mediated local translation of α -CaMKII could be reducing synaptic changes at the spinal and supraspinal levels in the DMM model of OA. However, the majority of the work investigating these changes has been done at the level of the brain. It is not fully understood how changes in local translation may affect other levels of the CNS. This inhibition of local translation could also affect BDNF's ability to increase synaptic strength (Kang & Schuman, 1996). This may underlie the therapeutic reversal of chronic joint pain by cordycepin in the DMM model of OA.

The mechanisms that link BDNF to local protein translation have all been demonstrated in the central nervous system. However, the reduction of BDNF in the periphery was shown to reduce chronic joint pain in rats. The question remains as to whether or not effects on local protein translation occur in the periphery. Experiments in the hyperalgesic priming model for the chronification of pain have shown the effect of inhibitors of local protein translation administered to the periphery (Ferrari *et al*, 2013a). Both rapamycin and cordycepin were found to inhibit hyperalgesic priming when administered to the peripheral terminals of nociceptors (Ferrari *et al*, 2013a). It is therefore possible that changes in local protein translation at the level of the joint could be involved in the chronic pain associated with OA.

6.3.4 Considerations

Despite the promising results of both BDNF and cordycepin in reducing OA joint pain there are still several factors for consideration. The development of a novel therapeutic into an established and effective analgesic is not an easy task (Woolf *et al*, 2010). It is therefore important to reflect on the potential limitations of these pre-clinical experiments.

Firstly, it is important to note the route of administration for both TrkB/fc chimera and cordycepin. TrkB/fc chimera was administered by intra-articular injection into the injured joint. Whether the effects of TrkB/fc chimera on pain behaviour are a result of local effects in the joint is yet to be ascertained. An appropriate control experiment for this would be to inject TrkB/fc chimera into the contralateral knee joint and to determine if there are any changes to the animals' behaviour. TrkB/fc was also only administered once, this was in order to investigate the acute effects of the compound. Future experiments should also investigate the effects of repeated administration of TrkB/fc. Cordycepin, on the other hand, was administered by oral gavage every two days for two weeks. Oral dosing has the advantage of allowing for systemic administration of a compound to investigate its effect on pain. It also is more translatable to the routes of administration that may be used in the clinic. However, systemic administration does not provide information as to the site of action of the drug.

Both cordycepin and TrkB/fc chimera were administered in a therapeutic manner when both pain behaviour and joint pathology have been well established. This study design was chosen for both treatments because it is potentially the most clinically relevant time point for treatment. Pain from the affected joint is more often than not the reason patients seek medical attention. However, this is not to say that preventative studies are not a useful pre-clinical tool. Compounds which could prevent the onset of both joint pathology and pain could have substantial future applications, especially with recent advances in biomarkers of the disease (Mobasheri *et al*, 2017).

The two treatments investigated in this thesis are similar in that they appear to have poly-target effects. The high affinity receptor for BDNF, TrkB, is found in a wide variety of cell types (Shibayama & Koizumi, 1996, Yan *et al*, 1997). Whilst cordycepin inhibits poly-adenylation, a process mediated by CPEB which has been found in a variety of cell types (Richter *et al*, 2007). These treatments could therefore have effects on multiple sites within the OA joint, including neurons, resident cells, and infiltrating cells. There are advantages to treatments which have multiple targets, for example with cordycepin affecting both pain behaviour and joint pathology in the DMM model. However, the chances of potentially negative side effects as a result of the treatment are increased as a consequence of the poly-target nature of the compounds.

6.4 Selection of Animal Models for Pre-clinical Investigations of Therapeutics for Chronic Joint Pain.

The need to carefully consider which animal model of OA is most appropriate for the questions being asked is well established (Little & Zaki, 2012). In this thesis three different models of OA were used, the MIA and MNX models in the rat, and the DMM model in the mouse. When investigating the role of BDNF in mediating chronic joint pain the MIA model in the rat was the initial model chosen. The MIA model has well established and robust pain outcomes (Sagar *et al*, 2011). Unlike spontaneous and surgically induced models of OA, the MIA model is not fully representative of the joint pathology changes seen in the human disease (Barve *et al*, 2007). However, the MIA model was chosen as a suitable model to investigate the initial effects of BDNF on joint pain, as intra-articular injection of MIA has a lesser burden on the animal when compared to MNX surgery.

Due to the translational limitations of the MIA model it was important to confirm the initial findings of these experiments in a second model of OA. The MNX model was used to replicate the TrkB/fc chimera intervention study. As a surgical model the resulting joint pathology could be seen as more representative of post-traumatic OA. The pain outcomes of the MNX model are also well established (Bove *et al*, 2006). Confirmation of the effects of TrkB/fc chimera in the MNX model suggest that the contribution of BDNF to joint pain are generalizable to the disease and not the induction method.

When designing the therapeutic cordycepin study a more slowly progressing model OA pathology was selected. This was partly due to previous research suggesting cordycepin may have osteoprotective effects (Dou *et al*, 2016), which may be relevant to OA pain. The DMM model in the mouse has the advantage of being representative of post-traumatic OA in the human, as well as being slow progressing. This means that the effects of drugs at different stages of OA can be investigated more easily. The DMM model is well established in the mouse which is advantageous for the future use of genetically modified mice, however the trade-off is that pain behaviour in mice is harder to measure than in the rat.

It is important for researchers to consider the divergent outputs from the large variety of animal models available in order to optimise the translatability of their research. This is perhaps not surprising considering the heterogeneous nature of the disease that these models are attempting to represent. In many cases it may be preferable to use more than one model in more than one species to fully investigate therapeutic targets of interest.

6.5 Future Directions

Now that the traditional DMM model has been shown to produce both significant joint pathology and pain behaviour, the model can be used as a tool for understanding the mechanisms underlying chronic joint behaviour. The slow progressing nature of the model lends itself to several interesting possibilities. One such possibility is the potential use of microCT to investigate pathological changes *in vivo* (Piscaer *et al*, 2008). There are some issues to consider with

the potential use of this technique; including the effect of repeated exposure to anaesthesia and the potential economic costs. However, being able to investigate the development of OA *in vivo* should help reduce the number of animals needed for experiments.

It would also be highly interesting to use the DMM model to investigate the changing levels of various pain mediators at a series of time points before, during and after the onset of pain. For example, longitudinal expression and localisation of the neurotrophins, BDNF, and NGF.

The slowly progressing time course of the DMM model is particularly useful for pharmacological intervention studies. It presents the opportunity to give interventions before, during, and after the onset of joint pathology and pain. This will enable investigation of both therapeutic and preventative treatments for OA induced joint pain. The DMM model has already been used to investigate the therapeutic potential of cordycepin. This study showed that cordycepin reduced weight bearing asymmetry in the mouse. However, it is still not known whether therapeutic administration of cordycepin reversed or reduced joint pathology in the model. In order to address this it is important to investigate the joint pathology in mice at a variety of timepoints before the 16 week end point.

The DMM model would also be useful for continuing the research into BDNF's role in peripheral joint pain. Data presented herein showed an acute reduction in pain behaviour following administration of TrkB/fc chimera. Despite being highly selective for BDNF, TrkB also binds with other neurotrophins including NT4 (Segal, 2003). There is lots of

evidence in the literature which has shown reductions in BDNF following administration of TrkB/fc chimera (Endo *et al*, 2009, Yajima *et al*, 2005). However, the results from the ELISA analysis of the synovial fluid following TrkB/fc administration were inclusive. This could be due to the methodology of collecting the synovial fluid by joint wicking diluting the sample too much. Therefore, future experiments could contemplate using magnetic capture to assess intra-articular levels of the neurotrophins (Yarmola *et al*, 2016). Showing that the TrkB/fc chimera is decreasing intra-articular levels of BDNF should be the first priority.

If it is possible to show that TrkB/fc chimera reduced BDNF levels then it would be interesting to see the effects of a longer lasting anti-BDNF treatment, for example a monoclonal antibody, in a model of OA. Due to its long time course the DMM model would be ideal for investigating the impact of a longer duration anti-BDNF treatment. Administering the anti-BDNF treatment systemically would also indicate whether an anti-BDNF treatment might have future clinical relevance. Previously my group has used rats as its primary model species. Establishing the DMM model in the mouse has opened up the possibility of using new and exciting techniques developed in this species. Mice are an ideal model species due to the relatively low costs of genetic modifications that are possible. Indeed, in the DMM model a recent paper has used Designer Receptors Exclusively Activated by a Designer Drug (DREADD) technology to investigate the effects of peripheral activation of inhibitory GPCRs on DMM induced joint pain (Miller *et al*, 2017). This technique relies on the binding of engineered GPCRs to synthetic molecules to activate or inhibit neurons. Miller *et al* genetically modified mice to express an

inhibitory GPCR on sensory neurons expressing the Na_v1.8 channel (Miller *et al*, 2017). The development of *in vivo* calcium imaging in mice also allows for the optical recording of neuronal activity in living mice (Grewe *et al*, 2010). Utilizing state of the art techniques in the DMM model will allow researchers to more thoroughly investigate neuronal changes during and after the development of joint pain.

Chapter 7: Appendices

7.1 Osteophytosis in Two Murine Models of OA

Aim: To investigate changes in osteophytosis in mice 16 weeks after either DMM or modified DMM surgery.

Methods: Sixteen weeks after either DMM or modified DMM surgery mice were euthanised and then their knee joints were harvested. Six sections from the ipsilateral knee joints were then stained with haematoxylin and eosin (**2.6.2**) before being scored for osteophytosis (**2.6.3**).

Results: There was no significant difference in osteophytosis seen between DMM and sham operated animals 16 weeks following surgery (**Fig. 7.1**). Sixteen weeks after either modified DMM or sham surgery there was no evidence of any osteophytes in any of the sections scored (6 sections per animal, 8 animals per group).

Conclusions: There was a small but significant increase in osteophyte size 16 weeks following DMM surgery when compared to sham surgery. However, there was no evidence of osteophytosis following modified DMM.

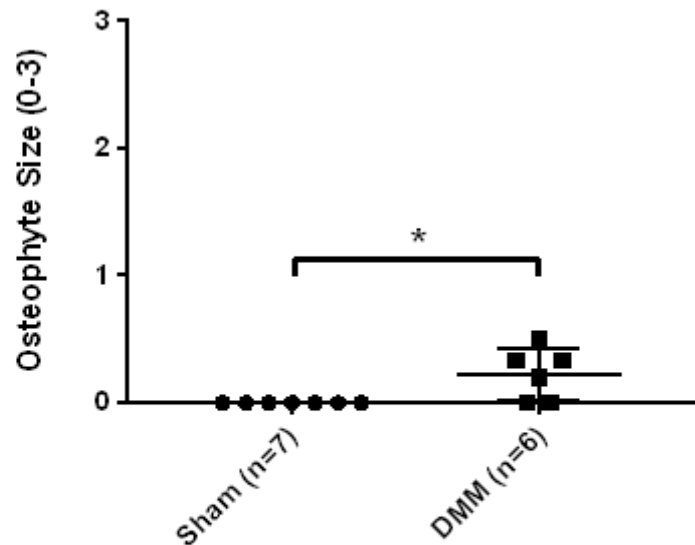


Figure 7.1: Adult male C57BL/6 mice underwent either destabilisation of the medial meniscus (DMM) (n=6) or sham (n=7) surgery. Osteophyte size was scored 16 weeks post-model induction. Data analysed by Mann Whitney U test. * = $p < 0.05$ DMM vs sham.

7.2 Repeated Intra-articular Injection of NGF in C57BL/6 mice

Aims: To quantify effects of repeated intra-articular injection of NGF on localised pain on loading (weight-bearing asymmetry) and distal (hindpaw withdrawal thresholds) pain behaviour in mice.

Methods: Mice received an intra-articular injection of either NGF (10 μ g/5 μ l) or saline (5 μ l), four days later mice received another intra-articular injection of either NGF or saline. Pain behaviour was assessed for 3 days following injection.

Results: Intra-articular injection of NGF produced robust weight-bearing asymmetry (**Fig. 7.2.A**) compared to saline and lowered hindpaw withdrawal threshold (**Fig. 7.2.B**) compared to saline. Intra-articular injection of NGF 96 hours later resulted in a comparable

magnitude of weight-bearing asymmetry irrespective of whether the earlier injection was saline or NGF. Similarly, a second NGF exposure produced comparable lowering of hindpaw withdrawal thresholds irrespective of earlier treatment.

Conclusions: The data here suggests that repeated intra-articular injection of NGF does not result in a prolonged hyperalgesic response. This may be linked the lack of IB4 positive cells that innervate the knee joint (Ivanavicius *et al*, 2004). IB4 positive cells have been shown to be necessary for hyperalgesic priming (Joseph & Levine, 2010).

7.3 Information Regarding the Synovial Samples used for Gene Expression Experiments

Table 7.1: Details of the synovial sections used for the gene expression work. The first three columns show the details for the low inflammation group, and the last three columns show the details of the high inflammation group.

Inflammation Score	Sex	Age	Inflammation Score	Sex	Age
1	M	81	3	M	72
1	F	67	3	F	62
1	F	65	3	F	57
1	M	74	3	M	64
1	F	73	3	F	72
1	M	79	3	M	76
1	M	65	3	M	74
0	M	71	3	M	63

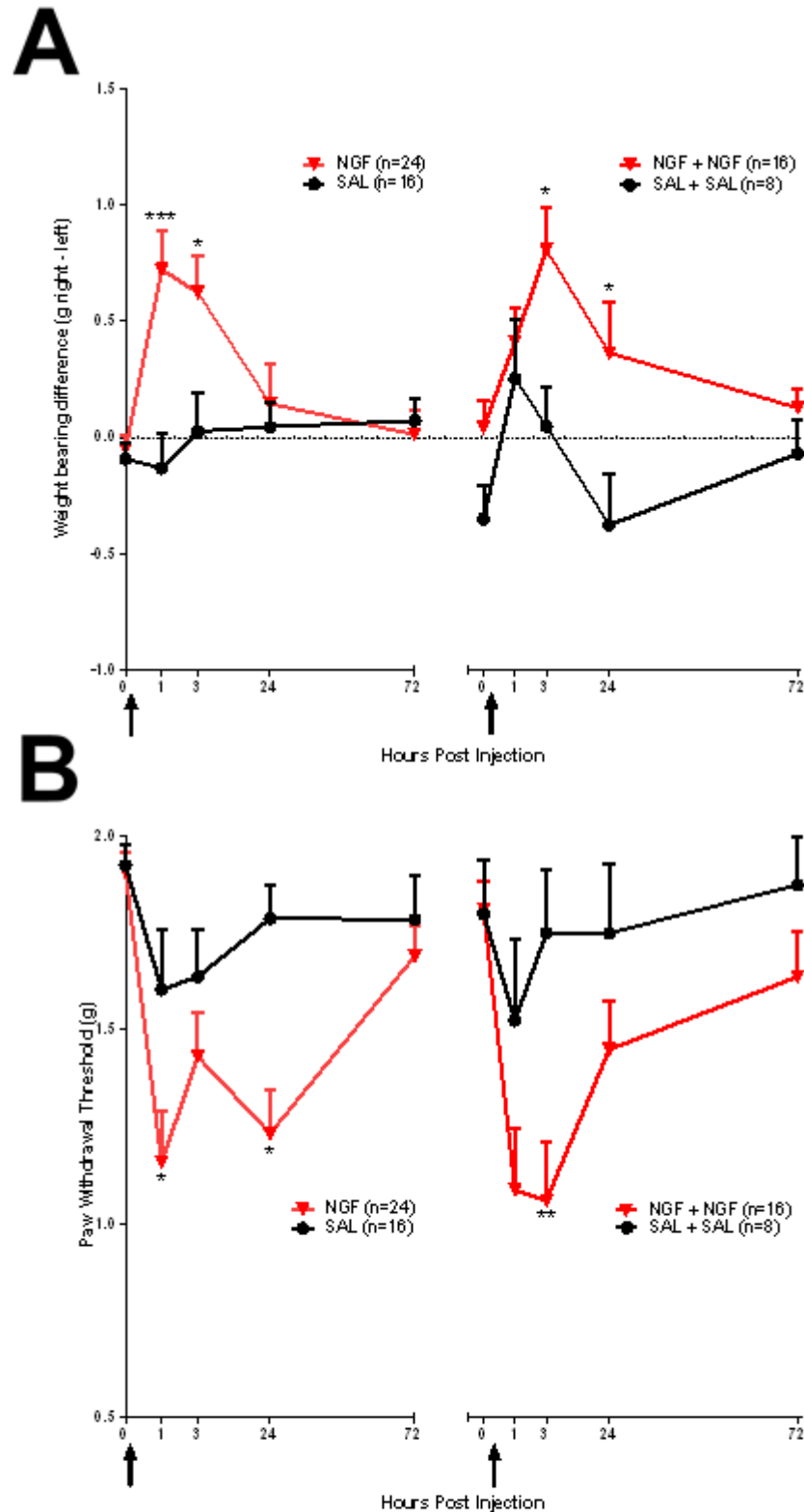


Figure 7.1: Adult C57BL/6 mice received two intra-articular injections into the knee joint of either 10µg/5µl NGF (n=24) or 5µl saline (n=16) followed by another injection of either 10µg/5µl NGF or 5µl saline 4 days later. Both the first and second injection of NGF resulted in significant weight bearing asymmetry (Fig.1.A) and lowered ipsilateral hindpaw withdrawal thresholds (Fig.1.B). There was no significant difference between the magnitudes of the response to NGF following the first or second injection. Data were analysed via 2 way ANOVA with Bonferroni correction. *p<0.05, **p<0.01 & ***p<0.001 versus the saline control.

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