Inflammatory and Post-Inflammatory Nociception in a Model of Arthritis

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Abstract

Pain associated with rheumatoid arthritis (RA) is described as one of the most debilitating symptoms of the condition, often persisting when joint inflammation is pharmacologically controlled. With current treatment options for RA-associated joint pain proving insufficient, a greater understanding is required of nociceptive signalling mechanisms, both in the presence and absence of overt joint inflammation. Animal models of RA pain by recapitulating clinical and pathological features of the human condition provide an important tool for delineating mechanisms contributing to pain in RA. This thesis, utilizing the mouse K/BxN serum-transfer model of inflammatory arthritis in addition to in-vitro experimentation using cultured peritoneal macrophages and dissociated dorsal root ganglion (DRG) neurons, aims to investigate mechanisms leading to heightened nociceptive signalling either in the presence and absence of joint swelling.

Following passive immunization with K/BxN serum, mechanical hypersensitivity presents alongside joint swelling, concomitant to greater calcitonin gene-related peptide (CGRP) expression from DRG neurons and increased cFos expression centrally, from superficial laminae dorsal horn neurons, indicative of neuronal activation. Following serum transfer, persistent hyperalgesia that outlasts joint swelling occurs concomitant to greater macrophage presence in K/BxN DRG. Lipid mediator profiling of lumbar DRG, the site of sensory neuron cell bodies innervating the ankle joints, revealed mechanical hyperalgesia alongside joint swelling (5 days post-immunization) is associated with greater pro-inflammatory PGD₂ levels in the K/BxN DRG, whereas persistent hyperalgesia which outlasts joint swelling (25 days post-immunization) occurs concurrent with reduced pro-resolution Maresin-1 (MaR1) levels in the K/BxN DRG. Following serum transfer, repeated, but not acute, systemic treatment with MaR1 either during or post-joint swelling caused sustained reversal of hyperalgesia, reduced pro-inflammatory macrophage presence in lumbar DRG (PNS) and reduced dorsal horn microglial activation (CNS). In-vitro experiments revealed that when applied to cultured peritoneal macrophages challenged with pro-inflammatory LPS, MaR1 reduced pro-inflammatory cytokine (TNFα & NOS2) expression and miR-155, with no effect on anti-inflammatory signalling. Moreover, calcium imaging demonstrated that MaR1 application inhibits neuronal activation of DRG neurons.
exposed to capsaicin in a dose dependant manner, with MaR1’s action dependant on GPCR_i/o signalling. Assessment of miRNA expression highlighted dysregulation of miRNAs in K/BxN DRG, where in the absence of joint swelling, hyperalgesia is associated with up-regulation of miR-706 and down-regulation of miR-709 expression. Further in-vitro investigation highlighted that following capsaicin exposure, DRG neurons exhibit increased miR-706 and miR-21 expression, which is unaffected by MaR1 treatment. Taken together, the findings of this thesis demonstrate that in the K/BxN serum transfer model of inflammatory arthritis, there are distinct neuro-immune signalling mechanisms relating to mechanical hyperalgesia either in the presence or absence of joint swelling. Specifically, in the K/BxN DRG, where cell bodies of nociceptors reside, when joint swelling has resolved persistent hyperalgesia is associated with increased pro-inflammatory macrophage presence, increased miRNA dysregulation, and reduced pro-resolution MaR1 levels. MaR1 supplementation leads to long-lasting anti-hyperalgesic actions. Although I also observe direct inhibition of capsaicin-sensitive sensory neuron activation, this MaR1-mediated anti-hyperalgesia is likely through reduction of pro-inflammatory macrophage signalling to neurons in the DRG. Collectively, the findings of this thesis highlight the crucial role neuro-immune interactions play in maintaining persistent nociceptive states associated with RA, whereby further investigation of aberrant pro-resolution mechanisms can inform novel therapeutic strategies against arthritis joint pain.
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Abbreviations

µg  Microgram
µl  Microlitre
µM  Micromolar
1-Ak  1-Ak MHC Class II
2-AG  2-Arachidonoylglycerol
2-AG-3P  2-Arachidonoylglycerol-3-phosphate
5-HT  5-Hydroxytryptamine (Serotonin)
AA  Arachidonic acid
aa-COX-2  Aspirin-acetylated cyclooxygenase 2
ABC  Avidin biotin complex
ACC  Anterior cingulate cortex
ACPA  Anti-cyclic citrullinated peptide antibodies
AEA  N-arachidonoylthanolamine
AGO  Argonaute
AIA  Antigen-induced arthritis
AMPA  α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMY  Amygdala
ANOVA  Analysis of variance
APCs  Antigen-presenting cells
ARG1  Arginase 1
ASIC  Acid-sensing ion channels
ATP  Adenosine triphosphate
BBB  Blood brain barrier
bFGF  Basic fibroblast growth factor
BG  Basal ganglia
BLT  Leukotriene B receptor
BMDMs  Bone marrow-derived macrophages
BSA  Bovine Serum Albumin
C. elegans  C. (Caenorhabditis) elegans
C5a  Complement 5a
\( \text{Ca}^{2+} \) Calcium ions
CAAP1 Caspase activity and apoptosis inhibitor 1
CAIA Collagen antibody induced arthritis
CamKII \( \text{Ca}^{2+}/\text{calmodulin-dependent protein kinase II} \)
cAMP Cyclic adenosine monophosphate
CAP Capsaicin
CB1/2 Cannabinoid receptor type 1/2
CCI Chronic constriction injury
CCR2 C-C chemokine receptor type 2
CCR4 Carbon catabolite repressor 4
CCR6 C-C chemokine receptor 6
Cdca4 Cell division cycle associated 4 gene
CFA Complete Freud's adjuvant
CGRP Calcitonin gene-related peptide
ChemR23 Chemerin receptor 23
CIA Collagen induced arthritis
CII Type II collagen
citHSP60 Citrullinated heat shock protein 60
CNS Central nervous system
COX Cyclooxygenase
cPLA\(_2\) Cytosolic phospholipase A\(_2\)
CR Calretinin
CTLA4 Cytotoxic T-lymphocyte-associated protein 4
CX3CR1 C-X3-C motif chemokine ligand 1
CX3CL1 C-X3-C motif chemokine receptor 1
CX43 Connexin 43
CYP450 Cytochrome P450
CysLT Cysteinyl leukotriene
D25 Day 25 post-immunization
D5 Day 5 post-immunization
DAG Diacylglycerol
DAMPs Damage-associated molecular patterns
DCP1 mRNA-decapping enzyme 1
DDX6  DEAD box helicase DEAD box protein 6
DHA  Docosahexaenoic acid
DMARDs  Disease modifying anti-rheumatic drugs
DMEM  Dulbecco’s Modified Eagle’s Medium
DP1-2  Prostaglandin D receptors 1-2
DPA  Docosapentaenoic acid
DRG  Dorsal root ganglion
dsRNA  Double-stranded miRNA
eCBs  Endocannabinoids
ECM  Extracellular matrix
EEG  Electroencephalographic
eIF4F  Eukaryotic translation initiation factor 4F
EP  PGE$_2$ receptors
EPA  Eicosapentaenoic acid
EPSCs  Excitatory post-synaptic currents
EPSP  Excitatory post synaptic potentials
ERK  Extracellular signal-regulated kinase
FBS  Fetal bovine serum
FCεRI  Immunoglobulin E receptors
FcγRI  Fc gamma receptors
FGFR1  Fibroblast Growth Factor Receptor 1
FITC  Fluorescein isothiocyanate
FLS  Fibroblast-like synoviocytes
fMRI  Functional magnetic resonance imaging
FP  Prostaglandin F receptor
FPR2  Formyl peptide receptor 2
Fura-2 AM  Fura-2-acetoxymethyl ester
G6PI  Glucose-6-phosphate isomerase
GABA  Gamma aminobutyric acid
GAGs  Glycosaminoglycans
GDNF  Glial-derived neurotrophin factor
GFAP  Glial fibrillary acidic protein
GFP  Green fluorescent protein
GlyR  Glycine receptor
GM-CSF  Granulocyte–macrophage colony-stimulating factor
GPCR  G-protein coupled receptors
GPR32  G protein-coupled receptor 32
GRK2  G-protein receptor kinase 2
GW  Glycine–tryptophan
GWAS  Genome-wide associated studies
H  Hours
H2  Histamine receptor 2
HBSS  Hank’s balanced salt solution
HLADR  Human leukocyte antigen related
HpETE  Hydroperoxy eicosatetraenoic acid
IB4  Isolectin B4
Iba 1  Ionized calcium-binding adapter molecule 1
IC  Immune complex
ICAM-1, 2  Intercellular adhesion molecule 1 or 2
IFN-c  Interferon-c
IFN-γ  Interferon-γ
IgG  Immunoglobulin G
IgIVg  Intravenous immunoglobulin
IL  Interleukin
IL-1R1  Interleukin-1 receptor 1
iNOS  Inducible nitric oxide
IP  Prostaglandin I receptor
IP3  Inositol 1,4,5-trisphosphate
iPLA2  Calcium independent cytosolic Phospholipase A2
IR  Immunoreactivity
JAK  Janus kinase
JNK  Jun N- terminal kinase
K+  Potassium ions
KCl  Potassium chloride
Kv7  Potassium voltage-gated type 7
LC/MS/MS  Liquid chromatography mass-spectrometry
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<td>MLS</td>
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<td>Metalloproteases</td>
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<td>MRP</td>
<td>Myeloid-related protein</td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response 88</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-acylphosphatidylethanolamine-hydrolyzingphospholipase l</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NK1</td>
<td>Neurokinin 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA2</td>
<td>Phospholipase A2</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
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<td>Phosphorylated p38</td>
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<tr>
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<td>PTPN22</td>
<td>Protein tyrosine phosphatase non-receptor type 22</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>PWT</td>
<td>Paw withdrawal thresholds</td>
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<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of NF-κB ligand</td>
</tr>
<tr>
<td>RET</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>RF</td>
<td>Rheumatoid factor</td>
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<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
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<td>RNA polymerase</td>
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<td>RNAse</td>
<td>Ribonuclease</td>
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<td>Reactive oxygen species</td>
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<tr>
<td>rRNAs</td>
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<td>RT qPCR</td>
<td>Real-time quantitative polymerase chain reaction</td>
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<tr>
<td>RvD</td>
<td>D-series resolvins</td>
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<tr>
<td>RvE</td>
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<td>RVM</td>
<td>Rostral ventromedial medulla</td>
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<tr>
<td>S</td>
<td>Seconds</td>
</tr>
<tr>
<td>SI</td>
<td>Primary somatosensory cortex</td>
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<tr>
<td>SNL</td>
<td>Spinal nerve ligation</td>
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<td>Small nuclear RNAs</td>
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<tr>
<td>SP</td>
<td>Substance P</td>
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<tr>
<td>SPMs</td>
<td>Specialised pro-resolving lipid mediators</td>
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<tr>
<td>Spry2</td>
<td>Sprouty2</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>STAT4</td>
<td>Signal transducer and activator of transcription 4</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
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<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
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<tr>
<td>TNFR1</td>
<td>Tumour necrosis factor α receptor 1</td>
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<tr>
<td>TNRC6</td>
<td>Trinucleotide repeat containing gene 6 proteins</td>
</tr>
<tr>
<td>TP</td>
<td>Thromboxane receptor</td>
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<tr>
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<td>Tumour-necrosis factor receptor family associated factor</td>
</tr>
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<td>TrKA</td>
<td>Tyrosine kinase receptor A</td>
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<tr>
<td>tRNAs</td>
<td>Transfer RNAs</td>
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<tr>
<td>TRPA1</td>
<td>Transient receptor potential ankyrin 1</td>
</tr>
<tr>
<td>TRPV1</td>
<td>Transient receptor potential vanilloid 1</td>
</tr>
<tr>
<td>TSA</td>
<td>Tyramide solution amplification</td>
</tr>
<tr>
<td>TTX-r</td>
<td>Tetrodotoxin-resistant</td>
</tr>
<tr>
<td>TXAS</td>
<td>Thromboxane A synthase</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VSCCs</td>
<td>Voltage sensitive calcium channels</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight by volume</td>
</tr>
<tr>
<td>WDR</td>
<td>Wide dynamic range neurons</td>
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Published Papers

Chapter 1:
General Introduction
1.1 Mechanisms of Pain

1.1.1 Primary Afferent Fibres and Sensory Neurons

The ability to sense pain is an essential aspect of our wellbeing, protecting us from injury and potentially harmful stimuli in the environment. Pain can be defined as an unpleasant sensory and emotional experience which is associated with actual or potential tissue damage (International Association for the Study of Pain). Nociception represents the first stage of pain perception and is the process by which intense thermal, mechanical, or chemical stimuli are detected by a subpopulation of peripheral nerve fibres called nociceptors (Basbaum et al., 2009). These neurons are pseudo-unipolar with a single axon that bifurcates creating one branch which projects to the periphery and another branch projecting into the central nervous system (CNS), either at the level of the spinal cord or brainstem (Dubin and Patapoutian, 2010).

In the periphery, nociceptors have specialized free nerve endings that are widely located in the skin, muscle, joint capsule, bone and viscera. These are finely tuned to detect a wide variety of noxious stimuli through the activation of modality-specific sensory transduction molecules. Nociceptors consist of four main functional components; i) peripheral terminals which transduces external inputs via action potential generation, ii) axons whereby these action potentials are propagated, iii) the soma which maintains neuronal function and integrity and iv) central terminals which allow for synaptic transmission via the release of neurotransmitters across the synaptic cleft to post-synaptic neurons of the CNS (Marmigere and Ernfors, 2007). All sensory afferents use glutamate as their primary fast neurotransmitter and thus have an excitatory action on their postsynaptic targets in the CNS. Cell bodies of sensory neurons innervating the head and face reside in trigeminal ganglia, near the base of the skull, whereas sensory nerves innervating the rest of the body reside in dorsal root ganglia (DRG) just outside the spinal cord.

Based on conductive properties and level of myelination, nociceptors can be categorised into three separate classes: Aβ, Aδ and c-fibres. Aβ-fibres are the largest diameter (> 10 μm) myelinated afferents with low mechanical threshold, transmitting non-painful, tactile, and proprioceptive information. Aδ fibres represent a smaller diameter (> 2 - 6 μm) subset of myelinated afferents which can also convey innocuous mechanical inputs, but predominantly communicate initial well localised noxious
mechanical stimulation and noxious heat signalling, via either Type I or Type II Aδ fibres respectively (Basbaum et al., 2009). C fibres are the most abundant sensory neuron class, representing over 50% of all somatosensory neurons. These small diameter (0.2 - 1.4 μm) fibres are unmyelinated and therefore slowly conducting (~2 m/s). C-fibres respond to predominantly noxious stimuli including pruritogens, tissue damage, chemical irritants, noxious temperatures and mechanical stimuli. These high activation thresholds fibres can be divided further into two broad categories: peptidergic and non-peptidergic. Many of the peptidergic neurons express neuropeptides including NGF, substance P and CGRP, in addition to ion channels including the heat-sensitive capsaicin receptor and transient receptor potential cation channel subfamily V member 1 (TRPV1) and NGF receptor TrkA. The non-peptidergic population, making up around 30% of DRG neurons, express the glial-derived neurotrophin factor (GDNF) sensitive receptor, receptor tyrosine kinase (RET). These RET-positive neurons also bind isolectin B4 (IB4) and express the ionotropic purinergic receptor P2X3, as well as Mas-related G-protein coupled receptor member D (MRGPRD), a sensory neuron-specific G protein-coupled receptor (Peirs and Seal, 2016).

Genetic deletion of the heat-sensitive TRPV1 in mice impairs response to heat only at high noxious temperatures, indicating the existence of additional heat sensors (Voets et al., 2004, Dhaka et al., 2007). Cold sensitivity is mediated by fibres expressing TRPA1 projecting to lamina I of the spinal dorsal horn. Additionally, TRPM8 channels which can be activated by cooling and menthol, play a critical role in low temperature detection as selective ablation of TRPM8-expressing cells completely prevents noxious cold sensitivity (Knowlton et al., 2013).

Unbiased classification of DRG nociceptors, using single-cell RNA sequencing of sensory neurons, established neuronal subsets based on function and identified 3 main sensory afferent categories: i) Noxious cold-sensing neurons that are further subdivided in Trpm8.1, Trpm8.2, Trpm8.3, ii) Mechano-noxious heat sensitive neurons which are further subdivided in peptidergic C fibres PEP1.1, PEP1.2, PEP1.3, PEP1.4 and Aδ peptidergic fibre PEP2; and iii) Noxious itch-mechano-heat sensitive neurons including nonpeptidergic C fibres NP1, NP2, NP3 (Usoskin et al., 2015).
Primary afferents terminate with a specific distribution pattern that is determined by their functional class, whereby myelinated low-threshold mechanoreceptive Aβ afferents arborize in an area extending from lamina II–V, whereas nociceptive Aδ and C afferents innervate superficial laminae I and II (Figure 1.1). The peptidergic and nonpeptidergic subsets of c-fibre afferents can also be distinguished further, with peptidergic afferents projecting to the most superficial laminae; I and outer II of the dorsal horn with non-peptidergic afferents targeting inner lamina II (Todd, 2010).

1.1.2 The Dorsal Horn of the Spinal Cord

From the periphery, sensory information is conveyed to the spinal cord via first order sensory neurons whose central afferent terminals synapse with second order dorsal horn neurons. In the spinal cord dorsal horn, the central terminals of sensory neurons are organized based on size, density and neuronal pattern to form 10 separate ventro-dorsal oriented laminae (Rexed, 1952). Sensory processing occurs in laminae I–VI of the spinal cord; with motor signalling in laminae VII–IX of the ventral horn and lamina X surrounding the spinal cord central canal (Peirs and Seal, 2016).

The dorsal horn is largely composed of excitatory (75%) and inhibitory (25%) interneurons which innervate localised regions within the spinal cord. Excitatory interneurons release the neurotransmitter glutamate, whereas inhibitory interneurons are mainly GABA-ergic. Although, many inhibitory neurons also are glycine-ergic, suggesting this subset of inhibitory neurons can co-release both GABA and glycine (Todd, 2010). In the dorsal horn, both primary afferent terminals and second order projection neurons contain receptors for excitatory and inhibitory neuromodulators allowing for the regulation of neurotransmitter release from primary afferents as well as direct modulation of projection neuron excitability (Todd, 2010).

Laminae I–II, known collectively as the superficial laminae, mostly consist of local interneurons. However, the largest number of projection neurons in the dorsal horn are found in lamina I and can be defined based on their expression for the substance P receptor, neurokinin 1 (NK1) (Mantyh et al., 1997, Todd et al., 2002). These projection neurons are mainly targeted by nociceptive afferents responding to noxious input (Todd et al., 2002). Interneurons are found to be densely packed in Lamina III,
where these PKC-γ expressing cells receive innocuous input from myelinated Aβ fibres. Further input from non-nociceptive Aβ fibres is also directed towards neurons in the deeper laminae, IV to VI, where interneurons are greater in size in comparison to superficial dorsal horn interneurons (Todd et al., 2002, Todd, 2010). Within the deeper laminae, mostly of lamina V, innocuous and noxious sensory input from monosynaptic A-fibres converges with polysynaptic C-fibres. These neurons, known as wide dynamic range neurons (WDR), enable a response to a broad range of stimulus intensities (Figure 1.1)(D’mello and Dickenson, 2008).

Communication between primary afferent terminals and second order neurons in the dorsal horn is dependent on release of neurotransmitters into the synaptic cleft via the exocytosis of vesicles from the pre-synaptic membrane (Dubin and Patapoutian, 2010). All primary afferent central terminals express the excitatory neurotransmitter glutamate, which is stored within synaptic vesicles of pre-synaptic terminals. Following membrane depolarisation and associated increases in intracellular calcium influx, glutamate is released from sensory neuron terminals (Merighi et al., 1991), where the neurotransmitter can then activate glutamate receptors present on the post-synaptic membrane such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and ionotropic N-methyl-D aspartate (NMDA) (Okuse, 2007).

In the spinal cord, glutamate-mediated monosynaptic rapid transmission is dependent on AMPA receptors, with NMDA receptors playing a greater role in synaptic plasticity (Hosli et al., 1983, Mayer et al., 1987). Following noxious stimulation of primary afferents, glutamate-mediated activation of AMPA receptors on the post-synaptic second-order dorsal horn neuron causes an influx of Na⁺ ions and associated depolarisation of the post synaptic neuron. Although NMDA receptors are also responsive to glutamate, receptor activity is tonically inhibited by a magnesium ion (Mg²⁺) block. However, following sustained membrane depolarisation this Mg²⁺ ion block is removed, allowing for glutamate-mediated NMDA receptor activation and Ca²⁺ ion influx which, in addition to membra depolarisation, leads to calcium/calmodulin dependent intracellular signalling pathway in post-synaptic neurons. This process contributes to long term potentiation and central sensitisation mechanisms (Bleakman et al., 2006).

Additionally, from peptidergic C-fibres, alongside glutamate the neuropeptides substance P and CGRP can be released. Following noxious stimulation substance P
and CGRP are released from central terminals of primary afferents, mostly exerting effects via NK1 receptors and CGRP receptors, respectively present on post-synaptic membrane surface of second order dorsal horn neurons (Malcangio and Bowery, 1996, Mantyh et al., 1997).

1.1.3 Supraspinal Transmission and Modulation of Pain

From the dorsal horn, nociceptive information is relayed via projection neurons to higher brain regions, of which there are 2 main ascending pathways; spinoparabrachial and spinothalamic. Within a subpopulation in superficial lamina I, projection neurons are distributed extensively. Contrastingly, very few projection neurons are present within lamina II, with sparse distribution also occurring in the deeper laminae III-VI. The importance of projection neurons was highlighted by studies whereby selective ablation of NK1 positive projection neurons using the neurotoxin saporin attenuates chronic pain behaviour in models of inflammatory and neuropathic pain (Mantyh et al., 1997). The spinoparabrachial pathway involves dorsal horn lamina I-derived projection neurons signalling to parabrachial nucleus, periaqueductal grey (PAG) and nuclei of the thalamus. Subsequently, from these regions third order neurons project to regions associated with the affective and emotional aspects of the painful experience such as the hypothalamus and amygdala (Hunt and Mantyh, 2001). The ascending spinothalamic pathway originates from the deeper laminae of the dorsal horn (laminae III-VI) projecting to the thalamic nuclei and onwards to the primary somatosensory cortex conveying information regarding the emotional-discriminative aspects of the painful experience (Peirs and Seal, 2016, Hunt and Mantyh, 2001). There is a third additional ascending pathway, originating from laminae II interneurons, whereby non-peptidergic IB4+ c-fibres synapse onto projection neurons located in lamina V which target neurons in the amygdala, hypothalamus, and globus pallidus. This ascending pathway in addition to targeting regions involved in the autonomic-aversive and emotional-affective pain processing can also project to motor regions (Braz et al., 2005).

Nociceptive transmission can also be modulated by descending pathways from the brain to the spinal cord of which there are two main monoamine-mediated pathways; nucleus raphe magnus-derived serotonergic pathway and noradrenergic pathways of the locus coeruleus and adjacent pontine regions (Bannister and Dickenson, 2017). Descending pathways originating with the periaqueductal grey (PAG) project to the
nucleus raphe magnus (NRM) in the rostral ventromedial medulla (RVM). The NRM can then relays signals via monosynaptic connections with neurons of the superficial dorsal horn, modulating spinal neuronal responses to noxious stimulation (Ossipov et al., 2014, Schweinhardt and Bushnell, 2010). These RVM-derived descending projections can have both facilitatory and inhibitory effects on synaptic transmission via Serotonin/5-HT receptor and α2 adrenoceptor signalling respectively (D’mello and Dickenson, 2008, Todd, 2010).

In response to noxious stimuli, functional magnetic resonance imaging (fMRI) studies in humans have demonstrated the coordinated activation of several brain areas, including the thalamus, anterior cingulate cortex (ACC), insular cortex, primary and secondary sensory cortices, prefrontal cortex, basal ganglia, cerebellum, and amygdala (Figure 1.1)(Peirs and Seal, 2016). This network of brain regions involved in both sensory discriminative and emotional-affective aspects of pain is termed the pain matrix, with machine learning algorithms which assess fMRI patterns of activity across the pain matrix showing a high predictive capacity for distinct forms of pain, within the pain matrix (Krishnan et al., 2016).
Figure 1.1: Overall organization of somatosensory circuits.
(A) Primary sensory afferent neurons are activated by a variety of stimuli (bottom left) at their peripheral terminals. In response to stimuli-mediated activation, signals from the periphery are transmitted by primary afferent fibres along their axons to the dorsal root ganglia (DRG) where primary afferent neurons cell bodies are located and projecting the signal then onto central terminal of sensory neurons in the spinal cord dorsal horn. (B) Primary afferent neuronal populations exhibit differing characteristics; Aβ (blue) have large-sized cell soma and thickly myelinated fibres, Aδ DRG neurons (purple) are medium-sized and thinly myelinated fibres, C-fibres DRG neurons (red) are unmyelinated and display the smallest sized soma. (C) In the DH (right), large diameter non-nociceptive Aβ fibres have central terminals which terminate in the deeper laminae (III-V), synapsing onto wide dynamic range (WDR) and non-nociceptive (NN) neurons. Located in the most superficial laminae (lamina I-II), the central terminals of C-fibres synapse onto nociceptive-specific (N) second-order projection neurons, alongside central terminals of Aδ primary afferents, which can also terminate in the deeper laminae of DH onto synapsing onto wide-dynamic range (WDR) neurons (lamina V). (D) Projection neurons send information to the brainstem and thalamus and then on to several brain regions implicated in sensory-discriminative (upper left, light brown) and emotional (upper left, dark brown) sensory perception. ACC, anterior cingular cortex; SI (II), primary (secondary) somatosensory cortex; PAG, periaqueductal gray area; PB, parabrachial nucleus; AMY, amygdala; PFC, prefrontal cortex; BG, basal ganglia. (Adapted from Peirs et al., 2016)
1.1.4 Chronic Pain

Although nociceptive pain typically lasts for the duration of noxious stimuli presentation or until healing of stimuli-induced tissue damage, pain can persist after resolution of these conditions developing into a maladaptive pathological state known as chronic pain. Chronic pain can broadly be divided into neuropathic and inflammatory pain, with mechanistic changes in both the PNS and CNS leading to enhancement and persistence of nociceptive transmission.

Neuropathic pain occurs typically in response to direct physical trauma of the nervous system, neuronal exposure to toxins or following disease such as herpes zoster infection and diabetes causing damage to nerves of the PNS and CNS. This condition can be described as pathologic pain, as neuropathic pain serves no physiological purpose, and the pain could be in the form of continuous sensation or episodic incidents. Neuropathy is often associated with allodynia, whereby a nociceptive response from a stimulus that is deemed as non-painful in normal conditions.

Inflammation is a protective mechanism evoked by injury, infection or irritation and is characterized by five key components: redness, heat, swelling, pain and loss of function (Ji et al., 2016). Inflammatory pain occurs due to the release of pro-nociceptive inflammatory mediators which sensitize and activate peripheral sensory neurons. These pro-inflammatory mediators are released from damaged cells, as well as from immune cells which have infiltrated to the site of injury creating an inflammatory soup. Inflammation can also be neuron derived, whereby activation of peripheral nociceptors triggers the release of neuropeptides CGRP and substance P in a process known as neurogenic inflammation. These pro-nociceptive inflammatory processes, by creating an increased pain response to normally noxious stimuli (hyperalgesia), aid the healing process and limit further damage to injury site. However, in chronic inflammatory conditions, such as arthritis, diminished resolution of inflammation and persistent states of hyperalgesia are observed where the presence of pro-nociceptive mediators leads to sensitization in both PNS and CNS (Schaible et al., 2002).

In chronic pain settings, pain is no longer protective and can either be i) spontaneous, ii) elicited by normally innocuous stimuli (allodynia), iii) heightened or prolonged in
response to noxious stimuli (hyperalgesia) or iv) spread beyond the site of injury (secondary hyperalgesia). These persistent pain states are broadly due to alterations in the activation thresholds of neurons and can be mediated by changes in plasticity in both the periphery (PNS) and centrally (CNS), known as peripheral and central sensitization, respectively (Schaible et al., 2002).

1.1.5 Peripheral Sensitization

Peripheral sensitization involves a reduction in activity threshold of nociceptors, typically as a result of changes in the chemical milieu surrounding the primary afferent nerve. This may be due to cell damage and mediator release from a variety of proximal cell populations to the injury/inflammation, such as endothelial cells, peripheral nerve terminals and immune cells (Ji et al., 2014). Commonly referred to as the inflammatory soup, these pro-inflammatory mediators include: prostanoids, histamine, cytokines, chemokines, neuropeptides, ATP, growth factors in addition to extracellular proteases and protons (Figure 1.2) (Pinho-Ribeiro et al., 2017).

Following their release, these pro-inflammatory mediators can act directly on cell surface receptors and ion channels present on the primary afferent free nerve ending. Subsequently, activation of these ionotropic receptors (i.e. ATP P2X3 receptor), G-protein coupled receptors (i.e. receptors for prostaglandins, and neuropeptides), and tyrosine kinase receptors (i.e. growth factor receptors) leads to the induction of several intracellular signalling cascades including protein kinase A (PKA), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), and the MAPKs: extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal (JNK) via second messengers such as Ca2+ and cAMP (Gold and Gebhart, 2010, Ji et al., 2014).

Activation of these kinases and respective intracellular pathways leads to the phosphorylation of various proteins and a substantial increase in the transcription of neuropeptides, growth factors, voltage gated ion channels (e.g. Nav1.7, Nav1.8, & Nav1.9) and transduction channels such as transient receptor potential ion channel A1 and V1 (TRPA1 and TRPV1) present on the nociceptor surface (Pinho-Ribeiro et al., 2017, Schaible et al., 2011). These sensitization mechanisms lead to an increase in the basal sensitivity of nociceptors to both noxious and innocuous stimuli,
consequently increasing neuronal excitability and action potential generation. Heightened sensory neuron excitability, mediated by peripheral sensitization mechanisms, leads to enhanced nociceptive communication to the spinal cord and therefore greater central nociceptive transmission (Woolf and Ma, 2007).

1.1.6 Immune Cell Contribution to Peripheral Sensitization

Following tissue or nerve damage activation of resident immune cells, in combination with infiltrating immune cells, contribute to the release of immune mediators and sensitization of nociceptors, at both their peripheral terminals and cell bodies of the DRG. Immune cells are shown to play a critical role in the development of peripheral sensitization, particularly mast cells, neutrophils, and macrophages.

1.1.6.1 Mast cells

Mast cells are immune cells resident to peripheral nerves playing a key role in in the inflammatory response and sensitization of nociceptors (Pinho-Ribeiro et al., 2017), participating in the inflammatory response through synthesis and secretion of cytokines in close proximity to peripheral nerves (Leon et al., 1994, Chatterjea and Martinov, 2015). Mast cells become activated in response to binding of high-affinity immunoglobulin E receptors (FCεRI) present on their membrane surface (Turner and Kinet, 1999). Upon activation, mast cells degranulate and release cytokines such as IL-5, TNFα, IL-6, in addition to 5-HT and histamine which act through receptors on sensory neuron peripheral terminals, leading to nociceptive sensitization (Pinho-Ribeiro et al., 2017) (Figure 1.2). Mast cell-derived histamine has been shown to bind H1 and H2 receptors on nociceptive neurons, leading to increased expression of Nav1.8 channels and greater sensitivity to mechanical and thermal stimuli (Yue et al., 2014). Similarly, mast cell derived 5-HT can bind to the neuronal 5-HT2 receptors, leading to PKC activation and subsequent increased expression of neuronal acid-sensing ion channels (ASICs), which sense extracellular protons and mediate increased nociceptive signalling (Qiu et al., 2012). Mast cell derived mediators, such as leukotriene B4 (LTB4) also possess chemoattractant properties leading to recruitment of other immune cells, particularly neutrophils, to the inflammatory site,
facilitating greater pro-inflammatory mediator release at the injury site and exacerbating the inflammatory response (Moalem and Tracey, 2006). Mast cells are also known to be a source of pro-nociceptive nerve growth factor (NGF), with depletion of mast cells resulting in diminished NGF production in complete Freud’s adjuvant (CFA) treated rats (Lopes et al., 2017). Moreover, the contribution of mast cells to peripheral sensitization is demonstrated by localised subcutaneous treatment of neuropathic rats with H2 receptor antagonist cimetidine, which reduced development and maintenance of mechanical and thermal hyperalgesia following partial nerve ligation (Zuo et al., 2003).

1.1.6.2 Neutrophils

Neutrophils are a crucial element of the inflammatory response and the most abundant leukocytes, comprising around 70% of white blood cells in mammals (Dancey et al., 1976). Neutrophils under basal conditions are found in blood circulation. However, following injury or tissue damage, an increase of inflammation-induced adhesion molecules causes extravasation at the injury site; a process mediated by Integrin α4β1 expressed neutrophils vascular cell adhesion molecule-1 (VCAM-1). Integrin α4β1 is also involved in neutrophil tethering, rolling and firm adhesion to endothelial cells (Ley et al., 2007), allowing neutrophil recruitment and migration to site of inflammation where these phagocytotic cells accumulate (Nauseef and Borregaard, 2014, Perkins and Tracey, 2000). The migration of neutrophils is dependent upon gradients of chemoattracts including CXCL1 and LTB4, as well as presence of complement 5a (C5a), with antagonism of the C5a receptor leading to reduced hyperalgesic effects in the zymosan, carrageenan and LPS models of peripheral inflammation (Levine et al., 1984, Levine et al., 1985a, Ting et al., 2008). Once at the site of inflammation, neutrophils contribute to neuronal sensitization via the release of pro-inflammatory mediators including IL-1β, TNFα, IL-6 which, when bound to their respective receptors, can sensitize nociceptors leading to heightened nociceptive states (Pinho-Ribeiro et al., 2017). It has also been shown that activation of neutrophils with IL-1β leads to the production of pro-inflammatory mediator PGE2. Although the lifespan and associated proinflammatory action of neutrophils is short (24 hours post-injury), these immune cells can perpetuate the inflammatory response by further releasing chemoattractants
such as CCL3 and CCL4, promoting monocyte/macrophage recruitment to the injury site (Ley et al., 2007).

### 1.1.6.3 Macrophages

Macrophages are large phagocytic cells of the innate immune system which are found in all tissues, where they display great anatomical and functional diversity. Macrophages can be derived from myeloid progenitor cells of the bone marrow which differentiate to pro-monocytes and eventually monocytes. These monocytes are then released into the bloodstream where they continue to develop and mature into functionally distinct inflammatory or resident monocytes (Mosser and Edwards, 2008). Following migration from circulation into the tissue, resident monocytes differentiate to resident macrophages (Zhang and Mosser, 2008). Tissue resident macrophages regulate tissue homeostasis by responding to changes in physiology as well as challenges from the outside environment. During these homeostatic adaptations, macrophages of different phenotypes can also be recruited from monocyte reservoirs of blood, spleen and bone marrow, and suspectedly from resident tissue progenitors or through local proliferation (Wynn et al., 2013). CD115+ CX3CR1+ resident macrophages can be differentiated from inflammatory monocytes, which are low in CX3CR1, and instead possess high levels of chemokine receptor CCR2 (David and Kroner, 2011, Zhang and Mosser, 2008).

Functional classifications of macrophages have traditionally been binary when referring to inflammatory states. These include the activated macrophage and alternatively activated macrophage categories; M1 (classically activated pro-inflammatory macrophages) and M2 (alternatively activated anti-inflammatory macrophages). These two states are defined by responses to the cytokine interferon-c (IFN-c) and activation of Toll-like receptors (TLRs), and to interleukin-4 (IL-4) and IL-13, respectively. Monocyte differentiation into M1 macrophages can also induced by interferon-γ (IFN-γ) typically released from natural killer cells and Th-1 lymphocytes. M1 phenotype macrophages exhibit greater production of pro-inflammatory mediators such as IL-1β, IL-6 and TNFα as compared to M2 anti-inflammatory macrophages which contrastingly display comparatively greater levels of anti-inflammatory cytokines.
IL-10 and transforming growth factor (TGF)-β, in addition to upregulation of anti-inflammatory ARG1 (Orecchioni et al., 2019).

As opposed to M1 activated macrophages, M2 macrophages exhibit an anti-inflammatory and pro-resolving function, promoting wound healing and tissue repair (Gordon, 2003). In addition, M2 macrophages have a high phagocytosis capacity, producing extracellular matrix components, angiogenic and chemotactic factors, and IL-10 (Martinez & Gordon, 2014; Mosser et al., 2008). M2 activation of macrophages can be induced by a wide variety of stimuli including immune complexes, complements, apoptotic cells, macrophage colony stimulating factor (MCSF), interleukin-4 (IL-4), IL-13, IL-10 and tumour growth factor beta (TGF-β) (Murray et al., 2014; Roszer, 2015). Anti-inflammatory mediators such as IL-4 or IL-13 can be secreted by innate and adaptive immune cells, including mast cells, basophils, and TH-2 lymphocytes (Stein & Keshav., 1992; Doyle et al., 1994). Based on the applied stimuli and the achieved transcriptional changes, the M2 macrophages have been classified into subdivisions; M2a, M2b, M2c and M2d. M2a, also known as alternatively activated macrophages, become activated following exposure to IL-4 and IL-13 which binds IL4 receptors present on macrophage cell surface. The M2b phenotype is induced by immune complexes and lipopolysaccharide activation via Toll-like receptors on the macrophage surface, with the M2c phenotype induced by glucocorticoids and TGF-β mediated signalling (Gordon, 2003; Martinez et al., 2008). Induced by the TLR antagonists, M2d macrophages lead to the release of IL-10 and vascular endothelial growth factors (VEGF) (Ferrante & Leibovich, 2012).

The anti-inflammatory profile of alternatively activated macrophages is characterized by high expression of mannose receptor (CD206), as well as pro-fibrotic factors including TGF-β, insulin-like growth factor 1 (IGF-1) and ARG1. These factors actively suppress inflammation and help promoting tissue repair, with increased ARG1 activity resulting in production of polyamines and collagen known to favour tissue remodelling and wound healing (Mantovani et al., 2013). In addition, further markers and effectors associated with M2 polarization include STAT6, SOCS1 (suppressor of cytokine signalling 1), PPARγ (peroxisome proliferator-activated receptor gamma), CD163 and matrix metalloproteases (MMPs) (Viola et al., 2019).
Following infection, injury or tissue damage, macrophages become rapidly activated by pathogen associated molecular patterns (PAMPs) binding to cell surface receptors, as well as inflammatory mediators produced by other immune cell populations such as neutrophils. Activation of toll-like receptors on macrophages, by lipopolysaccharide (LPS), represents a well-established mechanism of macrophage activation eliciting an upregulation of cell surface receptors, secretion of a range of pro-inflammatory mediators and facilitation of enhanced phagocytic activity (Zhang and Mosser, 2008). In models of inflammatory pain, macrophages have been shown to contribute to nociceptive states via the release of proinflammatory mediators, whereby loss of peritoneal macrophages leads to a reduction in zymosan-induced pain behaviours (Ribeiro et al., 2000). Moreover, pharmacological blockade of pro-inflammatory TNFα and IL-1β also reduced zymosan-induced nocifensive behaviours, indicating that pro-inflammatory mediators known to be produced by activated macrophages play a key role in chronic inflammatory nociceptive signalling (Ribeiro et al., 2000). Additionally, macrophage-derived pro-inflammatory mediators such as TNFα, IL-1β, IL-6 and prostaglandins are thought to directly contribute to neuron hyperexcitability (Woolf and Ma, 2007, Pinho-Ribeiro et al., 2017). Studies demonstrate that stimulation of P2X4R in macrophages is shown to trigger calcium influx and p38 MAPK phosphorylation which results in cytosolic PLA2 activation and ultimately COX-dependent release of pro-inflammatory PGE2 (Ullmann et al., 2010). Moreover, transfer of activated macrophages to the paws of naïve mice has been demonstrated to elicit mechanical hypersensitivity in naïve mice in P2X4R dependant manner (Ullmann et al., 2010). Macrophages have also been shown to contribute towards the development of peripheral sensitization in models of neuropathic pain whereby, following chemotherapy-induced neuropathy, as a result of chemoattractant CX3CL1 release from endothelial cells, there is infiltration of monocyte/macrophages to the sciatic nerve associated with development of allodynia, with transient monocyte/macrophage depletion demonstrating an anti-hyperalgesic effect in neuropathic animals (Old et al., 2014).
Figure 1.2: Immune cell mediated peripheral sensitization of nociceptor sensory neurons

During inflammation, tissue-resident and recruited immune cells secrete molecular mediators that act on the peripheral nerve terminals of nociceptor neurons leading to peripheral sensitization of the sensory neuron. In these neurons, specific cytokine, lipid, and growth factor receptor intracellular signalling pathways lead to phosphorylation and/or gating of ion channels Nav1.7, Nav1.8, Nav1.9, TRPV1, and TRPA1. Activation of these ion channels results in increased action potential generation and nociceptive sensitivity. Upon degranulation, mast cells can release Interleukin 5 (IL-5), serotonin (5-HT), histamine, and nerve growth factor (NGF) that act on IL-5R, 5-HT2, histamine receptor 2 (H2) and TrkA receptors, respectively present on the nociceptor terminal subsequently facilitating peripheral sensitization. During inflammatory states, primary sensory neurons are also sensitized by TNFα, IL-1β, and IL-6 produced by mast cells, neutrophils and pro-inflammatory M1 macrophages. In anti-inflammatory states the excitability of the primary afferents can be suppressed by M2 macrophage-derived IL-10 and TGF- β. These act via their respective cell surface receptors, IL-10R and TGF- βR, present on nociceptive neurons to inhibit MAPK phosphorylation and inhibit peripheral sensitization (Chen et al., 2020). However, during inflammatory states, macrophages can become skewed towards a pro-inflammatory M1 phenotype, leading to diminished macrophage-mediated IL-10 release and reduced IL-10 induced suppression of nociceptive signalling. TNFα receptor 1 (TNFR1) activation leads to phosphorylation of Nav1.9 channels. Activation of IL-1 receptor 1 (IL-1R1) increases TRPV1 expression by nociceptors, while IL-6 binds gp130 on nociceptors resulting in enhanced responsiveness to heat and reactive chemicals. Prostaglandin E₂ (PGE₂) released by macrophages and other innate
immune cells can also sensitizes nociceptor neurons through PGE$_2$ receptors 1–4 (EP1–4) present on peripheral terminal of sensory neurons (Pinho-Ribeiro et al., 2017).

1.1.7 Central Sensitization

In the spinal cord, following altered nociceptive transmission from the periphery, a variety of plastic changes occur which further enhance nociceptive signalling by facilitating an increase in responsiveness of nociceptive neurons in the CNS. As with peripheral sensitization, this phenomenon may manifest as allodynia, hyperalgesia or secondary hyperalgesia (Latremoliere and Woolf, 2009). Increased activity from primary sensory neurons leads to an increase in release of glutamate, as well as other neuromodulators (SP, CGRP and ATP) to second order nociceptive neurons of the spinal cord (Todd, 2010). Persistently increased release of these neurotransmitters and neuromodulators at nociceptor central terminals induces frequent excitatory post synaptic potentials (EPSPs) from second order neuron leading to sufficient temporal summation of EPSPs to induce removal of Mg$^{2+}$ ion blockage from NMDA receptors. Subsequently, NMDARs are free to be activated by extracellular glutamate which facilitates an increase in intracellular Ca$^{2+}$ influx and resultant post-synaptic depolarisation (Latremoliere and Woolf, 2009). Additionally, Ca$^{2+}$ influx leads to activation of Ca$^{2+}$-dependant kinases in the dorsal horn neuron, such as PKA, PKC and MAPK, triggering intracellular signalling pathways which result in increased phosphorylation of glutamate receptors (AMPA and NMDA), in addition to other receptors and ion channels present on the neuronal membrane. Moreover, as a result of Ca$^{2+}$-dependant intracellular signalling there is also an increase in synthesis and trafficking of receptors and ion channels to the membrane surface (Latremoliere and Woolf, 2009).

In addition to increased excitatory firing in the spinal cord, disinhibition is also known to contribute to central sensitization, whereby there is a reduction in input from inhibitory GABA-ergic and glycinergic interneurons of the spinal cord. Under basal conditions, inhibitory interneurons continuously release GABA and/or glycine to decrease excitatory output from nociceptive dorsal horn neurons (Basbaum et al., 2009). Studies have identified that dorsal horn neurons are relieved from glycinergic neurotransmission by the inflammatory mediator prostaglandin E$_2$ (PGE$_2$), which by
activating PGE$_2$ receptors on inhibitory interneurons leads to PKA-dependent phosphorylation and inhibition of dorsal horn α3 subunit containing glycine receptors (Zeilhofer, 2005).

1.1.8 Glia in Chronic Pain

1.1.8.1 Astrocytes

Astrocytes, representing 20-40% of glial cells, are critical for the maintenance of homeostasis in the CNS (Herculano-Houzel, 2014). These glial fibrillary acidic protein (GFAP)-expressing cells are physically coupled to each other by gap-junction proteins, allowing the free exchange of ions and small cytosolic components between adjacent astrocytes. Under physiological conditions, astrocytes wrap around the soma of neurons providing metabolic support by reducing glutamate spillover. In addition, astrocytes can aid the regulation of physiological levels of extracellular potassium ions (K$^+$), glutamate and water leading to modulation of neuronal network activity (Ji et al., 2019, Simard and Nedergaard, 2004).

The activation of astrocytes, often referred to as astrogliosis, has been demonstrated in the spinal cord dorsal horn in chronic pain models (Garrison et al., 1991, Ji et al., 2013). Moreover, proliferation of dorsal horn astrocytes is associated with maintenance of chronic pain, with inhibition of astroglia proliferation reducing mechanical allodynia in SNL model of neuropathic pain (Tsuda et al., 2011). GFAP expression is observed in all major astrocytic branches and processes. However, during astrogliosis, GFAP is upregulated (Lee et al., 2008). Astrogliosis is also characterized by a reduction in the complexity of astrocytic processes, thickening of the main processes, upregulation of connexin 43 (CX43) and activation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) signaling (Ji et al., 2019). The activation of these signaling pathways results in the release of astroglial mediators. Activated astrocytes are known to release pro-inflammatory chemokines such as CXCL1 and CCL2, as well as glutamate and ATP. These mediators may act on dorsal horn microglia, which express receptors for ATP, including P2X purinoreceptor 4 (P2RX4), P2RX7 and P2Y purinoreceptor 12 (P2RY12) (Chen et al., 2018a), leading to microglial activation (microgliosis) and subsequently microglial-mediated release of pro-inflammatory cytokines such as TNFα and IL-1β which can
sensitize dorsal horn neurons. Moreover, these proinflammatory cytokines can also feedback to astrocytes, activating intracellular pJNK and pERK pathways, and facilitating further pro-inflammatory chemokine release. Astrocytes can directly induce central sensitization through the release of pro-inflammatory CXCL1, CCL2 and IL-1β, which bind to neuronal CXCR2, CCR2 and IL-1 receptors, respectively. Activation of these neuronal receptors promotes ERK signaling in primary afferent central terminals and second-order neurons of the dorsal horn and intracellular leading to central sensitization (Gao et al., 2009, Ji et al., 2019).

1.1.8.2 Microglia

Microglia are macrophage-like cells which are resident to, and heterogeneously distributed in, the central nervous system. These cells actively maintain homeostatic conditions by undergoing morphological changes in response to mediators in the cellular environment detected by their ramified processes. In response to inflammatory insult or injury, microgliosis occurs whereby glial cells become activated, undergoing morphological changes such as shortening of processes and increased cell body size. This process is coupled with the proliferation and hypertrophy of glial cells (Chen et al., 2018a).

Microglia can become activated through ligand binding to their cell surface receptors such as P2X7, CX3CR1 and TLR4 (Chen et al., 2018a). Activation of TLR4 receptors can lead to microglial release of extracellular ATP. Extracellular ATP is known to bind to P2X7 receptors present on microglial cell surface (Clark et al., 2010). Additionally, nociceptive signalling from dorsal horn neurons can also lead to the exocytotic release of ATP (Masuda et al., 2016). Microglial P2X7 activation causes the intracellular phosphorylation of p38 MAPK and release of inflammatory mediator IL-1β, which can then bind to IL1R present on terminals of second order dorsal horn neurons, facilitating central sensitization.

Inflammatory insult can lead to hyperexcitability of primary sensory neurons and release of caspase-6 from the central terminals of primary afferents. For example, following inflammatory formalin injection, neuronal release of caspase-6 is shown to cause microglial activation via Pp38 MAPK and subsequent release of TNFα at the spinal cord synapse (Berta et al., 2014). Pre-synaptically, TNF receptor activation
leads to glutamate release, increasing nociceptive signalling by binding to second order dorsal horn neurons (Chen et al., 2018). Post-synaptically, extracellular TNF binds to TNF receptors also present on second order neurons, resulting in ERK phosphorylation, positive modulation of AMPA and NMDA receptor activity and ultimately central sensitization of neurons facilitating nociceptive signalling (Chen et al., 2018a).

Activation of microglia via p38 phosphorylation (P-p38) plays a critical role in inflammatory pain states (Svensson et al., 2003b). In the formalin model of inflammatory pain, a transient increase in P-p38 was observed in the ipsilateral dorsal horn up to 20 minutes after injection, with inhibition of p38 phosphorylation significantly reducing formalin-induced nocifensive behaviours (Svensson et al., 2003b). Similarly, in the carrageenan model of inflammatory pain, inhibition of p38 phosphorylation blocked the onset of carrageenan-induced thermal hyperalgesia. The spinal cord phosphorylation of p38 was microglia specific, with activation of microglia via P-p38 causing cFos activation and increased PGE$_2$ production, a pro-inflammatory mediator which facilitates central sensitization (Svensson et al., 2003b, Svensson et al., 2003a).

### 1.2 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disorder primarily affecting the joints. Around 1% of the population worldwide are thought to suffer from RA, with greater incidence found in females who are two- to threefold more likely to develop RA as compared to males (Firestein, 2003, Alamanos et al., 2006). There are several genetic and environmental risk factors associated with the development of RA, with multiple factors likely required to trigger the pathology.

Regarding genetic susceptibility, Gregerson et al. (1987) have demonstrated a specific amino acid motif commonly encoded by some alleles of the class II human leukocyte antigen related (HLADR) locus, especially $HLA$-$DRB1^\ast01$ and $HLA$-$DRB1^\ast04$, which are significantly associated with the risk of developing RA. Since this discovery, using large genome-wide associated studies (GWAS) over 100 loci have been identified as associated with RA pathogenesis (Okada et al., 2014). The second greatest genetic risk association to RA is from protein tyrosine phosphatase non-receptor type22 (PTPN22) which encodes the T-cell receptor regulator, lymphoid tyrosine
phosphatase. Other known RA-associated genes involved in immune cell signalling include cytotoxic T-lymphocyte-associated protein 4 (CTLA4), tumour-necrosis factor receptor family associated factor (TRAF1), Signal transducer and activator of transcription 4 (STAT4) and C-C chemokine receptor 6 (CCR6) (Edilova et al., 2020, Smolen et al., 2018).

Currently the presence of anti-cyclic citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF) represent the two main clinical diagnostic markers for RA. The presence of circulating ACPAs, RF and circulating pro-inflammatory cytokines and chemokines can be detected up to 10 years before clinical disease onset (Smolen et al., 2018). RF is a high-affinity autoantibody against the Fc portion of immunoglobulin. ACPAs are antibodies to autoantigens modified by citrullination through deamination of arginine to citrulline. These antibodies are observed in roughly two-thirds of RA patients, yet only 2% of healthy individuals (Assmann et al., 2014).

Lung stress, instigated by smoking or dust inhalation, is also a risk factor for RA development. Moreover, in HLA-DRB1 patients, a history of smoking is a strong indicator of RA development (Alpizar-Rodriguez and Finckh, 2017). Alternative environmental risk factors for RA include obesity and exposure to infectious diseases including Epstein-Barr virus (Edilova et al., 2020, Smolen et al., 2018).

1.2.1 Pathogenesis of Rheumatoid Arthritis

It has been hypothesised that environmental stressors, such as cigarette smoke, can activate mucosal cells promoting citrullination, via induction of peptidyl arginine deiminases, as well as promoting other post-translational modifications such as acetylation or carbamylation. Moreover, RA-associated citrullination has been shown to occur in response to leukotoxin which increases calcium influx into neutrophils (Konig et al., 2016). These post-translational modifications can be made to a variety of intracellular proteins such as histones, as well as matrix proteins including fibronectin, collagen and fibrinogen.

Following post-translational modification these altered peptides bind to MHC protein heterodimers causing the formation of neo-epitopes presented by antigen-presenting cells to T cell, which become activated (Figure 1.3). Subsequently, T-cell activation
leads to B-cell stimulation and synthesis of a range of autoantibodies, including RF and ACPAs (Smolen et al., 2018) (Figure 1.3). Citrullination-independent mechanisms, via acetylation or non-enzymatic carbamylation, are also likely to turn self-proteins into targets for autoantibody (Trouw et al., 2013).

Although the presence of ACPAs and RF is frequently observed in RA and can be used as a diagnostic and prognostic indicator, their formation is not sufficient for RA disease pathology and synovitis, which requires immune complex formation and complement activation in order to increase vascular permeability and influx of inflammatory cells into the synovium (Smolen et al., 2018). Alternate autoantibodies which recognize immunoglobulins such as glucose-6-phosphate isomerase (G6PI), proteoglycans and other joint autoantigens are thought to also contribute to pathogenesis via immune cell activation. In early RA, activation and infiltration of predominantly CD4+ T cells and macrophages to the synovium leads to characteristic mild swelling of joints on both hands and several proximal interphalangeal joints.

1.2.2 Synovial Hyperplasia

The synovium represents the soft tissue surrounding the joint and is comprised of an outer layer of fibroblasts, adipocytes, blood vessels and scattered immune cells, with an inner layer of macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS). The synovium contains synovial fluid which functions to allow the cartilage surface to operate within a low-friction environment, in addition to providing nutrients to the cartilage, which lacks its own blood supply (Mapp, 1995). Free trafficking of cells and proteins into the synovial fluid occurs via the leaky inner (intimal) synovial lining (Castor, 1960, Smolen et al., 2018). In RA, MLS and FLS of the intimal layer become activated leading to the production of inflammatory cytokines in the synovium. Activation of MLS leads to production of IL-1β, IL-6 and TNFα, whereas FLS activation leads to production of proinflammatory prostaglandins and leukotrienes, altered microRNA expression patterns, as well as the increased production of matrix-degrading enzymes such as metalloproteases (MMPs) (Stanczyk et al., 2008, Tu et al., 2018). In the outer layer of the synovium, RA is associated with an infiltration of CD4+ T cells into the sublining (Ziff, 1974). Additionally, in the synovium, B cells and plasma cells are present producing RF and ACPAs, alongside antigen-presenting
follicular dendritic cells, macrophages and mast cells which are also distributed through the synovial sublining. These activated and infiltrating synovial cells exacerbate the inflammatory response by producing pro-inflammatory cytokines which act in a paracrine or autocrine manner, with macrophage-derived cytokines activating adjacent FLS, T cells and dendritic cells further contributing to synovial hyperplasia.

Pro-inflammatory cytokines TNFα, IL-6 and IL-1β are key mediators of cell migration and inflammation in RA. IL-6 is known to act directly on neutrophils via binding of membrane-bound IL-6R. Studies assessing fibroblasts from patients with RA demonstrate the role of IL-6 in promoting neutrophil recruitment by activated fibroblasts, with recruitment inhibited in the presence of anti-IL-6 antibodies (Lally et al., 2005). Neutrophils are the most abundant leukocytes in inflamed joints (Cecchi et al., 2018), and bind immune complexes on the synovium via their Fcγ receptors. This receptor interaction triggering degranulation and production of reactive oxygen species (ROS) (Cecchi et al., 2018, Cedergren et al., 2007). In RA pathology, this enhanced ROS generation by activated neutrophils at the site of inflammation causes endothelial dysfunction and tissue injury.

As a major source of cytokines, chemokines and degradative enzymes, macrophages play a key role in RA pathogenesis. In joints of RA patients, macrophages with a M1 phenotype promote inflammation and tissue damage, secreting pro-inflammatory cytokines such as TNFα, IL-1β, IL-8, IL-15 and macrophage migration inhibitory factor (MIF) (Mcinnes et al., 2016, Kinne et al., 2000). The synovial macrophages of RA patients have been shown to express lower levels of the M2 macrophage indicator CD209 (Yang et al., 2020). Immunohistological examination of frozen sections of pannus tissue taken from the joints of patients with RA has revealed that the phenotypes of macrophages in the synovial lining differ from macrophages accumulating at the cartilage junction. In RA joints, accumulation of monocytes and macrophages positive for pro-inflammatory markers MRP-8 and MRP-14 proteins was observed in the sublining layer, whereas mature macrophages positive for late inflammatory macrophage markers were more abundant in the lining layers, indicating a distinction between infiltrating (sublining) and tissue-resident (lining) macrophage phenotypes (Udalova et al., 2016, Salisbury et al., 1987).
1.2.3 Cartilage Degradation

Cartilage degradation is a hallmark of RA pathology and facilitated by the invasion of immune cells into the synovium. Cartilage acts as a key component of synovial joints, consisting of chondrocytes and a dense extracellular matrix (ECM). This ECM is synthesized by chondrocytes and contains type II collagen and glycosaminoglycans (GAGs) (Guo et al., 2018). Macrophages, neutrophils and mast cells contribute to joint damage in rheumatoid arthritis through the release of cytokines and MMPs, with cartilage destruction much attributed to cadherin-11 positive FLS which produce MMPs (Kiener et al., 2009). FLS isolated from RA patients, but not healthy individuals, aggressively invade into cartilage explants placed in immunodeficient mice (Muller-Ladner et al., 1996). MMPs are the mediators of cartilage damage, synthesized by FLS and can promote disassembly of the type II collagen network, with membrane-type I MMP thought to be the predominant proteinase that degrades the collagenous cartilage matrix (Sabeh et al., 2010). Articular cartilage has limited regenerative properties. Moreover, in the inflamed RA joint, due to the influence of ROS and synovial cytokines such as IL-1β and IL-17, chondrocytes undergo apoptosis further reducing the regenerative ability of the articular cartilage (Mcinnes and Schett, 2011). These processes ultimately lead to the destruction of the surface cartilage observed in radiographs as a narrowing of the joint space and formation of pannus, an invasive and destructive front of abnormal synovial tissue attached to the articular surface.

1.2.4 Bone Erosion

Another key hallmark of RA pathology is localised bone erosion, which presents in 75% of RA patients within a year of diagnosis (Van Der Heijde, 1995). Bone erosion is associated with joint inflammation whereby the production of synovial cytokines promotes differentiation of bone-reabsorbing osteoclasts and their invasion into the periosteal surface adjacent to articular cartilage (Gravallese et al., 1998, Mcinnes and Schett, 2011). By binding their respective receptors, osteoclast differentiation can be potentiated by macrophage colony-stimulating factor (M-CSF), receptor activator of NF-κB ligand (RANKL) produced by T-cells, in addition to macrophage and FLS-derived inflammatory cytokines associated with RA including TNFα, IL-6, IL-1β and IL-17 (Schett and Teitelbaum, 2009). Studies demonstrate that bone erosion can be
perturbed by clinical inhibition of TNFα, IL-6, and RANKL, whereas in RA patients RANKL inhibition only reduces bone damage, without effecting inflammation or cartilage degradation (Schett et al., 2009). In addition to synovial cytokines, ACPAs are thought to contribute to bone erosion through their binding to citrullinated peptides expressed on osteoclasts and osteoclast precursors, subsequently triggering osteoclast activation. Alternatively, ACPAs may contribute to perturbed bone-producing mechanism int eh joint, with studies demonstrating ACPA-mediated osteoblast via cell-surface binding to citrullinated heat shock protein 60 (citHSP60) with anti-citHSP60 positively associated with joint damage in RA patients (Lu et al., 2016).
Figure 1.3: Mechanisms involved in initiation and progression of rheumatoid arthritis

(A) Environmental and genetic risk factors of rheumatoid arthritis (RA) can potentiate post-translational modifications in the mucosa such as by citrullination or carbamylation. (B) These modified peptides are presented by antigen-presenting cells (APCs) leading to activation of T-cells and B-cells of the adaptive immune response in lymphoid tissues and subsequent formation of autoantibodies such as anti-cyclic citrullinated peptide antibodies (ACPA) and rheumatoid factor (RF). (C) Fibroblast-like synoviocytes (FLS), macrophage-like synoviocytes, APCs and macrophages can be activated locally and produce a range of inflammatory factors. The autoimmune response elicited by the immune system triggers
synovial inflammation. However, this synovial inflammation may require a second hit, such as immune complex formation and complement activation, to induce or increase cytokine production and synovial vascular leakage in the joint. (D) Paracrine and autocrine actions of cytokines, along with persistent adaptive immune responses, can perpetuate the disease and ultimately lead to cartilage and bone destruction. Abbreviations: CCL19, CC-chemokine ligand 19; CCL21, CC-chemokine ligand 21; GM-CSF, granulocyte–macrophage colony-stimulating factor; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; RANKL, receptor activator of nuclear factor-κB ligand; RF, rheumatoid factor; TCR, T cell receptor; TNF, tumour necrosis factor. (Smolen et al., 2018)

1.2.5 Current Treatment for Rheumatoid Arthritis

Current pharmacological intervention strategies for RA include the use of disease modifying anti-rheumatic drugs (DMARDs), which can be broadly characterised into two main groups: Synthetic and biologic DMARDs. The conventional synthetic DMARD, methotrexate, is the most widely used first-line treatment for symptomatic RA, used as either a mono- or combination therapy alongside glucocorticoids (Smolen et al., 2016). Methotrexate is a modified form of vitamin B₉ (folate) which works in various capacities to inhibit adenosine signalling, block ROS formation, downregulate adhesion-molecule expression, modify cytokine expression profiles and downregulate the production of pro-inflammatory eicosanoids and MMPs (Brown et al., 2016).

Traditionally, for RA treatment methotrexate is administered as a 5–25 mg weekly regimen, with dosing conditional to the disease state and side effects. The drug can be administered orally and via subcutaneous injection, with subcutaneous treatment demonstrating greater bioavailability but greater side effects as compared with oral (Schiff and Sadowski, 2017). Alternatively, the immunosuppressant Leflunomide can be used to reduce joint inflammation in RA patients. Leflunomide can inhibit dihydroorotate enzymes essential for producing DNA and RNA, particularly within activated proliferating lymphocytes (Cherwinski et al., 1995). Additionally, Leflunomide treatment is known to dampen early T-cell and B-cell signalling via inhibition of tyrosine kinase activity (Herrmann et al., 2000). Sulfasalazine is another conventional DMARD used to treat RA-induced inflammation. Sulfasalazine is known to modulate RANK
thereby inhibiting osteoclast formation, via modulatory effects of the receptor activator of nuclear factor κB (RANK), promote macrophage apoptosis, inhibit TNFα expression and suppress B-cell function (Lee et al., 2004, Rodenburg et al., 2000, Hirohata et al., 2002). Another DMARD designed for use in RA, hydroxychloroquine, works to reduce the overall inflammatory response by modulating T helper cells and antigen-presenting macrophages interactions, subsequently dampening pro-inflammatory cytokines production and joint inflammation.

Although alternative treatments are available for RA treatment, including Rituximab (B cell inhibitor) and Tocilizumab (anti-IL-6 antibody), effective biologic treatments for clinical RA are largely inhibitors of the pro-inflammatory cytokine TNFα used as monotherapies or in combination with methotrexate. When produced by activated monocytes, macrophages, and T lymphocytes, TNFα acts on TNF receptors 1 and 2 (TNFR1/2) activating intracellular signalling for NF-κB, ERK and RANKL pathways. Treatment with TNFα inhibitors in RA has been shown to modulate a number of processes associated with RA pathology including local bone destruction, endothelial cell activation, MMP production, chondrocyte/osteoclast activation, as well as production of other inflammatory cytokines (Guo et al., 2018). Therefore, when conventional DMARDs display diminished efficacy or tolerability TNFα-inhibitors represent an alternative biologic DMARDs therapy for RA patients. Moreover, it is estimated that following treatment with etanercept, an anti-TNFα recombinant protein, 50-75% of RA patients achieve clinical remission (Guo et al., 2018).

1.3 Pain in Rheumatoid Arthritis

RA can be characterised by joint inflammation, cartilage degradation and bone erosion. However, for people with RA, pain is the predominate symptoms for which they seek medical care, with approximately 70% of RA patients identifying pain relief as their lead priority, as compared to reduction of other RA-related symptoms (Ten Klooster et al., 2007). Arthritis joint pain is known to occur in response to mechanical stimulation of the joint, for example during joint movement or weight-bearing. In addition, spontaneous pain may occur in RA while the joint is at rest. Triggers of pain in RA vary between individuals as well as differing temporally, dependant on early and
late-stage RA pathology. Severity of RA-associated joint pain can also vary during or between inflammatory flares.

People living with RA frequently experience transient increases in joint pain, swelling, and other symptoms such as stiffness and fatigue that indicate increased inflammation and worsening of their RA. These episodes described as flares vary widely in frequency, duration, and intensity. Flares are generally expected to be reversible, though elevated RA disease activity persists in some cases. Although joint inflammation may be present during RA flares, not all RA flares are associated with overt joint swelling or increases in erythrocyte sedimentation rate, used as an indicator of inflammation. This suggests that painful flares might sometimes be distinct from inflammatory flares (Mcwilliams and Walsh, 2017, Bartlett et al., 2012). RA patients describe pain during these inflammatory episodes as throbbing and sharp, as opposed to a dull aching joint pain felt when inflammation is in remission (Roche et al., 2003).

In the RA joint, during inflammatory periods, increased production of pro-nociceptive mediators has been identified in the synovium, as well as synovial fluid. Inflammatory mediators such as prostaglandins, proinflammatory cytokines (i.e. TNFα, IL-1β, IL-6) and growth factors such as NGF are produced by the inflamed synovium, resulting in sensitization of peripheral nerve terminals (Mcwilliams and Walsh, 2017). Sustained nociceptive input can lead to alterations in central pain processing, with central mechanisms also thought to contribute to pain in RA. In agreement, Edwards et al., (2009), identified increased pain sensitivity to mechanical pressure, noxious heat and noxious cold stimuli in RA patients as compared to non-arthritic individuals. Increased RA-associated pain sensitivity was not only observed in inflamed joints of the hand, but also distal non-articular sites such as the trapezius and quadriceps muscles, indicating augmented central pain processing mechanism (Edwards et al., 2009). Moreover, in the spinal cord of RA patients, pain evoked by intradermal capsaicin injection led to pain responses distant from inflamed joints in people with RA, but not healthy controls (Shenker et al., 2008). In addition, supraspinal pain processing is likely affected in RA, with greater electroencephalographic (EEG) activity reported in people with RA following painful intranasal CO₂ stimuli (Hummel et al., 2000).

Pain leads to significant psychological distress for people with RA, who report worse mental health and display higher instances of anxiety and depression in comparison to non-arthritic individuals (Edwards et al., 2011, Walsh and Mcwilliams, 2014).
Moreover, poor mental health is seen as a predictors of poor pain outcomes in RA (Odegaard et al., 2007). Together, this indicates that pain in RA has a significant psychological component, with pain informing an individual’s mood and conversely a negative mood exacerbating RA pain.

1.3.1 Pharmaceutical Treatment Pain in RA

As previously discussed, current treatments for RA largely involve DMARDs such as methotrexate and biologic inhibitors of cytokine signalling such as etanercept. While these drugs show efficacy at controlling the inflammatory symptoms of RA, a significant proportion of patients experience maintained pain when disease activity is low or in remission (Lee et al., 2011). Analgesic agents such as non-steroidal anti-inflammatory drugs (NSAIDs) can be prescribed to help manage pain in RA patients, however due to substantial gastrointestinal toxicity these treatments are inappropriate for long-term use (Sarzi-Puttini et al., 2014). Moreover, although effective as a short-term analgesic, NSAIDs prove ineffective in reducing chronic RA-related pain (Walsh and Mcwilliams, 2014). For pain related to inflammatory flare ups, biologic treatments such as TNFα blockers, are shown to reduce RA associated joint pain, typically within 2–4 weeks of treatment initiation in parallel with reductions in synovitis, where the dampening of inflammation, and associated peripheral and central sensitization, are thought to be the main mechanisms for improving RA pain (Keystone et al., 2004). However, for a proportion of patients TNFα blockers trigger significant adverse effects including upper respiratory tract infections (Alldred, 2001). In addition, alternative treatments for pain in RA, such as topical application of capsaicin and use of tricyclic anti-depressants have shown limited antinociceptive efficacy (Durham et al., 2015). Together, these findings highlight the urgent need to improve current understanding of nociceptive mechanisms underpinning pain in RA, in order to identify novel therapeutics for RA-associated joint pain.

1.3.2 Animal Models of RA Pain

With limited possibilities for experimentation in the human condition, animal models recapitulating key aspects of RA-associated joint pain have proven important for delineating nociceptive mechanisms facilitating the initiation and maintenance of RA
pain. Nociceptive signalling has been investigated in several rodent models of chronic immune-mediated articular inflammation whereby pain-like behaviours have been spontaneously observed in genetically mutated animals such as K/BxN transgenic mice, or induced through exposure to collagen, antigens or via serum-transfer (Nieto et al., 2015, Boettger et al., 2008, Christianson et al., 2010), summarised in Table 1.1. These models share similarities to human RA displaying synovial hyperplasia, joint immune cell infiltration, pannus formation, bone erosion and cartilage destruction. Alternatively, joint pain has also been studied independent of inflammation in rodent models by systemic injection of autoantibodies against citrullinated proteins (ACPA) (Wigerblad et al., 2016). Male B10.RIII mice and BALB/c mice injected intravenously with ACPA IgG from patients with ACPA+ RA, but not from patients with ACPA− RA or healthy individuals, led to the pronounced mechanical and thermal hyperalgesia. Mechanical hypersensitivity was also observed in mice 3 days following immunization with monoclonal mouse ACPA (Wigerblad et al., 2016). Although 7 days following injection ACPA were readily detected in skin, ankle joint, tibial bone marrow and plasma and to some extent in DRG, ACPA did not induce clinical signs of joint inflammation. Stimulation of DRG neurons with ACPA revealed that these autoantibodies do not directly sensitize nociceptors. However, ACPA was found to bind to CD68+ osteoclasts, facilitating the release of chemokine CXCL1. Pharmacological inhibition of CXCR1/2 by Reparixin led to partial reversal of ACPA-induced mechanical and thermal hypersensitivity. Therefore, it is likely that pro-nociceptive effects of ACPA largely involve facilitation of osteoclast-mediated CXCL1 signalling to sensory neurons innervating the joint, subsequently instigating peripheral sensitization of joint nociceptors without inducing an articular inflammatory response (Wigerblad et al., 2016).
<table>
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<tr>
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<th>CIA model</th>
<th>AIA model</th>
<th>CAIA model</th>
<th>K/BxN model</th>
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<tbody>
<tr>
<td><strong>Inflammatory Trigger</strong></td>
<td>CII/adjuvant</td>
<td>mBSA/adjuvant</td>
<td>mAb</td>
<td>Serum</td>
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<tr>
<td><strong>Feature</strong></td>
<td>Autoimmune</td>
<td>(Auto)immune†</td>
<td>Anti-CII antibodies</td>
<td>Anti-GPI antibodies</td>
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<tr>
<td><strong>Arthritis Type</strong></td>
<td>Polyarthritis</td>
<td>Monoarthritis</td>
<td>Polyarthritis</td>
<td>Polyarthritis</td>
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<tr>
<td><strong>Immune Response</strong></td>
<td>Adaptive</td>
<td>Adaptive</td>
<td>Innate</td>
<td>Innate</td>
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<tr>
<td><strong>Similarities to human RA</strong></td>
<td>-Adaptive immune activation</td>
<td>-Adaptive immune activation</td>
<td>-Antibodies against joint-specific epitopes</td>
<td>-Synovial inflammation</td>
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<td>-Infiltrating immune cells</td>
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<td></td>
<td>-Cartilage &amp; Bone destruction</td>
<td>-Chronic inflammation</td>
<td>-Joint hyperalgesia in absence of swelling, unperturbed by NSAID treatment</td>
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<td><strong>Time course of clinical arthritis†</strong></td>
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<td>D3-D21</td>
<td>Mouse strain:</td>
<td>D2-D18</td>
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<td>BALB/c: D9-D27</td>
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<td>CBA:  D6-D30</td>
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<td>QB:  D6-D16</td>
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<td><strong>Duration of mechanical hyperalgesia†</strong></td>
<td>D7-D28*</td>
<td>D7-D28*</td>
<td>Mouse strain:</td>
<td>D2-D28*</td>
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<td>QB:  D3-D30*</td>
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<td><strong>References</strong></td>
<td>(Nieto et al., 2015)</td>
<td>(Boettger et al., 2008)</td>
<td>(Bas et al., 2012, Nandakumar and Holmdahl, 2007)</td>
<td>(Christianson et al., 2010)</td>
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*Study endpoint
†Days following inoculation

Table 1.1: Inflammatory models of RA-associated joint pain
The collagen induced arthritis (CIA) model is the most frequently utilized experimental model of arthritis and is induced by intradermal immunization with an emulsion of complete Freund’s adjuvant and type II collagen (CII) to the base of the tail (Ingliš et al., 2007). The injection of CII initiates an adaptive immune response leading to production of anti-CII antibodies and chronic inflammatory arthritis primarily within joint structures of the fore- and hind paws including the synovium and articular cartilage, in addition to initiating inflammation of surrounding tissues. Joint pathology in the CIA model begins 11 days post-immunization and is strain dependent, with greatest efficacy in Wistar and Sprague-Dawley rats, as well as DBA/1 mice (Nieto et al., 2015, Courtenay et al., 1980). Much like the human RA condition, CIA involves activation of B-cells and T-cells of the adaptive immune system, in addition to persist hyperalgesia, which lasts at least 28 days post-immunization (Bas et al., 2016, Nieto et al., 2015). In the periphery, following CIA induction, increased TNFα levels are observed in serum and joints, with anti-TNFα therapy reducing hind paw hyperalgesia (Ingliš et al., 2007, Bas et al., 2016). Prostaglandins have also been implicated in joint inflammation, with COX-2 inhibitor celecoxib reducing hyperalgesia in CIA model (Ingliš et al., 2007). Studies have also demonstrated that alongside mechanical hyperalgesia joint nociceptors exhibit increased CGRP expression in the DRG (Nieto et al., 2015). Together, these studies highlight in the CIA model that inflammatory mediator-induced peripheral sensitization mechanisms at the joint contribute to hyperalgesia. Nieto et al. (2015) demonstrate that following collagen immunization although heat hyperalgesia develops alongside joint swelling, from day 7 post-immunization, mechanical hypersensitivity is observed prior to joint swelling concomitant to increased dorsal horn microgliosis, indicating a key role for central microglial-mediated mechanisms in the initiation of heightened nociceptive signalling (Nieto et al., 2015). In the spinal cord, following microglial activation, the cysteine protease cathepsin-S is released from microglia and can cleave neuronal-derived cytokine fractalkine, which in its soluble form subsequently activates CX3CR1 receptors present on microglia. This CX3CR1-mediated microglial activation resulting in intracellular p38 MAPK signalling and ultimately release of pro-inflammatory inflammatory mediators, which can then sensitize nociceptive dorsal horn neurons. Clark et al. (2012) highlight that these dorsal horn cathepsin-S/Fractalkine mediated signalling mechanisms are critical to hyperalgesia in the CIA model, with cathepsin-S inhibition or neutralization of
fractalkine resulting in diminished dorsal horn microglial activation and reduced hyperalgesia, without effect on joint inflammation. Collectively, these findings highlight the critical role that central microglial mediated mechanisms play in maintaining nociceptive signalling in the CIA model.

The antigen-induced arthritis (AIA) model represents a pre-clinical monoarthritis model of RA-associated joint pain, initiated by intradermal injection of an exogenous antigen mix containing ovalbumin or methylated bovine serum albumin (mBSA) in CFA. 3 weeks following inoculation, animals are injected in the knee joint with the same antigen, which induces a localised adaptive immune response and inflammation within the injected knee joint (Bas et al., 2016). AIA-induced inflammation is transient, peaking 3 days post-inoculation and lasting for several weeks. This inflammation is characterised by antigen-specific T-cell and immune complex formation in the affected joint (Van Den Berg et al., 2007). Alongside transient joint inflammation, AIA induces transient mechanical and thermal hyperalgesia specific to the affected joint. (Pinto et al., 2010, Bas et al., 2016).

Peripherally, induction of AIA leads to promotion of IL1-R1 expression in the DRG, in addition to increased TNFα production in the affected arthritic joint (Boettger et al., 2008). Increased TNFα production contributes to the pain-like behaviour in the AIA model, as treatment with systemic anti-TNFα blockers leads to a reduction in both mechanical and thermal hyperalgesia (Boettger et al., 2008). In addition, pro-inflammatory IL-6 is found to be elevated in inflamed AIA joints. Local injection of soluble gp130 into the inflamed AIA causes inactivation of IL-6 signalling in the joint and subsequently has a significant anti-hyperalgesic effect indicating that IL-6 mediated peripheral sensitization of the joint afferents contributes to hyperalgesia in the AIA model (Boettger et al., 2010a). Similarly, IL-17 is also shown to be elevated in periarticular tissue of the AIA joint, with neutralising intraarticular and intraperitoneal injection of monoclonal antibodies against IL-17 leading to reduction of AIA-induced mechanical hyperalgesia (Pinto et al., 2010). These findings indicate that in the AIA model, IL-17 contributes to hyperalgesia through peripheral sensitization of joint nociceptors. In the DRG, where cell bodies of nociceptors are present, studies have observed significant infiltration of macrophages into the ipsi- and contralateral lumbar DRGs following AIA induction, as well as de novo expression of vascular cell adhesion molecule-1 (VCAM-1), which is known to be involved in macrophage infiltration.
Moreover, neutralization of TNFα with etanercept or infliximab treatment after induction of AIA significantly reduced both macrophage infiltration and expression of VCAM-1 expression. TNFα inhibition also decreased mechanical hyperalgesia in the inflamed joint without modulation of joint inflammation indicating a key role for TNFα signalling in maintaining heightened nociceptive states following AIA induction (Segond Von Banchet et al., 2009). In the CNS, following AIA induction, blockade of TNFα using spinal application of etanercept has been shown to reduce spinal cord hyperexcitability, indicating that TNFα signalling, likely derived from microglial contribute to central sensitization of dorsal horn neurons and enhanced nociceptive signalling in the AIA model (Boettger et al., 2010b).

Acute transient arthritis can also be induced in rodents by immunization with anti-CII antibodies. Unlike the CIA model, whereby anti-CII antibodies are produced from B-cells and T-cells of the adaptive immune system, in the collagen antibody induced model of RA (CAIA) anti-CII antibodies are systemically injected into naïve mice. Subsequently, 3-5 days following antibody injection lipopolysaccharide is administered via intraperitoneal injection leading to clinical signs of arthritis which resemble active CIA pathology such as synovial inflammation, cartilage destruction, bone degradation and infiltration of immune cells to the joints (Bas et al., 2016). However, there is significant variation in susceptibility to CAIA between mice strains with DBA/1, BALB/c and CBA mice showing high susceptibility and little RA pathology observed in C57BL/6 mice (Nandakumar and Holmdahl, 2007). Clinical signs of arthritis are transient, peaking 12 days after CAIA immunization, then returning to baseline by day 30. However, mechanical hypersensitivity precedes and outlasts transient inflammation, with observations of hind paw hyperalgesia up to 54 days post-CAIA immunization (Fernandez-Zafran et al., 2019). Moreover, inhibition of COX-1/2 activity with diclofenac treatment reversed CAIA-induced hyperalgesia only during the inflammatory phase and not when hyperalgesia presents in the absence of joint swelling indicating that there is a time-dependent contribution of prostaglandin signalling to heightened nociceptive signalling in the CAIA model (Bas et al., 2012). Assessment of arthritic joints in the inflammatory phase of the CAIA model highlight greater levels of pro-inflammatory TNFα in both synovial fluid and synovial tissue. Moreover, in the inflammatory phase CAIA-induced mechanical hypersensitivity can be diminished by systemic treatment of TNFα blockers (Bas et al., 2016). These findings indicate that
in the CAIA model during inflammatory states, pro-inflammatory TNFα signalling to peripheral afferent fibres of the joint likely facilitates peripheral sensitization of primary afferents and associated hyperalgesia. Although, due to systemic administration, the effects of TNFα inhibition on nociceptive CNS mechanism cannot be excluded. In the CNS, following CAIA-immunization, studies indicate increased astrocyte and microglial activity in males and females associated with hind paw hyperalgesia both in the presence and absence of clinical signs of arthritis (Bas et al., 2012, Fernandez-Zafra et al., 2019). However, intrathecal administration of the glial inhibitors minocycline and pentoxifylline reversed mechanical thresholds in male, but not in female mice, indicating sexual dimorphisms in the contribution of CNS glial signalling to central sensitization and hyperalgesia following CAIA immunization (Fernandez-Zafra et al., 2019).

Similar to the CAIA model, the K/BxN serum transfer model of arthritis utilizes the passive transfer of antibodies in order to initiate an innate immune response and clinical signs of arthritis in the absence of T and B cell activation. In the K/BxN serum transfer model, IgG against glucose-6-phosphate isomerase (G6PI) is obtained from transgenic, constitutively arthritic, K/BxN mice and injected systemically to naïve mice causing transient clinical signs of arthritis which peak within days of injection, subsiding to near baseline around two weeks post-immunization. However, persistent mechanical hyperalgesia is observed which outlasts joint swelling (Christianson et al., 2010). Similar to the CAIA model, following immunization with K/BxN serum, systemic administration of ketorolac (COX 1/2 inhibitor) and etanercept (TNFα inhibitor) led to reductions in mechanical sensitivity only in the presence of joint swelling, showing little efficacy when hyperalgesia presents in the absence of joint swelling (Christianson et al., 2010, Park et al., 2016). These findings indicate that pro-nociceptive prostaglandin and TNFα signalling only play a role in heightened nociceptive signalling during joint swelling, with prostaglandin and TNFα independent mechanisms likely facilitating hyperalgesia in the absence of joint swelling. Moreover, akin to the CAIA model, following K/BxN serum transfer greater spinal cord dorsal horn microgliosis is associated with mechanical hyperalgesia both in the presence and absence of joint swelling, indicating central glial-mediated mechanisms contribute to mechanical hyperalgesia following serum transfer.

However, as compared to the CAIA model, K/BxN serum transfer is a more robust model of RA-associated joint pain, inducing transient clinical arthritis and persistent
nociceptive signalling in a wider number of mice strains including BALB/c, CBA and C57BL/6 (Ji et al., 2001). A review of currently identified inflammatory and nociceptive mechanisms involved in K/BxN serum transfer arthritis are detailed in section 2.1. Although people with RA describe persistent pain to be one of the most debilitating symptoms of the condition, current treatment options for RA-associated joint pain are insufficient. Therefore, a deeper understanding of nociceptive signalling mechanisms is required in order to develop novel therapeutics for RA-associated joint pain. Animal models of RA pain recapitulate aspects of the human condition and represent important tools for further investigation of nociceptive mechanisms contributing to RA-associated joint pain, both in the presence and absence or joint swelling.

1.4 General Aims of Thesis

The aims of this thesis are to investigate peripheral (PNS) and central (CNS) processes underlying heightened nociceptive mechanisms both associated and dissociated from clinical signs of arthritis. In order to examine these topics, I have:

1. Investigated whether persistent mechanical hyperalgesia in the K/BxN serum transfer model, in the presence or absence of joint swelling, was associated with an altered inflammatory response in the DRG (PNS), as well as altered nociceptive neuronal and microglial activation in the spinal cord dorsal horn (CNS). - Chapter 2

2. Examined if following K/BxN serum transfer, alongside persistent hyperalgesia aberrant bioactive lipid mediator signalling is observed in the DRG, either in the presence or absence of joint swelling. After bioactive lipid mediator profiling, I explored the effect of restorative lipid mediator treatment on hind paw mechanical hypersensitivity, DRG immune cell infiltration and spinal cord DRG microgliosis following immunization with K/BxN serum. - Chapter 3

3. Assessed the ability of restorative lipid mediator treatment to modulate pro-inflammatory and pro-resolution mechanisms in cultured macrophages, as
well as pro-nociceptive mechanisms of cultured DRG sensory neurons. - Chapter 4

4. Determined if following serum transfer, alongside persistent hyperalgesia either in the presence or absence of joint swelling, there is aberrant miRNA signalling in the K/BxN DRG. Furthermore, I assessed the ability of restorative lipid mediator treatment in modulating pro-inflammatory and pro-nociceptive miRNA signalling in separately cultured peritoneal macrophages and DRG sensory neurons. - Chapter 5
Chapter 2:
Characterisation of Nociceptive Processing in the K/BxN Serum Transfer Model of Arthritis
2.1 Introduction

2.1.1 K/BxN Serum Transfer Model of Arthritis

With limitations to extensive experimental study of RA patients in the clinic, pre-clinical animal models provide a useful tool to unmask the mechanisms behind RA and pain in RA. The murine K/BxN serum transfer model robustly mimics aspects of arthritis observed in the clinic including cartilage loss, bone erosion and allodynia in affected limbs (O’Brien et al., 2016, Koziczak-Holbro et al., 2009, Christianson et al., 2010).

This model was fortuitously discovered in 1996 by the Mathis and Benoist laboratories. These groups of immunologists had originally set out to investigate the selection specificity of R28; a T cell hybridoma capable of recognising bovine pancreas ribonuclease. In order to do this, they used a KRN transgenic mouse line, which was crossed with various other mouse lines in the hope of creating a strain where R28 protein was specific to only H-2k allele derived A<sub>k</sub> T-cell receptor (TCR). However, there was poor selection for this transgene-encoded T cell receptor. Interestingly, though, whilst breeding these various mice, by luck, Mathis/Benoist did find that TCR transgenic KRN mice on a C57BL/6, when crossed with autoimmune prone non-obese diabetic (NOD) mice (Kouskoff et al., 1996) produced offspring which developed a joint disease similar to RA by 4-5 weeks of age. These arthritic mice were known as K/BxN mice.

Typically, T-cells in KRN mice recognise bovine ribonuclease peptide (RNAse 42-56), which are present on 1-Ak MHC class II molecules of antigen presenting cells such as dendritic cells, B-lymphocytes and macrophages (Figure 2.1) (Kouskoff et al., 1996, Roche and Furuta, 2015). However, when crossed with NOD mice, KRN T-cell receptors bind to NOD-derived Ag7 MHC class II molecules due to the presence of a self-antigen: Glucose-6-phosphate isomerase (G6PI). These G6PI-bound MHC Class II molecules are also present on B-cells found in the blood (Figure 2.1). The binding of KRN TCR-containing T-cells and G6PI-bound B-cells in the blood of K/BxN mice leads to activation of the adaptive immune response and differentiation of B-cells into plasma cells. These plasma cells actively produce IgG antibodies against G6PI (Kouskoff et al., 1996).
Under normal conditions, KRN T-cell receptors (KRN TCR) recognize bovine ribonuclease peptide (RNAse 42-56) present on 1-Ak MHC Class II (1-Ak) molecules of antigen presenting cells (APCs) such as dendritic cells and macrophages. However, in K/BxN transgenic mice, KRN TCR bind with high affinity to Glucose-6-phosphate isomerase (G6PI) which is self-bound to 1-Ag7 MHC Class II molecules on K/BxN B-Cells (1-Ag7). This binding induces an adaptive immune response whereby G6PI-bound B-cells differentiate into plasma cells which produce arthritogenic anti-G6PI IgG.

Transgenic K/BxN mice develop severe, chronic arthritis 4 weeks from birth which does not subside (Kouskoff et al., 1996). Furthermore, anti-G6PI IgG antibodies from K/BxN transgenic mice when injected systemically, lead to induction of a transient arthritogenic inflammatory response specifically in the joints of a wide range of mouse strains (Monach et al., 2007). This specificity is due to extracellular G6PI lining the cartilage surface (Matsumoto et al., 2002). G6PI is a ubiquitously expressed protein, known to be elevated in serum of RA patients (Soleimani et al., 2019), which under normal circumstances acts as an intracellular glycolytic enzyme necessary for gluconeogenesis (Schulz et al., 2014).
IgG are large plasma cell-derived glycoproteins around 150 kDa in weight (Tabrizi et al., 2010). When injected into the peritoneal cavity of naïve mice purified anti-G6PI IgG can freely migrate into systemic circulation via the blood vessels of the peritoneum (Al Shoyaib et al., 2019) and easily diffusing to various tissues throughout the body (Tabrizi et al., 2010). Under normal conditions, without pre-existing breakdown of the blood brain barrier (BBB), it is widely believed that there is low uptake of IgG, around 0.1% in animal models, from circulation across the blood brain barrier (Tabrizi et al., 2010, Villasenor et al., 2016). Furthermore, investigations into intravenous immunoglobulin (IVIg) show that only less than 0.01% of systemically injected IVIg dose is detectable in the cortex of mice, with localization mainly within micro vessels and much less frequently in neurons (St-Amour et al., 2013). Therefore, as evidence points away from clear translocation of systemic IgG across the BBB to the brain, from herein I shall focus on the effects of anti-G6PI antibody from the perspective of the periphery.

Following diffusion to systemic circulation, anti-G6PI IgG binds G6PI present in blood plasma, forming G6PI/anti-G6PI IgG immune complexes. PET tracing of injected radio-labelled anti-G6PI IgG highlighted that as early as 10 minutes after injection anti-GPI IgG is localized specifically in joints of hind paw and fore limbs, with little evidence of accumulation in organs such as kidneys, liver and spleen (Wipke et al., 2002). Interestingly, using immunohistochemistry, it has also been shown that in naive mice although there is no difference in G6PI expression between joint and other tissues, there is an accumulation of G6PI specifically deposited at the articular surface, with GPI observed lining the cartilage surface (Matsumoto et al., 2002). Together these observations indicate an extracellular accumulation of G6PI in normal joints, which provides explanation for a joint specific response to systemic anti-G6PI injection.

The pathogenesis of joint inflammation in the K/BxN serum transfer model is thought to occur via two key mechanisms; immune cell-mediated Fc receptor signalling originating in the blood and complement signalling directly at the joint (Christensen et al., 2016, Matsumoto et al., 2020).
2.1.2 Immune cell-mediated Joint Inflammation in K/BxN Serum Transfer Arthritis

2.1.2.1 Neutrophils, mast cells and Fc Receptor Signalling

Neutrophils are the most abundant granulocytes found in circulation, produced in the bone marrow, they mature and migrate into blood circulation. They form a key component of the innate immune response, by circulating in the blood and mobilizing to sites of infection or inflammation (Rosales, 2018).

Neutrophils are crucial for the initiation of K/BxN serum transfer arthritis, with no clinical signs of arthritis observed in neutrophil deficient mice, or in mice deficient in Fc receptors, present on neutrophils (Wipke et al., 2002). Within 10 minutes of purified serum injection, G6PI/anti-G6PI immune complexes form in the blood, freely binding Fc receptors present on neutrophils (Figure 2.2). The knockout of FC receptors leads to complete resistance to joint inflammation after transfer of anti-GPI serum (Wipke et al., 2004). In murine species four Fc receptor subtypes have been identified; FcR I, II, III, IV, of which FcγRIV binds G6PI with the greatest affinity. However, FcRIII plays the most important role in localisation of arthritogenic antibodies to the joint, with complete lack of joint accumulation in FcRIII-deficient mice (Wipke et al., 2004) Moreover, FcγRIII-deficient mice show both delayed and attenuated arthritis pathogenesis (Corr and Crain, 2002).

In the blood, the binding of G6PI/anti-G6PI immune complexes to membrane bound FcγRIII leads to activation of neutrophils which results in the release of vasoactive mediators and local increase of vascular permeability and immune complex access to the perivascular space of the joint (Wipke et al., 2004). Specifically, neutrophils increase permeability through release of chemokines and the self-activation of leukocyte function-association antigen 1 (LFA-1) (Distasi and Ley, 2009). LFA-1 engages with endothelial cells expressing ligands intercellular adhesion molecule 1 or 2 (ICAM-1, 2) and activates ICAM-1 signalling, which increases permeability through the formation of intracellular gaps (Ma et al., 2019). These processes are crucial to arthritis development as mice deficient in β2-integrins, including ICAM-1, showed no signs of arthritis following K/BxN serum transfer (Watts et al., 2005). In addition, the release of inflammatory mediators including TNFα also, which induces increases in of endothelial permeability, by binding TNFR1 and 2 receptors present on endothelial...
surface, resulting in intercellular gap formation and increased endothelial permeability (Distasi and Ley, 2009).

Following increased vascular permeability, unbound immune complexes from the blood diffuse into the perivascular space, where they are free to bind FcγR receptors present on mast cells (Figure 2.2), which are tissue resident granulocytes known to be activated in rheumatic diseases (Suurmond et al., 2016). Although sharing some overlapping function with neutrophils, mast cell activation is important in K/BxN serum induced arthritis, with mast cell deficient mice displaying greatly reduced signs of clinical arthritis and reduced ability of immune complexes to migrate to the (Wipke et al., 2004).

Resident mast cells, upon immune complex binding, become activated leading to degranulation and subsequent release of inflammatory mediators including histamine, which greatly increases vascular permeability and causing vasodilation which allows circulating immune complexes and G6PI IgG antibodies to diffuse into the joint cavity (Figure 2.2) (Rajasekaran et al., 2009, Nent et al., 2013). Following K/BxN induction, mast cells produce TNFα and IL-1β (Nigrovic et al., 2007), with IL-1β being critical for disease progression as no clinical arthritis is observed in IL-1β deficient mice (Ji et al., 2002b).
Stage 1: Access of antibody into the joint

(a) After systemic injection of purified anti-Glucose-6-Phosphate Isomerase IgG antibody (anti-G6PI IgG), in the blood, anti-G6PI IgG binds to endogenous Glucose-6 Phosphate Isomerase (G6PI), forming immune complexes.

(b) Immune complexes then bind to Fc gamma receptors (FcγR) on the surface membrane of neutrophils.

(c) This leads to the release of inflammatory mediators from neutrophils, such as TNFα, increasing vascular permeation of the endothelium allowing the diffusion of immune complexes and anti-G6PI IgG into the perivascular space.

(d) Immune complexes in the perivascular space then bind to FcγR, present on the surface membrane of mast cells, causing mast cell degranulation and further release of inflammatory mediators.

(e) This induces transient increase in vascular permeability and allows immune complexes and anti-G6PI IgG to diffuse from perivascular space to the joint cavity.

Stage 2: Recognition of target antigen in the joint

(f) In the synovial fluid immune complexes are free to bind FcγR present on the cartilage surface (Wipke et al., 2004)
2.1.2.2 Complement Signalling

Availability of anti-G6PI antibodies to the joint leads to the formation of anti-G6PI/G6PI immune complexes with cartilage expressed G6PI and the activation of the alternative complement pathway which produces the anaphylatoxin, C5a (Wipke et al., 2004). The alternative complement signalling system forms part of the innate immune response and is critical for arthritic disease pathogenesis (Ji et al., 2002a), resulting in macrophage and neutrophil attraction to the joint (Solomon et al., 2005). However, microPET studies, in C5 deficient mice highlight that complement signalling is not necessary for anti-G6PI antibody accumulation at the joint (Wipke et al., 2004). This provides further evidence that that complement signalling occurs after Fc-receptor mediated increases in joint vascular permeability in what is known as the innate effector stage of K/BxN serum transfer arthritis (Christensen et al., 2016).

In this effector stage, within 1 hour of serum transfer, anti-G6PI antibodies form immune complexes in the joint with endogenous G6PI protein present on the cartilage surface (Wipke et al., 2002, Sadik et al., 2018). Subsequently, G6PI/anti-G6PI immune complexes bind synovial derived complement proteins, C3 and factor B. This binding facilitates the cleavage of complement component (C5) to C5a (Figure 2.3). Complement protein C5a is then able to bind C5a receptors present on neutrophil and mast cell, which have been attracted to the synovial tissue. Further activation of neutrophils and mast cells causing a subsequent release of inflammatory mediators such as IL-1β, IL6, LTB4 and TNFα. The release of pro-inflammatory cytokines from activated granulocytes induces the release of chemokines from endothelial cells, pro-inflammatory activation of macrophages and subsequent joint inflammation and tissue damage. The release of pro-inflammatory cytokines and mediators, act as chemo-attractants for neutrophils leading to migration of activated neutrophils from the blood to the joint (Chen et al., 2018b), via retrograde LTB4 mediated signalling pathways (Christensen et al., 2016). Neutrophil migration to the inflamed joint is likely to also by attributed to L-selectin. L-selectin is a cell adhesion molecule that is expressed on most circulating leukocytes, allowing circulating neutrophils to tether and adhere to the endothelium, before transmigrating across the endothelium towards the inflammatory stimulus (Strausbaugh et al., 1999b). Inhibition of L-selectin leads to inhibition of neutrophil accumulation at the site of inflammation and decreased plasma...
extravasation associated with inflammation (Strausbaugh et al., 1999a, Strausbaugh et al., 1999b, Levine et al., 2006).

Figure 2.3: Complement signalling in the arthritic joint following K/BxN serum transfer

(1) Following systemic K/BxN serum injection, anti-G6PI IgG antibodies move from the blood to the joints, where they freely bind G6PI present on the synovium and cartilage surface forming immune complexes. (2) In the synovial fluid, complement proteins C3 and factor B bind to these immune complexes triggering the cleavage of complement component 5 (C5) to C5a. (3) C5a binds to C5a receptors on neutrophils, mast cells, macrophages, and endothelial cells leading to production of pro-inflammatory mediators including TNFα and IL-1β and chemokines. Release of pro-inflammatory mediators in the joint cavity facilitates inflammation, damage to joint tissue including (4) pannus formation, (5) further infiltration of immune cells to site of inflammation including macrophages, mast cells and neutrophils and (6) bone erosion due to osteoclast activation (Adapted from Wipke et al., 2004)

2.1.2.3 Macrophages in K/BxN arthritis development

Although neutrophils are critical for the initiation of arthritic disease pathology (Monach et al., 2007), within days of activation these cells undergo apoptosis and are cleared
from the joint by macrophages (Chatfield et al., 2018, Misharin et al., 2014, Bratton and Henson, 2011). Therefore, macrophages are involved in the prolonged maintenance of joint inflammation following K/BxN serum transfer. Macrophages have been shown to have an evolving role in the arthritic joint. A seminal paper by Misharin et al. (2014) demonstrated the crucial role to which monocyte /macrophage signalling plays in the progression and resolution of inflammation in the K/BxN joint.

Following tissue injury, inflammatory Ly6C^+ CCR2^+ monocytes are usually recruited from peripheral blood to aid tissue repair (Boniakowski et al., 2018). However, this is not the case in the K/BxN joint. Indeed, using clodronate-mediated depletion of monocytes, Misharin et al. (2014) identified a necessary role for patrolling Ly6C^-CX3CR1^+ monocytes circulating in the blood. The depletion of monocytes resulted in complete prevention of arthritis and neutrophil recruitment to joint, following K/BxN serum transfer (Misharin et al., 2014). Analysis of individual immune cell types in K/BxN synovium identified that 2 days following serum transfer, prior to widespread neutrophil recruitment to the synovium at day 7, blood-derived patrolling Ly6C^- monocytes are recruited to the joint where they preferentially mature into MHC II^-CX3CR1^+ macrophages. In the joint, these newly recruited macrophages are found alongside tissue resident macrophages (Misharin et al., 2014). Other studies have investigated the importance of chemokine-receptor expressing immune cells showing that, following K/BxN serum transfer, CXCR2, but not CX3CR1 is important, but not critical for arthritis with CXCR2 deficient mice displaying attenuated joint swelling (Jacobs et al., 2010).

In the instigation and resolution of inflammation macrophages can have differing roles, distinctly described as being either pro-inflammatory (M1) or anti-inflammatory (M2) (Orecchioni et al., 2019, Gautier et al., 2012). However, M1/M2 polarization of macrophages can be distinguished by a several markers and genes (Orecchioni et al., 2019, Gautier et al., 2012). Misharin et al. (2014) analysed over 50 genes indicative of M1 or M2 macrophage phenotype in sorted tissue resident and recruited macrophages, they observed that tissue resident macrophages throughout serum transfer displayed a more M2 repair phenotype. Whereas, recruited macrophages in the inflamed synovium, at day 7, displayed a more M1 phenotype, expressing more pro-inflammatory genes. However, by day 14 synovial recruited macrophages had shifted towards an M2 repair phenotype.
In addition, by selective depletion of macrophage populations, this study showed loss of recruited MHC II⁺ macrophages had no effect on the time course of clinical arthritis following serum transfer, whereas depletion of tissue-resident MHC II⁻ macrophages in K/BxN joint led to sustained and increased arthritis (Misharin et al., 2014). This indicated that, in addition to M2 phenotype recruited macrophages in the synovium, the resolution of joint inflammation also involves tissue resident macrophages. Misharin et al.’s (2014) study highlights that macrophages play a pivotal and complex role, facilitating and resolving inflammation in the joint.

2.1.3 Innervation of the joint by nociceptive fibres under inflammatory states

Synovial joints are known to be innervated by sensory and sympathetic nerves. Postganglionic sympathetic fibres terminate near articular blood vessels, regulating blood flow to the joint (Mapp, 1995). Sensory nerves, innervating the joint can be either large diameter myelinated nerve fibres encoding proprioceptive information or small in diameter (<5µm) responding to nociceptive stimuli (Mapp, 1995). Sensory nerves which innervate the joint contain Aβ, Aδ and C-fibres, with free nerve endings identified in all joint structures with the exception of normal cartilage (Schaible et al., 2009).

Under normal condition, the synovium, soft tissue surrounding the joint capsule, contains a good supply of both thinly myelinated or unmyelinated nerve fibres, postganglionic sympathetic fibres and C fibres respectively. Nociceptive nerves are not usually active in the joint, responding primarily to tissue damage. However, during inflammatory states, these primary afferent fibres are susceptible to sensitization by inflammatory mediators released from immune cells, likely leading to an increase of mechanosensitivity of joint nociceptors (Schaible et al., 2009). Following chronic inflammation in rheumatoid arthritis, an absence of nerve fibres in the superficial synovium has been observed, possibly reflecting damage to the peripheral terminals of nerve fibres caused by mediators released during the inflammatory response (Mapp, 1995).

Inflammation is not only perpetuated by immune cells. Neurogenic inflammation can also occur whereby activation of sensory nerves triggers inflammation through the release of substances pro-inflammatory neuropeptides such as calcitonin-gene
related product (CGRP) and substance P (Levine et al., 2006). These neuropeptides are synthesised in the dorsal root ganglion cells and transported peripherally from the cell bodies to the peripheral terminals, where they are released from the terminals of sympathetic nerves and nociceptors in the joint in response to neuronal activation (Levine et al., 1985b). These neuropeptides act directly on vascular endothelial and smooth muscle cells; CGRP produces vasodilation effects whereas SP increases capillary permeability leading to plasma extravasation and oedema (Chiu et al., 2012). In addition to neuropeptide release from C fibres, there is also evidence for a contribution of sympathetic postganglionic terminals, releasing peptides that act on postcapillary venules, causing plasma extravasation and vasodilatation (Mcdougall, 2006). In the rheumatic joint, there are less neuropeptides present in the synovium reflecting an increased release of these inflammatory neuropeptides (Mapp, 1995).

Joints are encased in a fibrous capsule containing synovial fluid. During neurogenic inflammation, neuropeptide mediated vasodilation of synovial blood vessels causes increased permeability to plasma proteins, which can leak from vasculature and accumulate in the intra-articular space. This accumulation of plasma proteins promotes fluid exudation into the joint with subsequent oedema formation (Mcdougall, 2006). This oedema causes an increase in intra-articular pressure which is likely to further activate nociceptors in the joint.

### 2.1.4 Pain in K/BxN serum transfer arthritis

Although the immunopathology leading to joint inflammation has been widely studied in the K/BxN serum transfer model, fewer groups have focussed on the pathophysiology of nociceptive signalling in this model of inflammatory arthritis. The first investigation into nociceptive states in the K/BxN serum transfer model was performed by Christianson et al. (2010), who characterised pain-like behaviours in the model; to tactile pressure and noxious temperatures. They found that following serum transfer, alongside inflammatory infiltrate in the joint (day 6 after serum transfer) and bony erosions (day 28 after serum transfer), there is also development of nociceptive hypersensitivity (Christianson et al., 2010, Park et al., 2016). Within 24 hours of serum induction, there was overt redness and joint swelling of the forepaws and hind paws,
with significant mechanical hypersensitivity present from day 2 to day 28 after serum transfer.

Clinical signs of arthritis in fore- and hind paws was shown to be transient, peaking on day 6 and subsiding by day 18 following serum transfer. The peak of clinical arthritis scoring was associated with a transient mild hypoalgesia (Christianson et al., 2010). In addition to noxious cold, studies have also assessed responses to noxious heat, observing transient hypersensitivity associated only with joint swelling (Borbely et al., 2015). Joint swelling and peripheral noxious heat sensitivity are transient in the K/BxN serum transfer model. However, remarkably, Christianson et al. (2010) showed mechanical allodynia to persistent, from day 2, beyond the resolution of joint swelling until day 28, the end of study. However, the group have since showed that serum induced mechanical hypersensitivity is still observed 40 days following K/BxN induction (Christianson et al., 2011). These findings indicate a clear disconnect between joint swelling and hind paw allodynia in the K/BxN serum transfer model.

The synovial tissue surrounding the joint is innervated by Aβ, Aδ- and C nociceptive fibres, with free nerve endings present in all structures of the joint except cartilage. Following joint inflammation, primary afferent fibres are susceptible to sensitization by inflammatory mediators, likely leading to an increase of mechanosensitivity of joint nociceptors (Momin and Mcnaughton, 2009). Peripheral sensitization of nociceptors is likely to be due to the release of pro-inflammatory mediators from infiltrating immune cells in the joint (Schaible et al., 2009, Pinho-Ribeiro et al., 2017, Samad et al., 2001, Lopshire and Nicol, 1997). As early as 1 day following serum transfer, neutrophil and macrophage mediate increases in cytokines occur in the synovial fluid of the K/BxN joint (Chou et al., 2010). IL-1β is produced by macrophages and neutrophils, present in the synovial fluid, closely situated to articular nerve fibres innervating joint structures (Chou et al., 2010, Mailhot et al., 2020). IL-1β, by binding to IL-1 receptors, sensitizes nerve peripheral terminals in addition to directly activating nociceptors (Binshtok et al., 2008). Mast cell derived histamine can binds histamine 1-2 receptors present on nociceptive terminals, resulting in phospholipase C activation and downstream release of Ca^{2+} from the endoplasmic reticulum. Intracellular Ca^{2+} release, via action with calmodulin, then can suppress potassium voltage-gated type 7 (Kv7) channels leading to heightened neuronal excitability (Obara et al., 2020, Rosa and Fantozzi, 2013). Analysis of lipid mediators in the inflamed K/BxN joint have revealed greater levels of
pro-inflammatory lipid mediators prostaglandin D$_2$ and prostaglandin E$_2$ (Norling et al., 2016). Although the study did identify the cellular source of prostaglandins, studies have shown these pro-inflammatory lipids to be macrophage-derived, with increasing COX production in macrophages following pro-inflammatory macrophage stimulation (Williams and Shacter, 1997, Osma-Garcia et al., 2016). Activation of EP receptors, present on nociceptors, triggers the release of intracellular Ca$^{2+}$ from the endoplasmic reticulum via inositol 1,4,5-trisphosphate (IP3) production. In addition, when activated EP2 and EP4 receptors stimulate cAMP production by activating adenylyl cyclase. cAMP then activates protein kinase A (PKA), causing phosphorylation of various signalling proteins, which alter the functions of ion channels present on nociceptor membrane and promoting increased electrical excitability of the sensory neuron (Momin and Mcnaughton, 2009, Jang et al., 2020).

As described above, collectively, these immune cell-derived specific cytokines, mediators and lipids all bind to specific receptors present on peripheral nociceptors causing intracellular signalling pathways leading to phosphorylation and gating of ion channels related to nociceptive firing. In turn, these mechanisms cause an increase in action potential generation and nociceptor sensitivity (Momin and Mcnaughton, 2009, Pinho-Ribeiro et al., 2017) (Figure 2.4).

The cell bodies of peripheral nociceptors that innervate all hind paw joint structures, converge in the lumbar dorsal root ganglia, with the exception of joint cartilage which does not receive innervation (Schaible, 2014). A recent study by Mailhot et al. (2020) highlighted that as oppose to receptor presence at peripheral terminals, away from the site of inflammation, around 10% of DRG neurons are proposed to express IL1R, the receptor for pro-inflammatory IL-1β. The conditional deletion of IL1R in TRPV1 positive neurons leads to the absence of allodynia from day 2-5 following serum transfer, without altering joint inflammation and swelling (Mailhot et al., 2020). Macrophages in the DRG are known to express IL-1β. Therefore, in addition to macrophages at the joint, it is possible that in K/BxN serum transfer model macrophage mediated IL-1β signalling to DRG neurons is key to the development of mechanical hypersensitivity, via macrophage derived IL-1β sensitization and excitation of cell bodies of nociceptors innervating the joint (Binshtok et al., 2008). These studies indicate that during states of peripheral joint inflammation neuro-immune signalling to peripheral nociceptors
plays a critical role, which may lead to long lasting sensory neuron alterations associated with heightened nociception in the absence of joint swelling (Figure 2.4).

Neuroimmune interactions in the spinal cord are known to play a key role in the maintenance of chronic pain states in both patients and animal model of RA (Bersellini Farinotti et al., 2019, Nieto et al., 2015). Following insult and noxious stimulation, microglia are activated displaying upregulation of microglial markers such as ionized calcium-binding adaptor molecule-1 [IBA1]. Christianson et al. (2010) report an increase in microglial presence in the superficial laminae of the spinal cord when mechanical hypersensitivity is present either with (day 6 after serum transfer) or without joint swelling (day 28 after serum transfer). Recent studies suggest that following peripheral inflammatory insult persistent mechanical nociceptive signalling is conveyed from nociceptive c-fibres to calretinin positive second order neurons in laminae II of the dorsal horn (Peirs et al., 2020). Therefore, following K/BxN serum transfer it is expected that spinal cord neuronal circuits associated with mechanical hypersensitivity would also be present in superficial laminae of the dorsal horn, as well as corresponding microglial populations facilitating nociceptive signalling.

Astrocytes are widely regarded as support cells of the CNS. The close contact of astrocytes with neurons and synapses also allows them to regulate the external chemical environment during synaptic transmission (Ji et al., 2013). Astrocyte activation in the spinal cord occurs following noxious stimulation resulting increased intracellular Ca^{2+} in astrocytes and upregulation of glial fibrillary acidic protein (GFAP) (Ji et al., 2013). Only at day 6 following serum induction, was there an increase in astrocyte presence as compared to controls. However, only spinal cord microglia, and not astrocytes were shown to have increased presence at day 28 after serum induction (Christianson et al., 2010).

These results indicate that, in the absence of K/BxN serum induced joint swelling, spinal cord microglial mediated glial mechanisms are associated with maintenance of mechanical hypersensitivity, possibly facilitating sensitization of spinal cord neurons.
Figure 2.4: Schematic summarizing changes at the peripheral nerve terminals, DRG and spinal cord dorsal horn facilitating heightened nociceptive signalling in K/BxN serum transfer arthritis

During peak joint swelling, there is infiltration of immune cells including neutrophils, mast cells to the site of inflammation. Patrolling Ly6C- monocytes are also recruited to the joint where mature into M1 pro-inflammatory macrophages. In the K/BxN joint, recruited immune cells release pro-inflammatory mediators such as histamine, TNFα, IL-1β and PGE₂ at site of inflammation which respectively act on Histamine receptors (H1/2), TNFα receptor 1 (TNFR1), IL-1 receptor 1 (IL1R) and PGE₂ receptors 1–4 (EP1–4) present on peripheral nerve terminals of nociceptor neurons to produce peripheral sensitization of the sensory afferent fibre. In the DRG, where peripheral afferents cell bodies are present, IL-1 receptors are observed levels are also observed in the DRG, in neurons also positive for TRPV1 and P2X3 (white arrows). Over time, as joint swelling subsides, neutrophils are cleared from the joint and M1 macrophages undergo a phenotype switch to a pro-resolution M2 phenotype. In the dorsal horn of the spinal cord, peripheral C fibres synapse with second order calretinin (CR) -positive neurons in superficial laminae I-II. Whilst joint swelling is present with mechanical hypersensitivity evidence suggests an increase in microglia in the dorsal horn, compared to controls. Microglial activation is associated with increased pro-inflammatory PGE₂ and TNFα release, contributing to central sensitization and exacerbation of excitatory glutamatergic signalling. As joint swelling subsides and mechanical hypersensitivity persists, greater microglial presence is still observed. Little is currently known regarding neuro-immune interactions in the DRG when mechanical hypersensitivity is present in the absence of joint swelling (Adapted from (Pinho-Ribeiro et al., 2017, Misharin et al., 2014, Mailhot et al., 2020, Chen et al., 2018a))

2.1.4.1 Sex Differences in nociception in the K/BxN serum transfer model

Studies have claimed sex differences in severity of allodynia with females showing reduced post-inflammatory tactile allodynia as compared to males after serum transfer (Woller et al., 2019). Activation of TLR4 on glial cells leads to intracellular
phosphorylation of p38 MAPK and release of ATP. Extracellular ATP can then activate membrane bound P2X7 receptor, on glial cells, which results in further p38 phosphorylation and release of pro-inflammatory IL-1β (Clark et al., 2010). Studies show that knockout of TLR4 does not affect K/BxN serum transfer induced joint inflammation. However, importantly, unlike wildtype mice, TLR4 knockout mice exhibit significant reversal in mechanical hypersensitivity and diminished appearance of glial activation markers after resolution of peripheral inflammation (Christianson et al., 2011). Therefore, it is possible that allodynia in the absence of joint swelling is dependent on TLR4 spinal cord mechanisms, which are inherently specific to male mice.

2.1.4.2 Effect of anti-nociceptive treatment in K/BxN serum transfer arthritis

Akin to observations in the clinic, whereby DMARDs and NSAIDs show little efficacy at reducing chronic joint pain associated with RA (Smolen et al., 2016, Mcwilliams and Walsh, 2017), there is disparity in analgesic efficacy in K/BxN serum transfer arthritis either in the presence or absence of joint swelling. When administered on day 4, during K/BxN joint inflammation, mechanical allodynia can be attenuated with non-steroidal anti-inflammatory drugs (NSAIDs) such as Ketorolac, or using anti-TNF antibody Etanercept (Christianson et al., 2010, Park et al., 2016). Ketorolac works to reduce pro-inflammatory prostaglandin production by inhibiting COX1/2, whereas Etanercept reduces the activity of TNFα production by competitively inhibiting binding to TNF receptors (Haraoui and Bykerk, 2007). Both Ketorolac and Etanercept prove ineffective when administered 28 days after serum transfer. On the other hand, gabapentin, which inhibits calcium mediated neurotransmitter release through effects on α2δ-1 subunits, can cross the BBB and act directly on the CNS to suppress nociception, was effective at either day 4 or day 28 following serum induction. These studies highlight that distinct nociceptive mechanisms may exist between heightened nociceptive states during, and in the absence of joint swelling.
2.1.5 Aims

The K/BxN serum transfer model of inflammatory arthritis represents a useful model for the study of pain RA with key features including i) transient joint swelling, ii) hind paw mechanical hypersensitivity which persists beyond the resolution of joint swelling and iii) ineffective analgesic action of NSAIDs when administered where mechanical hypersensitivity presents in the absence of joint swelling. These features mirror observations in the clinic, with experimental evidence highlighting a clear dissociation between in the model between hind paw mechanical hypersensitivity and joint swelling in this model.

The aims of the present chapter were to:

1) Investigate the robustness of transient joint swelling and persistent mechanical hypersensitivity in the K/BxN serum transfer model, by measured clinical signs of arthritis, joint swelling and assessment of hind paw mechanical thresholds following serum transfer.

2) Assess whether K/BxN serum-induced mechanical hypersensitivity, in the presence or absence of joint swelling, correlated to changes in inflammatory response in the DRG (PNS) by measuring CGRP (a marker for peptidergic sensory neurons – upregulated following inflammation) and F4/80+ macrophage presence following serum transfer.

3) To Identify changes in activation of microglial cell populations (using Iba1/ P-p38 as markers) and nociceptive activation of second-order dorsal horn neurons (cFos as a marker) in the dorsal horn spinal cord (CNS) associated with mechanical hypersensitivity, either in presence or absence of joint swelling in K/BxN serum transfer model.
2.2 Materials and methods

2.2.1 Animals

All experiments were performed on adult male C57BL/6 mice (Envigo, UK), aged between 10-12 weeks old and weighing approximately 25g. Mice were housed in groups of up to 5 per standard cage with ad libitum access to food and water. All animals were kept at room temperature with a 12h light/dark cycle. All experiments were carried out in a non-sterile environment. All studies were carried out with appropriate ethical approval and performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and local care and use guidelines. Approval for these studies was provided by King’s Animal Welfare and Ethical Review Body, London, United Kingdom.

2.2.2 Induction of K/BxN Serum Transfer Model

K/BxN serum and control serum were purchased from Professor Perretti, Queen Mary, University of London. Prior to purchase, all serum batches were tested on naïve mice to ensure arthritogenic potency. Mice received 50µl intraperitoneal injection of serum on days 0 and 2. Control mice were given equal volume of control serum obtained from KRN/C57 mice.

2.2.3 Assessment of Joint swelling

2.2.3.1 Visual signs of clinical arthritis

Clinical signs of arthritis were evaluated using a 12-point scoring system (Norling et al., 2016). Each limb was scored separately; 0-3 points per limb with the following criteria: 0 – no sign of redness/swelling; 1 – redness/swelling observed in either ankle/wrist, pad or any of the digits; 2 – redness/swelling in two regions; 3 – redness/swelling seen in all limb regions. Scores for all 4 limbs were combined to give a total score, with a maximum score of 12 per animal.
2.2.3.2 Ankle thickness measurements

Ankle thickness (mm) was measured using a mini digital thickness gauge with flat anvils and compared to baseline thickness normalised for each individual animal. Each hind paw was placed between the callipers and measured at the widest point of the ankle joint, known as the malleolus. Once both sides of the calliper touched the ankle measurements were read immediately, without application of further pressure, with care taken not to disturb any possible ankle oedema. As K/BxN-induced arthritis is induced systemically, left and right ankle measurements were averaged for each animal. The experimenter was blinded to animal treatment groups.

2.2.4 Mechanical thresholds

2.2.4.1 von Frey filament test

Hind paw mechanical withdrawal thresholds were assessed by applying a series of calibrated von Frey filaments (0.02-1.0g; North Coast Medical) to the plantar surface of the hind paw. On each day of testing, mice were habituated for at least 30 min in individual transparent Plexiglas boxes with a wire mesh bottom, allowing access to the underside of the plantar surface. Filaments were then applied at increasing intensity until a withdrawal response was achieved or application of 1.0g filament failed to elicit a withdrawal response. Changes in hind paw mechanical withdrawal thresholds were calculated using the Dixon up and down method (Chaplan et al., 1994).

Specifically, a mechanical force is applied to the plantar surface of the hind paw with a series of calibrated von Frey filaments with logarithmically incremental stiffness (0.04, 0.07, 0.16, 0.4, 0.6 and 1.0g). When the animal is still, the von Frey filaments is presented perpendicular to the plantar surface with enough force to cause slight bending against the paw. The filament is held for approximately 5 sec. A positive response is recorded when a sharp withdrawal of the paw is observed or if there is flinching behaviour upon removal of the filaments from the paw. A brief interval of a few seconds is left between application of von Frey filaments, to allow resolution of any behavioural responses to the previous stimuli. Testing is initiated with the 0.07 g filament. If there is a lack of response to the initially selected filament, the next filament of greater stiffness is presented. If there is paw withdrawal in response to a filament, the next lowest filament is used. Subsequent responses to the continued presentation
of stimuli are varied sequentially up or down, based on the animal's response (Chaplan et al., 1994). 50% withdrawal threshold calculation by the Dixon method requires a minimum of 5, up-to a maximum of 9 responses. From the resulting pattern of responses, the 50% withdrawal threshold can be interpolated. All behavioural experiments were conducted under the same environmental conditions and at the same time of day. Mice were randomly assigned to treatment groups, with the experimenter blinded to treatments.

2.2.5 Tissue Preparation and Immunohistochemistry

2.2.5.1 Perfusion and cryoprotection

On day 5 and day 25 following control or K/BxN serum transfer, mice were deeply anaesthetised with sodium pentobarbital (60mg/300µl/mouse i.p.) (Euthasol; Merial). Thereafter, mice were transcardially perfused with 0.9% saline solution (w/v) until the liver was cleared of blood. Next, the animal was perfused with 4% paraformaldehyde (PFA, VWR Chemicals) with 1.5% picric acid in 0.1M phosphate buffered solution (PBS). After fixation, lumbar spinal cord and L3, 4 and L5 dorsal root ganglia were dissected bilaterally, post-fixed for 2 hr in 4% PFA at 4°C, and then transferred to 20% sucrose in 0.1M PBS for cryo-protection for 48 hr at 4°C, before being embedded in OCT Compound (VWR Chemicals) and snap frozen in liquid nitrogen. Samples were then stored at -80°C.

For immunohistochemistry, dorsal root ganglion sections were cut in transverse sections (10µm thickness) using a cryostat (Bright Instruments) and thaw mounted onto Superfrost Plus microscope slides (Thermo-Scientific). Lumbar spinal cord sections were cut in transversely (20µm thickness), serially sectioned in sets of 8 sections per slide to ensure each slide contained a similar cross section from L4-L5 regions. All sections were thaw mounted on Superfrost Plus microscope slides.

2.2.5.2 Immunohistochemistry

Following drying, sections were rehydrated in 0.1 M PBS for 10 min. Sections were then blocked with 1% Bovine Serum Albumin (BSA)(Sigma-Aldrich) in 0.1% TritonX-100 in PBS (pH 7.2) for 1 hr and then incubated overnight (at least 16 h) with primary
antibodies. All primary antibodies used are outlined Table 2.1. Sections are then washed 3 times for 10 min in 0.1% PBS-T followed by incubation for 2 hr in the appropriate secondary antibodies (Alexa-Flour 488 or Alexa-Flour 546 conjugated (Life Technologies)). Slides were then coverslipped with Vectashield Mounting Medium containing DAPI (Vector Laboratories). All antibodies were prepared in 0.1 M phosphate buffered saline (PBS) with 0.1% BSA and 0.1% Triton X-100 (Sigma) (PBS-T) and incubated at room temperature.

For spinal cord slices undergoing P-p38/Iba 1 co-staining, tyramide solution amplification (TSA) was used. Firstly, frozen slide mounted spinal cord sections were washed 3 times for 10 min in 0.1 M PBS with 1% BSA and 0.2% Triton X-100 (Sigma) (0.2% PBS-T). Following this, sections were incubated overnight (at least 16 h) with rabbit anti-Phospho-p38 (P-p38; 1:100; Cell Signalling). Sections were then washed 3 times for 10 min in 0.2% PBS-T followed by incubation for 1.5 hr at room temperature in secondary antibody; biotinylated donkey anti-rabbit (1:400). Sections were then washed a further 3 times for 10 min in 0.2% PBS-T prior to incubation in Avidin Biotin Complex kit (ABC; Perkin Elmer) for 30 min. Sections are then washed 3 times for 10 min in 0.2% PBS-T and incubated for 10 min in biotynyl tyramide diluted 1:75 in amplification diluent (Perkin Elmer). Sections are washed 3 times for 10 min in 0.2% PBS-T, then incubated for 2 hr in Extra-Avidin conjugated FITC (1:500; Sigma). Sections are again washed 3 times for 10 min in 0.2% PBS-T before overnight incubation in with second primary antibody; rabbit anti-Iba 1 (1:1000; Wako). Following day, sections are washed 3 times for 10 min in 0.2% PBS-T before incubation with secondary antibody, Alexa-Flour 546 conjugated donkey anti-rabbit (1:1000, Life Technologies)) for 2 h. Sections are then washed a subsequent 3 times for 10 min. Slides were then coverslipped with Vectashield Mounting Medium containing DAPI (Vector Laboratories).

Unless stated otherwise, all antibodies were prepared in 0.2% PBS-T and incubated at room temperature. For negative controls, the primary antibodies were omitted; this resulted in the absence of staining.
Quantification of immunoreactivity

Images for immunofluorescence analysis were visualised and captured using a Zeiss Axioplan 2 fluorescence microscope. Subsequently acquired images were analysed using ImageJ software (1.50i). Cells in the DRG were analysed for calcitonin-gene related protein (CGRP) immunoreactivity, a marker for sensory afferent activation. The number of neurons displaying immunoreactivity for CGRP were counted and expressed as the percentage of total neurons within the image. For each image areas of individual cell bodies and intensity of immunoreactivity was measured using ImageJ. Determination of neurons was based on cell morphology and soma area, CGRP is primarily expressed in small and medium-sized neurons (Natura et al., 2005). Therefore, all large sized cell bodies (greater than 900µm² in area; as previously defined (Hansen et al., 2016) were excluded from classification. A single cell body was considered high in CGRP immunoreactivity when the mean grey level intensity was 20% greater than a known negative cell body within the same image. The known negative cell in each image was defined as the cell with the lowest mean grey level intensity. The percentage of cell bodies expressing a high level of CGRP-IR was

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Marker</th>
<th>Raised Species</th>
<th>Concentration Used</th>
<th>Manufacturer</th>
</tr>
</thead>
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<tr>
<td>CGRP</td>
<td>Sensory afferent activation</td>
<td>Sheep</td>
<td>1:800</td>
<td>Enzo</td>
</tr>
<tr>
<td>F4/80</td>
<td>Monocytes / Macrophages</td>
<td>Rat</td>
<td>1:400</td>
<td>Abcam</td>
</tr>
<tr>
<td>cFos</td>
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<td>Rabbit</td>
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<td>Cell-Signalling</td>
</tr>
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<td>Microglia</td>
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<td>Wako</td>
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<tr>
<td>P-p38</td>
<td>Microglial Activation</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Cell Signalling</td>
</tr>
</tbody>
</table>

Table 2.1: Primary antibodies used during immunohistochemistry

2.2.5.3 Quantification of immunoreactivity

Images for immunofluorescence analysis were visualised and captured using a Zeiss Axioplan 2 fluorescence microscope. Subsequently acquired images were analysed using ImageJ software (1.50i). Cells in the DRG were analysed for calcitonin-gene related protein (CGRP) immunoreactivity, a marker for sensory afferent activation. The number of neurons displaying immunoreactivity for CGRP were counted and expressed as the percentage of total neurons within the image. For each image areas of individual cell bodies and intensity of immunoreactivity was measured using ImageJ. Determination of neurons was based on cell morphology and soma area, CGRP is primarily expressed in small and medium-sized neurons (Natura et al., 2005). Therefore, all large sized cell bodies (greater than 900µm² in area; as previously defined (Hansen et al., 2016) were excluded from classification. A single cell body was considered high in CGRP immunoreactivity when the mean grey level intensity was 20% greater than a known negative cell body within the same image. The known negative cell in each image was defined as the cell with the lowest mean grey level intensity. The percentage of cell bodies expressing a high level of CGRP-IR was
calculated from the total cell numbers in each section, with 6 sections analysed per animal.

For quantification of F4/80 staining in the DRG, F4/80\(^+\) profiles, indicative of number of macrophages, were manually counted within four boxes/fields of view (10\(^4\) \(\mu m^2\)) randomly positioned across a single DRG section. At least four sections per animal were assessed. Experimenter was blinded to treatment groups during image analysis.

When analysing cFos, a marker for nociceptive neuronal activation, immunoreactivity within the spinal cord, the number of positive profiles were counted within three fixed boxes/fields of view (2.25 x 10\(^4\) \(\mu m^2\)) placed within medial, central and lateral portions of the superficial dorsal horn (Laminae I-II), then subsequently placed in the following k/bxn transfer, but within deeper laminae (laminae III-IV).

P-p38 and Iba1 spinal cord immunoreactivity were analysed by counting positive profiles within three fixed boxes (4 x 10\(^4\) \(\mu m^2\)) placed within medial, central and lateral portions of the superficial dorsal horn.

For analysis of cFos, Iba1 and pp38 immunoreactivity, the number of respective positive profiles were counted within each box and summed to give a total number of positive profiles for each individual section. A minimum of three sections were analysed per animal and then averaged to give the mean number of positive profiles per animal. 4 animals were studied per experimental group, with final data presented as mean per experimental group.

For all immunohistochemistry analyses DAPI staining was used to confirm cellular staining.

2.2.6 Data and Statistics

All data are presented as means ±SEM, where \(n\) is the number of biological replicates (individual mice), with differences between means considered as statistically significant when \(P<0.05\). All statistical analysis for behavioural, immunohistochemical and qPCR data was conducted using out with GraphPad Prism (v8.0.0; GraphPad Software, USA). For any single comparison between two groups unpaired Student’s t-test (two groups using different samples) was used. Multiple comparison of data used
one-way ANOVA followed by post-hoc Tukey test if more than two groups are present or two-way repeated measure ANOVA followed by post-hoc Tukey test for statistical analysis of behavioural testing and clinical scoring. Strength of correlations between datasets was assessed using R squared values calculated within GraphPad.
2.3 Results

2.3.1 Hind paw mechanical hypersensitivity persists after joint swelling has subsided in K/BxN serum transfer model

Persistent nociceptive behaviour in K/BxN mouse model of inflammatory arthritis has previously been shown to occur both in the presence and absence of overt joint swelling (Christianson et al., 2010). To investigate the robustness of our model, I first measured joint swelling and hind paw mechanical thresholds.

I observed that the administration of control serum failed to elicit signs of clinical arthritis (Figure 2B, C). Instead, ankle joint thickness was significantly increased 2 days after K/BxN serum transfer and peaked at day 6 compared to control serum treated mice (CON: 0.13 ±0.03 mm, K/BxN: 0.88 ±0.09 mm) (Figure 2A). After which, K/BxN serum transfer ankle thickness slowly subsided to near baseline by day 17 (0.18 mm ±0.08), where it remained until end of study (day 25). Similarly, mice exhibited significant fore and hind paw joint swelling after serum transfer (Figure 2B, C), which peaked at day 5 (CON: 0.10 ±0.10, K/BxN: 7.70 ±0.91) (Figure 2B). In line with ankle thickness measurements, following peak swelling, overall joint swelling slowly subsided and approached full recovery from day 17 onwards (0.25 ±0.25) (Figure 2D). Alongside joint swelling, mechanical hypersensitivity of the hind paw was observed, which persisted beyond the resolution of overt joint swelling, remaining highly significant relative to mice treated with control serum, at day 25 (CON: 0.94 ±0.02 g, K/BxN: 0.45 ±0.07 g) (Figure 2D).

Together, this data highlights that K/BxN serum transfer arthritis is associated with mechanical hypersensitivity which persists beyond the resolution of arthritic joint swelling and clinical scores.
**Figure 2.5:** K/BxN serum transfer arthritis is associated with mechanical hypersensitivity which persists beyond the resolution of arthritic joint swelling

(A) Clinical signs of arthritis were assessed using a 12-point clinical arthritis scoring of male mouse paws up to 25 days following K/BxN serum transfer (2 x 50μl intraperitoneal injections on days 0 and 2). Control animals were administered with non-arthritic control serum.  (B) Ankle thickness was measured prior to serum transfer and continually monitored up to 25 days after serum transfer. All measurements are compared to individual day 0 baselines. (C) Images displaying fore paw and hind paw joint swelling 4 days after K/BxN serum transfer as compared to controls. (D) Assessment of mechanical hypersensitivity using paw withdrawal thresholds to von Frey filaments applied to the hind paw following K/BxN serum transfer.  

*P<0.05 or ***P<0.001 compared to same day control, Two-Way RM ANOVA, post-hoc Tukey.

Data are expressed as mean ± SEM; n = 10 mice per group.
2.3.2 No direct correlation between severity of joint swelling and level of allodynia in K/BxN serum transfer model

It is often noted that in the clinic severity of joint pathology in rheumatoid arthritis patients poorly correlates with perceived levels of pain (Mcwilliams and Walsh, 2017). To evaluate if this observation is mirrored in the pre-clinical K/BxN model of rheumatoid arthritis, paw withdrawal thresholds were correlated to clinical scores for individual mice at peak joint swelling (day 5). Interestingly, I observed no significant correlation ($r^2:0.01$, $p=0.5$) (Figure 2.6A) between paw withdrawal thresholds and clinical arthritis scores at peak joint swelling. Furthermore, no correlation was observed between paw withdrawal thresholds at day 25 and clinical scores at peak swelling (day 5) ($r^2:0.04$, $p=0.2$) (Figure 2.6B). This indicates that severity of peak joint swelling does not predict future mechanical allodynia in K/BxN serum transfer arthritis. Interestingly however, when correlating mechanical hypersensitivity at day 5 and day 25 within individual K/BxN serum treated mice, our data highlight that no mice display spontaneous recovery from allodynia (Figure 2.6C).

These data suggest although there is no correlation between severity of clinical arthritis and mechanical allodynia in K/BxN serum transfer arthritis, mechanical hypersensitivity is robustly and consistently chronic in this model.
Figure 2.6: Correlations between K/BxN serum associated mechanical allodynia and joint swelling severity

(A) Graph plotting individual clinical scores and paw withdrawal thresholds (PWT) at peak joint swelling (Day 5) after K/BxN serum transfer. (B) Paw withdrawal thresholds 25 days after serum transfer as compared to previous day 5 clinical scores for individual K/BxN serum treated animals. (C) Comparing day 5 and subsequent day 25 paw withdrawal thresholds for individual K/BxN serum treated mice. For all graphs each point relates to an individual K/BxN serum treated animal, with linear regression lines denoting correlation between recorded variables.
2.3.3 Following K/BxN induction, CGRP immunoreactivity is increased in lumbar dorsal root ganglia neurons at peak joint swelling

After observing a behavioural phenotype in response to K/BxN serum induction, I next investigated whether K/BxN serum-induced inflammatory joint swelling, coupled with mechanical hypersensitivity, correlated to changes in neuronal nociceptive activation from peripheral nerve terminals. Calcitonin gene related peptide (CGRP) is used as a marker of peptidergic sensory neurons and following peripheral inflammation CGRP-expressing population increases significantly (Nieto et al., 2015). Therefore, using immunohistochemistry I assessed CGRP presence in the lumbar dorsal root ganglia (L4-5), where cell bodies of neurons innervating the hind paw are present. At day 5 after K/BxN serum transfer, a significantly greater percentage of DRG neurons displayed high CGRP immunoreactivity (49.7% ± 6.7) as compared to same-day controls (28.9% ± 6.8) (Figure 2.7 A-C). However, although a trend is observed, no significant difference was observed in CGRP immunoreactivity in K/BxN or control DRG, 25 days after serum transfer when mechanical hypersensitivity occurs in the absence of overt joint swelling.

Following K/BxN transfer, our results indicate that inflammation associated hind paw allodynia, 5 days after serum transfer, results in up-regulation of CGRP in DRG. However, at the experimental power used in this experiment, I do not observe significant increases in CGRP content in the DRG at day 25. It is possible that other spinal cord mediated mechanisms may contribute at this later timepoint.
Figure 2.7: CGRP expression is upregulated in lumbar DRG neurons at peak joint swelling, 5 days after K/Bxn serum transfer

(A-B) Representative images from lumbar DRG sections depicting single neuronal cell bodies displaying high CGRP-IR (White arrowheads) 5 days after either control (A) or K/Bxn (B) serum transfer. *P<0.05 or ***P<0.001 compared to same day control group, One-Way ANOVA, post-hoc Tukey. Data are expressed as mean ± SEM; n = 4 mice per group. All scale bars, 50µm. (C) Quantification of the total percentage of DRG neuronal cell bodies which express high CGRP immunoreactivity (IR) after K/Bxn serum transfer.
2.3.4 Macrophage presence is greater in lumbar DRG 25 days after K/BxN serum induction

The infiltration of non-classical (patrolling) Ly6C⁻ monocytes into the joints is crucial for the initiation, progression and resolution of joint swelling in K/BxN serum transfer model of inflammatory arthritis, possibly associated with the sensitisation peripheral terminals of nociceptive fibres via release of inflammatory mediators (Misharin et al., 2014). In this study I investigated immune cell presence distant from the arthritic joint and focused on the lumbar DRG, where the cell bodies of sensory neurons innervating the ankle joints are located (Figure 2.8A, B).

I performed immunohistochemical staining for F4/80, a marker for murine macrophage populations. Macrophage presence was observed in DRG following injection with either K/BxN or non-arthritic control serum (Figure 2.8A, B). Quantification of F4/80 staining at day 5, when swelling and mechanical hypersensitivity were both present, highlighted no difference in DRG macrophage number between K/BxN serum treated (3.85 ±0.49/10⁴μm²) and same day control group (4.1 ±0.4/10⁴μm²) (Figure 2.8B). However, at day 25 when mechanical hypersensitivity is still significantly maintained but joint swelling has subsided the number of macrophages was greater in DRG of K/BxN-treated (7.1 ±0.3/10⁴μm²) than in control serum-treated mice (4.6 ±0.1/10⁴μm²) (Figure 2.8B).

These data suggest that monocytes/macrophages numbers are greater in the lumbar DRG at timepoints associated with persistent pain after joint swelling has subsided (day 25) in the K/BxN serum transfer model.
Figure 2.8: Increased macrophage presence in lumbar DRG 25 days after K/BxN serum induction

(A) Representative images of F4/80+ profiles (arrows) (macrophages) presence in DRG day 5 and day 25 after transfer with either K/BxN or control serum. Scale bars, 50 μm. ** P<0.01 compared to same-day controls, One-Way ANOVA, post-hoc Tukey. Data are expressed as mean ± SEM; n = 4 mice per group. (B) Quantification of F4/80 positive macrophage profiles in the lumbar DRG at peak joint swelling (day 5) or post-joint swelling (day 25) as compared to same day controls.
2.3.5 Neuronal activation in superficial laminae of spinal cord dorsal horn occurs during K/BxN induced joint swelling

It is likely that for mechanical hypersensitivity to be maintained following K/BxN serum transfer, there would be increased nociceptive signalling in the spinal cord. With the aim to assess activation of second-order dorsal horn neurons in the spinal cord following K/BxN serum transfer I used immunohistochemical staining for cFos in lumbar spinal cord sections (L4,5). Regions were analysed separately at superficial laminae (I-II), where nociceptive fibres terminate and deeper laminae (III-IV) where proprioceptive fibres terminate at timepoints where mechanical hypersensitivity is observed in presence (day 5) or absence (day 25) of swelling. At peak peripheral joint swelling after K/BxN serum transfer, average number of cells in superficial laminae of dorsal horn displaying Fos (protein that results from cFos activation) was significantly greater (16.3 ± 4.4) compared to same day controls (6.6 ± 0.5) (Figure 2.9A-D). In contrast, no difference between K/BxN and control treated groups was observed in dorsal horn superficial laminae 25 days after serum transfer (Figure 2.9A). Moreover, when assessing cFos immunoreactivity in laminae III-IV, no difference was observed between K/BxN serum treated group and same-day controls either 5 or 25 days after serum induction (Figure 2.9D). As afferent nociceptive activity is confined to superficial laminae of the dorsal horn where c-fibres terminate, our results suggest recruitment of c-fibres at day 5.

These results suggest that K/BxN-associated mechanical hypersensitivity at peak joint swelling is associated with activation of dorsal horn neurons in superficial laminae, which become undetectable over time with the resolution of peripheral joint swelling. It is possible that cFos, an early gene marker of neuronal activation, may not be sufficient to detect maintained persistent nociceptor activity 25 days after K/BxN serum transfer.
Figure 2.9: Neuronal activation in superficial laminae of spinal cord dorsal horn occurs during K/BxN induced joint swelling

(A) Representative images displaying cFos+ cells in lumbar dorsal horn sections at both 5 and 25 days after serum induction. Scale bar = 100µm. (B-D) Quantification of cFos+ staining in the lumbar dorsal horn in K/BxN and control serum treated 5 and 25 days after K/BxN serum transfer, as compared to controls. Dorsal horn neuronal cFos staining was quantified in (B) superficial laminae I-II, (C) laminae III-IV and (D) cumulative total across dorsal horn laminae I-IV. * = P<0.05 compared to same-day controls, One-Way ANOVA, post-hoc Tukey. Data are expressed as mean ± SEM; n = 4 mice per group.
2.3.6 Increased microgliosis in superficial laminae of the dorsal horn following K/BxN transfer arthritis

Microglia in the dorsal horn of spinal cord are known to be involved in the formation and maintenance of chronic pain states. Moreover, in the collagen-induced model of inflammatory arthritis (CIA), pain behaviours is association with spinal cord microgliosis, both prior to and during the observation of peripheral joint inflammation (Nieto et al., 2016), suggesting that microglial activation can be considered a surrogate marker of central sensitization. Therefore, I assessed if dorsal horn microglial response occurs in K/BxN serum transfer arthritis.

At 5 days following serum induction, greater numbers of microglia (Iba1+ cells) were observed in the K/BxN treated group (19.5 ± 0.9/ 12x10^4 µm^2) as compared to same day controls (12.8 ±1.4/ 12x10^4 µm^2). Moreover, a 30% increase in superficial laminae microglial number was observed 25 days following K/BxN serum induction, as compared to same-day controls (Figure 2.10A, B).

In addition to microglia density, I also assessed microglial activation and used phosphorylation of P38 MAPK as this protein kinase is known to contribute to chronic pain mechanisms and becomes activated selectively in dorsal horn microglia following peripheral damage (Ji and Suter, 2007). At 5 days after serum transfer, I observed greater P-p38/Iba1 co-localisation in K/BxN treated as compared to same-day controls. This observation was sustained at day 25 after serum transfer, where increased K/BxN serum-induced microgliosis occurred compared to same day controls (Figure 2.10A, C). With microgliosis occurring in conjunction with hind paw mechanical hypersensitivity, but independent of joint swelling, this process may reflect a maintained heightened nociceptive state in K/BxN serum transfer arthritis.
Figure 2.10: Microglial activation in lumbar superficial laminae of the dorsal horn following induction of K/BxN serum transfer arthritis

Quantification of microglia (Iba1⁺) staining in the lumbar superficial laminae of the dorsal horn in K/BxN and control serum treated animals at both (A) 5 days and (B) 25 days post K/BxN serum transfer as compared to same day controls. Data are expressed as mean ± SEM; n = 4. ** = P<0.01 and * = P<0.05 compared to same day controls, One-Way ANOVA, post-hoc Tukey test. (C) Representative images displaying Iba1⁺ (red) and phospho-p38⁺ cells (green) in lumbar dorsal horn sections 25 days after immunization with either K/BxN or control serum. Scale bar = 200 μm
2.4 Discussion

In this chapter I initially validated that passive immunization with K/BxN serum results in acute paw swelling in fore- and hind-paws in the K/BxN serum transfer model, which occurs concomitant with mechanical hypersensitivity, persisting beyond the resolution of arthritic joint swelling. In addition, I provide evidence that in the lumbar DRG, where cell bodies of nociceptors innervating the hind paw reside, only during peak joint inflammation is there an upregulation of neuronal CGRP. These findings are consistent with the observations that in sensory neurons expression of neuropeptides, namely CGRP and substance P, is directly controlled by nerve growth factor (NGF) which is produced at enhanced concentrations in inflamed tissues (Donnerer et al., 1992). Furthermore, in the K/BxN model of inflammatory arthritis 25 days after serum transfer, I show that monocytes/macrophages are present in large numbers in lumbar DRG. In the CNS, only when concomitant with joint swelling, K/BxN serum-induced mechanical hypersensitivity is associated with activation of spinal cord dorsal horn neurons in superficial laminae. Whereas, increased spinal cord dorsal horn microgliosis occurs independent of joint swelling, but consistently related with hind paw mechanical hypersensitivity suggesting that neuronal activity induces spinal cord microgliosis which may reflect heightened nociceptive states in K/BxN serum transfer arthritis.

In behavioural studies, mice exhibited increased swelling of fore- and hind-paws as a direct consequence of passive immunization with K/BxN serum. This joint swelling was transient and subsided to near baseline levels in weeks following serum transfer. These findings are in agreement with several studies which also show peak signs of clinical arthritis around 5 days following K/BxN serum induction, reducing to near baseline levels by day 25 post-injection (Christianson et al., 2010, Park et al., 2016). This demonstrates that clinical arthritis following K/BxN serum induction is both robust and reproducible. In line with clinical signs of arthritis, ankle thickness increased following K/BxN serum induction subsides to near baseline levels 3 weeks post injection. Although ankle thickness does not fully return to baseline. With no observed redness of the hindpaw at these timepoints, this observation is likely the result of new pannus formation at the ankle joint, causing prolonged ankle thickness beyond resolution of overt swelling (Monach et al., 2007).
In the K/BxN serum transfer model I confirm hind paw mechanical hypersensitivity to occur alongside joint swelling which persists beyond resolution of joint swelling. Mechanical hypersensitivity beyond joint swelling is chronic following K/BxN serum induction, in line with studies where mechanical thresholds were also maintained from day 3 to end of study (Christianson et al., 2010, Park et al., 2016). Although I show incidence of K/BxN serum induced clinical arthritis strongly associates with hindpaw, allodynia, there is no direct correlation between severity of joint swelling with level of hindpaw allodynia.

In K/BxN serum transfer model, joint inflammation is indicative of immune cells infiltration to the joint (Misharin et al., 2014). These pro-inflammatory immune cells release inflammatory mediators, which act on nociceptive terminals likely leading to peripheral sensitization and increased mechano-sensitivity of joint nociceptors following serum transfer. However, within days of activation infiltrating neutrophils undergo apoptosis and are cleared from the joint by macrophages. Moreover, 3 weeks after serum induction, macrophages at the joint are known to favour an anti-inflammatory (M2) phenotype (Misharin et al., 2014). 25 days after serum transfer, an absence of overt joint swelling but maintained mechanical hypersensitivity, may be indicative of distinct mechanisms mediating heightened nociceptive signalling without joint swelling, which are less reliant on peripheral sensitization of nerve terminals. Following neuronal activation, the neuropeptide CGRP is synthesised in DRG neuronal cell bodies transported peripherally and released from nociceptive terminals in the joint, causing vasodilation and neurogenic inflammation (Levine et al., 1985b). In the CIA model of inflammatory arthritis, development of allodynia is associated with an increase in CGRP–expressing neurons in lumbar DRGs, likely due to the presence of NGF in the inflammatory soup of inflamed arthritic joints driving neuropeptide expression (Nieto et al., 2015). Similarly, I observe in the K/BxN serum transfer model that mechanical hypersensitivity, when concomitant with joint swelling, features upregulation of CGRP in the DRG. However, when mechanical hypersensitivity occurs after joint swelling has subsided there is no longer significant CGRP upregulation in the DRG neurons. These finding may indicate that following K/BxN induction, peripheral sensitization of joint nociceptors occurs. Subsequently, activation of sensory neuron innervating the inflamed joints is enhanced, resulting in CGRP-induced neurogenic inflammation and further peripheral sensitization of nociceptors. As joint swelling subsides, alternate CGRP-independent mechanisms may be at play,
possibly in the CNS, resulting in maintained nociceptive signalling and persistent mechanical hypersensitivity.

In the CIA model, when examining across all lumbar DRG neuronal populations, upregulation of CGRP-expressing neurons was only observed when allodynia occurred in the presence of clinical arthritis (Nieto et al., 2015). However, when only assessing either flour-gold labelled sensory neurons innervating ankle joint or fluorogold labelled sensory neurons expressing pERK (marker of neuronal activation), DRG neuron CGRP-expression was upregulated when allodynia was observed either prior to or in the presence of clinical arthritis (Nieto et al., 2015). In our K/BxN model, I observe a trend towards CGRP upregulation in DRG when mechanical hypersensitivity occurs in the absence of joint swelling. Therefore, discrepancies between DRG CGRP upregulation when mechanical hypersensitivity is seen either in the presence or absence of joint swelling may be due to detection sensitivity. Future study could address this issue by assessing pERK/CGRP double labelling in retrograde labelled joint innervating DRG neurons, examining neurogenic inflammation associated specifically with joint nociceptor activation in the K/BxN model.

Joint inflammation following K/BxN serum transfer is critically dependant on the infiltration of non-classical Ly6C- monocytes which mature into macrophages at the joint (Misharin et al., 2014). During peak joint swelling, these infiltrating monocyte/macrophages likely contribute to nociception by releasing pro-nociceptive mediators sensitising peripheral terminals of sensory neurons. Specifically, in the inflamed K/BxN joint II-1β expressing macrophages are shown to co-localise with articular nociceptors, likely facilitating nociceptive signalling (Mailhot et al., 2020). The cell bodies of nociceptors innervating the joint are found in the lumbar dorsal root ganglia. I show macrophages to be present in DRG of control mice only administered non-arthritogenic control serum indicating that under basal conditions resident macrophages do populate the DRG, possibly undertaking a regulatory role similar to resident M2 phenotype macrophages in the naïve mouse joint. Following serum transfer, when mechanical hypersensitivity presents alongside joint swelling induction (day 5), I do not observe any change in DRG macrophage presence. This result indicates that DRG macrophages are not involved in the heightened nociceptive states during joint swelling, and peripheral sensitization is more likely a result of previously
evidenced pro-inflammatory M1 macrophage signalling to nociceptive terminals at the joint (Misharin et al., 2014, Mailhot et al., 2020).

Interestingly, however, I do observe increased macrophage presence in the K/BxN lumbar DRG associated with persistent pain after joint swelling has subsided (day 25). Future experiments could build upon these findings and assess if the severity mechanical hypersensitivity correlates with the prominence of macrophage presence in the DRG 25 days after serum transfer. Previous studies show, following serum transfer when joint swelling has subsided, the majority of both infiltrated and resident joint macrophages display a pro-resolution M2 phenotype, unlikely to produce pro-nociceptive mediators which sensitize nerve terminals (Misharin et al., 2014). Away from the joint, in the absence of joint swelling, newly present macrophages in the K/BxN DRG may play a role in the maintenance of persistent pain states and be less dependent on peripheral sensitization at nociceptor nerve terminals.

Other studies show infiltration of macrophages to the DRG, in the acute phase of antigen-induced arthritis (AIA) in the rat knee joint, which correlates to reduced mechanical thresholds (Segond Von Banchet et al., 2009, Massier et al., 2015). Infiltrating macrophages in the AIA DRG are detected in close proximity to sensory neuron cell bodies (Massier et al., 2015). Similarly, 25 days after K/BxN serum transfer, I observe macrophages to be largely surrounding neuronal cell bodies. This close cellular proximity enables neuro-immune communication, facilitating increased nociceptive signalling from sensory neuron via pro-nociceptive mediators. In the AIA model, a significant proportion of infiltrating DRG macrophages display an inflammatory-like phenotype showing pro-inflammatory IL-6 positivity (Massier et al., 2015). As K/BxN DRG macrophages are associated with persistent mechanical hypersensitivity in the absence of joint swelling, it is possible that these macrophages also possess a pro-inflammatory phenotype, signalling to proximal nociceptor cell bodies and exacerbating nociceptive signalling. In the K/BxN joint, infiltrating macrophages are shown to undergo a phenotypic switch from M1 proinflammatory to M2 anti-inflammatory in the weeks following serum induction (Misharin et al., 2014). Therefore, further investigation is needed to clarify the phenotype of K/BxN DRG macrophages and their potential effect on nociceptive signalling when persistent mechanical hypersensitivity occurs in the absence of joint swelling.

In the AIA model, increased DRG macrophage presence is due to infiltration of blood derived monocyte/macrophages, which have adhered to the endothelium and
migrated through vessel walls into DRG tissue (Segond Von Banchet et al., 2009). When mechanical hypersensitivity presents in the absence of joint swelling, increased macrophage presence in K/BxN DRG may occur via a similar mechanism, whereby monocyte/macrophages infiltrate lumbar DRG tissue. Alternatively, it is possible that the observed increase in macrophage presence may be a result of the proliferation of resident DRG macrophages. Yu et al. (2020) show that following neuropathic injury, expansion of DRG macrophages is critical to the maintenance of mechanical allodynia, occurring independent of macrophage infiltration, and instead involves local proliferation from resident macrophages (Yu et al., 2020). Further work could investigate if K/BxN DRG macrophage expansion is the result of infiltrating or proliferative macrophages using markers against either cell proliferation (Ki67+) or the chemokine receptor CCR2, indicative of macrophage infiltration. As currently unknown, future studies may also look to establish the effects of K/BxN serum on the integrity of the blood-spinal cord barrier, which may be indicative of spinal cord immune infiltration.

Dorsal horn cFos expression is a strong indicator of nociceptive input into the spinal cord from primary afferents (Harris, 1998). Following K/BxN serum transfer, I show mechanical hypersensitivity at peak joint swelling is associated with activation of cFos in dorsal horn neurons. These results are in agreement with studies highlighting a marked increase in the number of fos positive laminae I neurons, in the dorsal horn during the acute inflammatory stage of the AIA model. Superficial laminae fos activation returns to baseline over time, coinciding with resolution of inflammation (Abbadie et al., 1994, Abbadie and Besson, 1992). Similarly, I show 25 days following serum transfer, neuronal cFos activation no longer differs compared to controls. cFos has frequently been used as an immediate early gene, with expression occurring rapidly and transiently, within minutes after a particular stimuli (Gao and Ji, 2009). Studies show in formalin model of acute inflammatory pain, cFos expression begins 30 mins after hindpaw injection and returns to baseline after 24 hours (Bullitt et al., 1992). Whereas, in the chronic constriction injury (CCI) model of neuropathic pain, fos induction diminishes within 2 weeks, although allodynia persists for many weeks, beyond spinal cord fos detection (Catheline et al., 1999). I find mechanical hypersensitivity to be present up to 25 days after serum transfer, indicating that the spinal cord is likely continually receiving nociceptive input from peripheral afferents. Therefore, in this instance cFos may not be the most accurate indicator of sustained
nociceptive input to the spinal cord. Instead, pERK activation may be used in the future, which has been suggested as more closely associating with pain-related behaviour (Gao and Ji, 2009).

Following K/BxN serum transfer when mechanical hypersensitivity is associated with joint swelling, spinal cord cFos activation occurs particularly in superficial laminae I-II of the dorsal horn, and not in deeper laminae III-IV. These results are in line with studies highlighting that after exposure to noxious stimuli, Fos or cFos expressing neurons are commonly concentrated within laminae I and II with fewer activation in neurons of in laminae III-IV (Coggeshall, 2005, Abbadie and Besson, 1992, Bullitt et al., 1992). Our investigations have focused on dorsal horn laminae I-IV. However, in response to carrageenan administration, rat lumbar spinal cord neurons have also shown increased Fos-like immunoreactivity in deeper laminae V,VI and ventral horn (Honoré & Besson, 1995). Evidence suggests that following inflammatory carrageenan injection, mechanical allodynia is conveyed in the spinal cord by calretinin neurons in dorsal horn laminae II, which robustly upregulate cFos (Peirs et al., 2015), as oppose to neuropathic-induced mechanical allodynia which is conveyed via deeper laminae of the spinal cord. Laminae II calretinin neurons receive input from c-fibres in the periphery (Peirs et al., 2020). Together with our findings, this indicates that following K/BxN serum transfer, joint swelling-associated mechanical hypersensitivity is likely mediated by c-fibre activation. However, as Aδ nociceptive fibres are also known to terminate superficial laminae of the dorsal, specifically in laminae I, their contribution cannot be entirely ruled out (Basbaum et al., 2009). Moreover, although I observe an association between joint swelling-associated mechanical hypersensitivity and cFos activation at day 5, my studies do not assess correlation between cFos activation and severity of mechanical hypersensitivity and joint swelling, which could further elucidate the connection between cFos activation and induction of mechanical hypersensitivity. In my assessment of cFos activation at Day 25 post-serum immunization, there is greater variation between control samples, as compared to day 5. This variation may have led to a reduction in effect of change between K/BxN and control groups at this time-point. Future experiments could use greater number of samples to increase the power of the study and address the robustness of these results. cFos is a marker of early neuronal activation, when
mechanical hypersensitivity persists beyond joint swelling c-fibre activation may continue, but may no longer be identified using cFos as a marker. Additionally, I show that mechanical hypersensitivity either in the presence or absence of joint swelling occurs alongside an enhanced microgliosis response in the lumbar spinal cord dorsal horn. These findings are in agreement with previous studies which also reported increased dorsal horn microglial presence when mechanical hypersensitivity is present either with (day 6 after serum transfer) or without joint swelling (day 28 after serum transfer) (Christianson et al., 2010). In the CIA model of inflammatory arthritis, spinal cord microgliosis was shown to correlate with the time course of mechanical hypersensitivity, occurring both prior to and during joint swelling (Nieto et al., 2016). Together, with our findings, this highlights that in these pre-clinical models of inflammatory arthritis microgliosis is associated with heightened nociceptive signalling to the dorsal horn, independent of overt joint inflammation. I observed the majority of microglia to be within the dorsal horn superficial laminae, where dorsal horn neurons receive noxious input from primary afferents. These dorsal horn neurons can activate resident spinal cord microglia, via exocytotic release of neuron-derived ATP (Masuda et al., 2016). Microglial activation via p38 phosphorylation is a common pathway after the activation of microglia cell surface receptors such as P2X7 and TLR4 (Clark et al., 2010, Ji et al., 2013). Following K/BxN induction, I observe increased p38 phosphorylation in dorsal horn microglia when mechanical hypersensitivity occurs in presence or absence of joint swelling. Microglial p38 phosphorylation is known to increase the synthesis and release of various pro-inflammatory microglial mediators, such as TNFα, IL-1β, IL-6, PGE2. (Ji et al., 2013, Clark et al., 2010, Chen et al., 2020). Specifically, phosphorylation of microglia p38 in the carrageenan model of inflammatory pain caused increased pro-inflammatory PGE2 production facilitating central sensitization (Svensson et al., 2003a).

Collectively, these findings highlight that in arthritic pain models, as with other chronic pain models, peripheral sensory neuron activation and subsequent signalling to spinal cord resident microglia drives microgliosis, promotes microglia-neuron communication and consequent maintenance of chronic pain states. Future works in the K/BxN model may look to correlate neuronal activation in second order dorsal horn neurons receiving noxious input to activation of proximal microglia, further highlighting the importance of neuro-immune interactions in maintenance of pain states. Similarly, experiments performed by (Qi et al., 2016) used immunohistochemical staining to
assess Fos and Iba-1 activation in the same dorsal horn section in CFA-exposed rats. They found that in the superficial laminae Fos-IR neurons were surrounded by Iba-1-IR processes, forming a large number of close contacts indicative of neuron-glial crosstalk (Qi et al., 2016).

2.4.1 Chapter Key Findings

In this chapter, I provide preclinical evidence showing:

- Persistent mechanical hypersensitivity in K/BxN serum transfer model of arthritis, which occurs in the presence of joint swelling and persists beyond the resolution of clinical signs of arthritis.
- Peripherally, in the K/BxN DRG, when mechanical hypersensitivity presents alongside joint swelling there is an increased pro-inflammatory CGRP expression from peptidergic sensory neurons. Whereas only when mechanical hypersensitivity occurs following resolution of joint swelling is there greater macrophage presence in K/BxN DRG.
- Centrally, in spinal cord dorsal horn following serum transfer, when mechanical hypersensitivity presents alongside joint swelling there is increased activation of early response gene cFos in second-order dorsal horn neurons conveying noxious information. Whereas, either in the presence or absence of joint swelling mechanical hypersensitivity is associated with increased microgliosis in K/BxN dorsal horn.

These data suggest that heightened neuro-immune communication both in the lumbar DRG (PNS) and spinal cord dorsal horn (CNS), mediated by macrophages and microglia respectively, may underlie persistent nociceptive hypersensitivity following K/BxN serum transfer.

2.4.2 Future Direction Leading to Chapter 3

Existing therapies for rheumatoid arthritis-associated pain largely consist of non-steroidal anti-inflammatory drugs (NSAIDs). These show limited efficacy in alleviating chronic pain, which may persist when joint swelling is pharmacologically controlled.
Mirroring observations in the clinic, following K/BxN serum transfer pharmacological blockade of COX1/2, preventing production of pro-inflammatory prostanoids, no longer acts as an effective analgesic target when mechanical hypersensitivity presents in the absence of joint swelling. In the inflamed K/BxN joint, coinciding with joint macrophage infiltration, there is increased pro-inflammatory and pro-nociceptive bioactive lipid mediator production. Thus far, I detect an increase in K/BxN DRG macrophage populations when mechanical hypersensitivity is dissociated from joint swelling. It is possible that distal to site of joint inflammation, dysregulation of bioactive lipid mediators in DRG may also occur and facilitate neuro-immune mediated exacerbation of nociceptive signalling. Moreover, lipid mediator dysregulation may also be distinct in K/BxN DRG when mechanical hypersensitivity occurs either in the presence (Day 5) or absence (Day 25) of joint swelling.

Thus, in the next chapter (chapter 3), I looked to determine bioactive lipid mediator profiles in K/BxN DRG when mechanical hypersensitivity occurs either in the presence (Day 5) or absence (Day 25) of joint swelling. Based on these findings, the effect of restorative lipid mediator treatment was assessed following K/BxN serum transfer, investigating changes to i) hind paw mechanical hypersensitivity, ii) DRG macrophage populations and iii) spinal cord dorsal horn microgliosis, identified in the current chapter.
Chapter 3:
The Contribution of Bioactive Lipid Mediators to Persistent Hyperalgesia in the K/BxN Serum Transfer Model of Arthritis
3.1 Introduction

3.1.1 Bioactive lipid mediators associated with inflammation

Inflammation is a biological mechanism triggered in various tissue types in response to the invasion of pathogens or tissue damage to the host. The recognition of molecules containing pathogen-associated or damage-associated molecular patterns (PAMPs or DAMPs) by cells of the innate immune system, results in the production of pro-inflammatory mediators including pro-inflammatory cytokines and bioactive lipid mediators. Following injury and infection, the acute phase of inflammation is characterized by the rapid influx of blood granulocytes, typically neutrophils, to the site of damage. This is quickly followed by monocytes which mature into inflammatory macrophages and are known to produce pro-inflammatory eicosanoids (Chiurchiu et al., 2018). These eicosanoids promote the cardinal signs of inflammation; including redness, swelling, heat, loss of function and pain in the affected region (Lawrence et al., 2002). During this acute inflammatory process, these recruited leukocytes undergo a temporal shift where they cease to produce pro-inflammatory eicosanoids and instead begin to release specialised pro-resolving lipid mediators (SPMs) derived from omega-3 polyunsaturated fatty acids. These SPMs promote the resolution of inflammation, repair of tissue and alleviate pain (Serhan and Levy, 2018). However, if resolution mechanisms are impaired or there is exacerbation of inflammatory response, chronic inflammation occurs. Chronic inflammation is characterised by a persistent production of pro-inflammatory lipids, such as eicosanoids and leukotrienes, which drive the continued recruitment of immune cells to the site of damage. This process is common to a number of autoimmune, cardiovascular neurodegenerative disorders in which bioactive lipid mediators play a key role (Perretti et al., 2017).

The initiation of inflammation is mediated by arachidonic acid-derived lipid mediators. During homeostatic function, calcium independent cytosolic Phospholipase A₂ (iPLA₂) constitutively generates low levels of free fatty acids including arachidonic acid (Dennis and Norris, 2015). However, following cell surface receptor activation by PAMPs/DAMPs, intracellular calcium influx leads to the recruitment of calcium dependant cytosolic phospholipase A₂ (cPLA₂) to the membrane. At the membrane, cPLA₂ facilitates the hydrolysis of the sn-2 ester bond of cellular phospholipids, allowing their release of arachidonic acid (AA) (Balsinde et al., 2002). AA can then be
metabolized by mechanistically distinct enzyme pathways initiated by cyclooxygenase 1 and 2 (COX-1 & COX-2) promoting prostanoid production, 5-lipoxygenase (5-LOX) for leukotriene formation and 15-lipoxygenase (15-LOX) inducing lipoxin formation (Figure 3.1) (Leuti et al., 2020).

3.1.1.1 Prostaglandins

Prostaglandins are produced in most tissue and cell types either constitutively, by physiological stimuli, or in response to noxious stimuli (Yao and Narumiya, 2019). Homeostatic production of prostanoids occurs via action of COX-1, whereas COX-2 expression is induced in response to inflammatory stimuli (Mitchell et al., 1993). As a result of COX 1/2 enzymatic action, AA is converted to PGG2, followed by PGH2, a common precursor for prostanoids. Subsequently, PGH2 is catalysed to PGD2, PGE2, PGF2α, PGI2 and TXA2 by their respective synthases, outlined in Figure 3.1. The action of each prostanoid is exerted via association to their cognate G-protein coupled receptors (GPCRs) of which 9 have been identified; prostaglandin D receptors 1-2 (DP1-2), prostaglandin E receptors 1-4 (EP1-4), prostaglandin F receptor (FP), prostaglandin I receptor (IP) and thromboxane receptor (TP) (Bos et al., 2004) (Figure 3.1). These 9 GPCR prostanoid receptors can be classified further into 3 distinct groups based on their structure and downstream signalling pathways: being either relaxants, contractile or inhibitory. The relaxant group of receptors consists of IP, DP, EP2 and EP4 which all elevate intracellular cAMP levels (Yao and Narumiya, 2019). Whereas inhibitory EP3 and DP2, are thought to reduce intracellular cAMP. EP1, FP and TP form the contractile group which are associated with activation of Ca2+ and PKC signalling (Aoki and Narumiya, 2012).

Preclinical studies using prostanoid receptor-deficient mice identified prostaglandin signalling to be important for acute inflammation in the carrageenan-induced model of paw oedema. Each prostanoid receptor was assessed individually, with IP-receptor deficient mice displaying a 50% reduction in carrageenan-induced paw swelling compared to wild types (Murata et al., 1997, Narumiya and Furuyashiki, 2011). Moreover, studies assessing mice deficient in EP2, EP3, or IP showed reduced
exudative pleural effusion 1–5 hours following carrageenan injection (Yuhki et al., 2004). Together, these studies verify the important role prostanoids signalling, particularly mediated by PGE₂ or PGI₂, plays in acute inflammation.

Among eicosanoids, prostanoids represent a key area for inflammatory research, mostly due to the findings that non-steroidal anti-inflammatory drugs (NSAIDs) effectively reduce signs of acute inflammation, by blocking the action of COX-1/2 both in animals models and in the clinic (Mitchell et al., 1993, Mitchell and Warner, 1999). However, important to note, when administered for chronic inflammatory conditions, such as RA, NSAIDs are no longer effective, suggesting that although involved in the initiation of acute inflammation prostanoid production is less involved in the maintenance of chronic of inflammatory disease. Although prostanoids may play a key role in the development of chronicity and transition from acute to chronic inflammatory states by amplifying pro-inflammatory cytokine production from leukocytes during the immune response. (Chiurchiu et al., 2018).

Macrophages are known to be one of the main cellular sources of prostaglandins, including PGE₂, during inflammation. In inflammatory states, macrophage-derived PGE₂ is thought to act in an autocrine manner by amplifying pro-inflammatory action in activated macrophages (Yao and Narumiya, 2019). This claim is substantiated by work demonstrating that lipopolysaccharide (LPS), a potent activator of macrophages, can trigger expression of pro-inflammatory IL-1β, IL-6 and COX-2 in cultured murine macrophages (Oshima et al., 2011). Cox inhibition, by Celecoxib, or antagonism of Prostaglandin E₂ receptor 4 (EP4), attenuates this action suggesting that a positive feedback initiated by LPS-mediated endogenous PGE₂-EP4 receptor signalling in macrophages (Oshima et al., 2011). Moreover, in-vitro studies suggest that Prostaglandin E₂ receptor 2 (EP2) signalling from macrophages can activate intracellular NF-κB signalling cascade resulting in increased expression of pro-inflammatory COX-2 and chemokine CCL2 (Aoki et al., 2017). CCL2 is a known chemo-attractant regulating migration and infiltration of monocytes/macrophages (Shahrara et al., 2008). Together, these data suggest a role for aberrant PGE₂ signalling in chronic inflammation facilitating further immune cell recruitment to inflamed tissues.
As well as acute inflammatory processes, prostaglandins have been implicated in chronic inflammatory signalling. In the CIA model of rheumatoid arthritis, Honda et al. (2006) observed that prostaglandin I$_2$ receptor (IP) deficient mice had significantly reduced clinical signs of arthritis, as compared to wildtype mice. Furthermore, IP-deficient mice also displayed lower levels of pro-inflammatory cytokines IL-1$\beta$ and IL-6 in arthritic paws, as compared to wildtype (Honda et al., 2006). These findings suggest that, in addition to PGE$_2$, prostaglandin I$_2$ may also act to exacerbate inflammatory states by further intensifying pro-inflammatory cytokine signalling. Prostaglandins have also been shown to enhance neutrophil migration during chronic inflammatory states. In the AIA model of rheumatoid arthritis, PGE$_2$ induced significant neutrophil migration to the inflamed joint, by enhancing production of pro-inflammatory cytokine IL-17 production and subsequent chemokine signalling leading to neutrophil attraction (Lemos et al., 2009).

3.1.1.2 Leukotrienes

The biosynthesis of Leukotriene from AA is catalysed in a two-step process by 5-LOX. AA is initially oxidized to 5-HPETE, with further dehydration of 5-HpETE by 5-LOX to form the common leukotriene precursor leukotriene A$_4$ (LTA$_4$) (Radmark and Samuelsson, 2009). LTA$_4$ can then be converted to LTB$_4$ by LTA$_4$ Hydroxylase (LTA$$_4$$H). As a ligand LTB$_4$, can bind G-protein coupled receptors (GPCRs) BLT$_1$ and BLT$_2$, which are present in various immune cell populations (Yokomizo, 2015). In addition, to producing LTB$_4$, LTC$_4$ synthase can act to conjugate glutathione to LTA$_4$, in a process which forms LTC$_4$, which is a ligand for CysLT1/2 receptors (Evans, 2003) (Figure 3.1). In addition to 5-LOX, another lipoxygenase isoform, 15-LOX, is also known to metabolise AA to the intermediary product 15-HpETE. 15-HpETE can be further catalysed by 5-LOX to produce lipoxins; LXA$_4$ and LXB$_4$ (Figure 3.1). It is important to note that although lipoxins are an AA-derived eicosanoid, unlike prostanoids and leukotrienes which are associated with pro-inflammatory signalling, lipoxins are anti-inflammatory in action (Serhan and Savill, 2005).
During inflammatory states, leukotrienes are known to facilitate migration of neutrophils to inflamed tissues (Dennis and Norris, 2015). As opposed to constitutive eicosanoid COX signalling, 5-LOX-mediated leukotriene mechanisms are specific to inflammation and cause increased vascular permeability and greater leukocyte recruitment to the site of tissue damage (Distasi and Ley, 2009, Lammermann et al., 2013). The 5-LOX enzyme is selectively expressed in bone marrow–derived cells such as neutrophils, monocytes, macrophages, dendritic cells, and mast cells (De Caterina and Zampolli, 2004), limiting leukotriene production to these cell types. Lammermann et al. (2013) identified that, following injury, neutrophil-derived leukotriene B4 (LTB$_4$) promotes the recruitment of neutrophils from distant regions to the damage site in a feed-forward manner. Moreover, neutrophil-derived LTB$_4$ is shown to be critical in the development of chronic joint inflammation in animal models of rheumatoid arthritis (Chen et al., 2006, Griffiths et al., 1995). Activation of LTB$_4$ receptors, BLT1/2, present on macrophages initiates intracellular calcium influx. This influx initiates a Ca$^{2+}$-mediated signalling cascade which triggers further PLA$_2$-mediated production of LTB$_4$. This autocrine macrophage-mediated signalling results in an aggravation of the inflammatory state (Dixit et al., 2014).

Together, these studies highlight an integral role for neutrophil and macrophage mediated leukotriene production, particularly LTB$_4$, in the formation and maintenance of inflammation.
Figure 3.1: Biosynthetic pathways and receptors for selected AA-derived bioactive lipids

AA-derivatives (black); Receptors (blue); Enzymes (red) Abbreviations - AA: arachidonic acid; BLT: leukotriene B receptor; COX: cyclooxygenase; CysLT: cysteiny1 leukotriene; DP: prostaglandin D receptor; EP: prostaglandin E receptor; FP: prostaglandin F receptor; HpETE: hydroperoxy eicosatetraenoic acid; IP: prostaglandin I receptor; LOX: lipoxygenase; LT: leukotriene; LTA4H: leukotriene A4 hydrolase; LTC4S: leukotriene C4 synthase; LX: lipoxin; PG: prostaglandin; PGDS: prostaglandin D synthase; PGES: prostaglandin E synthase; PGFS: prostaglandin F synthase; PGIS: prostaglandin I synthase; PLA: phospholipase ATP; TP: thromboxane receptor TXAS: thromboxane A synthase. (Leuti et al., 2020)
3.1.2 Bioactive lipid mediators associated with resolution of inflammation

Acute inflammation was previously thought to resolve passively, whereby following initiation of inflammation released pro-inflammatory mediators would naturally catabolise. However, it is now thought that endogenous specialized pro-resolving mediators (SPMs) are released from the very same immune cells involved in the production of pro-inflammatory lipid mediators. These cells undergo a class switch in order to counterbalance inflammatory signalling and facilitate the resolution of inflammation (Perretti et al., 2017).

SPMs are derived from either omega-6 arachidonic acid (AA), omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or docosapentaenoic acid (DPA). These fatty acids are biosynthesised using a combination of COXs and LOXs, the same enzymes necessary for eicosanoid production. The SPM research field is rapidly advancing, with frequent discoveries of new SPMs. The SPMs, so far discovered, can be divided into six main classes: AA-derived Lipoxins (LXA4 and LXB4); EPA-derived E-series resolvins (RvE1–3); DHA-derived D-series resolvins (RvD1–6); protectins (PD1/NPD1); maresins (MaR1 and MaR2); and DPA-derived 13-series resolvins (Leuti et al., 2020).

3.1.2.1 Lipoxins

Lipoxins, LXA4 and LXB4, are synthesised from omega-6 arachidonic acid, by direct conversion by 15-LOX into 15-hydroperoxyeicosatetraenoic acid (15-HpETE), which is subsequently converted to lipoxins by 5-LOX. Alternatively, lipoxins can be synthesised similarly to leukotrienes by 5-LOX conversion of AA to LTA4, via 5-HpETE production. LTA4 metabolism and is then skewed towards 12-LOX activity, forming lipoxins (LXA4 & LXB4) instead of producing leukotrienes. Lipoxins have been shown to target formyl peptide receptor 2 (FPR2), a G-protein coupled receptor also known as ALX (Chiang et al., 2006)(Figure 3.2). ALX, the receptor target for lipoxins, is present on neutrophils, macrophages, T cells (Perretti et al., 2017).

Following initiation of inflammation, lipoxins actively stop signals for further immune infiltration to site of inflammation, whereby formerly pro-inflammatory leukocytes undergo class switching to self-limit the inflammatory response (Buckley et al., 2014). Studies show that prostaglandin D2 or E2, responsible for inflammation induction,
subsequently stimulate anti-inflammatory circuits by inducing 15-LOX in neutrophils. This class switch in eicosanoid production, stops LTB₄-stimulated neutrophil signalling more immune infiltration and increase lipoxin production (Levy et al., 2001). Lipoxins further limit neutrophil influx to the inflammatory site and stimulate efferocytosis; the phagocytosis of apoptotic cells and cellular debris by resolving macrophages (Maderna et al., 2010). In addition to evoking efferocytosis, Lipoxin A₄ has been shown to block LTB₄-mediated neutrophil extravasation and tissue injury (Lee et al., 1989).

Genetically modified mice lacking either 12/15-LOX activity or ALX/FPR2 in leukocytes display exacerbated disease severity and tissue damage in inflammatory arthritis (Kronke et al., 2009, Dufton et al., 2010). 5-LOX deficient mice exhibit intensified and prolonged infectious arthritis (Blaho et al., 2011, Norling et al., 2016). Collectively these studies highlight the importance of AA-derived lipoxin signalling to the resolution of inflammatory states.

3.1.2.2 DHA-derived resolvins

Resolvins are specialised pro-resolving lipid mediators biosynthesised from omega-3 poly-unsaturated fatty acids such as EPA and DHA. Omega-3 fatty acids are not greatly biosynthesised de novo in humans and therefore must be supplied in the diet, of which fish oils are rich in DHA and EPA (Ji et al., 2011). Resolvins are found to form in both rodents and humans and named due to their biosynthesis specifically in the resolution phase of inflammation (Serhan and Savill, 2005). These SPMs are potent, showing efficacy at pico-nanomolar concentrations by counteracting pro-inflammatory processes to promote efferocytosis, a process whereby apoptotic cells are cleared by phagocytes, and ultimately promoting the resolution of inflammation (Buckley et al., 2014).

DHA is the most common precursor in the biosynthesis of SPMs including D-series resolvins (RvD1-6), maresins (MaR1/2) and protectins (PD1 and PDX). Initially, either 12-LOX or 15-LOX catalyses the conversion of DHA to form 17-(S)-HpDHA or 14-(S)-HpDHA, respectively (Leuti et al., 2020). 17-(S)-HpDHA can then be oxidised by action of 5-LOX at the 4-Carbon or 7-Carbon position to form either 7- hydroperoxy-17S-HDHA (precursors to RvD1, RvD2 and RvD5) or 4-hydroperoxy-17S-HDHA.
(precursors to RvD3, RvD4 and RvD6). Whereas 14-(S)-HpDHA is converted by 12-LOX to form 13S,14S-epoxy-maresin which is hydrolysed to form either MaR1 or MaR2 (Leuti et al., 2020) (Figure 3.2).

Several DHA-derived resolvin receptors have been identified, with SPMs not all restricted to binding a single receptor; FPR2/ALX (RvD1 and RvD3), GPR32/DRV1 (RvD1, RvD3 and RvD5), GPR18 (RvD2) (Figure 3.2). SPM-receptor binding is thought to be context dependent as, RvD1 interacts with GPR32 for homeostatic functions, whereas, in response to an inflammatory stimulus, RvD1-ALX/FPR2 binding facilitates anti-neutrophil actions to resolve inflammation (Krishnamoorthy et al., 2010, Norling et al., 2012).

FPR2/ALX receptors are broadly expressed in neutrophils, monocytes, macrophages, immature dendritic cells, and at low levels in T and B cells (Leuti et al., 2020). Upon SPM binding, ALX/FPR2 receptors can dimerize to alter ligand-dependent intracellular signalling. The engagement of SPM ligands increases both ALX/FPR2 homodimerization and heterodimerization with FPR1 receptor. ALX/FPR2-FPR1 heterodimers mediate distinct downstream signalling events promoting phosphorylation of the JNK/caspase-3 pathway and proapoptotic signalling pathways (Cooray et al., 2013). Studies show ALX/FPR2-mediated receptor signalling to be key for the resolution of inflammation, with genetically modified mice lacking ALX/FPR2 displaying increased disease severity and tissue damage in inflammatory arthritis (Dufton et al., 2010).

Resolvin signalling in activated neutrophils and macrophages is associated with mechanisms necessary for the resolution of inflammation. In the carrageenan model, RvE1 was shown to block neutrophil recruitment to the site of inflammation(Campbell et al., 2011). In the K/BxN serum transfer model of inflammatory arthritis, 17(R)-RvD1, a metabolically stable epimer of RvD1, when administered at peak joint swelling led to reductions in arthritis severity, hind paw oedema and joint leukocyte infiltration (Norling et al., 2016). Moreover, 17(R)-RvD1 stimulated chondrocyte matrix production facilitating tissue repair (Norling et al., 2016). Together these data highlight that resolving signalling plays a key role promoting inflammatory resolution.

The resolvin receptor GPR32, also known as DRV1 is known to be present on various innate and adaptive immune cell types including neutrophils, epithelial cells, T helper
cells and tissue macrophages (Norling et al., 2012, Schmid et al., 2016). GPR32 is sensitive to the binding of low concentrations of RvD1 and following exposure to pro-inflammatory chemokines, RvD1 facilitates a reduction of neutrophil recruitment to endothelial cells (Norling et al., 2012). During the inflammatory response, following bacterial infection, RvD5 is shown to be a potent stimulator of phagocytosis by macrophages, correlating with surface expression of GPR32 (Werz et al., 2018).

Recent work by Flak et al. (2019) demonstrates that in addition to GPR32, DPA-derived RvD5 can bind orphan receptor GPR101, present on macrophages. Knockdown of this receptor from macrophages results in inhibition of RvD5 mediated cAMP upregulation, bacterial phagocytosis and efferocytosis. Moreover, in the K/BxN serum transfer, GPR101 knockdown inhibited RvD5 mediated protection from joint inflammation (Flak et al., 2019). This study highlights an important role for RvD5-GPR101 mediated signalling in the resolution of inflammation.

### 3.1.2.3 EPA-derived Resolvins

In addition to DHA and DPA, resolvins can be formed from the metabolism of EPA. This leads to production of E-series resolvins namely RvE1, RvE2 and RvE3 (Leuti et al., 2020) (Figure 3.2). Oxygenation of membrane-bound EPA by either acetylated COX-2 or CYP450 leads to production E-series resolving common precursor 18-R-hydroperoxy-eicosapentaenoic acid (18-(R)-HpEPE) (Leuti et al., 2020). Hereafter, enzymatic action of 12/15 LOX mediates the production of RvE3, whereas 5-LOX in combination with peroxidase and hydrolase mediate RvE1 and RvE2 formation, which can bind Chemerin receptor 23 (ChemR23) (Leuti et al., 2020)(Figure 3.2). ChemR23 is expressed on neutrophils and M1, but not M2, macrophages (Cash et al., 2013, Herova et al., 2015). In addition to binding ChemR23, much like D-series resolvins, E-series resolvins can antagonise BLT1, inhibiting the proinflammatory action of leukotrienes (Serhan and Levy, 2018). RvE1 is known to accelerate resolution of inflammation by promoting neutrophil apoptosis and macrophage repolarisation from proinflammatory M1 to pro-resolution M2 phenotype (Herova et al., 2015).
3.1.2.4 Protectins

The protectin PD1, is a DHA-derived lipid mediators formed by hydrolysis of common precursor, 17(S)-HpDHA, for which GPR37 is the known receptor (Bang et al., 2018). Studies using synthetic PD1 displayed a reduction in LTB₄ induced leukocyte recruitment following exposure to synthetic PD1 (Serhan et al., 2006). Protectins are potent at very low doses with PD1 inhibiting leukocyte infiltration across endothelial cells at doses as low as 1ng in mice, following acute inflammatory zymosan injection (Serhan et al., 2006).

3.1.2.5 Maresins

Recently discovered maresins, MaR1 and MaR2, are macrophage mediators with a key role in in the resolution of inflammation. Maresin-1 (MaR1) has displayed potent anti-inflammatory and pro-resolving actions by inhibiting neutrophil infiltration, stimulating macrophage phagocytosis and efferocytosis, as well as promoting macrophage class switching from proinflammatory (M1) to pro-resolution (M2) phenotype (Francos-Quijorna et al., 2017, Dalli et al., 2013, Serhan et al., 2012). MaR1’s pro-resolution features were clearly shown by Serhan et al., (2012), in the zymosan model of inflammatory peritonitis, where MaR1 treatment caused a reduction in neutrophil infiltration to inflammatory site. MaR1 was also shown in increase macrophage phagocytosis of apoptotic neutrophils (Serhan et al., 2012).

Work by Francos-Quijorna et al. (2017) highlighted that MaR1 effectively promotes resolution of inflammation and has neuroprotective qualities in the CNS, following spinal cord injury. Following injury, repeated MaR1 treatment led to a rapid increase in neutrophil and macrophage clearance from the site of damage, 7 days post-injury (Francos-Quijorna et al., 2017). Moreover, at the site of inflammation, MaR1 was shown to reduce pro-inflammatory cytokine expression as well as silence pro-inflammatory STAT and MAPK signalling pathways. Remaining macrophages at the injury site were shown to be redirected towards a pro-resolution M2 phenotype, assisting in phagocytosis of neutrophils (Francos-Quijorna et al., 2017). Similarly, cultured macrophages with an M1 phenotype, undergo a class switch from pro-
inflammatory M1 to anti-inflammatory M2 phenotype following incubation with MaR1, further highlighting the ability of MaR1 to promote resolution of inflammation (Dalli et al., 2013).

MaR1 has recently been shown to bind to leucine-rich repeat containing G protein–coupled receptor 6 (LGR6), expressed in phagocytes (Chiang et al., 2019). In cultured human macrophages, MaR1 was shown to initiate LGR6 coupling via a Gαs protein leading to downstream increases in intracellular cAMP levels. LGR6 was found to be present on the cell surface of immune cells including monocytes, macrophages and neutrophils, with in-vivo knockdown of LGR6 in mice resulting in inhibition of MaR1-mediated blockade of neutrophil recruitment and macrophage phagocytosis following inflammatory peritonitis (Chiang et al., 2019). In addition to immune cell effects MaR1 was also shown to instigate resolution of TRPV1-mediated nociceptive signalling from sensory neurons (Serhan et al., 2012). This anti-nociceptive feature is discussed in section 3.13.
Figure 3.2: Biosynthetic pathways and known receptors of specialized pro-resolving mediators

Ligand receptor binding (blue arrow); receptor antagonism (red line); Receptors (blue text); Enzymes (red text). Abbreviations- AA: Arachidonic acid; aa-COX-2: aspirin-acetylated cyclooxygenase 2; ALX/FPR2: Formyl peptide receptor 2; BLT1: Leukotriene B4 receptor 1; ChemR23: Chemerin receptor 23; COX: Cyclooxygenase; CYP450: Cytochrome P450; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; EPHX: Epoxide hydrolase; HpEPE: Hydroperoxyeicosapentaenoic acid; LGR6: Leucine-rich repeat containing G-protein coupled receptor 6; LOX: Lipoxygenase; LT: Leukotriene; LX: Lipoxin; MaR: Maresin; PD: Protectin; Rv: Resolvin. (Adapted from Leuti et al., 2020 & Ji et al., 2011)

### 3.1.2.6 Endocannabinoids

Endocannabinoids (eCBs) are bioactive lipids which are ubiquitous to all cells in the body and display immunomodulatory properties related to the resolution of inflammation. eCBs are not stored intracellularly and are instead produced on demand from the membrane lipids in response to GPCR or neuronal activation (Woodhams et al., 2015). eCBs form two major group; N-rachidonoyethanolamine (AEA) and 2-arachidonoylglycerol (2-AG), which are amide and ester derivatives of AA, respectively. AEA-derived eCBs are produced using the calcium dependent enzyme
N-acylphosphatidylethanolamine-hydrolyzingphospholipase D (NAPE-PLD). Whereas, 2-AG synthesis occurs via two separate routes; either the conversion of diacylglycerol (DAG) by DAG-lipase or the phospholipase C-mediated conversion of 2-Arachidonoylglycerol-3-phosphate (2-AG-3P) (Woodhams et al., 2015, Leuti et al., 2020).

AEA and 2-AG mainly acting on two sub-types of Gi/o-protein coupled cannabinoid receptors, CB1 and CB2. These receptors are expressed to various extents in immune cells, with CB2 being predominant both under physiological conditions and upon acute and chronic inflammation. 2-AG has been shown to be a full agonist of CB1 & CB2. Whereas, AEA is a partial agonist for both cannabinoid receptors, with preferential selectivity for CB1 over CB2. (Woodhams et al., 2015). In addition to cannabinoid receptors, at high concentrations, AEA is shown to be a full agonist for TRPV1 at higher concentrations (Zygmunt et al., 1999, Ross, 2003, Woodhams et al., 2015). The activation of eCB signalling has been associated with pro-resolution mechanisms in inflammation, with 2-AG treatment stimulating efferocytosis from macrophage-like cells following zymosan induced inflammation (Gokoh et al., 2007). In addition, eCBs are known to have anti-nociceptive properties, reducing nociceptive responses in carrageenan model of inflammatory pain (Elmes et al., 2004). Application of anandamide, a CB1/2 agonist, to inflamed paws in carrageenan model significantly inhibited paw oedema, protein extravasation and local CGRP release (Richardson et al., 1998). CB1 and CB2 receptors are present in the CNS and on terminals of peripheral sensory afferents. Moreover, CB1 is also found in nociceptive and non-nociceptive sensory neurons in the DRG and trigeminal ganglion (Piomelli and Sasso, 2014). These findings suggest that endocannabinoids signalling can promote resolution of inflammation by also attenuating neurogenic inflammation at the periphery.

3.1.3 Bioactive lipid mediators and pain
3.1.3.1 Pro-nociceptive lipid mediator signalling

Pro-inflammatory bioactive lipid mediators are long known to be involved in the peripheral sensitization of nociceptors, leading to an exacerbation of nociceptive responses. Ferreira et al. (1972) showed in humans that intradermal injection of PGE₁
causes increased pain sensitivity, with subsequent studies demonstrating hyperalgesia also evoked by PGE$_2$ and PGI$_2$ injection in rodents (Ferreira et al., 1978, Khasar et al., 2008). Prostaglandins produced at the site of inflammation readily activate specific G-protein-coupled prostaglandin receptors (Figure 3.2), which are present on the terminals of peripheral nociceptors. This leads to increased membrane excitability and subsequent secretion of neurogenic inflammatory mediators such as substance P and CGRP (Park and Vasko, 2005, Piomelli and Sasso, 2014, Jenkins et al., 2001). Cyclopentenone prostaglandins are a class of prostaglandin metabolites formed by dehydration of classical eicosanoids such as PGD$_2$, PGE$_2$ and PGI$_2$, exhibiting biological action through non-GPCR target proteins. This prostaglandin subset is shown to also facilitate sensory neuron CGRP and substance P release, from the central terminals of nociceptors. However, this function is abolished by pre-exposure of neurons to desensitizing levels of capsaicin, suggesting that TRPV1 signalling from nociceptors is necessary for prostaglandin-induced neuropeptide release (Materazzi et al., 2008). Mice deficient in prostaglandin receptors have normal baseline nociceptive behaviour, indicating that prostaglandins are primarily contribute to the peripheral sensitization of sensory neurons.

Peripheral sensitization has been shown to occur via modulation of TRPV1 receptors on sensory neurons, with PGE$_2$ and PGI$_2$ causing increased capsaicin-induced responses from DRG neurons (Moriyama et al., 2005). Furthermore, activation of EP1 and IP receptors by PGE$_2$ and PGI$_2$ respectively, can lower nociceptive thresholds to noxious heat (Moriyama et al., 2005). Together these results suggest sensitizing action of prostaglandins occurs via TRPV1 mediated mechanisms. EP2 and EP4 receptors are coupled to Gs proteins and increase cAMP via PKA signalling, leading to downstream signalling sensitizing neurons. Alternatively, studies have shown PGE$_2$ can also produce hyperalgesia via PKC-mediated signalling (Khasar et al., 2008, Natura et al., 2013). Natura et al. (2013) show that following pro-nociceptive PGE$_2$-induced activation of EP2 and EP4, DRG neurons are stimulated to express inhibitory EP3 receptors, which have anti-nociceptive actions. This highlights the context dependant function of PGE$_2$ signalling in regulating nociception.

PGE$_2$ has also been shown to modulate activity of neuronal HCN channels, which when activated by either hyperpolarisation or cAMP lead to depolarisation of the membrane and increased neuronal excitability (Emery et al., 2012). The McNaughton
group showed HCN1 receptors, expressed in large diameter neurons, were activated by PGE₂, with knockout of Hcn1 gene resulting in decreased PGE₂-mediated cAMP production from neurons and reduced cold allodynia following neuropathic injury (Momin et al., 2008). In the inflammatory carrageenan model, HCN2, another isoform of HCN channel, was shown to be activated by PGE₂ contributing to heat hypersensitivity (Emery et al., 2011).

PGE₂ has also been implicating in heightened nociceptive states. Work by Sousa-Valente et al. (2018) showed that in the MIA model of osteoarthritis, at the site of inflammation, there is delayed PGD₂ production by mast cells. This occurs in response to NGF-mediated TrKA activation of mast cells and enhancement of COX-2 production. Importantly, the inhibition of PGD₂ synthesis prevents MIA-induced mechanical hypersensitivity in TrkA KI mice (Sousa-Valente et al., 2018). The pro-nociceptive action of PGD₂ is known to occur via activation of PD1 and PD2 receptors present of sensory neurons, causing the enhancement of tetrodotoxin-resistant Na⁺ currents (Ebersberger et al., 2011). Therefore, following its release from mast cells, PGD₂ can directly sensitive peripheral neurons via activation of DP1 receptors, increasing Na⁺ channel mediated neuronal activation.

In addition to the periphery, prostaglandins have been shown to heighten nociception by central mechanisms (Malmberg and Yaksh, 1992). Malmberg and Yaksh (1992) showed that central blockade of prostaglandin production, by inhibition of COX-2 with intrathecal NSAIDs, can effectively attenuate thermal hyperalgesia induced by intrathecal NMDA, AMPA, or SP administration. EP2 receptors are known to be expressed in spinal cord neurons (Kawamura et al., 1997). Studies have demonstrated PGE₂, acting via an EP2-like receptor, can directly depolarizes spinal neurons (Baba et al., 2001). Additionally, PGE₂, injected intrathecally into the spinal canal caused profound neuronal sensitization, with increased glutamate release from peripheral afferent synapsing with second order neurons in the dorsal horn (Ferreira and Lorenzetti, 1996, Zeilhofer, 2007). Harvey et al., (2004) demonstrated that PGE₂ can also contribute to central sensitization by disrupting inhibitory glycine-mediated Cl⁻ influx at postsynaptic sites in the spinal cord. GlyR α3 subunits are distinctly expressed in the superficial dorsal horn of the spinal cord. Glycine-mediated activation of GlyR α3 subunits induces inhibitory post-synaptic currents via Cl⁻ influx. These inhibitory currents are reduced in the presence of PGE₂ in a PKA-dependent manner (Harvey
et al., 2004), As blocking phosphorylation of GlyR α3 abolishes PGE$_2$ action, it likely that PGE$_2$-mediated inhibition of GlyRs α3 occurs due to direct receptor phosphorylation. In addition to lacking PGE$_2$-mediated inhibition of glycinergic neurotransmission, GlyR α3 deficient mice also show reduced nociceptive sensitisation induced by both intrathecal PGE$_2$ injection or zymosan-induced peripheral inflammation (Harvey et al., 2004). In the K/BxN serum transfer model of inflammatory arthritis, Ketorolac, an inhibitor of COX1/2, effectively reduces mechanical hypersensitivity when administered concomitant with joint swelling (Park et al., 2016, Christianson et al., 2010). However, NSAID treatment is no longer effective when mechanical hypersensitivity presents in the absence of joint swelling. This indicates that COX- mediated prostaglandin production at the joint may not drive nociceptive hypersensitivity, beyond joint swelling.

### 3.1.3.2 Anti-nociceptive lipid mediator signalling

In recent years, in addition to anti-inflammatory actions of pro-resolution lipid mediators, there is evidence of an anti-nociceptive role for bioactive lipid mediators. Work by Xu et al., (2010) highlight the potent anti-hyperalgesic ability of RvD1 and RvE1 in reducing nociceptive signalling in both acute and persistent models of inflammation; with RvE1 effectively reducing CFA-induced heat hyperalgesia at doses 1000x lower than COX inhibitors. In the formalin model of acute inflammation, intrathecal RvE1 treatment led to attenuation of the second, but not first, phase of nocifensive behaviour thought to be mediated via central mechanisms within the spinal cord. Knockdown of ChemR23 receptors, present on TRPV1$^+$ DRG neurons and spinal cord neurons, indicate that RvE1 could mediate anti-nociceptive action via ChemR23 expressed in DRG and spinal cord neurons (Xu et al., 2010). ChemR23 is known to regulate ERK signalling pathways. Xu et al. (2010) observed that inhibition of ERK signalling blocked capsaicin-induced increases in spontaneous excitatory postsynaptic currents increase from spinal cord neurons, indicating a role for ERK in regulating presynaptic glutamate release in the spinal cord.

Intraplantar pre-treatment with RvE1 was shown to substantially attenuate hyperalgesia in acute inflammation by blocking ERK-mediated glutamate release in presynaptic terminals in response to TNFα stimulation and TRPV1 activation (Xu et
al., 2010). These anti-nociceptive effects occurred without altering baseline nociceptive thresholds indicating that resolvins counteract heightened nociceptive signalling rather than constitutively dampening neuronal activity.

In the adjuvant-induced arthritis model of chronic inflammatory pain, repeated systemic administration of Resolvin-D precursor, 17(r)HDoHE, led to a reduction in mechanical hypersensitivity when administered prior to chronicity of inflammation. 17(r)HDoHE treatment was also associated with decreased spinal cord and DRG phosphorylation of NF-κB p65 subunit, whose signalling regulates pro-inflammatory cytokine release. In addition, COX-2 expression, necessary for pro-inflammatory prostaglandin production, was also reduced in DRG and spinal cord as a result of 17(r)HDoHE treatment. Inhibiting both NF-κB and COX-2 induced pro-inflammatory cytokine release in DRG and spinal cord would reduce peripheral sensitization of nociceptors and central sensitisation of second order spinal cord neurons, respectively (Lima-Garcia et al., 2011). RvD1 has been shown to reduce inflammatory nociceptive signalling by attenuating peripheral activity of TRPA1, TRPV3 and TRPV4, without modulation of TRPV1 (Bang et al., 2010, Xu and Ji, 2011). Park et al. (2011) also identified distinct roles for resolvins regarding analgesic action, whereby RvD2 was shown to be a potent inhibitor of both TRPV1 and TRPA1 in primary sensory neurons, whereas RvE1 and RvD1 only selectively inhibited TRPV1 and TRPA1, respectively (Park et al., 2011). Together these results indicate that resolvin-mediated mechanisms can reduce pro-inflammatory signalling associated with sensitization of neurons.

During chronic pain development, central sensitization occurs whereby NMDA receptors present on spinal cord dorsal horn neurons become activated. This activation can be mediated by TNFα induced phosphorylation of ERK, which is inhibited by RvE1. Therefore, in addition to mediating peripheral sensitization, RvE1 at the spinal cord can modulate activity from post-synaptic spinal cord neurons reducing nociceptive signalling (Xu et al., 2010).

Recent work by Luo et al (2019) has demonstrated the presence of sexual dimorphism in the analgesic action of SPMs. Following neuropathic injury, as opposed to the sex independent analgesic action of RvD1 and RvD2, intrathecal RvD5 treatment only reduced mechanical allodynia in male mice (Luo et al., 2019). Moreover, RvD5 only
effectively reduced inflammatory formalin-induced second-phase of nocifensive behaviours in male but not female mice (Luo et al., 2019). This study indicates that as analgesic efficacy of specialised pro-resolving mediators exists between sexes, sexual dimorphisms may occur in mechanisms by which pro-resolution lipid mediators regulate nociceptive signalling.

DHA-derived neuroprotection D1 (NPD1) induces potent inhibition of inflammation and inflammatory pain via its multiple actions on neurons and immune cells (Xu et al., 2013, Bang et al., 2018). Bang et al. (2018) show in zymosan-induced model of inflammatory pain that NPD1, by binding GPR37 expressed on macrophages, modulates pro-inflammatory IL-1β release from macrophages and reducing subsequent peripheral sensitization of nerve terminals at the site of acute inflammation. Moreover, GPR37 activation, presumably mediated by NPD1, in macrophages was shown to be critical for the resolution of inflammation induced mechanical hypersensitivity (Bang et al., 2018). This study points towards a key role for SPMs in the attenuation of neuro-immune signalling contributing to the exacerbation of nociceptive states.

First acknowledged by Serhan et al. (2012) macrophage-derived SPM, Maresin-1, demonstrates potent anti-nociceptive actions. Intraplantar MaR1 treatment (10ng/mouse) effectively reduced capsaicin induced-nocifensive behaviours in mice. Intraperitoneal MaR1 treatment (40ng/mouse) was also shown to reduce chemotherapy-induced mechanical allodynia, following intraperitoneal injection of vincristine sulphate. Reversal of mechanical sensitivity was observed to 14 days after final vincristine injection. Moreover, in-vitro, perfusion of dissociated DRG neurons in culture with MaR1 was shown to reduce capsaicin-mediated currents from TRPV1+ neurons in a dose-dependent manner, with capsaicin-induced currents completely abolished with perfusion of 3ng/ml MaR1 (Serhan et al., 2012). The MaR1-mediated reduction in current was observed in a concentration dependant manner, with MaR1 exhibiting efficacy at 1ng/ml and 3ng/ml concentrations. This MaR1-mediated inhibition of capsaicin-induced current in TRPV1+ neurons was blocked by G-protein coupled receptor inhibitor pertussis toxin, indicating that MaR1 can act directly on TRPV1+ neurons inhibiting neuronal activity via GPCR mediated mechanisms (Serhan et al., 2012) Together, these results indicate that anti-nociceptive action of MaR1 may be mediated by TRPV1-associated neuronal mechanisms in both inflammatory and neuropathic pain.
In the carrageenan model of inflammation, as early as 1 hour after inflammatory injection, 3ng/mouse intrathecal MaR1 treatment reduce mechanical and thermal hyperalgesia. Moreover, MaR1 reduced capsaicin-induced CGRP release from nociceptive nerve terminals, in a dose-dependent manner, showing greatest efficacy when administered at 3ng/ml (Fattori et al., 2019).

Studies have highlighted the ability of MaR1 to induce pro-resolution class switching of macrophages associated with reductions in pro-inflammatory cytokine signalling (Dalli et al., 2013). Moreover, MaR1 reduces proinflammatory IL-1β and TNFα signalling from spinal cord glial cells following inflammation (Fattori et al., 2019). Together, these studies indicate that MaR1, as well as acting directly of nociceptive neurons, may also exhibit anti-nociceptive action by hindering immune-mediated pro-inflammatory cytokine signalling to either peripheral nerve terminals or spinal cord neurons, reducing peripheral and central sensitization, respectively.

### 3.1.4 Lipid mediators in K/BxN serum transfer arthritis

Aberrant resolution of inflammation is associated with chronic inflammatory disorders such as rheumatoid arthritis. In the K/BxN serum transfer model of inflammatory arthritis, 8 days after serum transfer arthritic paws were shown to have greater levels of pro-inflammatory prostanoids including TBX₂, PGD₂, PGE₂ and LTB₄ (Norling et al., 2016). Similarly, all these pro-nociceptive and pro-inflammatory prostanoids were observed in the synovial fluid of RA patients (Norling et al., 2016).

In the inflamed K/BxN paw, there is also seen a reduced local levels of pro-resolving lipid mediators D-series resolvins and MaR1 indicating that it is not only an exacerbation of pro-inflammatory signalling, but also disturbed SPM action at the site of inflammation which contributes to inflammatory state (Arnardottir et al., 2016). Supplementation of pro-resolution lipid mediator RvD3 to K/BxN treated mice reduced joint leukocyte infiltration and clinical signs of arthritis (Arnardottir et al., 2016). Similarly, 17R-RvD1 treatment at peak joint swelling also led to a suppression of joint leukocyte infiltration and reduced arthritis scores (Norling et al., 2016). These results indicate that tackling arthritis-associated SPM dysregulation could offer therapeutic possibilities for the treatment of joint inflammation in RA. Importantly, in addition to
being anti-inflammatory, SPMs have potent anti-nociceptive qualities, highlighted in section 3.1.3. Therefore, heightened joint nociception in K/BxN serum transfer model may be facilitated by dysregulation of bioactive lipid mediators, which could be corrected with SPM treatment.

3.1.5 Aims

Bioactive lipid mediators play a key role in regulating both pain and inflammation. Thus far in the K/BxN serum transfer model, I have observed mechanical hypersensitivity dissociated from joint swelling. During peak joint swelling, Norling et al. (2016) showed increased pro-inflammatory and pro-nociceptive prostanoids in arthritic paws. Additionally, pharmacological blockade of COX1/2, preventing prostanoid production, is no longer an effective analgesic target when mechanical hypersensitivity presents in the absence of joint swelling (Park et al., 2016). Therefore, it is possible that a distinct dysregulation of lipid mediators may be responsible for sensitization of nociceptors and subsequent heightened nociceptive states when joint swelling has subsided. Cell bodies of nociceptors innervating the joint reside in the lumbar DRG. Following serum transfer, I observed increased microglial activation in the dorsal horn of the spinal when mechanical hypersensitivity presents both in the presence and absence of joint swelling. However, I only observed increases in DRG macrophage population when mechanical hypersensitivity is dissociated from joint swelling. These observations highlight the possibility that in the absence of joint swelling, altered neuron-macrophage interactions in the K/BxN DRG may have been involved in the maintenance of pain states at this timepoint. Therefore, in the present chapter our rationale for assessing the DRG was to further investigate the role of neuro-immune signalling in the DRG for the maintenance of mechanical hypersensitivity. I began by assessing, in the K/BxN DRG, known pronociceptive lipid mediators as well as recently described pro-resolving lipid mediators, which carry antinociceptive actions.

The aims of the present chapter were to:

- Examine bioactive lipids mediator profiles in lumbar DRG at timepoints where mechanical hypersensitivity occurs either in the presence (Day 5) or absence
(Day 25) of joint swelling, following K/BxN serum transfer. Results from lipid mediator profiling will then be used to inform future experimental treatment of mechanical hypersensitivity and associated immune cell modulation in DRG and spinal cord following K/BxN serum transfer.

- Based on the results from lipid mediator profiling, assess the effect of restorative lipid mediator treatment on hind paw mechanical hypersensitivity in K/BxN serum transfer model when repeatedly administered during peak joint swelling (Days 5, 7, 9, 11) or once joint swelling has subsided (Days 19, 21, 23).

- Assess the effect of restorative lipid mediator treatment on leukocyte (CD45\(^+\)), pro-inflammatory macrophage (M1: F4/80\(^+\)CD206\(^+\)CD11c\(^+\)) and anti-inflammatory macrophage (M2: F4/80\(^+\)CD206\(^+\)CD11c\(^-\)) populations in the DRG when repeatedly administered during peak joint swelling (Days 5, 7, 9, 11) or once joint swelling has subsided (Days 19, 21, 23).

- Evaluate changes in activation of microglial cell population (using Iba1/P-p38 a markers) in the dorsal horn spinal cord following specialised lipid mediator treatment in K/BxN serum transfer model.

Investigate sex differences in hind paw mechanical thresholds and associated spinal cord microglial activation (Iba1/P-p38) in K/BxN serum transfer model following specialised lipid mediator treatment (Days 5, 7, 9, 11)
3.2 Materials and methods

3.2.1 Animals

All experiments were performed on either adult male or female (where specified) C57BL/6 mice (Envigo, UK) aged between 10-12 weeks old and weighing approximately 25g. All mice were kept in the same living conditions as outlined in section 2.2.1. All mice were administered either K/BxN or non-arthritogenic control serum using the same protocol as outlined in section 2.2.2.

3.2.2 Targeted LC-MS/MS–based lipidomics of lumbar dorsal root ganglia

On day 5 and 25 following serum induction, L4 and L5 dorsal root ganglia were dissected, briefly washed in PBS before being immediately frozen in liquid nitrogen. Samples were then taken for processing of metabololipidomics at Queen Mary Lipid Mediator Unit using liquid chromatography mass-spectrometry (LC-MS). All samples for LCMS were processed using the methods described by Dalli et al., (2018) and outlined in Allen et al. (2020) as below.

Prior to sample extraction, deuterated internal standards, representing each region in the chromatographic analysis (d4-LTB4, d8-5S-HETE, d4-PGE2, d5-LXA4, d5-RvD2, d5-LTC4, d5-LTD4 and d5-LTE4, 500 pg each) were added to facilitate quantification in cold methanol. Samples were gently homogenised using a glace dounce and kept at -20°C for a minimum of 45 min to allow protein precipitation. Samples were then centrifuged for 10 min at 4000 x g. Supernatants were subjected to solid phase extraction, methyl formate and methanol fractions collected, brought to dryness and resuspended in phase (methanol/water, 1:1, vol/vol) for injection on a Shimadzu LC-20AD HPLC and a Shimadzu SIL-20AC autoinjector, paired with a QTrap 6500 (ABSciex). For the methyl formate lipid mediators, an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 μm) was kept at 50°C and lipid mediators were eluted with a mobile phase consisting of methanol-water-acetic acid of 50:50:0.01 (vol/vol/vol) that was ramped to 80:20:0.01 (vol/vol/vol) from 2 min to 11 min, maintained till 14.5 min and then rapidly ramped to 98:2:0.01 (vol/vol/vol) for the next 0.1 min. This was subsequently maintained at 98:2:0.01 (vol/vol/vol) for 5.4 min, and the flow rate was maintained at 0.5 ml/min. QTrap 6500 was operated in negative
ionisation mode using a multiple reaction monitoring (MRM) method as previously described [7]. For the methanol lipid mediators, an Agilent Poroshell 120 EC-C18 column (100 mm x 4.6 mm x 2.7 μm) was kept at 50°C and conjugates were eluted with a mobile phase consisting of methanol-water-acetic acid of 55:45:0.5 (vol/vol/vol) that was isocratic for 1 min, ramped to 70:30:0.1 (vol/vol/vol) over 5 min, then to 80:20:0.5 (vol/vol/vol) for 2 min, then isocratic 80:20:0.5 (vol/vol/vol) for the next 3 min, and ramped to 98:2:0.5 (vol/vol/vol) over 3 min. This was subsequently maintained at 98:2:0.5 (vol/vol/vol) for 3 min. The flow rate was maintained at 0.6 ml/min. QTrap 6500 was operated in positive ionisation mode using a multiple reaction monitoring (MRM) method as previously described [6]. Each lipid mediator (LM) was identified using strict criteria including matching retention time to synthetic and authentic materials and at least 6 diagnostic ions. Calibration curves were obtained for each using synthetic compound mixtures and deuterium-labeled lipid mediator at 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, and 200 pg. Linear calibration curves were obtained for each lipid mediator, which gave r2 values of 0.98–0.99.

2D principal component analysis of lipid mediator profiles between groups was created by Queen Mary Lipidomic Unit using orthogonal partial least square to latent structures discriminant analysis (OPLS-DA) and following mean centering and unit variance scaling of lipid mediator levels.

Data regarding lipid mediator levels within groups for each bioactive arachidonic, eicosapentaenoic, docosahexaenoic and docosapentaenoic acid bioactive metabolome measured was then returned for further data analyses.

### 3.2.3 Systemic intraperitoneal administration of maresin-1

Prior to administration all Maresin-1 (MaR1) (Cayman Chemical) was taken to Professor Dalli (Queen Mary, University of London) where quality was tested using ultraviolet–visible spectroscopy to confirm MaR1 absorbance of 271 AU. On testing days, MaR1, under nitrogen, was freshly prepared for administration.

On each treatment day, MaR1 was freshly prepared from stock solution in ethanol and stored under nitrogen at -80°C without exposure to light. To prepare MaR1 10μl of stock solution (70 ng/μl) was added to 3.5ml saline (0.9% w/v). The solution was then
vortexed for 30 s followed by water bath sonication for 10 secs. These two processes were then repeated a further 2 times. Mice were then immediately injected intraperitoneally with 500µl of MaR1 solution (100ng / mouse). K/BxN controls were administered with intraperitoneally with saline.

Mice received MaR1 treatment either on days 5, 7, 9, and 11 after K/BxN serum injection, or alternatively, MaR1 treatment occurred on days 19, 21, and 23. All behavioural testing was performed 1 hr after MaR1 injection.

3.2.4 Flow cytometry of DRG tissue
3.2.4.1 Tissue preparation for flow cytometry

On day 25 following K/BxN induction, from each animal, C6-8 and L3-5 DRG were dissected bilaterally. DRG tissue was then placed in a Falcon tube and dissociated using 3 mg/mL Dispase II (Roche), 0.1% collagenase (Sigma-Aldrich) and 200 U/mL DNAse I (Roche) in F-12 medium (Life Technologies) for 45 min at 37°C. Dorsal root ganglia were centrifuged for 1 min at 2000 rpm. Medium was then removed and replaced with fresh F-12 medium. DRG are then triturated and centrifuged for a further 5 min at 800 rpm. The remaining supernatant is then removed and discarded. Cell pellets were then resuspended in 500 mL PBS (no calcium chloride or magnesium chloride) (Sigma) plus 1% BSA. A 30 mL aliquot of cell suspension was taken for cell counting to establish absolute cell numbers for each sample (subsequently described in Section 3.2.4.2). 200µl of each sample was then added to individual wells of a filtration plate. The filtration plate was then centrifuged for at 2000 rpm for 4 min at 4°C. Supernatant was then removed, discarded and replaced with 100µl Fc receptor blocker / well (anti-mouse CD16/CD32; BD Biosciences) (1:5 concentration in 1% BSA (Sigma-Aldrich) in sterile PBS). Cells were incubated for 20 min on ice. Following incubation cells were washed with 1% BSA (Sigma-Aldrich) in sterile PBS for 5 min, then centrifuged 2000 rpm for 5 min at 4°C. Cells were then incubation with a mix containing fluorochrome-conjugated antibodies for 30 min. These antibodies are outlined in Table 3.1 and are markers for leukocytes and macrophages with either a pro-inflammatory (M1) or anti-inflammatory phenotype (M2). All primary antibodies outlined in Table 3.1 were raised in mouse and used at 1:200 concentration. 1µl of antibody solution is added to each well, with cells left to incubate for 30 min at 4°C.
Cells were then washed with 1% BSA (Sigma-Aldrich) in sterile PBS, centrifuged at 800 rpm for 1 min. Cells were then washed and centrifuged once more. After centrifugation, supernatant was removed from each well and replaced with 200µl of 1% BSA (Sigma-Aldrich) in sterile PBS. Cells were then fixed in 50µl 4% paraformaldehyde (BD Biosciences) for 10 min before centrifuged at 2000 rpm for 4 min at 4°C. Then the supernatant was discarded and cells re-suspended and stored in new tubes containing 1% BSA (Sigma-Aldrich) in sterile PBS, in the dark at 4°C ready for analysis. Flow cytometry samples were then run using LSR Fortessa cell analyser (BD Bioscience) and analysed using FlowJo software (v10.1; BD Bioscience).

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Marker</th>
<th>Conjugated-Fluorophore</th>
<th>Manufacturer</th>
<th>Clone No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
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<td>Pacific Blue</td>
<td>BioLegend</td>
<td>30-F11</td>
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<tr>
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<td>Macrophages</td>
<td>PE</td>
<td>eBioscience</td>
<td>BM8</td>
</tr>
<tr>
<td>CD11b</td>
<td>Myeloid Cells</td>
<td>APC</td>
<td>eBioscience</td>
<td>M1/70</td>
</tr>
<tr>
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<td>M2 macrophages</td>
<td>PE-Cy7</td>
<td>BioLegend</td>
<td>C068C2</td>
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<tr>
<td></td>
<td>(anti-inflammatory)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11c</td>
<td>M1 macrophages</td>
<td>APC-eFlour780</td>
<td>eBioscience</td>
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<tr>
<td></td>
<td>(pro-inflammatory)</td>
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</tr>
</tbody>
</table>

Table 3.1: Fluorophore-conjugated primary antibodies used for flow cytometry

3.2.4.2 Cell counting

Prior to estimation of specific cell type number within a sample using flow cytometry, total cell number must be estimated. After cell lysis and trituration, 30µl cell suspensions were initially taken from a single DRG sample. This 30 µl cell suspension was then mixed with 200 µl Trypan Blue (Thermo-Fisher). Trypan Blue was used to
distinguish live cells suitable for counting. As Trypan Blue binds to intracellular proteins and cannot penetrate the cell membrane, all cells which are stained with Trypan Blue are dead with disrupted cell membrane integrity (Farah et al., 2010). Live cells are then counted using a Neubauer chamber, whereby:

\[ \text{Number of live cells} \times \text{dilution factor} \times \text{chamber size} = \text{estimated cell count} \]

All subsequent flow cytometry data obtained using FlowJo was normalised to the cell count for that particular sample.

3.2.5 Tissue Preparation and Immunohistochemistry

3.2.5.1 Perfusion, cryoprotection & Immunohistochemistry

At the end of behavioural experiments, 25 days after serum transfer, mice were culled and using terminal anaesthesia, after which dorsal root ganglia and lumbar spinal cord tissue was obtained for immunohistochemistry using the exact methods described in section 2.2.5 for perfusion and cryoprotection of tissue. Immunohistochemistry was performed on tissue sections using the same protocol stated in section 2.2.5. All primary antibodies used for immunohistochemistry are stated in Table 3.2. For negative controls, the primary antibodies were omitted; this resulted in the absence of staining. For all experiments directly comparing Iba1 immunoreactivity in males and females, all tissue was stained and imaged at the same time under the same conditions. Unless stated otherwise, all antibodies were prepared in 0.2% PBS-T and incubated at room temperature.

<table>
<thead>
<tr>
<th>Antibody Name</th>
<th>Marker</th>
<th>Raised Species</th>
<th>Concentration</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Rat</td>
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<td>Abcam</td>
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<tr>
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<td>Neurons</td>
<td>Rabbit</td>
<td>1:1000</td>
<td>Thermo-Fisher</td>
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<td>Rabbit</td>
<td>1:1000</td>
<td>Wako</td>
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<tr>
<td>P-p38</td>
<td>Microglial Activation</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Cell Signalling</td>
</tr>
</tbody>
</table>

Table 3.2: Primary antibodies used during immunohistochemistry
3.2.5.2 Quantification of immunoreactivity

For quantification of F4/80 staining (macrophage marker) in the DRG the exact method used was stated in section 2.2.5. F4/80+ profiles, indicative of number of macrophages, were manually counted within four field of view (10^4 µm^2) randomly positioned across a single DRG section. Four sections per animal were assessed. Experimenter was blinded to treatment groups during image analysis. As previously stated in section 2.2.5, P-p38 (activated microglia marker) and Iba1 (microglia marker) spinal cord immunoreactivity were analysed using by counting positive profiles within three fixed boxes (4 x 10^4 µm^2) placed within medial, central and lateral portions of the superficial dorsal horn. A minimum of three sections were analysed per animal. For all immunohistochemistry analyses DAPI staining was used to confirm cellular staining.

3.2.6 Data and statistics

All data are presented as means ±SEM, where n is the number of biological replicates (individual mice), with differences between means considered as statistically significant when P<0.05. All statistical analysis for behavioural, immunohistochemical and flow cytometry was conducted using GraphPad Prism (v8.0.0; GraphPad Software, USA). Multiple comparison of data used one-way ANOVA followed by post-hoc Tukey test. If more than two groups are present, two-way repeated measure ANOVA was used, followed by post-hoc Tukey test for statistical analysis of behavioural testing and clinical scoring.
3.3 Results

3.3.1 Lipid mediator profiles are distinct in lumbar DRG both 5 and 25 days after K/BxN serum transfer

Bioactive lipid mediators are known to play a key role in controlling the balance and transition between pro-inflammatory and pro-resolving states. In the K/BxN serum transfer model, it is known that during peak joint swelling increased pro-inflammatory lipid mediators are observed in the inflamed joint (Norling et al., 2016). Moreover, NSAIDs, blocking COX-1/2 production, are no longer effective analgesics when administered at later timepoints following K/BxN serum passive immunization (Park et al., 2016). Therefore, to elucidate potentially altered lipid mediator signalling after K/BxN serum transfer I investigated the presence of known pro-inflammatory lipid mediators, in addition to pro-resolution lipid mediators with known anti-nociceptive abilities in the DRG, where cell bodies of sensory neurons are present.

Lumbar DRG tissue samples 5- and 25-days following K/BxN serum transfer were dissected and later analysed at Queen Mary Lipid Mediator Unit using liquid chromatography mass-spectrometry (LC/MS/MS) based lipid mediator profiling of arachidonic, eicosapentaenoic, docosahexaenoic and docosapentaenoic acid bioactive metabolomes. Based on measurement of over 100 fatty acid derived molecules in lumbar DRG extracts, subsequent principal component analysis highlighted at both day 5 and day 25 after K/BxN serum transfer, DRG displayed distinct lipid mediator profiles, as compared to same day controls (Figure 3.3A, B). Moreover, when assessing all lipid mediators, pathway markers and further metabolites of the omega-3 and omega-6 bioactive metabolomes individually, via 2D principal component analyses, I see further distinction of lipid mediators at both day 5 and 25 following K/BxN serum induction, as compared to respective same day controls (Figure 3.3C-D).
Figure 3.3: Distinct lipid mediator profiles in lumbar DRG at day 5 and day 25 after K/BxN serum transfer

Orthogonal partial least square to latent structures discriminant analysis (OPLS-DA) was used to generate 2 principal component functions from bioactive lipid mediator levels. (A-B) 2D principal component analyses based on bioactive lipid mediator levels in lumbar DRG highlighting within group similarity and between group differences both (A) 5 days and (B) 25 days after K/BxN serum transfer (blue) compared to same-day control group (green). (C-D) 2D principal component analyses comparing all measured bioactive lipid mediators (C) 5 days and (D) 25 days after K/BxN serum transfer as compared to same-day controls. Coloured lipid mediators displaying a variable importance in projection coefficient \( \geq 1 \).
3.3.2 Increased Prostaglandin D<sub>2</sub> levels in DRG 5 days after K/BxN serum transfer

At day 5, with assessment of specific lipid mediators using LC/MS/MS, I observed the presence of proinflammatory leukotrienes and prostaglandins (Figure 3.4 A-C) in the DRG. Although, levels of leukotriene-B<sub>4</sub> (CON: 0.39 ±0.11 pg/sample, K/BxN: 0.55 ±0.22 pg/sample) and prostaglandin-E<sub>2</sub> (CON: 16.34 ±2.11 pg/sample, K/BxN: 17.21 ±3.35 pg/sample), did not differ between K/BxN and control serum treated groups. However, I observed a significant increase in pro-inflammatory prostaglandin-D<sub>2</sub> (41.67 ±2.51 pg/sample), as compared to same day controls (24.31 ±2.79 pg/sample) (Figure 3.4 B). When assessing pro-resolution lipid mediators at day 5, although the presence of resolvin-D1 (0.11 ±0.05 pg/sample), resolvin D2 (0.08 ±0.06 pg/sample) and maresin-1 (0.97 ±0.08 pg/sample) were observed in K/BxN DRG, levels did not differ as compared to respective same day controls (0.08 ±0.04, 0.12 ±0.04, 0.32 ±0.36)( Figure 3.4 D-F).

These results suggest that following K/BxN serum transfer arthritis, swelling-associated joint pain is also accompanied with increased pro-inflammatory prostaglandin D<sub>2</sub> in the lumbar DRG.
Figure 3.4: Increased pro-inflammatory prostaglandin-D2 levels in lumbar DRG is associated with mechanical allodynia concurrent with joint swelling

Quantification of liquid chromatography mass-spectrometry (LC/MS/MS) based lipid mediator profiling for selected (A-C) pro-inflammatory and (D-F) pro-resolution bioactive lipids 5 days after K/BxN serum transfer, as compared to same-day controls. * = \( P<0.05 \), Student’s t-test. Data are expressed as mean ± SEM; \( n = 5 \) mice per group; 2 DRGs per mouse.
3.3.3 Lower pro-resolution Maresin-1 levels observed in DRG 25 days after K/BxN serum transfer

When assessing pro-inflammatory lipid mediators in DRG 25 days after serum transfer, as also observed at day 5, I observe no significant K/BxN serum transfer-associated changes to proinflammatory mediators such as leukotriene B$_4$ (CON: 0.38 ±0.10 pg/sample, K/BxN: 0.38 ±0.09 pg/sample) or prostaglandin E$_2$ (CON: 22.68 ±3.65 pg/sample, K/BxN: 20.6 ±2.23 pg/sample) (Figure 3.5A-C). However, in contrast to our observation at day 5, no significant difference was observed in prostaglandin D$_2$ levels in the lumbar DRG between K/BxN (31.84 ±2.96 pg/sample) and control (39.97 ±5.97 pg/sample) serum groups (Figure 3.5B).

When assessing pro-resolution lipid mediators in DRG I observed no significant alteration to previously established anti-inflammatory lipid mediators resolvin D1 (CON: 0.13 ±0.01 pg/sample, K/BxN: 0.17 ±0.02 pg/sample) or resolvin D2 (CON: 0.15 ±0.09 pg/sample, K/BxN: 0.03 ±0.04 pg/sample) (Figure 3.5D,E), with various other pro-resolution lipid mediators also unaffected by K/BxN serum transfer. Surprisingly, however, 25 days after serum transfer, I observed a significant decrease in the levels of pro-resolution and anti-nociceptive lipid mediator maresin1 (1.13 ±0.58 pg/sample) versus non-arthritic control DRG (0.05 ±0.05 pg/sample) (Figure 3.5F).

These findings suggest a possible functional association between persistent allodynia dissociated from joint pain and decreased maresin-1 levels in the DRG.
Figure 3.5: Decreased pro-resolution maresin-1 levels in lumbar DRG is associated with mechanical allodynia in the absence of joint swelling

Quantification of liquid chromatography mass-spectrometry (LC/MS/MS) based lipid mediator profiling for selected (A-C) pro-inflammatory and (D-F) pro-resolution bioactive lipids 25 days after K/BxN serum transfer, as compared to same-day controls. * = P<0.05, Student's t-test. Data are expressed as mean ± SEM; n = 5 mice per group; 2 DRGs per mouse.
3.3.4 When administered during peak joint swelling systemic Maresin-1 treatment leads to reduction of mechanical hypersensitivity, which persists beyond treatment

Analysis of bioactive lipid mediators within the DRG highlighted lower levels of MaR1, 25 days after K/BxN serum transfer as compared to controls. In-vitro studies have shown macrophage-derived MaR1 to have pro-resolution properties in models of inflammation (Serhan et al., 2012). Moreover, in models of neuropathic pain, MaR1 treatment has been shown to have anti-nociceptive effects (Gao et al., 2018). Therefore, I investigated whether supplementation of 100ng MaR1 repeatedly administered via intraperitoneal injection altered both the time course of joint swelling and mechanical hypersensitivity in the K/BxN serum transfer model.

MaR1 was repeatedly administered, beginning at peak joint swelling on day 5, 7, 9, 11 after induction of K/BxN serum arthritis. When assessing clinical scores, initial systemic injection of MaR1 (100 ng/mouse, intraperitoneally) had no significant effect on joint swelling as compared to saline treated controls (Figure 3.6A). Although, after the third MaR1 treatment (Day 9) there was lower overall joint swelling in MaR1 treated (3.8 ± 0.5 clinical scores) as compared to saline treated (6.4 ± 1.1) arthritic animal, this difference was not significant. These results highlight that MaR1 administration does not effect the time-course and severity of K/BxN serum associated joint swelling.

When assessing mechanical hypersensitivity in K/BxN serum transfer mice after MaR1 treatment I observed 1 hour after treatment, on day 5, no acute effects of K/BxN serum transfer on hind paw withdrawal thresholds (K/BxN Saline: 0.40 ± 0.06 g; K/BxN-MaR1: 0.42 ± 0.05 g) (Figure 3.6). However, MaR1 significantly attenuated mechanical hypersensitivity after the third dose (day 9) (K/BxN Saline: 0.44 ± 0.05 g; K/BxN-MaR1: 0.70 ± 0.05 g) (Figure 3.6). This anti-alldynic effect of MaR1 treatment was maintained until day 25, 14 days after the final MaR1 administration on day 11 (K/BxN Saline: 0.49 ± 0.06 g; K/BxN-MaR1: 0.76 ± 0.03 g) (Figure 3.6).

These results highlight that although systemic administration of MaR1 does not have an acute effect on hind paw alldynia in K/BxN serum transfer model, repeated administration has long-lasting abilities to attenuate mechanical hypersensitivity in inflammatory arthritis.
Figure 3.6: Repeated maresin-1 treatment during overt joint swelling leads to sustained reversal of hind paw allodynia in the K/BxN serum transfer model

(A) Clinical scoring fore-paws and hind-paws following 100ng MaR1 or saline intraperitoneal administration on days 5, 7, 9, and 11 after K/BxN serum transfer, with control mice receiving non-arthritogenic control serum. (B) Reversal of mechanical hypersensitivity assessed using von Frey filaments following third and fourth doses of 100ng MaR1 on days 9 and 11 after serum transfer. Arrows indicate treatment days. ***P < 0.001 vs same day K/BxN-Saline group (closed circles), two-way ANOVA, post hoc Tukey. Data are mean ± SEM; n = 5 mice per group; 2 DRGs per mouse.
3.3.5 In the absence of overt joint swelling repeated Maresin-1 treatment attenuates allostynia after K/BxN serum transfer

Herein, I have shown pro-resolving lipid mediator MaR1 has effective anti-nociceptive action when administered during joint swelling in K/BxN serum transfer model. As K/BxN serum transfer model involved mechanical hypersensitivity at both peak joint swelling and the absence of joint swelling, it is possible that our findings regarding anti-nociceptive qualities may only be relevant during peak joint swelling, as is shown with NSAIDs (Park et al., 2016). Therefore, I next assessed if repeated MaR1 treatment is still effective when administered after overt joint swelling has subsided when hind paw allostynia is still present. 19 days after K/BxN serum transfer, clinical scores indicated joint swelling to be near baseline (K/BxN Saline: 0.25 ± 0.16 g; K/BxN-MaR1: 0.13 ± 0.13) (Figure 3.7A). Initial systemic MaR1 treatment, beginning day 19, showed no acute effects on mechanical hypersensitivity (K/BxN Saline: 0.42 ± 0.07 g; K/BxN-MaR1: 0.32 ± 0.06 g) (Figure 3.7B). However, recovery from allostynia was observed from second MaR1 dose (day 21) until the end of study day 25 (K/BxN Saline: 0.31 ± 0.05 g; K/BxN-MaR1: 0.60 ± 0.05g) (Figure 3.7B). These results highlight that, in the absence of joint swelling, repeated but not acute, MaR1 systemic treatment is effective in reducing arthritic hindpaw allostynia.
Figure 3.7: When overt joint swelling has subsided repeated maresin-1 treatment leads to sustained reversal of hind paw allodynia after K/BxN serum transfer

(A) Clinical scoring of hind paws following 100ng MaR1 or saline intraperitoneal administration on days 19, 21, 23 after K/BxN serum transfer, with control mice receiving non-arthritogenic control serum. (B) Reversal of mechanical hypersensitivity assessed using von Frey filaments following second and third doses of 100ng MaR1 on days 21 and 23 after serum transfer. Arrows indicate treatment days. **P < 0.01 ***P < 0.001 vs same day K/BxN-Saline group (closed circles), two-way ANOVA, post hoc Tukey. Data are mean ± SEM; n = 8 mice per group.
3.3.6 Systemic Maresin-1 treatment during joint swelling reduces pro-inflammatory macrophage number in the DRG

Previous studies have shown MaR1 to have potent anti-inflammatory actions, promoting pro-resolution mechanisms within neutrophils, monocytes and other immune cells (Serhan et al., 2012). With the knowledge that MaR1 is macrophage-derived and has anti-inflammatory properties, I investigated if, concomitant to its antinociceptive effects, MaR1 administration altered either macrophage presence or phenotype in the DRG after K/BxN serum transfer. Flow cytometry analysis of DRG cells, taken 25 days after serum transfer, showed greater numbers of leukocytes (CD45+ (Control: 1.59 ± 0.10 cells (x10³); K/BxN Saline: 3.55 ± 0.64 cells (x10³)) and macrophages (CD45+ CD11b+ F4/80+) (Control: 0.52 ± 0.07 cells (x10³); K/BxN Saline: 1.41 ± 0.30 cells (x10³)) in K/BxN-Saline treated DRG as compared to controls (Figure 3.8A-E), in line with previous immunohistochemical results. Interestingly, after MaR1 treatment beginning at peak swelling, there is a distinct reduction in number leukocytes (K/BxN Saline: 3.55 ± 0.64 cells (x10³); K/BxN-MaR1: 1.77 ± 0.24 cells (x10³)) and macrophages (K/BxN Saline: 1.41 ± 0.30 cells (x10³); K/BxN-MaR1: 0.63 ± 0.16 cells (x10³)) as compared to saline treated K/BxN animals (Figure 3.8A-E). Moreover, macrophages displaying an inflammatory M1 phenotype (CD45+ CD11b+ F4/80+ CD206- CD11c+) were reduced in number after repeated MaR1 treatment (55.0 ± 12.8 cells) as compared to saline treated K/BxN animals (123.3 ± 24.9 cells) (Figure 3.8F). With respect to pro-resolution M2 macrophages (CD45+ CD11b+ F4/80+ CD206+ CD11c+) however, after K/BxN serum transfer, no significant difference was shown between MaR1 (309.7 ± 76.3 cells) and saline groups 14 days after end of MaR1 treatment (210.7 ± 48.8 cells) (Figure 3.8G).

These results indicate that following MaR1 administration during peak swelling reversal of hind paw alldynia occurs in association with a reduction in proinflammatory macrophage number in the DRG.
Figure 3.8: MaR1 treatment during overt swelling is associated with decreased immune cell and M1 macrophage presence in DRG after K/BxN serum transfer

(A–C) Representative scatterplots of immune cells sorted from lumbar and cervical DRG dissected at day 25 after transfer of control serum (A), K/BxN serum (B) or K/BxN serum and MaR1 (C) (days 5, 7, 9, and 11 after K/BxN serum transfer). Cells were gated on CD45+, F4/80+, and CD11b+. Macrophages were defined as CD45+ F4/80+ CD11b+ and further analysed for M1 (CD11c+ CD206+) and M2 (CD11b+CD206+). (D–F) Bar charts representing numbers of leukocytes (CD45+ cells) (D), macrophages (CD11b+ F4/80+) (E) and M1 macrophages (CD206-CD11c+) (F) 14 days after last MaR1 dose; *P < 0.05, **P < 0.01, one-way ANOVA, post hoc Tukey. Data are mean ± SEM; n = 5 animals per group.
3.3.7 MaR1 associated reversal of hind paw allodynia in the absence of overt joint swelling occurs concomitant with decreased inflammatory macrophage presence in the DRG

In K/BxN serum transfer model, MaR1 thus far has displayed anti-nociceptive abilities when administered either in the presence or absence of overt joint swelling. When administered during peak joint swelling, MaR1 treatment leads to a reduction in inflammatory macrophage number in the DRG 25 days after serum transfer. Next, I investigated whether, much like its anti-nociceptive effects in-vivo, the anti-inflammatory effects of MaR1 on macrophages in the K/BxN DRG occurs irrespective of joint swelling.

Therefore, I conducted flow cytometry analysis of immune cells in DRG obtained 2 days after the last dose of MaR1 (days 19, 21 and 23 after serum transfer). I observed a greater number of leukocytes (CD45+) following K/BxN serum transfer (16.53 ± 3.50 cells (x10^3)) as compared to controls (6.48 ± 1.92 cells (x10^3)) (Figure 3.9A). This K/BxN-induced increase in DRG leukocyte number was halted MaR1 treatment (K/BxN Saline: 16.53 ± 3.50 cells (x10^3); K/BxN-MaR1: 8.90 ± 1.11 cells (x10^3)) (Figure 3.9A). This result was also observed when assessing macrophage (CD45+ CD11b+ F4/80+) in the DRG, where following later stage MaR1 treatment overall macrophage numbers were lower in DRG (0.25 ± 0.04 cells (x10^3)) as compared to saline treated K/BxN animals (0.73 ± 0.20 cells (x10^3)) (Figure 3.9B). Interestingly, when assessing pro-inflammatory macrophages (CD45+ CD11b+ F4/80+ CD206- CD11c+) after K/BxN serum transfer, I observe a marked decrease in M1 macrophage number as a result of later phase MaR1 treatment (K/BxN Saline: 151.2 ± 53.6 cells; K/BxN-MaR1: 44.7 ± 8.3 cells) (Figure 3.9C), with no effect on M2 macrophage number (Figure 3.9D).

These results highlight that as with MaR1’s anti-nociceptive properties in inflammatory arthritis, the anti-inflammatory effects of MaR1 in DRG are long-lasting and observed when MaR1 is administered either in the presence or absence of joint swelling after K/BxN serum transfer.
Figure 3.9: MaR1 treatment, in the absence of joint swelling, is associated with decreased immune cell and M1 macrophage presence in DRG after K/BxN serum transfer.

(A–D) Flow cytometry analysis representing numbers of leukocytes (A) (CD45+ cells), macrophages (B) (CD11b+ F4/80+), M1 macrophages (C) (CD206-CD11c+) and M2 macrophages (D) (CD206+CD11c-) in lumbar and cervical DRG dissected at day 25 after transfer of control serum, K/BxN serum or K/BxN serum and MaR1 (days 19, 21 and 23 after K/BxN serum transfer); *P < 0.05, **P < 0.01, one-way ANOVA, post hoc Tukey. Data are mean ± SEM; n = 5 animals per group.
3.3.8 Reduced macrophage presence in the DRG following repeated systemic MaR1 treatment following K/BxN serum transfer

Following macrophage infiltration to the DRG, neuronal sensitisation is known to occur via release of pro-inflammatory mediators (Simeoli et al., 2017, Santa-Cecilia et al., 2019). Immunohistochemical and flow cytometry results thus far indicate that increased macrophage presence occurs in the DRG 25 days after K/BxN serum transfer. Further quantitative flow cytometry analysis of K/BxN DRG highlighted that macrophage number can be reduced by repeated MaR1 treatment.

Using immunohistochemistry, I next looked to visually, as well as quantitatively, confirm the ability of MaR1 to reduce K/BxN serum induced macrophage presence in the DRG. I stained for F4/80 in the lumbar DRG 14 days after the final dose of MaR1 (days 5, 7, 9 and 11 after K/BxN serum transfer). This showed that macrophages appear to be largely associated with β-tubulin positive neuronal cell bodies (Figure 3.10A). As compared to controls (5.1 ± 0.5 cells/4 x 10^4µm^2), 25 days after serum transfer K/BxN treated animals displayed significantly greater DRG macrophage number (8.1 ± 0.2 cells/4 x 10^4µm^2) (Figure 3.10B). This increase in K/BxN serum-induced macrophage number was attenuated by repeated MaR1 administration (6.5 ± 0.2 cells/4 x 10^4µm^2). This further affirms our findings that MaR1’s anti-nociceptive effects in K/BxN serum transfer model are associated with reduced macrophage number in the DRG.
Figure 3.10: Repeated MaR1 treatment associated with a reduction in macrophage presence in DRG after K/BxN serum transfer

(A) Representative images of F4/80\(^+\) profiles (arrows) (green) (macrophages) presence in DRG, in addition to β-tubulin (red) (neurons) and DAPI staining (blue) (nuclear). Scale bars, 50 μm. (B) Quantification of F4/80\(^+\) monocyte/macrophage profiles in the lumbar DRG 25 days after transfer of control serum, K/BxN serum or K/BxN serum and MaR1 (days 5, 7, 9 and 11 after K/BxN serum transfer). * P < 0.05, *** P < 0.001, One-Way ANOVA, post-hoc Tukey. Data are expressed as mean ± SEM; n = 4 mice per group, 4 sections per DRG across 4 fields of view.
3.3.9 Reduced microglial activation in spinal cord dorsal horn following repeated systemic MaR1 treatment in inflammatory arthritis

Activation of spinal cord microglia has been known to occur concomitant with enhanced nociceptive processing in models of rheumatoid arthritis (Nieto et al., 2016). Moreover, in K/BxN serum transfer model of inflammatory arthritis, I observe increased microgliosis at timepoints associated and dissociated from joint swelling, days 5 and 25, respectively. Both timepoints are also associated with significant hind paw allodynia. As MaR1 treatment leads to a long-lasting reduction in K/BxN serum-induced hind paw allodynia, I investigated the ability of MaR1 treatment to have a long-lasting effect on microglial presence and activation in the spinal cord following K/BxN serum transfer.

On day 25, 14 days after final MaR1 dose (days 5, 7, 9 and 11 after K/BxN serum transfer), I used immunohistochemistry to stain for microglial marker Iba1 in the lumbar spinal cord. Quantification of Iba1 in the superficial laminae of the dorsal horn revealed a significant increase in Iba1 positive profiles after K/BxN serum transfer as compared to controls (Control: 14.8 ± 1.8 profiles 3x (4x10^4 µm^2); K/BxN Saline: 26.2 ± 1.5 profiles 3x (4x10^4 µm^2). However, after K/BxN serum transfer, repeated MaR1 treatment resulted in reduced microgliosis as compared to saline treated K/BxN animals (K/BxN-Saline: 26.1 ± 1.5 profiles 3x (4x10^4 µm^2); K/BxN-MaR1: 18.2 ± 2.5 profiles 3x (4x10^4 µm^2) (Figure 3.11A, B).

I also stained dorsal horn tissue for phosphorylated p38 MAPK (P-p38), a known marker of microglial activation. As previously observed, there was greater P-p38/Iba1 co-localisation 25 days after K/BxN serum transfer (15.5 ± 1.5 profiles 3x (4x10^4 µm^2)); as compared to controls (5.4 ± 1.2 profiles 3x (4x10^4 µm^2)). However, interestingly, this K/BxN-serum induced increase in p38 phosphorylation was lost following MaR1 treatment (K/BxN-Saline: 15.5 ± 1.5 profiles 3x (4x10^4 µm^2); K/BxN-MaR1: 9.1± 1.6 profiles 3x (4x10^4 µm^2) (Figure 3.11A, C)

Together these results indicate that following K/BxN serum transfer arthritis long-lasting anti-nociceptive effects of systemic MaR1 treatment are mirrored by long lasting reductions in microglial activation in the dorsal horn.
**Figure 3.11:** Systemic MaR1 treatment is associated with a reduction in microglial activation in spinal cord dorsal horn after K/BxN serum transfer

(A) Representative images of Iba1+ profiles (red) (microglia) and phospho-p38+ cells/Iba1+ profiles (green) in lumbar dorsal horn spinal cord sections 25 days after serum transfer. Scale bar = 200 µm (B) Quantification of Iba1+ microglia and phospho-p38+/Iba1+ activated microglial profiles in the superficial laminae of the dorsal horn 25 days after transfer of either control serum, K/BxN serum or K/BxN serum and MaR1 (days 5, 7, 9 and 11 after K/BxN serum transfer). * $P < 0.05$, ** $P < 0.01$ compared to same-day controls, One-Way ANOVA, post-hoc Tukey. Data are expressed as mean ± SEM; $n = 4$ mice per group.
3.3.10 Anti-nociceptive effects of systemic MaR1 in K/BxN serum transfer arthritis are not sex specific

Clinically, RA pain is known to disproportionately affect the female population whereas in pre-clinical models of RA pain, some studies have also shown disparities in nociceptive processing between sexes (Borbely et al., 2015). Moreover, recent evidence suggests that also the efficacy of anti-nociceptive bioactive lipid mediator treatments may differ between sexes (Luo et al., 2019). Therefore, using female mice, I looked to replicate our earlier experiments in males assessing the ability of MaR1 to attenuate persistent mechanical hypersensitivity following K/BxN serum transfer.

Much like previous results in males, following K/BxN serum transfer, clinical scores for both MaR1 and saline treated groups peaked around day 5 (K/BxN-Saline: 7.6±1.2; K/BxN-MaR1: 8.3±1.0), subsiding to near baseline at day 14 (K/BxN-Saline: 0.3±0.2; K/BxN-MaR1: 0.3±0.2). Repeated systemic MaR1 administration, beginning from peak joint swelling on day 5 until day 11 after induction of K/BxN serum arthritis, did not significantly alter the time course of clinical scores as compared to saline-treated K/BxN mice (Figure 3.12A).

When assessing hind paw mechanical hypersensitivity, I observed that initial systemic MaR1 administration on day 5 after serum transfer, was not acutely effective in altering mechanical thresholds as compared to saline treated K/BxN group (Figure 3.12B). However, repeated MaR1 administration was effective from the third (day 9) and fourth/final dose (day 11). Anti-nociceptive effect of MaR1 was sustained until day 25 following K/BxN serum transfer (K/BxN-Saline: 0.28±0.03; K/BxN-MaR1: 0.54±0.05).

These results in female mice indicate that the sustained anti-nociceptive effect of MaR1 is not sex specific in K/BxN serum transfer arthritis.
Figure 3.12: In female mice, repeated systemic MaR1 treatment during joint swelling leads to a long-lasting reversal of hind paw alldynia

(A) Clinical scoring of fore-paws and hind-paws from female mice following MaR1 or saline administration on days 5, 7, 9, and 11 after K/BxN serum transfer, with control mice receiving non-arthritogenic control serum. (B) Reversal of mechanical hypersensitivity assessed using von Frey filaments following third and fourth doses of MaR1 on days 9 and 11 after serum transfer until end of study. Arrows indicate treatment days. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs same day K/BxN-Saline group (closed circles), two-way ANOVA, post hoc Tukey. Data are mean ± SEM; n = 8 female mice per group.
3.3.11 MaR1-mediated reductions in dorsal horn microgliosis following K/BxN serum transfer arthritis are not sex specific

In addition to proposed differences in clinical pain outcomes, pre-clinically it has been proposed that the neuro-immune mechanisms contributing to pain differ between sexes, with much debate surrounding sex distinct mediation of pain processing by spinal cord microglia (Sorge et al., 2015). Therefore, I investigated whether sex differences occurred in spinal cord microgliosis following K/BxN serum transfer arthritis and subsequent MaR1 treatment.

I performed immunohistochemical analysis of spinal cord slices taken 14 days after final MaR1 treatment (days 5, 7, 9 and 11 after K/BxN serum transfer), assessing for the microglial marker, Iba1. As compared to same-day non-arthritis controls both male (Control: 12.4 ±1.3 profiles 3x (4x10^4 µm^2); K/BxN-Saline: 23.7 ±0.9 profiles 3x (4x10^4 µm^2)) and female groups (Control: 10.8 ±0.2 profiles 3x (4x10^4 µm^2); K/BxN-Saline: 22.2 ±2.5 profiles 3x (4x10^4 µm^2)) 25 after K/BxN serum transfer displayed increased microglial presence in the dorsal horn. This K/BxN-induced microgliosis was abolished by repeated MaR1 treatment with both males (16.8 ±0.9 profiles 3x (4x10^4 µm^2)) and females (14.9 ±2.5 profiles 3x (4x10^4 µm^2)) displaying significantly lower microglial presence as compared to saline-treated same-sex K/BxN groups (23.7 ±0.9 and 22.2 ±2.5 profiles 3x (4x10^4 µm^2), respectively) (Figure 3.13A, B). No significant difference was observed between sexes within each group. These results indicate that sex does not affect K/BxN serum transfer arthritis associated microgliosis. Moreover, following serum transfer, repeated MaR1 treatment can attenuate microgliosis significantly in both males and females.
Figure 3.13: After K/BxN serum transfer, female mice display MaR1-associated reductions in dorsal horn microglial activation comparable to male mice

(A) Representative images of Iba1⁺ profiles (red) (microglia) in lumbar dorsal horn spinal cord sections 25 days after serum transfer. Scale bar = 200 µm (B) Quantification of Iba1⁺ microglial profiles in the superficial laminae of the dorsal horn 25 days after transfer of either control serum, K/BxN serum or K/BxN serum and MaR1 (days 5, 7, 9 and 11 after K/BxN serum transfer) in both male and female mice. ***P < 0.01 versus same-sex control; # P < 0.05 versus same-sex saline-treated K/BxN group. Data are expressed as mean ± SEM; n = 4 mice per group.
3.4 Discussion

In the present chapter I provide evidence that following passive immunization with K/BxN serum there is an imbalance in bioactive lipid mediator levels between K/BxN DRG and controls. While mechanical hypersensitivity persists in the absence of joint swelling, though the expression of pro-inflammatory lipid mediators is largely unaltered, production of pro-resolving lipid mediator MaR1 is decreased in concomitance to i) increased lumbar DRG macrophage presence and ii) dorsal horn microgliosis. These features were all reversed following repeated systemic administration of MaR1, either during or after resolution of joint swelling. This suggests that a brief repeated-dosing protocol of MaR1 administration facilitates long-lasting effects on K/BxN serum-transfer–associated mechanical hyperalgesia and pro-nociceptive neuro-immune communication; both peripherally in the DRG and centrally at the spinal cord dorsal horn.

Bioactive lipid mediators are known to play a key role in both the initiation and resolution phases of the inflammatory response. In addition, aberrant lipid mediator function is linked to chronic inflammatory states and persistence of inflammatory features; whereby nociception is one of the most relevant in the context of arthritis. In the K/BxN model of inflammatory arthritis, several proinflammatory lipid mediators are elevated in the inflamed paw (Norling et al., 2016). In the lumbar K/BxN DRG, I too identified the presence of lipid mediators using LC-MS/MS–based LM profiling. Similar to the inflamed paw, I identified distinct bioactive lipid mediator profiles in the DRG associated with arthritic swelling in comparison to same-day controls. In addition, when persistent hyperalgesia occurs in absence of joint swelling, I also discovered dysregulation of lipid mediator profiles in K/BxN DRG, which differed to profiles at peak joint swelling. Together, these findings highlight that SPM-mediated mechanisms underlying sensitization of K/BxN DRG neurons differ when hyperalgesia occurs either in presence or absence of joint swelling.

Leukotriene B4 (LTB₄) is required for induction of K/BxN serum-induced inflammatory arthritis, with increased LTB₄ levels detected in K/BxN paw during arthritic joint swelling (Chen et al., 2006, Norling et al., 2016). Contrastingly in the DRG, distal to the site of inflammation, no change in LTB₄ levels were observed. LTB₄ production and binding to BLT1 and BLT2 receptors facilitates the recruitment of neutrophils which release...
pro-nociceptive mediators in the inflamed K/BxN joint (Chen et al., 2006). With little LTB$_4$ production and subsequent neutrophil recruitment in the K/BxN DRG, it is unlikely that LTB$_4$ contributes to maintenance of nociceptive signalling in the K/BxN DRG, distal to the inflammatory site. Contrastingly, pro-inflammatory prostaglandin D$_2$ (PGD$_2$) levels are elevated in K/BxN DRG when mechanical hypersensitivity presents alongside joint swelling. Albeit, at a lesser extent compared to inflamed K/BxN paws, which display PGD$_2$ levels 8 times greater than controls (Norling et al., 2016). Expectedly, this likely indicates lower overall immune cell infiltration and subsequent immune cell-mediated pro-inflammatory signalling in K/BxN DRG as compared to the paw during peak joint swelling.

PGD$_2$ is known to be pro-nociceptive, enhancing nociceptive transmission via PD1 and PD2 receptors present on sensory neurons (Sousa-Valente et al., 2018, Ebersberger et al., 2011). In the MIA experimental arthritis model, PGD$_2$ is produced by mast cells at the inflammatory site (Sousa-Valente et al., 2018). Prostanoids are produced by most cell types (Yao and Narumiya, 2019). As the DRG contains a mixed population of cell types, further investigation is required to determine the cellular source of PGD$_2$ in the K/BxN DRG when hyperalgesia occurs alongside joint swelling. When joint swelling has subsided, but mechanical hyperalgesia remains, PGD$_2$ levels were no longer heightened in the K/BxN DRG. In line with our results, following passive immunization, COX-1/2 inhibition although effective when mechanical hyperalgesia presents alongside joint swelling, treatment has little anti-hyperalgesic efficacy when administered in the absence of joint swelling (Park et al., 2016, Christianson et al., 2010). Together, this suggest that in the absence of joint swelling, alternate prostaglandin-independent mechanisms take place, away from the joint, which facilitate maintained nociceptive states.

Timely resolution of an inflammatory response is dependant of production of specialised pro-resolving lip mediators (SPMs), of which several have been shown to display anti-nociceptive properties in models of inflammatory arthritis (Serhan et al., 2012, Xu et al., 2010, Luo et al., 2019). Mechanical hypersensitivity concomitant with joint swelling is not associated with dysregulation of SPMs in the K/BxN DRG. This contrasts with findings in the inflamed K/BxN paw, where at peak joint swelling reduced D-series resolvins and MaR1 levels are observed (Arnardottir et al., 2016, Norling et al., 2016). Collectively, these findings indicate that following serum transfer,
there a key regional difference between SPM signalling at peripheral nerve terminals and nociceptor cell bodies in the DRG.

Studies have shown RvD1 and RvD2 to have anti-nociceptive abilities when administered repeatedly in models of inflammatory arthritis (Xu et al., 2010). Levels of D-series resolvins in K/BxN DRG are unaltered when mechanical hypersensitivity occurs in either presence or absence of joint swelling. Interestingly, following serum transfer, persistent mechanical hypersensitivity in the absence of joint swelling is associated with lower levels of MaR1. Studies demonstrate that macrophage-derived pro-resolving MaR1 exerts significant anti-inflammatory and anti-nociceptive actions both on immune cells and neurons, respectively (Serhan et al., 2012, Dalli et al., 2013). Therefore, it is probable that diminished MaR1 function in K/BxN DRG is linked to mechanical hyperalgesia in the absence of joint swelling.

Indeed, following repeated systemic MaR1 treatment I observe a reversal of mechanical hypersensitivity, when administered either during- or after joint swelling has subsided. In both instances, the long-lasting anti-hyperalgesic effects of MaR1 were observed beyond final treatment until the end of study. Similarly, in rat spinal nerve ligation (SNL) model of neuropathic pain repeated intrathecal MaR1 demonstrates long-lasting anti-nociceptive effects 2 days post-final treatment (Gao et al., 2018). Moreover, in vincristine-induced neuropathic pain model MaR1 remains anti-allodynic up to 2 weeks following vincristine treatment (Serhan et al., 2012). Although I observe sustained MaR1-mediated reversal of hyperalgesia there was no acute reversal of K/BxN-associated mechanical hypersensitivity, 1 hour after initial systemic intraperitoneal administration. Contrastingly, within hours of intrathecal administration, MaR1 exhibits anti-hyperalgesic effects on carrageenan-induced inflammatory pain and perioperative pain following tibia fracture (Fattori et al., 2019, Zhang et al., 2018). In-vitro studies demonstrate that MaR1 can directly dampen nociceptor activity, reducing capsaicin-mediated currents from cultured TRPV1+ primary sensory neurons in a dose dependant manner (Serhan et al., 2012). Intrathecal administration allows for higher MaR1 concentrations locally in the spinal cord, proximal to neurons conveying noxious inflammation, which may not be achieved by systemic administration. Therefore, to induce acute anti-nociceptive effects in-vivo, I suggest that MaR1 must be present in sufficient concentrations in close proximity to neurons conveying noxious information.
Following passive immunization, when hyperalgesia occurs in the absence of joint swelling, there is an increase in DRG macrophages displaying a pro-inflammatory M1 phenotype. M1 macrophages express numerous pro-inflammatory mediators including TNFα, IL-1β, IL-6 (Chen et al., 2020). Following resolution of joint swelling, the majority of macrophages in the K/BxN joint exhibit a pro-resolution M2 phenotype (Misharin et al., 2014). Therefore, in the absence of joint swelling, it is likely that these pro-inflammatory macrophages observed in K/BxN DRG mediate mechanical hyperalgesia via the release pro-inflammatory mediators which bind sensory neurons and facilitate peripheral sensitization.

Following serum transfer, I provide evidence that repeated MaR1 treatment facilitates a decrease in M1 macrophage presence in K/BxN DRG when administered either from peak joint swelling or after swelling has subsided. With no acute anti-nociceptive effect of systemic MaR1 administration, it is unlikely that direct MaR1-mediated neuronal mechanisms play a significant role modulating K/BxN-associated hyperalgesia in arthritic settings. Alternatively, our data suggest that the delayed anti-hyperalgesic effects of systemic MaR1 administration are due to this specialised pro-resolving mediator having a long-term effect on macrophage function. In particular, MaR1 can exert an autocrine function on macrophages in DRG resulting in reduction of both proinflammatory macrophage number and likely release of proinflammatory cytokines, which are known to sensitize sensory neurons and facilitate nociceptive signalling. Recently, a leucine-rich repeat containing G protein-coupled receptor (LGR6) expressed in immune cells, including macrophages, was shown as the MaR1 receptor. The knockdown of LGR6 reduces MaR1-mediated attenuation of zymosan induced inflammation (Chiang et al., 2019). Following passive transfer of K/BxN serum, MaR1 may instigate anti-nociceptive and anti-inflammatory actions via LG6R receptors present on macrophages in the DRG. Future experiments targeting LG6R activation and the receptor’s downstream cellular signalling mechanisms could help determine the role of MaR1 in anti-inflammatory and anti-nociceptive signalling.

MaR1 can induce M1 to M2 phenotype switching in cultured human blood-derived macrophages (Dalli et al., 2013). Although a MaR1-mediated reduction in M1 pro-inflammatory macrophages occurs, I do not observe an increase in pro-resolution M2 macrophages in K/BxN DRG. Macrophages can be typically defined as classically activated (M1) or alternatively activated (M2), with various cytokine and genetic...
markers used to define cells within either phenotype (Orecchioni et al., 2019, Murray et al., 2014). In the present study, CD11c and CD206 were used as established pro- and anti-inflammatory markers, respectively (Simeoli et al., 2017). However, it is increasingly thought that a macrophage’s existence may be dynamic and non-binary, creating a continuous spectrum of activation and functional states which extend beyond the classical M1/M2 nomenclature (Murray et al., 2014, Chen et al., 2020). Future work could form a comprehensive representation of macrophage phenotype in the K/BxN DRG following MaR1 treatment by investigating the expression of a gene panel containing numerous genes associated with pro-inflammatory (M1) and anti-inflammatory (M2) macrophage signalling.

Spinal cord microgliosis is a common feature of several arthritic pain models (Christianson et al., 2010, Nieto et al., 2015, Fattori et al., 2019). In the carrageenan model of inflammatory pain, intrathecal injection with DHA leads to marked reductions in mechanical allodynia as well as spinal cord microglial p38 MAPK activation (Lu et al., 2013). In the K/BxN serum transfer model, I show that repeated systemic administration of MaR1, a DHA metabolite, leads to attenuated mechanical hypersensitivity and reduced dorsal horn microglial activation. In the SNL neuropathic pain model, repeated intrathecal MaR1 suppressed microglia activation in association with a reversal of hind paw allodynia (Gao et al., 2018). Similarly, intrathecal MaR1 treatment decreases CFA-induced hyperalgesia and microglia activation in the spinal cord (Fattori et al., 2019). Moreover, Fattori et al. (2018) demonstrate that MaR1 treatment also decreased spinal cord production of pro-inflammatory TNF-α and IL-1β which can bind to dorsal horn neurons and facilitate central sensitisation, as well as intensify the activation of glial cells.

In the PNS, ourselves and others have demonstrated that MaR1 can act both on immune cells and directly on sensory neurons to diminish pro-nociceptive signalling (Fattori et al., 2019, Serhan et al., 2012). Therefore, following systemic MaR1 treatment, it is likely that K/BxN spinal cord neurons are receiving less nociceptive input from the periphery causing a subsequent reduction in dorsal horn glial cell activation. However, it is also possible that MaR1 acts directly on glial cells to reduce their pro-inflammatory activity. Resolvins are rapidly degraded in vivo resulting in a short biological half-life as compared to their precursors such as DHA and EPA (Yoo et al., 2013), indicating that rather than sustained direct neuronal modulation long-term
anti-nociceptive effects of resolvins are more likely dependent on altered neuro-immune interactions. Future work could address MaR1-glial interactions by assessing if pro-inflammatory signalling from isolated activated microglia is directly attenuated by MaR1 treatment.

The delivery method of MaR1 treatment may be important in deciphering the extent of the SPM’s anti-nociceptive capabilities. In the tibia fracture model of peri-operative pain, intravenous MaR1 administration (0 and 24 hr post-surgery) produced a sustained reduction in mechanical hypersensitivity, lasting from 3-5 days after surgery. Interestingly, intrathecal administration 1 hour after surgery elicited a short-lived analgesic effect, losing efficacy 5 hours post-treatment. In addition, systemic intraperitoneal administration of MaR1 reduced chemotherapy-induced mechanical allodynia up to 14 days after vincristine treatment (Serhan et al., 2012). Together, this suggests, if route of administration allows close proximity to neurons, MaR1 can have an acute direct effect on nociceptive firing of neurons. Whereas repeated systemic administration leads to longer lasting changes, more indicative of indirect alterations to neuronal firing, possibly via modulation of neuro-immune interactions (Zhang et al., 2018).

When administered via intraperitoneal injection, RvD1 and RvE1 have shown protective effects in neurological disease mouse models such as diffuse brain injury, indicating the effective ability of SPMs to cross the blood-blood barrier (Harrison et al., 2015). However, the specific ability of MaR1 across the blood-brain barrier requires further investigation.

An emerging body of evidence suggests sex can be a contributing factor to pain sensitivity, with sexual dimorphism observed in the neuro-immune mechanisms by which nociceptive information is conveyed. Mostly notably, the Mogil group highlighted distinct male microglia-mediated and female T-lymphocyte mediated mechanisms which facilitate pain hypersensitivity following SNI-induced neuropathic injury (Sorge et al., 2015).

Following serum transfer, no differences are seen between male and female mice in both the initiation and resolution of joint swelling. In agreement, other studies also observe that following passive immunization paw oedema, ankle thickness and overall signs of clinical arthritis do not differ based on sex (Borbely et al., 2015, Woller et al.,
I also do not observe sex differences in serum-induced persistent hyperalgesia, either in presence or absence of joint swelling. Previous studies also observe that the onset and development of mechanical allodynia are no different between sexes (Borbely et al., 2015, Woller et al., 2019). However, following passive immunization, it has been reported that as joint swelling subsides there is a partial recovery of mechanical allodynia only in female mice (Woller et al., 2019). The authors suggest that the transition from pre- to post-inflammatory allodynia is dependent on spinal TLR4 signalling mechanisms, which are absent in female mice following serum transfer (Woller et al., 2019). However, their findings demonstrate that female K/BxN mechanical withdrawal thresholds are still far below baseline levels, up to 30 days after serum transfer. This indicates that following passive immunization persistent mechanical allodynia still remains in female mice. Taken together, I believe that following serum transfer, persistent hyperalgesia induction is not sex specific and unlikely to be solely dependent on TLR4 related mechanisms.

Specialised pro-resolution lipid mediators (SPM) treatment is shown to have anti-nociceptive efficacy in models of inflammatory and neuropathic pain (Xu et al., 2010, Serhan et al., 2012). However, recent work has highlighted SPMs, specifically D-series resolvins, display sex specific differences in analgesic efficacy. Luo et al. (2019) demonstrate that Paclitaxel-induced neuropathic pain is attenuated similarly in both male and female mice, 1 hour following intrathecal injection of either RvD1 or RvD2. However, the anti-allodynic action of RvD5 was sex dependent. In models of chemotherapy-induced neuropathic pain and formalin-induced inflammatory pain RvD5 reduced mechanical allodynia in male, but not female, mice (Luo et al., 2019). I observe, following passive serum transfer, MaR1-mediated sustained reversal of hyperalgesia occurs similarly in both males and females. Moreover, MaR1-associated attenuation of dorsal horn microglial activation is comparable in males and females following serum transfer. Therefore, much like findings for RvD1 and RvD2, our results indicate that the anti-nociceptive and immune-modulatory effects of MaR1 are not sex dependent.

3.4.1 Chapter Key Findings

In this chapter I demonstrate that following K/BxN passive immunization:
- Mechanical hypersensitivity concomitant to joint swelling (Day 5) is associated with increased pro-inflammatory PGD₂ levels in the lumbar DRG. However, when persistent hyperalgesia occurs in the absence of joint swelling, PGD₂ levels are no longer elevated in the lumbar DRG while lower levels of pro-resolution MaR1 are observed.
- Repeated systemic treatment with MaR1 either during joint swelling (Days 5, 7, 9, 11) or post-joint swelling (Days 19, 21, 23) leads to:
  o Sustained reversal of nociceptive hypersensitivity
  o Reduced pro-inflammatory macrophage presence in lumbar DRG (PNS)
  o Diminished dorsal horn microglial activation (CNS)

These data suggest that aberrant pro-resolution mechanisms underlie nociceptive hypersensitivity following passive K/BxN serum transfer. These heightened nociceptive states be attenuated by MaR1 treatment which modulates pro-inflammatory neuro-immune signalling from macrophages in the DRG (PNS) and microglia in the dorsal horn spinal cord (CNS).

### 3.4.2 Future Direction Leading to Chapter 4

Thus far I have demonstrated that, following passive immunization, hyperalgesia in the absence of joint swelling is associated with diminished MaR1 levels in the DRG, with repeated systemic MaR1 treatment leading to a sustained reversal of mechanical hypersensitivity. The anti-hyperalgesic effects of MaR1 are also associated with decreased pro-inflammatory macrophage presence peripherally in the DRG, where sensory neuron cell bodies reside. Neuro-immune communication in the DRG contributes to the maintenance of chronic pain states. Moreover, MaR1 can effectively act directly on both macrophages and neurons. Therefore, MaR1-mediated anti-hyperalgesia may be mediated by modulation of inflammatory macrophage-derived signalling to DRG neurons, or alternatively by direct dampening of nociceptor firing.

Thus, in the next chapter (chapter 4), using in-vitro experimentation, I looked to further determine anti-inflammatory and anti-nociceptive effects of MaR1 treatment on neuronal and macrophage signalling. Firstly, in cultured macrophages, I assessed the ability of MaR1 to modulate pro-inflammatory and pro-resolution macrophage signalling following inflammatory LPS challenge. Secondly, using dissociated DRG
neurons, I investigated the ability of MaR1 to directly alter capsaicin-induced activation of TRPV1+ DRG neurons, and if these abilities are reduced by blockade of GPCR-signalling pathways.
Chapter 4:
Anti-Nociceptive and Anti-Inflammatory Actions of Maresin-1 in Neurons and Macrophages
4.1 Introduction

4.1.1 Macrophage-mediated cytokine signalling in inflammatory nociception

As discussed in section 1.1.6.3, macrophages can be polarised to either a M1 (pro-inflammatory) or M2 (anti-inflammatory) phenotype. Polarization of M1 macrophages can be induced by proinflammatory cytokines, such as TNFα, or by microbial products such as lipopolysaccharide (LPS). Following challenge with inflammatory LPS, M1 macrophages exhibit greater inducible nitric oxide (iNOS) signalling and enhanced production of nitric oxide (NO); known to inhibit cell division. Whereas M2 macrophages were shown to promote arginine metabolism via arginase to produce ornithine, which is involved in cell proliferation and healing (Mills et al., 2000). These results indicate that macrophage M1 and M2 phenotypic responses influence inflammation in opposite manners.

Activation of M1 macrophages induces production of TNFα (Gordon and Taylor, 2005), an inflammatory cytokine known to play a key role in several pain models, inducing inflammatory hyperalgesia (De Oliveira et al., 2011). In CFA model of inflammatory pain, significantly greater levels of TNFα are observed in the inflamed paws. Moreover, anti-TNFα treatment with etanercept prior to CFA-induction significantly reduced inflammatory hyperalgesia (Inglis et al., 2005). Similarly, in the adjuvant-induced model of inflammatory arthritis, neutralizing TNFα leads to rapid attenuation of mechanical and thermal hypersensitivity in the inflamed knee (Boettger et al., 2008).

TNFα can act on several signalling pathways, including the activation of intracellular nuclear factor kappa B (NF-κB) pro-inflammatory signalling pathway, via binding of cell surface receptors TNFR1 and TNFR2. These are single-spanning type I transmembrane proteins which are present on both peripheral DRG neurons and spinal cord neurons, as well as immune cells (Cook et al., 2018, Zhang et al., 2011). Following CFA-induced inflammatory hyperalgesia, it is suggested that increased TNFα in the DRG, likely derived from macrophages, acts both with direct action on neurons via TNFR1 and also by facilitating the accumulation of macrophages in the DRG via a TNFR2-mediated pathway (Inglis et al., 2005) (Figure 4.1). Peripheral activation of tetrodotoxin-resistant (TTX-r) sodium channels, such as Naᵥ1.8 and Naᵥ1.9, is critical during inflammatory nociceptive signalling (Cook et al., 2018). TNFα
has been shown to increase Na$^+$ currents from TTX-r channels in isolated murine DRG neurons, with no change observed in TNFR1 knockout mice (Jin and Gereau, 2006). As TNFα is predominantly released by macrophages, these studies further highlight the ability of peripheral neuro-immune communication to enhance nociceptive signalling during inflammation. Moreover, administration of TNFα to isolated rat skin was shown to enhance heat-evoked CGRP release from nociceptor terminals, indicating that neuronal-mediated TNFα signalling leads to enhancement of neurogenic inflammation (Opree and Kress, 2000).

In addition to TNFα, M1 inflammatory macrophages are also known to produce pro-inflammatory cytokine Interleukin-6 (IL-6). IL-6 is a glycoprotein which binds to IL-6R and transduces intracellular signalling via gp130 subunit (Matsuda et al., 2019) (Figure 4.1). Functionally, IL-6 is known to promote neutrophil maturation and activation, in addition to facilitating monocyte to macrophage maturation. However, as well as modifying immune cells, IL-6 release is also shown to be pro-nociceptive and heighten nociceptive transmission in neurons. DRG neurons express the signal transducer gp130 which is shown to be involved in the maintenance of mechanical hyperalgesia in inflammatory states, as mice deficient in gp130 display do not display mechanical hyperalgesia induced in antigen-induced arthritis (Von Banchet et al., 2005, Ebbinghaus et al., 2015). Additionally, IL-6 application to C-fibres leads to direct peripheral sensitization and increased response to mechanical stimulation within 1 hour of administration (Brenn et al., 2007), further highlighting the pro-nociceptive action of IL-6. In mice which were gp130 deficient specifically in sensory neurons, antigen-induced arthritis was less pronounced with lower clinical scores and no significant mechanical allodynia (Ebbinghaus et al., 2015). In addition, gp130 deficient mice showed reduced CGRP release from neurons, indicating that inhibition of IL-6 signalling also reduces peripheral neurogenic inflammation. Therefore, IL-6 signalling in these neurons mediates both nociceptive and inflammatory responses (Ebbinghaus et al., 2015).

Together, these studies point to a crucial role for pro-inflammatory cytokines released from activated M1 macrophages in the propagation of nociceptive signalling (Figure 4.1).
4.1.2 Immune mediated anti-inflammatory action of MaR1

The attenuation of pro-inflammatory cytokine signalling can be facilitated by MaR1, a macrophage derived SPM. Endogenous production of MaR1 occurs during the resolution phase of inflammation, with exogenous MaR1 treatment shown to have anti-inflammatory and anti-nociceptive actions.

Following zymosan-induced peritonitis, MaR1 has demonstrated anti-inflammatory action at doses as low as 0.1ng/mouse, reducing neutrophil infiltration and promoting increased macrophage efferocytosis of apoptotic neutrophils (Serhan et al., 2012). Culture of human blood-derived isolated monocytes with GM-CSF, as well as stimulation with Interferon-γ and LPS skews cell maturation towards macrophages with a pro-inflammatory M1 phenotype. Dalli et al. (2013) showed that in human macrophages, cultured to exhibit this pro-inflammatory M1 phenotype, MaR1 incubation caused a shift towards a pro-resolution M2 phenotype reducing CD54 and CD80 expression (M1 markers) and promoting upregulation of CD163 and CD206 (M2 markers). This shift in phenotype likely results in the reduction of pro-inflammatory signalling and subsequent mediator release. Cultured bone marrow-derived macrophages exposed to LPS exhibit increased NF-κB p65 nuclear translocation indicative of increased pro-inflammatory gene expression and TNFα release. Yang et al. (2019) demonstrated that co-incubation of macrophages with MaR1 attenuated this LPS-induced function, reducing both NF-κB p65 translocation and TNFα levels (Figure 4.1). Moreover, MaR1 treatment abolished LPS-induced expression of M1 marker, CD86. Interestingly, no alterations were observed in anti-inflammatory marker, CD206, following LPS-MaR1 treatment. These results indicate that the majority action of MaR1 following LPS challenge in cultured macrophages was to modulate M1 signalling, but not boost pro-resolution M2 signalling (Yang et al., 2019). These findings are in agreement with in-vivo experiments by Francos- Quijorna et al. (2017), who utilising a spinal cord injury model demonstrated that MaR1 treatment reduces pro-inflammatory cytokines including CxCL1, CxCL2, CxCL3 and IL-6 in the contused spinal cord, without promotion of pro-resolution mediators. This, again, suggests MaR1 preferentially attenuates pro-inflammatory cytokine signalling (Francos-Quijorna et al., 2017). In addition, MaR1 caused a significant reduction in macrophage number to site of inflammation, 7 days after spinal cord injury. Macrophages that remained in the spinal cord were skewed away from pro-inflammatory M1 towards a
pro-resolution M2 phenotype; displaying decreased expression of iNOS, a pro-inflammatory cytokine enzyme and concomitant increases in anti-inflammatory Arginase-1, following MaR1 treatment (Francos-Quijorna et al., 2017).

MaR1 has also been shown to inhibit inflammatory signalling in the brain where following injury induced by orthopaedic surgery, hippocampal microglia exhibit an activated morphology 24 hours after surgery, which is abolished by pre-treatment with MaR1. MaR1 treatment leads to a more ramified microglial morphology, less indicative of activation (Yang et al., 2019). These studies indicate a strong anti-inflammatory effect of MaR1 on immune cells, particularly macrophages, which may result in reduced sensitization and activation of neurons.

Discussed in section 3.1.2.5, Chiang et al., (2019) recently demonstrated that MaR1 administration leads to enhanced efferocytosis and phagocytosis from human macrophages in a LGR6-dependent manner via Gs/cAMP signalling, suggesting MaR1 exerts pro-resolution action on immune cells via binding to LGR6 receptor.

### 4.1.3 Neuro-immune led anti-inflammatory & anti-nociceptive action of MaR1

MaR1 is also known to modulate noxious-like activation in sensory neurons. Following MaR1 incubation, dissociated DRG neurons showed reduced capsaicin-mediated currents from TRPV1+ neurons (Serhan et al., 2012). The MaR1-mediated reduction in current was observed in a concentration dependant manner and was blocked by Gα-protein coupled receptor inhibitor pertussis toxin. These findings indicate that MaR1 acts directly on TRPV1+ neurons inhibiting neuronal activity via GPCR mediated mechanisms (Serhan et al., 2012) (Figure 4.1).

Similar to the DRG, MaR1 also attenuated capsaicin-induced TRPV1 currents in trigeminal ganglion derived nociceptive neurons. This action could be blocked by pertussis toxin (Park, 2015). In these studies, the application of TRPV1 agonist capsaicin to trigeminal nuclei slices evoked a significant increase in the frequency of spontaneous excitatory post-synaptic potentials (EPSCs) in lamina II neurons. These EPSCs were subsequently blocked by application of MaR1, suggesting that MaR1 can modulate nociceptive signalling in trigeminal ganglion through direct inhibition of nociceptive peripheral neurons (Park, 2015).
Interestingly, these anti-nociceptive mechanisms do not appear to be unique to MaR1. Another specialised pro-resolving lipid mediator, RvE1, also displays anti-nociceptive action in TRPV1+ DRG sensory neurons, completely inhibiting capsaicin-induced intracellular calcium influx at 3nM concentrations (Jo et al., 2016). This function, much like that of MaR1, is completely abolished by blockade GPCRi/o mechanisms (Jo et al., 2016). MaR1 has recently been found to act via the GPCR, LGR6, whereas RvE1 is known to activate another GPCR, the chemerin 23 receptor (ChemR23) (Chiang et al., 2019, Jo et al., 2016). This indicates that although lipid mediator binding to neuronal receptors can be SPM-specific, shared GPCR-mediated signalling mechanisms may exist between pro-resolving mediators to dampen nociceptive signalling.

Following spinal nerve ligation, repeated intrathecal MaR1 treatment from days 3-5 after injury led to suppression of injury-induced microglial activation in the spinal cord dorsal horn and inhibition of NF-κB activation and translocation (Gao et al., 2018). Moreover, following MaR1 treatment, lower levels of SNL-induced inflammatory cytokines were observed in the dorsal horn; namely TNFα, IL-1β and IL-6. These results indicate a central action for MaR1, to reduce pro-inflammatory microglial-mediated signalling in the spinal cord. Attenuation of these pro-inflammatory mechanisms would result in reduced central sensitization of neurons by inflammatory cytokines (Gao et al., 2018). Intrathecal MaR1 administration has also been shown to reduce activation of NF-kB signalling and promote the resolution of pro-inflammatory mediators IL-1β and TNFα, dose-dependently, in the spinal dorsal horn following lumbar disc herniation (Wang et al., 2020b).

Intrathecal MaR1 treatment has been shown to reduce carrageenan-induced neutrophil and macrophage recruitment to the paw. Additionally, MaR1 inhibited NF-κB p65 phosphorylation and downstream production of pro-inflammatory cytokines IL-1β and TNFα (Fattori et al., 2019). In response to inflammatory CFA injection, intrathecal MaR1 administration was also shown to reduce DRG neuronal activation. MaR1 inhibited upregulation of NaV1.8 sodium channel and TRPV1 receptor expression, attenuating CFA-induced peripheral sensitization of nociceptors (Fattori et al., 2019). The release of CGRP from DRG neurons in response to capsaicin was attenuated by MaR1, in a dose dependant manner, at concentrations as low as 3ng/ml (Fattori et al., 2019).
Together, these results demonstrate the anti-nociceptive action of MaR1 on peripheral neurons, as well as MaR1’s anti-inflammatory role reducing both CGRP-mediated inflammatory signalling and immune infiltration to inflammatory site (Figure 4.1). This MaR1-mediated inhibition of pro-inflammatory signalling from immune cells and inhibition of nociceptive signalling from DRG neurons would lead to reduced sensitization of neurons and associated nociceptive signalling.
(1) Following injury or inflammatory insult, M1 inflammatory macrophages infiltrate into the surrounding microenvironment releasing a series of cytokines and other inflammatory factors such as TNFα and IL-6, via intracellular NF-κB signalling pathways. (2) Extracellular TNFα can act on neuronal TNFR1 and facilitate activation of TTX-resistant Na⁺ channels via phosphorylation of MAPKs and activation of protein kinase A (PKA). This activation leads to kinases enhancing activity of ion channels. In addition, TNFα can act on macrophages to propagate inflammation via TNFR2-mediated pathways. (3) Extracellular IL-6 can bind to neuronal IL-6R, transducing intracellular signalling via gp130 subunit. Subsequently, Janus kinase (JAK) present in the cytoplasm is phosphorylated and activate downstream modulators such as protein kinase C (PKC), which facilitates peripheral sensitization of nociceptors through effects on ion channels (e.g., TRPV1 or Na⁺ channels), increases neuronal activation and neuronal CGRP release. (4) Pro-inflammatory cytokine release from macrophages can be attenuated by Maresin-1 via the inhibition of NF-κB phosphorylation and subsequent reductions in primary response genes encoding cytokines such as TNFα and IL-6. MaR1 also facilitates a macrophage phenotype switch from M1 to M2. (5) MaR1 is also known to inhibit TRPV1-mediated responses and CGRP release from neurons following capsaicin exposure, evidently via GPCRi/o mediated mechanisms. (Adapted from (Yang et al., 2019, Chen et al., 2020, Goncalves Dos Santos et al., 2019))

Figure 4.1: Schematic depicting known maresin-1 actions on pro-inflammatory M1 macrophages and TRPV1-sensitive nociceptive DRG neurons
4.1.4 Aims

Thus far I have demonstrated that, following K/BxN serum transfer, repeated systemic MaR1 treatment is associated with a sustained reversal of mechanical hypersensitivity and reduced inflammatory macrophage number in the lumbar DRG. Alongside immune cells, the cell bodies of peripheral nociceptors reside in the DRG, with active neuro-immune crosstalk known to occur during pain states. MaR1 is a potent macrophage-derived SPM, whose action is not limited to a specific cell type. Therefore, it is possible that the anti-nociceptive effects of MaR1 may be attributed to either macrophage-mediated modulation of pro-inflammatory signalling or direct alterations to neuronal transmission. Although studies have previously observed MaR1’s inhibitory function on capsaicin-induced TRPV1 currents in the trigeminal ganglion (Park, 2015), none as yet have addressed MaR1’s potential action on calcium influx in the DRG.

The aims of the present chapter were to:

- Investigate the ability of MaR1 to modulate mRNA expression of pro-inflammatory (IL-6, TNFα & NOS2) and pro-resolution (Arg1, Mrc1 & IL-4) markers in cultured peritoneal macrophages challenged with LPS.
- Examine the direct ability of MaR1 to alter activation of TRPV1-mediated nociceptive neurons, by measuring capsaicin-induced intracellular Ca\(^{2+}\) influx in cultured DRG neurons.
- Assess how MaR1-mediated modulation of intracellular Ca\(^{2+}\) influx, in DRG neuron exposed to capsaicin, is affected by inhibition of GPCR signalling; by pre-incubating cultured DRG neurons in the presence of pertussis toxin (G\(_{\text{i/o}}\)-coupled GPCR blocker).
4.2 Materials and Methods

4.2.1 Animals

All experiments were performed on adult male C57BL/6 mice (Envigo, UK), aged between 10-12 weeks old and weighing approximately 25g. All mice were kept in the same living conditions as outlined in section 2.2.1.

4.2.2 Primary Culture of Peritoneal Macrophages

Prior to lavage, mice were euthanised via cervical dislocation and stored temporarily on ice. The peritoneal cavity was exposed using sterile surgical scissors and macrophages collected via lavage of the peritoneal cavity with 1% penicillin/streptomycin in sterile saline (0.9% w/v). Macrophage solution was then centrifuged 2400 rpm for 7 min. Supernatant was then removed and replaced with 5ml macrophage medium consisting of red phenol-free complete Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; Thermo-Fisher), 1% pen/strep (Gibco; Thermo-Fisher), and 1% sodium pyruvate (Gibco; Thermo-Fisher). Cell suspension was then vortexed for 15 seconds. To a 12-well plate, macrophages were seeded at 1x10^6 cells/well. Cells were then placed in an incubator to adhere overnight at 37°C. Following incubation, non-adherent cells were removed by washing with macrophage medium 3 times for 5 min/wash. Adherent macrophage cells were then covered a final time with macrophage medium.

For experiments assessing effect of varying LPS concentrations on mRNA expression, macrophages were then incubated for 3 hr with 1, 10 or 100 ng/ml lipopolysaccharide from Escherichia coli (O111:B4; Sigma-Aldrich) (LPS) in macrophage medium. Controls were incubated in macrophage medium alone.

For subsequent experiments assessing effects of MaR1 on mRNA expression, following 100 ng/ml LPS for 3 hr, either 3 ng/ml MaR1 or macrophage medium vehicle was added to cells for an additional 5 hours. Culture media were then removed, with cell lysates obtained using lysis buffer provided by mirVana miRNA Isolation Kit (0.5% 2-Mercaptoethanol; 50% Thiocyanic acid, compound with guanidine (1:1)) (Invitrogen) followed by acid phenol extraction. Small RNA-enriched fractions were then isolated from total RNA using mirVana miRNA Isolation Kit (Invitrogen). After purification, RNA
was eluted using RNase-free water. Purity and concentration of RNA samples were then estimated using Nano-Drop ND-100 Spectrophotometer (ThermoFisher Scientific); establishing that nucleic acid samples were sufficiently pure for analysis if 260/280 nucleic acid ratio was above 1.8.

Total RNA fractions were diluted to 30 ng/µl. cDNA from total RNA samples was formed using Superscript IV one-step RT-PCR System (Invitrogen) according to manufacturer’s protocol.

### 4.2.3 Real-time Quantitative Polymerase Chain Reaction

Total and small RNA-enriched fractions were isolated using mirVana miRNA Isolation Kit (Invitrogen) and RNA-eluted using RNase-free water. Purity and concentration of RNA samples were estimated using Nano-Drop ND-100 Spectrophotometer (Thermo-Fisher Scientific).

For quantification of mRNA levels, PCR was performed using a LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche) in a LightCycler 480 (Roche). All primer sequences for quantification of mRNA expression are reported in Table 4.1.

The relative quantities of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method, where CT represents the threshold cycle, normalised to expression within control groups and using Actb as a housekeeper gene.
<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acbt</td>
<td>5'-GGCTGTATTCCTCCCTCATCG</td>
<td>5'-CCAGTTGGAACAAATGCCATGT</td>
<td>NM_007393.5</td>
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<td>Arg1</td>
<td>5'-GTGAAGAACCCACGCTCTGT</td>
<td>5'-CTGGTTGTCAGGGGAGTGTT</td>
<td>NM_007482.3</td>
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<td>Il4</td>
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<td>5'-CATGATGCTCTTTAGGGCTTT</td>
<td>NM_021283.2</td>
</tr>
<tr>
<td>Il6</td>
<td>5'-GGCTGTATTCCTCCCTCATCG</td>
<td>5'-CCAGTTGGAACAAATGCCATGT</td>
<td>NM_031168.2</td>
</tr>
<tr>
<td>Nos2</td>
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<td>5'-CTCAAGGTCAGCTGGT</td>
<td>NM_010927.4</td>
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<tr>
<td>TNFa</td>
<td>5'-GTGCCTATGTCTCAGGCTCT</td>
<td>5'-TGTTTGTGAGTGAGGTTG</td>
<td>NM_013693.3</td>
</tr>
</tbody>
</table>

Table 4.1: Sequences of mRNA primers used for real-time qPCR analysis. Primers were provided by Sigma Aldrich.

4.2.4 Preparation of dorsal root ganglia neuronal cell cultures

From male C57BL/6 mice, DRG tissue were dissected aseptically and placed in Hibernate A (Gibco; Thermo-Fisher) supplemented with 0.5 mM L-Glutamine (Gibco; Thermo-Fisher) and 2% B-27 Supplement (50X) (Gibco; Thermo-Fisher). Dorsal root ganglia were then dissociated in 2 mg/ml papain in Hank’s balanced salt solution (HBSS) for 30 min and placed in incubator (37°C and 5% CO₂). After incubation, cell suspension is centrifuged 800 rpm for 1 minute. Supernatant was then removed and replaced with 2.5 mg/ml collagenase in HBSS. Cells were then incubated for a further 30 min at 37°C. After incubation, cell suspension was centrifuged 800 rpm for 1 minute. Cells were then triturated and resuspended in Neurobasal A (Gibco; Thermo-Fisher) supplemented with 0.5 mM L-Glutamine (Gibco; Thermo-Fisher) and 2% B-27 Supplement (50X) (Gibco; Thermo-Fisher).

For calcium imaging, following resuspension, cells were then seeded onto laminin-coated glass coverslips, with care taken to ensure cells are placed in the centre of the
coverslip. Cells are then incubated for 30 minutes at 37°C before overnight incubation in Neurobasal medium, 1ml/well and experiments would take place the following day. For experiments involving pertussis toxin (PTX) before incubation, before calcium imaging, cells were incubated with 500 ng/ml PTX (EMD Millipore) in Neurobasal medium for 18 hours at 37°C, with controls incubated in Neurobasal medium plus sterile H2O vehicle.

4.2.5 Calcium imaging

Calcium imaging was performed as previously described by Allen et al. (2020). On the experimental day, DRG neurons were loaded with Fura-2 AM dye (3 mM) for 30 min in the presence of pluronic acid F-127 0.02% (Invitrogen) and incubated at 37°C. Following incubation, coverslips were imaged using Nikon Eclipse Ti-E inverted microscope at 10x magnification and subsequently analysed using ImageJ software (Version 1.50i). For preliminary experiments establishing effect of MaR1 on KCl-induced responses, cells were continuously superfused with either HBSS, 500 nM capsaicin, 30 mM KCl or 25 ng/ml MaR1. For all subsequent experiments, cells were continuously superfused with either HBSS, 500 nM capsaicin, 50 mM KCl, or 0.3 to 3ng/ml MaR1. All solutions were superfused at a flow rate of 2 ml/min. Capsaicin and KCl responses were quantified as the difference between (ΔF340/F380 ratio immediately before application of agent, and peak calcium after agent application).

For preliminary experiments establishing effect of MaR1 on KCl-induced responses, KCl (50mM) was applied for 30 sec, washed with HBSS for 120 sec, then exposed to either 25ng/ml MaR1 or HBSS buffer for a further 120 secs. During the final 30 secs of exposure, cells are additionally superfused with KCl (50mM) in the presence of either 25ng/ml MaR1 or HBSS.

Ca²⁺ influx via TRPV1 channels can cause desensitization on the channels themselves, representing a feedback mechanism protecting the nociceptive neurons from toxic Ca²⁺ overload (Touska et al., 2011). Following capsaicin dependant Ca²⁺ influx, acute desensitization of TRPV1 is reported to be modulated by Ca²⁺-activated enzymes, including Ca²+/calmodulin-dependent serine/threonine phosphatase 2B,
calcineurin, which lead to dephosphorylation of TRPV1 receptors (Docherty et al. 1996, Mohapatra and Nau, 2005). Proposely, dependent on duration of capsaicin exposure and external Ca$^{2+}$ concentration, acute desensitization can cause reduction within the initial few seconds following capsaicin exposure to the cell for the first time. Therefore, capsaicin (500 nM) was applied to cells 6 times for 15 seconds at 5-minute intervals to determine if desensitization of TRPV1 had occurred at our chosen concentration and exposure length. After the sixth capsaicin application, cells are exposed to 50 mM KCl to confirm cell viability. In MaR1/Capsaicin experiments, coverslips are continuously superfused with 0.3- to 3 ng/ml MaR1 in HBSS for 1 minute before, and 1 minute after, fourth capsaicin application. During all experiments involving MaR1, for each coverslip MaR1 was freshly prepared from stock solution in ethanol stored under nitrogen at -80°C without exposure to light.

### 4.2.6 Data and Statistics

All data are presented as means ±SEM. n is the number of experimental replicates, with the exception of preliminary calcium imaging experiments assessing effect of MaR1 on KCl-induced Ca$^{2+}$ responses. In these experiments n is number of total cells analysed under each condition from cells pooled from 2 animals. For all other calcium imaging experimental replicates, DRG were pooled from 5 animals and separated into wells of approximately 10,000 cells/well as described by Simeoli et al., (2017), with at least 100 cells analysed per well. For each experimental replicate, 3 wells were analysed and averaged per experimental condition.

For all experiments, differences between means are considered as statistically significant when \( P<0.05 \). All statistical analysis was conducted using GraphPad Prism (v8.0.0; GraphPad Software, USA). Multiple comparison of data used one-way ANOVA followed by post-hoc Tukey test, except for experiments assessing LPS concentration dependant changes to mRNA expression where post-hoc Dunnett test was used to comparing all results directly to control group.
4.3 Results

4.3.1 Cytokine expression in macrophages challenged with lipopolysaccharide

Our findings thus far have highlighted the role MaR1 can play in attenuating pro-inflammatory M1 macrophage presence in the DRG following K/BxN serum transfer. Activated M1 macrophages produce pro-inflammatory cytokines which lead to heightened nociceptive processing via the upregulation of inflammatory mediators (Misharin et al., 2014). As MaR1 treatment can reduce mechanical hypersensitivity and DRG M1 macrophage presence in the K/BxN serum transfer model of inflammatory arthritis, I investigated if in-vitro MaR1 can modulate both pro-inflammatory and anti-inflammatory cytokine signalling in activated macrophages.

The endotoxin lipopolysaccharide (LPS) is commonly used to activate monocytes and macrophages, generating a pro-inflammatory response and potentiating the production of inflammatory mediators (Clark et al., 2010). Firstly, I looked to establish the appropriate concentration of LPS required to evoke activation of peritoneal cultured macrophages. Following 24 hours in culture, peritoneal macrophages were challenged with varying concentrations of LPS for 3 hours, after which mRNA expression levels of pro-inflammatory and anti-inflammatory cytokines were assessed using qPCR.

When assessing anti-inflammatory cytokine mRNA expression, I observed a significant decrease in Arg1 expression at both 10 ng/ml (0.28 ± 0.22) and 100ng/ml (0.36 ± 0.13), as compared to controls incubated in medium alone (1.04 ± 0.04) (Figure 4.2A). Moreover, Interleukin-4 mRNA expression, known to be associated with M2 macrophage phenotype (Orecchioni et al., 2019), was also reduced in peritoneal macrophages following LPS challenge. However, this phenomenon was only observed when cells were incubated at 100 ng/ml LPS, the highest concentration tested (100ng/ml LPS: 0.61 ± 0.08; Control: 1.15 ± 0.05) (Figure 4.2B). Interestingly, LPS incubation did not lead to changes in anti-inflammatory Mrc1 mRNA expression at any concentration tested (Figure 4.2C).

Assessment of pro-inflammatory cytokine expression revealed as little as 1ng/ml LPS incubation was sufficient to produce over 2000-fold increase in Nos2 mRNA expression (2517.04 ± 357.44) as compared to medium only controls (1.425 ± 0.07) (Figure 4.2D). This potency to induce Nos2 expression was maintained at both
10ng/ml (3282.6 ± 1076.8) and 100 ng/ml LPS concentrations (3120.2 ± 645.3). Although not significant at low concentrations (1 ng/ml), mRNA expression for proinflammatory IL-6 was also greatly increased in peritoneal macrophages following incubation in either 10ng/ml (490.8 ± 180.0) and 100ng/ml (608.3 ± 162.6) LPS as compared to control medium (1.858 ± 0.39) (Figure 4.2E). mRNA expression for inflammatory cell signalling protein TNFα was less pronounced with an effective increase observed in peritoneal macrophages after challenge with 100ng/ml LPS (100ng/ml LPS: 12.27 ± 3.58; Con: 0.93 ± 0.04) (Figure 4.2F).

These results indicate that, as expected, LPS affects cytokine signalling to varying degrees dependant on the signalling marker assessed. However, overall, LPS most effectively influences pro-inflammatory and anti-inflammatory cytokine expression in peritoneal macrophages when administered at higher doses. Therefore, for future experiments, to induce a consistent pro-inflammatory state, the highest concentration of 100ng/ml LPS should be used.
Figure 4.2: Altered cytokine expression in macrophages challenged with lipopolysaccharide

Cultured peritoneal macrophages incubated with or without varying concentrations of lipopolysaccharide (LPS) (1, 10, 100 ng/ml for 3 hr). Quantification of mRNA for (A-C) anti-inflammatory or (D-F) pro-inflammatory cytokines assessed by qPCR. *P < 0.05 compared with medium only controls; one-way ANOVA, post hoc Dunnett. Data are mean ±SEM; n = 4 experiments, cells pooled from 5 animals per experiment.
4.3.2 MaR1 attenuates pro-inflammatory signalling in cultured macrophages following challenge with LPS

Our findings thus far demonstrate that peritoneal macrophages, when challenged with high doses of LPS, exhibit a rise in pro-inflammatory cytokine signalling. Therefore, using qPCR, I investigated the ability of MaR1 in altering pro-inflammatory cytokine expression in peritoneal macrophages.

Following LPS challenge, 5-hour incubation with MaR1 (3ng/ml) led to a marked decrease in mRNA expression of proinflammatory cytokines TNF-α (LPS Veh: 13.4 ± 1.5; LPS MaR1: 8.8 ± 1.48) and Nos2 (LPS Veh: 2800.4 ± 679.4; LPS MaR1: 1111.5 ± 287.4) in peritoneal macrophages (Figure 4.3A-C). For all pro-inflammatory markers tested MaR1 had no effect when incubated with unchallenged macrophages.

These results indicate that following inflammatory insult MaR1 can effectively attenuate the pro-inflammatory macrophage phenotype, with no effect on unchallenged macrophages.

4.3.3 MaR1 has no effect expression of anti-inflammatory signalling markers in cultured macrophages challenged with LPS

I have shown that high doses of LPS exposure can lead to not only changes in pro-inflammatory signalling, but a decrease in anti-inflammatory cytokine mRNA expression in peritoneal macrophage. It is known that MaR1 treatment of bone marrow-derived macrophages can skew phenotype from M1 (pro-inflammatory) to M2 (anti-inflammatory). Therefore, using qPCR, I next investigated if MaR1 alters mRNA expression for anti-inflammatory cytokines.

After cultured peritoneal macrophages had been challenged with LPS, I exposed cells to MaR1 (3ng/ml) for 5 hours. As expected, LPS challenge resulted in decreased mRNA expression of anti-inflammatory cytokine markers Arg1 (LPS Veh: 0.39 ± 0.05; Medium Veh: 1.04 ± 0.02), Mrc1 (LPS Veh: 0.38 ± 0.06; Medium Veh: 1.01 0.09), and Il4 (LPS Veh: 0.47 ± 0.10; Medium Veh: 1.02 ±0.06) as compared to unchallenged controls (Figure 4.4). However, LPS-induced reductions in anti-inflammatory mRNA expression were not attenuated by MaR1 treatment for any of the anti-inflammatory
markers tested. Moreover, MaR1 alone had no effect anti-inflammatory signalling in unchallenged peritoneal macrophages.

Therefore, our results indicate that pro-resolution action of MaR1 in peritoneal macrophages challenged with LPS, is likely due to a reduction in pro-inflammatory signalling rather than an enhancement of anti-inflammatory M2 signalling.
**Figure 4.3: MaR1 reduces expression of pro-inflammatory markers in macrophages challenged with lipopolysaccharide**

Cultured peritoneal macrophages incubated with or without lipopolysaccharide (LPS) (100 ng/ml for 3 hr) followed by MaR1 (3ng/ml) or vehicle for 5 hr. (A-C) Quantification of mRNA expression for pro-inflammatory cytokines and (D) pro-inflammatory miRNA markers assessed by qPCR. *P < 0.05, **P < 0.01, ***P < 0.01 compared with medium/vehicle controls, # P < 0.05; one-way ANOVA, post hoc Tukey. Data are mean ±SEM; n = 4-6 experimental replicates, cells pooled from 5 animals per experiment.

**Figure 4.4: MaR1 does not alter expression of anti-inflammatory markers in macrophages challenged with lipopolysaccharide**

Cultured peritoneal macrophages incubated with or without lipopolysaccharide (LPS) (100 ng/ml for 3 hr) followed by MaR1 (3ng/ml) or vehicle for 5 hr. (A-C) Quantification of mRNA expression for anti-inflammatory cytokines assessed by qPCR. *P < 0.05, **P < 0.01, ***P < 0.01 compared with medium/vehicle controls; one-way ANOVA, post hoc Tukey. Data are mean ±SEM; n = 4-6 experimental replicates, cells pooled from 5 animals per experiment.
4.3.4 MaR1 does not attenuate potassium chloride-mediated intracellular calcium ion influx in cultured DRG neurons

Thus far I have shown MaR1 to be a potent pro-resolution lipid mediator with significant anti-allodynic effects in K/BxN serum transfer model of inflammatory arthritis. Moreover, in K/BxN DRG, MaR1 attenuates pro-inflammatory macrophage signalling at timepoints where hind paw allodynia is dissociated from joint swelling. However, DRG tissue contains a mixed population of cell types where both neurons and immune cells are present. Moreover, during pain states, at the level of the DRG there is heightened neuro-immune cell communication. Therefore, it is possible that administration of MaR1 may effect macrophage and neuronal cell populations, either separately or in concert with each other.

To investigate the possible neuronal effects of MaR1 I used calcium imaging. These experiments enable the quantification of intracellular calcium ion influx, indicative of neuronal activation in cultured DRG neurons. Stimulation of DRG neurons with potassium chloride (KCl) (50mM, 30 s) robustly induces cellular depolarization and calcium ion influx. Therefore, during calcium imaging experiments, KCl is often used as a positive control, indicative of neuronal viability. As the effect of MaR1 on neuronal function is relatively unknown, I first investigated if MaR1 has any effect on KCl-mediated Ca\(^{2+}\) influx in DRG neurons.

Following a 30 second pulse of KCl (50mM) cultured DRG neurons were exposed to high MaR1 concentrations (25ng/ml / 120s). When KCl was applied in the presence of MaR1, there was no effect on DRG Ca\(^{2+}\) influx (Figure 4.5B, D). As expected, HBSS control buffer also did not alter KCl-mediated calcium responses (Figure 4.5A, C). A sensitization ratio was used to quantify change in calcium influx between KCl pulses for DRG neurons, highlighting a steady KCl-mediated calcium response following MaR1 exposure (0.98 ± 0.00), comparable to HBSS controls (0.99 ± 0.00) (Figure 4.5D). These initial findings show MaR1 has no effect on KCl-mediated neuronal responses, and therefore KCl can be used a positive control for neuronal viability in future calcium imaging experiments involving MaR1 incubation.
Figure 4.5: MaR1 does not attenuate KCl-mediated intracellular Ca$^{2+}$ responses in cultured DRG neurons

(A-B) Representative calcium imaging traces of cultured DRG neurons loaded with fura-2-AM (3µM, 30 min) then exposed to potassium chloride pulses (KCl; 50mM, 30 s). Neurons were incubated with either (A) HBSS vehicle control (Veh; 120 s) or (B) Maresin-1 (MaR1; 25ng/ml, 120 s). KCl (50mM, 30 s) was again applied in the presence of either MaR1 or Vehicle. (C-D) Quantification of peak amplitude responses of DRG neurons to first KCl application and second KCl application, in the presence of either (C) HBSS vehicle or (D) MaR1. (E) For each cell, the sensitization ratio was calculated as the amplitude of the first over the second KCl response. Data are mean ±SEM; n = cells analysed under each condition; Control (248), MaR1 (201)
4.3.5 Repeated capsaicin exposure does not significantly attenuate intracellular calcium responses in cultured DRG neurons

Capsaicin is an agonist and activator of TRPV1 receptors, present on primary afferent nociceptors. Following inflammation and injury TRPV1-mediated signalling from DRG neurons occurs in a signalling cascade which results in neuronal activation and intracellular calcium ion influx. However, capsaicin application can induce desensitization of TRPV1 channel, which may lead to decreased Ca\(^{2+}\) influx. Therefore, prior to investigating the effect of MaR1 on TRPV1-mediated neuronal signalling, it was important for us to establish if capsaicin-induced TRPV1 desensitization occurs in our system.

Therefore, Fura-2-AM pre-loaded cultured DRG neurons, were exposed to consecutive applications of capsaicin (500nM) at 15 second intervals and intracellular calcium influx measured. Between 6 pulses of capsaicin, I observed a marked and consistent intracellular Ca\(^{2+}\) response from KCl-responsive DRG neurons (Figure 4.6A-B). There was no significant difference in neuronal responses between capsaicin pulses 1-6, however a small change in neuronal calcium influx was observed between third and fourth capsaicin pulses (3\(^{rd}\) pulse: 5.53 ± 1.12; 4\(^{th}\) pulse: 5.20 ± 1.13) (Figure 4.6B). Therefore, subsequent investigation of MaR1’s effects on TRPV1-mediated neuronal responses occurred between third and fourth capsaicin application.
Figure 4.6: Capsaicin-induced intracellular Ca\textsuperscript{2+} influx is not attenuated in DRG neurons following repeated exposure

(A) Representative calcium imaging trace of cultured DRG neuron loaded with Fura-2-AM (3µM, 30 min) then exposed to 6 capsaicin pulses (500 nM, 15 s/pulse) at 5 min intervals, followed by final application of KCl (50mM, 15 sec). (B) Quantification of calcium responses after 6 consecutive applications of capsaicin. Data are mean ±SEM; n = 3 experimental replicates
4.3.6 MaR1 attenuates TRPV1-mediated responses of nociceptive DRG neurons

Having established that capsaicin application induces a robust TRPV1-mediated response in nociceptive DRG neurons, I next investigated if this nociceptive response can be attenuated by potent pro-resolution lipid mediator MaR1.

Using calcium imaging experiments, cultured DRG neurons were exposed to 5 pulses of capsaicin (500nM; 15 s intervals), with neurons incubated in either MaR1 or HBSS during 4th capsaicin pulse (Figure 4.7A, B). Although ineffective at doses below 1ng/ml, addition of MaR1 resulted in a significant reduction in intracellular Ca$^{2+}$ influx when administered at 1 ng/ml (0.44 ± 0.06) or 3 ng/ml (0.32 ± 0.08) concentrations, as compared to HBSS alone (1.04 ± 0.18) (Figure 4.7C).

These results indicate that MaR1 can significantly attenuate TRPV1 mediated nociceptive signalling from DRG neurons, likely attributing to the anti-allodynic effect of MaR1 in pain states (1ng/ml: $P<0.05$, 3ng/ml: $P=0.01$, as compared to HBSS controls).
Figure 4.7: Capsaicin-induced intracellular Ca$^{2+}$ influx is attenuated in DRG neurons by MaR1

(A) Representative calcium imaging trace of cultured DRG neurons exposed to either HBSS buffer control (90 sec) or MaR1 (3ng/ml, 90 sec) during the fourth of six capsaicin pulses (500 nM, 15 s/pulse, at 5 min intervals) (B) Capsaicin response ratio (4/3) in the presence of MaR1 (0.3-3ng/ml, 90 sec) or HBSS alone. *$P < 0.05$, **$P < 0.01$ compared with HBSS control; one-way ANOVA, post hoc Tukey. Data are mean ±SEM; n = 4 experimental replicates. Each replicate consists of 3 wells analysed (100 cells analysed/well) per experimental condition. DRG cells for all wells pooled from 5 mice.
4.3.7 Pertussis toxin abolishes MaR1-mediated inhibition of TRPV1 sensitive nociceptive neurons

Previous studies have proposed that the inhibition of neuronal TRPV1 signalling by MaR1 is due to its interaction and modulation of GPCR function (Serhan et al., 2012). To test this hypothesis, prior to calcium imaging experiments, I cultured DRG neurons in the presence of the $G_{i/o}$-coupled GPCR blocker pertussis toxin (PTX) (500ng/ml, 18h).

Following culture in the presence of PTX, DRG neurons were exposed to 5 pulses of capsaicin (500nM; 15 s intervals), with neurons incubated in 3 ng/ml MaR1 during 4\textsuperscript{th} capsaicin pulse. Remarkably, PTX pre-treatment led to a reduction in MaR1’s ability to attenuate capsaicin-induced intracellular calcium influx, as compared to pre-incubation with control buffer (PTX: 0.85 ± 0.05; Buffer: 0.33 ± 0.07) (Figure 4.8A-C), bringing capsaicin responses in either the presence or absence of MaR1 to comparable levels (Figure 4.8B).

Together these results highlight that MaR1 can interact directly with TRPV1 sensitive DRG neurons to attenuate nociceptive activation. MaR1-mediated reduction of nociceptive neuronal signalling may occur via interactions with GPCR signalling pathways.
Figure 4.8: Reduction of capsaicin-induced calcium responses by MaR1 is blocked by disruption of GPCRi/o mechanisms

(A-B) Calcium imaging traces of cultured DRG neurons which have been preincubated in either (A) control buffer or (B) pertussis toxin (PTX) (GPCRi/o inhibitor) (500ng/ml, 18 hr), then exposed to MaR1 (3ng/ml, 90 sec) during the fourth of six capsaicin pulses (500 nM, 15 s/pulse, at 5 min intervals) (C) Capsaicin response ratio (4/3) in the presence of MaR1 (3ng/ml, 90 sec) in neurons preincubated with PTX or control buffer for 18 hr **P < 0.01; unpaired student t-test. Data are mean ±SEM; n = 4 experimental replicates. Each replicate consists of 3 wells analysed (100 cells analysed/well) per experimental condition. DRG cells for all wells pooled from 5 mice.
4.4 Discussion

In this chapter I offer in-vitro evidence that MaR1, a macrophage-derived pro-resolving lipid mediator, produces distinct anti-inflammatory and anti-nociceptive effects on macrophages and neurons, respectively. Firstly, by demonstrating that co-incubation of peritoneal macrophages with MaR1 leads to a reduction in pro-inflammatory cytokine signalling following challenge with LPS. Secondly, when applied to TRPV1+ DRG neurons, MaR1 can directly attenuate capsaicin-induced intracellular Ca\(^{2+}\) influx; with MaR1-mediated effects dependant on neuronal GPCR signalling. These findings affirm that MaR1 has multifaceted function modulating nociceptive signalling by i) indirectly reducing immune-mediated production of pro-inflammatory cytokines which sensitize nociceptive neurons and ii) directly reducing intracellular neuronal signalling capabilities of nociceptive sensory neurons. Both these functions of MaR1 are likely dependent on intracellular GPCR-mediated mechanisms.

Lipopolysaccharide (LPS) is widely recognized as a potent activator of monocytes/macrophages causing an acute inflammatory response by triggering the release of inflammatory mediators, via TLR4-mediated signalling mechanisms (Wollenberg et al., 1993). Therefore, expectedly, I observe that peritoneal macrophage exposure to LPS elicits increased expression of pro-inflammatory cytokines IL-6 and TNFα. These findings are in line with previous studies also showing increased IL-6 and TNFα expression in peritoneal macrophages following LPS exposure (Wollenberg et al., 1993, Takashiba et al., 1999). These increases in pro-inflammatory mRNA expression levels are indicative of greater cytokine release, with studies demonstrating that following LPS challenge murine peritoneal macrophages exhibit increased IL-6 and TNFα protein levels in macrophage culture supernatant (Amura et al., 1997). In addition to IL-6 and TNFα expression, increased macrophage iNOS production is also associated with a M1 pro-inflammatory phenotype. In cultured macrophages, I find in agreement with previous studies increased iNOS expression following LPS challenge (Mills et al., 2000). Conversely, LPS also decreased expression of anti-inflammatory mediators, Arg1 and IL-4, following peritoneal macrophage challenge with LPS. Together, these findings highlight that LPS exposure not only triggers the expression of pro-inflammatory mediators from peritoneal macrophages, but in addition the endotoxin also reduces anti-inflammatory macrophage signalling.
In human peritoneal macrophages, MaR1 has been shown to induce macrophage switching from pro-inflammatory (M1) to anti-inflammatory (M2) phenotype (Dalli et al., 2013). Our findings demonstrate using peritoneal macrophages that MaR1 can attenuate LPS-induced inflammatory conditions indicated by reduction of TNFα and iNOS expression. In agreement, other studies utilizing aged bone marrow-derived macrophages demonstrated that following LPS exposure (10ng/ml), co-incubation with MaR1 (3.6ng/ml) induces an anti-inflammatory phenotype with reduced iNOS, IL-6, TNFα and IL-1β mRNA expression (Huang et al., 2020). Differing from Huang et al. (2020), I do not find significant MaR1-mediated reduction in IL-6 expression from LPS-exposed macrophages, although I do observe a trend. This may be due to experimental differences in incubation time, as following LPS exposure, in our studies macrophages were incubated with MaR1 for 5 hours as oppose to the protocol by Huang et al. (2020) featuring a longer 12-hour MaR1 incubation post-LPS exposure. Similar to our findings, bone-marrow derived macrophages incubated with MaR1 display reduced TNFα cytokine expression following LPS challenge (Yang et al., 2019). Production of pro-inflammatory IL-6, TNFα and iNOS are thought to be controlled by the intracellular NF-κB pathway in macrophages (Dorrington and Fraser, 2019, Lopez-Collazo et al., 1998). Indeed, MaR1 decreases p65 nuclear translocation in cultured macrophages following LPS challenge (Yang et al., 2019).

Together, with our results, these findings would agree with the hypothesis that MaR1 reduces pro-inflammatory cytokine signalling from M1 macrophages via modulation of NF-κB pathways. However, future experiments could look to confirm this by assessing MaR1-mediated changes to p65 nuclear translocation and pro-inflammatory cytokine signalling in the same cultured macrophage population following LPS challenge.

Following MaR1 incubation, I observed no change in expression of anti-inflammatory cytokines (Arg1, Mrc1 & IL-4) in macrophages exposed to LPS. In agreement, Yang et al. (2020) found that following LPS challenge MaR1 did not promote anti-inflammatory CD206 expression. Together with our previous results these findings indicate that in peritoneal macrophages challenged with LPS MaR1 largely exhibits a pro-resolution action by reducing pro-inflammatory M1 signalling, as oppose to the enhancement of anti-inflammatory M2 signalling. Conversely, a recent study demonstrated that following anti-inflammatory IL-4 treatment, skewing bone-marrow derived macrophages to a M2 phenotype, MaR1 has an additive effect further
increasing Arg1 and TGF-β expression (Huang et al., 2020). These findings would indicate that, under certain conditions, MaR1 may influence cultured macrophages to promote anti-inflammatory signalling.

All experiments I conducted assessed the ability of MaR1 alter cytokine expression 3 hours after initial exposure to LPS. However, it is feasible that the time course of cytokine expression may differ between both proinflammatory and anti-inflammatory cytokines following LPS exposure. Therefore, to gain an understanding of the effects of MaR1 on peak cytokine expression, a time course experiment would be required prior, to assess the optimum LPS incubation period required for each cytokine studied.

As previously stated in section 4.1.2, the LGR6 receptor known to be present on human and murine macrophages, has recently been discovered as the MaR1 receptor (Chiang et al., 2019). Future work may look to assess if MaR1-mediated inhibition of pro-inflammatory cytokine signalling is attenuated following knockdown of the LGR6 receptor in macrophages challenged with LPS. Here it would be expected that LGR6 loss of function would inhibit MaR1 anti-inflammatory action.

When applied to neurons, potassium chloride (KCl) is known to evoke cellular depolarization and elevate neuronal intracellular calcium levels (Dolmetsch et al., 2001, Tan and Mcnaughton, 2016). KCl-induced intracellular Ca^{2+} influx is known to be mediated in neurons by L-type voltage sensitive calcium channels (VSCCs), N- and P/Q-type Ca^{2+} channels, and NMDA receptors (Dolmetsch et al., 2001, Xia et al., 1996). I show that the presence of MaR1 does not inhibit these processes indicative of neuronal activation in dissociated DRG neurons. Therefore, our results suggest that MaR1 does not alter L-type VSCCs, calcium channels or NMDA receptor function in neurons. Moreover, I observe no initial change in neuronal activation following application of MaR1 alone to DRG neurons. Similarly, studies in the trigeminal ganglion highlight that MaR1 treatment also has no effect on basal neuronal synaptic transmission from lamina II neurons, with MaR1 treatment leaving frequency and the amplitude of spontaneous excitatory postsynaptic currents unaltered (Park, 2015).

TRPV1 is a polymodal channel sensitive to various stimuli including noxious heat, mechanical stimuli, and ligands such as capsaicin, with high TRPV1 expression found in the plasma membrane of nociceptive primary sensory neurons (Caterina and Julius, 2001). Patch-clamp recordings in dissociated DRG neurons have shown that MaR1
has an inhibitory effect on capsaicin-induced TRPV1 currents, in a dose-dependent manner (Serhan et al., 2012). This inhibitory function is also observed in the trigeminal ganglion, where MaR1 also inhibits capsaicin-induced TRPV1 currents, with no action on TRPA1 mediated-mechanisms (Park, 2015). Similarly, I observe a dose-dependent attenuation of TRPV1-mediated calcium influx from dissociated DRG neurons. Together, these results indicate that MaR1, in both the trigeminal ganglion and DRG, acts on sensory neurons to inhibit nociceptive signalling. Further experimentation with pure neuronal cultures could help clarify if MaR1-mediated neuronal effects can be singularly attributed to the SPM’s direct action on neurons and not glial cells which may be present within dissociated cultures.

Following CFA injection into the hind paw, capsaicin-induced neuronal activity is greater in TRPV1+ DRG neurons as compared to naïve animals (Fattori et al., 2019). Furthermore, this heightened TRPV1+ neuronal activity can be attenuated by MaR1 treatment. Future experiments could similarly address if following passive transfer of K/BxN serum, when hyperalgesia presents in the absence of joint swelling, whether TRPV1+ DRG neurons display heightened capsaicin-induced neuronal activity which could be attenuated by MaR1 treatment.

Previous studies show that pre-treatment of dissociated DRG neurons with pertussis toxin also prevents inhibitory action of MaR1 on TRPV1 currents (Serhan et al., 2012). Similarly, in trigeminal ganglion neurons, inhibition of GPCRi/o signalling completely blocked MaR1’s inhibition of capsaicin-induced TRPV1 currents (Park, 2015). My work builds upon these findings and provides a greater mechanistic understanding of MaR1-mediated inhibition by demonstrating that MaR1-mediated attenuation of capsaicin-induced neuronal activity is disrupted via a reduction in capsaicin-induced calcium influx in DRG neurons. This process is inhibited by disruption of GPCRi/o signalling.

Together these results highlight that GPCR signalling pathways are crucial for the anti-nociceptive action of MaR1 on sensory neurons. Although LGR6 presence has been confirmed in macrophages, its presence is yet to be established in DRG neuronal populations. Therefore, future experiments could look to prove the receptors presence on sensory neurons and assess if MaR1-mediated inhibition of TRPV1+ neuronal activation is perturbed by specific inhibition of LGR6 function in dissociated DRG
neurons. Overall, I indicate that MaR1-mediated reduction in pro-inflammatory cytokine release likely attenuates nociceptive signalling by reducing macrophage-neuron communication, pro-nociceptive mediator release and subsequent sensitization of neurons conveying noxious information.

4.4.1 Chapter Key Findings

In this chapter I have determined using *in-vitro* experimentation that:

- Application of MaR1 reduces pro-inflammatory TNFα & NOS2 signalling in peritoneal macrophages challenged with LPS, without effect on anti-inflammatory signalling
- Capsaicin-induced activation of DRG neurons is inhibited by MaR1 treatment, in a dose dependent manner
- MaR1-mediated inhibition of neuronal activation in capsaicin-sensitive neurons is perturbed by blockade of GPCRi/o signalling

These data suggest, in agreement with previous studies, that MaR1 has dual abilities acting both on activated macrophages to dampen pro-inflammatory cytokine signalling and also directly reducing nociceptive sensory neuron activation. These GPCR-mediated mechanisms of MaR1 on nociceptive neuro-immune signalling likely contribute to the anti-hyperalgesic effects of MaR1 administration in models of arthritic joint pain.

4.4.2 Future Direction Leading to Chapter 5

Our findings thus far have demonstrated that MaR1 exhibits clear anti-inflammatory and anti-nociceptive action on cultured macrophages and dissociated capsaicin-sensitive DRG neurons, respectively. Moreover, in the K/BxN serum transfer model of inflammatory arthritis, repeated systemic MaR1 treatment can alleviate hind paw
hyperalgesia dissociated from joint swelling, as well as decreasing concomitant inflammatory M1 macrophage presence in the K/BxN DRG.

MiRNAs are known to be involved in the modulation of nociceptive signalling, influencing both pro- and anti-inflammatory neuro-immune communication. In the DRG, following passive immunization, dysregulation of DRG miRNA signalling may occur contributing to heightened nociceptive states either in the presence or absence of joint swelling. Moreover, the anti-nociceptive and anti-inflammatory action of SPMs, including MaR1, are also likely to involve modulation of miRNA signalling, consequently altering nociceptive neuro-immune communication.

Therefore, in the next chapter (chapter 5), I firstly looked to identify possible dysregulation of miRNA expression in the K/BxN DRG when mechanical hyperalgesia occurs either in the presence or absence of joint swelling. Secondly, I assessed expression of newly identified K/BxN dysregulated miRNAs and previously known pro-nociceptive and pro-inflammatory miRNAs in cultured macrophages and DRG neurons exposed to capsaicin. Finally, I examined if this modulation of pro-inflammatory and pro-nociceptive miRNA signalling in cultured neurons or macrophages could be modified by MaR1 treatment.
Chapter 5:
The Contribution of MicroRNA Signalling to Inflammatory and Nociceptive Mechanisms Associated with Persistent Hyperalgesia
5.1 Introduction

First discovered in the 1990’s by the Ambros and Ruvkun groups in C. (Caenorhabditis) elegans, miRNAs have since been shown to be important regulators of protein expression which are highly conserved between organisms (Lee et al., 1993, Wightman et al., 1993). miRNAs are endogenous non-coding RNA molecules, usually between 18-25 nucleotides in length (Tahamtan et al., 2018). Although less than 0.02% of total RNA content are composed of miRNAs in cells, it has been estimated that approximately 60% of all protein-coding genes are regulated by miRNAs.

miRNAs function as post-transcriptional regulator of gene expression, whereby the binding of miRNA to its target mRNA typically leads to mRNA degradation or translational inhibition. However, there is also evidence to suggest that miRNAs occasionally can enhance RNA stability causing upregulation of specific mRNA targets (Figure 5.1).

The majority of miRNA interactions occur at the seed sequence. This is a conserved sequence mostly situated at positions 2-7 from the miRNA 5’-untranslated region (UTR) end. It recognises 3’-UTR motifs in target mRNAs, which commonly possess AU-rich regions (Montague-Cardoso and Malcangio, 2020).

In silico analysis of miRNA targets estimate an average of 200 mRNA targets per miRNA, and conversely, each mRNA may be regulated by multiple miRNAs (Mahesh and Biswas, 2019). Therefore, the regulation of miRNA is thought to be combinatorial in nature, increasing the effectiveness of miRNA signalling (Cursons et al., 2018). Moreover, miRNAs can be present in clusters whereby they are expressed as polycistronic units; encoding several genes of which share a functional relationship within a particular metabolic pathway (Montague-Cardoso and Malcangio, 2020).

5.1.1 miRNA biosynthesis and function

miRNAs are transcribed by the action of RNA polymerase (RNA pol) II and III, as primary miRNAs (pri-miRNAs). These pri-miRNAs are polyadenylated and feature a cap structure, with a N7-methylated guanosine (m^7G) linked to the first nucleotide of the RNA. Pri-miRNA, which can be several thousand bases long, must first be cleaved by Drosha, an RNase III enzyme present in the cell nucleus, to form a stem loop-
structure known as the precursor miRNA (pre-miRNA), which is around 70 nucleotides in length (Tahamtan et al., 2018) (Figure 5.1).

Thereafter, pre-miRNAs can then be exported from the nucleus to the cytoplasm via the export receptor exportin 5 (Exp5). Once in the cytoplasm, Dicer, a RNase III-type enzyme, facilitates the formation of a shorter 20–25 nucleotide, double-stranded miRNA (dsRNA), from the stem of the pre-miRNA (Figure 5.1).

Finally, in a process termed RNA-induced silencing complex (RISC) loading, the Dicer-produced dsRNA is modulated further by a member of the Argonaute (AGO) protein family. AGO proteins select one strand to become the mature miRNA, known as the guide strand, while discarding the other, passenger strand. AGO proteins loaded with miRNAs dissociate from Dicer and form RISC.
Figure 5.1: The biogenesis and function of miRNAs

Abbreviations: long-non-coding RNAs (lncRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), RNA inducing silencing complex (RISC), RNA Polymerase II (RNA Pol II), RNA Polymerase III (RNA Pol III) (Tahamtan et al., 2018).

Following binding, mature miRNA then guides RISC to complementary sequences at target mRNA 3'UTR motifs, complementary to the miRNA seed sequence. Once mature miRNA binds to its target mRNA, the AGO protein recruits a member of the GW protein family, which have multiple glycine–tryptophan (GW) repeats (Figure 5.2). In mammals these GW proteins are known to be trinucleotide repeat containing gene 6 proteins (TNRC6) (Treiber et al., 2019). These proteins contain an AGO-binding
domain and a silencing domain. The silencing domain contains tryptophan residues, which are required for interaction with the de-adenylase complexes poly(A)-specific ribonuclease 2 (PARN2)–PARN3 or carbon catabolite repressor 4 (CCR4) (Figure 5.2). Collectively, these shorten the poly(A) tail of the mRNA. A short or absent poly(A) tail is a signal for mRNA decapping, a largely irreversible and highly regulated step of mRNA decay. This process is mediated by mRNA-decapping enzyme 1 (DCP1)–DCP2 complex, as well as DEAD box helicase protein 6 (DDX6), which is recruited to the 5’end of the mRNA, removing the 7-methylguanylate cap (m7G) and allowing the unprotected 5’end to be degraded (Treiber et al., 2019) (Figure 5.2). DDX6 is crucial for the translational repression and stimulation of decapping. Both of these mechanisms are prerequisites for mRNA decay.

**Figure 5.2: Mechanisms of microRNA function**

Abbreviations: Argonaute protein (Ago), trinucleotide repeat-containing gene 6 proteins (TNRC6), poly(A)-specific ribonuclease 2/3 (PARN2/3), carbon catabolite repressor 4 (CCR4), DEAD box helicase DEAD box protein 6 (DDX6), mRNA-decapping enzyme 1/2 complex ((DCP1/2), 7-methylguanylate cap (m7G), eukaryotic translation initiation factor 4F (eIF4F) open reading frame (ORF) (Treiber et al., 2019).
5.1.2 miRNA signalling is involved in inflammatory pain models

Since their discovery miRNAs have been shown to play a critical regulatory role in a diverse number of biological processes. miRNAs have been implicated in the regulation of inflammatory responses, through their expression in immune cells (Tahamtan et al., 2018). The initiation, spread and eventual resolution of inflammation are all mediated by miRNAs, which either exacerbate or reduce inflammation, depending on the miRNA target. In the CFA model of inflammatory pain, miRNA dysregulation was shown to occur in trigeminal ganglion within 4 hours of CFA injection into the masseter muscles of the face in rats (Bai et al., 2007). Inflamed trigeminal ganglion displayed a downregulation of miR-10a, miR-29a, miR-98, miR-99a, miR124a, miR-134 and miR-183. Later studies by Zhao et al. (2010) identified a functional role for miRNAs in inflammatory nociceptive signalling whereby knockout of dicer, preventing miRNA formation, in Nav1.8 expressing nociceptors of the DRG leads to abolition of CFA-induced thermal hyperalgesia and mechanical allodynia. Moreover, Nav1.8-sensitive neurons from dicer-null mice showed limited increases in excitability following exposure to multiple inflammatory mediators such as, PGE$_2$, histamine, ATP and 5-HT (Zhao et al., 2010). These findings therefore suggest that miRNAs are directly contributed to inflammatory pain signalling from nociceptive neurons.

Following CFA-induced inflammation, expression of miR-1 and miR-16 in the DRG is consistently reduced up to 7 days after induction (Kusuda et al., 2011). Whereas, miR-206 expression was only reduced in a time-dependent manner, at days 1 and 7 post-CFA injection relative to controls (Kusuda et al., 2011). Moreover, when assessing spinal cord expression, all 3 miRs showed increased expression, acting oppositely as compared to the DRG. These findings indicate that the role of miRNAs in inflammatory pain signalling is varied, with some miRs showing differing functions depending on their expression either in peripheral or central nervous system.

Ca$^{2+}$/calmodulin-dependent protein kinase II (CamKII) is a family of protein kinases that are regulated by the intracellular Ca$^{2+}$ concentration through the Ca$^{2+}$/calmodulin complex (Lopez-Gonzalez et al., 2017). CamKIIy, a protein of this family, has been implicated in inflammatory pain mechanisms and is thought to be regulated by miRNAs (Pan et al., 2014). In the mouse CFA model of inflammatory pain, miR-219 expression
is reduced in spinal neurons, with concomitant upregulation of spinal CamKIIγ expression, known to be target of miR-219 (Pan et al., 2014). An up-regulation of miR-219 leads to a decrease of CamKIIγ and significant reduction of thermal hyperalgesia and mechanical hypersensitivity (Pan et al., 2014). Additionally, in the spinal cord, aberrant miR-124 signalling, suggestively from microglia, has been implicated in chronic inflammatory pain signalling. Six days after intraplantar injection of carrageenan, intrathecal miR-124 treatment can reverse carrageenan induced-persistent thermal hyperalgesia (Willemen et al., 2012). Inflammatory hyperalgesia and neuropathic pain are associated with decreased levels of the serine–threonine kinase G protein heterotrimeric receptor kinase 2 (GRK2) in spinal microglia/macrophages. Intrathecal miR-124 treatment can decrease ratio of M1/M2 phenotype markers in spinal cord microglia/macrophages and reduce persistent hyperalgesia of GRK2-deficient mice following intraplantar IL-1β administration (Willemen et al., 2012). In cultured macrophages exposed to LPS, miR-149 is shown to be a modulator of inflammatory response, with over-expression of miR-149 leading to reduced production of inflammatory mediators such as NF-κB, TNFα and IL-6; as well as MyD88, an adaptor protein associated with downstream activation of intracellular NF-κB signalling (Xu et al., 2014). Together, these findings highlight an important role for miRNAs in the central regulation of inflammatory nociceptive signalling via modulation of pro-inflammatory mediator production from neurons and immune cells.

5.1.2.1 miR-155

In addition to the aforementioned miRs, several studies indicate that miR-155, may act as a master regulator of inflammation playing a key role regulating several critical pathways that coordinate innate immune responses and chronic inflammatory processes, such as NF-κB signalling (Marques-Rocha et al., 2015). Bone marrow derived murine macrophages, when cultured in either the presence of inflammatory LPS or TNFα and interferon-β, express miR-155 (Marques-Rocha et al., 2015, O'Connell et al., 2007).

Ship1 is believed to be a target for miR-155 in macrophages (Mashima, 2015). In blood-derived human monocytes, induction of miR-155 expression was shown to repress Ship1 expression. Moreover, in rheumatoid arthritis patients, an inverse
relationship was observed between the expression of miR-155 and Ship1 in monocytes and CD68+ macrophages taken from synovial biopsies (Kurowska-Stolarska et al., 2011). It was observed that overexpression of miR-155 in cultured human CD14+ monocytes also triggered the production of cytokines and chemokines strongly implicated in rheumatoid arthritis synovitis; namely TNFα, IL-6, IL-1β, and IL-8 (Kurowska-Stolarska et al., 2011). In agreement, following miR-155-transfection, greater pro-inflammatory TNFα and IL-1β were produced in cultured human monocyte-like THP1 cells, in response to pro-inflammatory monosodium urate monohydrate treatment (Jin et al., 2014). Studies using murine bone marrow-derived macrophages (BMDMs) to determine miRNA expression in M1 and M2 polarizing conditions highlighted that miR-155 was upregulated in M1-polarized macrophages, exposed to LPS and interferon-γ (Zhang et al., 2013), indicating that miR-155 expression is of particular importance during inflammatory macrophage signalling. Together these studies indicate that miR-155 acts as a positive regulator of cytokine production in monocytes and macrophages, with implications for inflammatory disorders such as rheumatoid arthritis.

Mann et al. (2017) conducted experiments using mice reconstituted with bone marrow transduced with GFP miR-155. 4 months after reconstitution, mice were analysed for CD11b+ peripheral blood monocyte-derived macrophages, revealing that miR-155 overexpression led to increased and longer-lasting NF-κB activity in CD11b+ peripheral blood monocyte-derived macrophages. Moreover, mice overexpressing miR-155 also showed increased pro-inflammatory IL-6 serum levels, as compared to controls (Mann et al., 2017). The study showed both Ship1 and Socs1 mRNA and protein levels are higher in miR-155−/− derived macrophages, following LPS stimulation. These proteins were shown to be primary targets of miR-155 in macrophages, attenuating PI3K-AKT signalling, resulting in downstream reductions in pIKKα/β phosphorylation and subsequent amplification in NF-κB activity following LPS stimulation (Mann et al., 2017).

miR-155, and its downstream targets, have also been associated with heightened nociceptive states. In the rat chronic constriction injury model (CCI) of neuropathic pain, miR-155 is shown to be up-regulated in the spinal cord microglia, 2 days post injury with high levels maintained throughout a period of 16 days, targeting spinal Socs1 expression (Tan et al., 2015). CCI induces mechanical allodynia, thermal
hyperalgesia, and the expression of proinflammatory cytokines IL-1β, IL-6 and TNFα, which were all attenuated by intrathecal treatment with miR-155 inhibitor. This anti-allodynic effect of MiR-155 inhibition was perturbed by knockdown of Socs1. In addition, inhibition of miR-155 also suppresses spinal cord NF-κB and p38 MAPK activation (Tan et al., 2015). Together these results indicate that miR-155 inhibition can induce a suppression of NF-κB and p38 MAPK activation through Socs1, resulting in exacerbation of CNS nociceptive signalling (Lopez-Gonzalez et al., 2017).

In the collagen-induced arthritis (CIA) model, where mechanical hypersensitivity is present both prior to and in conjunction with joint swelling (Nieto et al., 2015), miR-155 deficient mice do not develop clinical signs of arthritis at any timepoint (Bluml et al., 2011). CIA induction requires an adaptive immune response, miR-155 deficiency after induction of CIA, was shown to prevent autoreactive B and T cell generation and inhibit of Th17 polarization. Moreover, miR-155 deficient mice display decreased serum levels of pro-inflammatory IL-6 and IL-17 following the induction of CIA. Following passive transfer of K/BxN serum, inducing an innate immune response, miR-155 KO mice showed significantly reduced local bone destruction (Bluml et al., 2011). However, severity of joint inflammation was similar to that in WT mice, indicating miR-155 may not play a vital role in K/BxN joint swelling. Together, this study shows that miR-155 plays differing roles dependent on the pre-clinical model of arthritis, with greatest involvement in the adaptive immune reactions leading to autoimmune arthritis.

5.1.3 miRNA mediated neuro-immune communication in pain models

Following injury or inflammatory insult, pro-inflammatory mediators produced by M1 inflammatory macrophages, such as IL-6 and TNFα can bind to receptors present on cell surface of nociceptors, leading to neuronal sensitization and an exacerbation of nociceptive signalling, as previously outlined in Figure 4.1. The extracellular release of miRNAs provides a method of neuro-immune communication, facilitating the regulation of nociceptive signalling. miRNAs, due to their negative charge, do not easily cross the cell membrane and are rapidly degraded in vivo (Aagaard and Rossi, 2007). Far more efficient is the release of extracellular miRs as part of exosomal cargo. Exosomes are extracellular vesicles which are secreted by all cell types;
including both macrophages and neuronal cell bodies (Montague-Cardoso and Malcangio, 2020).

Simeoli et al., (2017) showed that capsaicin stimulation of nociceptive DRG neurons incites release of miR-21-5p, let7b, miR-124 and miR-134 in the exosomal fraction. This miR release from DRG neurons was shown to be TRPV1-dependant and occurred alongside significant increases in Dicer activity, essential for mature miRNA formation. Subsequently, these exosomes derived from sensory neurons were shown to be readily phagocytosed by cultured peritoneal macrophages, causing an increase in Nos2, reductions in Mrç1 mRNA expression and reduced mRNA expression of Sprouty2 (Spry2), a known miR-21 target. These expression changes are prevented by miR-21 antagomir. Additionally, transfection of peritoneal macrophages with miR-21 led to increased levels of pro-inflammatory iNOS protein and NF-κB subunit p65, as well as reduced anti-inflammatory Mrç1 and Arg1 mRNA expression. In agreement with these intracellular changes, extracellular levels of pro-inflammatory TNFα and IL-6 were greater from miR-21-transfected macrophages as compared to controls (Simeoli et al., 2017). Moreover, miR-21-transfected macrophages were shown to be more polarized towards M1 (CD206−CD11c+) phenotype, compared to controls. These in-vitro findings indicate that neuronal-exosome derived miR-21, when phagocytosed by macrophages, enable a shift towards a proinflammatory M1 phenotype (Simeoli et al., 2017). In vivo intrathecal administration of miR-21 antagomir, to mice following SNI injury led to prevention of mechanical hypersensitivity and reduced inflammatory macrophage presence in the lumbar DRG (Simeoli et al., 2017).

Together, Simeoli et al., (2017) show that following neuronal activation, release of neuronal-derived exosomes containing miR-21-5p can alter DRG macrophage phenotype, which are more likely to release pro-nociceptive cytokines. Subsequently, once released in the DRG, these cytokines are known to act on nociceptors causing peripheral sensitization and enhanced nociceptive signalling. This study highlights an important role for miRs in facilitating neuron-macrophage communication and nociceptive signalling in the periphery.
Neuronal miRNA action in inflammatory pain

miRNAs can modulate nociceptive signalling via regulation of downstream intracellular genes targets in neurons. In addition, it has been shown that miRNAs can also directly activate sensory neurons (Park et al., 2014).

Toll-like receptor 7 (TLR7) is expressed by small diameter nociceptive DRG neurons. The exposure of dissociated DRG neurons to miRNA lethal-7b (let-7b), induces robust inward currents in small-diameter DRG neurons in a TLR7-dependant manner. This inward current is abolished in TRPA1−/− mice. Moreover, let-7b application can evoke action potentials in DRG neurons, which are eliminated in TLR7-deficient neurons. These results indicate that let-7b causes excitation of sensory neurons via activation of TLR7 and TRPA1; which are mediators of sensitization and nociceptive signalling (Montague-Cardoso and Malcangio, 2020). Knockdown of MyD88, an essential intracellular component of TLR7 signalling, did not affect the activation of TLR7+TRPA1+ neurons by exogenous let-7b, indicating let-7b mediated nociceptor activation is not mediated by intracellular signalling, and instead is likely facilitated by increased TRPA1-mediated ion influx (Park et al., 2014).

Formalin is known to excite sensory neurons by directly activating TRPA1, with DRG neurons from TRPA1 KO mice unresponsive to formalin (Mcnamara et al., 2007). In the formalin model of inflammatory pain, inhibition of endogenous let-7b release reduces TRPA1-mediated inward currents in DRG neurons, following intraplantar formalin injection. Park et al. (2014) showed that intraplantar pre-treatment with let-7b inhibitor reduces formalin-induced spontaneous pain behaviour in both phases. This suggests a key role for endogenous let-7b in inflammatory nociceptive signalling. Moreover, intraplantar injection of let-7b produces rapid and transient spontaneous nocifensive behaviour lasting less than 5 minutes post-injection. Let-7b-mediated nocifensive behaviour was abolished in TLR7 knockout mice, reduced in Trpa1−/− or Myd88−/− mice and reduced by pre-treatment with a TRPA1 antagonist (Park et al., 2014). These findings suggest that through regulation of TRPA1 currents in nociceptors, extracellular miR-let-7b can mediate formalin-induced inflammatory pain (Park et al., 2014). As let-7b can also expressed by macrophages, neuro-immune communication via let-7b signalling may play a role in mediating inflammatory pain signalling (Chen et al., 2020).
5.1.5 Effects of specialised lipid mediators on miRNA signalling in models of inflammation

As well as being involved in mediating inflammation and inflammatory pain mechanisms, miRNAs have also been implicated in the resolution of inflammation, specifically the molecular mechanisms by which specialised pro-resolving lipid mediators control inflammation and promote resolution. Twelve hours following induction of zymosan-induced inflammation, peritoneal exudates displayed dysregulation of several miRNAs, namely miR-21, miR-146b, miR-208a, miR-203, miR-142–3p, miR-142–5p, miR-219, and miR-302d (Recchiuti et al., 2011). At the peak of zymosan-induced inflammation, both miR-21 and miR-208a were significantly up-regulated in peritoneal exudates. However, while miR-21 remained up-regulated up to 48 hours following induction, during resolution of inflammation, miR-208a expression gradually declined at 24 and 48 hr (Recchiuti et al., 2011). These results highlight that miRNA expression is temporally regulated during acute inflammation and resolution.

Intraperitoneal administration of pro-resolving lipid mediator resolvin-D1 (RvD1) (300ng/ mouse) led to significantly reduced leukocyte infiltration 12 hr post-zymosan injection. RvD1 also significantly up-regulated miR-146b and miR-219 in peritoneal exudates, while simultaneously reducing miR-208 and miR302d expression. Twelve hours post-zymosan induction, RvD1 induced significant upregulation of miR-21, miR-146b, and miR-219 (Recchiuti et al., 2011). Human macrophages overexpressing recombinant RvD1 receptors ALX/FPR2 or GPR32, highlighted that the same miRNAs were significantly regulated by RvD1. Although no significant changes were observed for miR-142 at 12 hr, miR-142–3p expression was significantly decreased at 24 and 48 h, during resolution of inflammation, suggesting that RvD1 temporally controls specific sets of miRNAs (Recchiuti et al., 2011). In silico analysis identified gene targets for RvD1-regulated miRNAs, highlighting that miR-146b targets inflammatory NF-ĸB signalling. As RvD1 also reduced nuclear translocation of NF-ĸB, these results indicate that resolvin-regulated specific miRNAs can target genes involved in promoting resolution.

Maresin-1 has also been suggested to exert its biological functions by regulating miRNA signalling (Jin et al., 2018). In the CIA model in DBA/1 mice, repeated intravenous MaR1 administration caused decreased pro-inflammatory TNFα, IFN-γ,
IL-1β and IL-6 cytokines in serum, as well as reduced IL-17. IL-17 is secreted by Th17 cells which promote inflammation by inducing the production of pro-inflammatory cytokines. Contrastingly, Treg cells mediate an anti-inflammatory response by secreting IL-10 and TGF-β, which are increased in CIA blood serum following MaR1 treatment. CD4+ T cells can differentiate into either anti-inflammatory Treg cells or pro-inflammatory Th17 cells. 49 days following CIA induction, the ratio of Treg/Th17 cells was elevated in the MaR1-treated mice in a dose-dependent manner, indicating a T-cell population shift towards anti-inflammatory signalling (Jin et al., 2018).

Naive CD4+ T-cells, isolated from peripheral blood mononuclear cells of RA patients, when treated with MaR1 exhibit downregulation of pro-inflammatory miR-146a and miR-155, while also demonstrating upregulation of miR-21. MiR-21 decreased the proportion of Th17 cells, while it increased the proportion of Treg cells. Moreover, miR-21 was associated with decreased IL-17 expression, while increasing IL-10 and TGF-β levels.

Together these results indicate MaR1 can influence Treg/Th17 balance in rheumatoid arthritis and promote resolution of inflammation via promotion of miR-21 expression and inhibition of pro-inflammatory miR-155 and miR-146 in T-cells. This opens up the possibility that MaR1 may exert pro-resolution effects observed in other immune cells, such as macrophages, via miRNA-mediated signalling mechanisms.

5.1.6 Aims

Thus far I have demonstrated that, following K/BxN serum transfer, hind paw mechanical hypersensitivity occurs dissociated from joint swelling and concomitant with increased inflammatory M1 macrophage presence in the DRG. MiRNA are known contribute to both pro-inflammatory and anti-inflammatory immune cell signalling, influencing pro-nociceptive and anti-nociceptive neuro-immune communication, respectively. In the K/BxN serum transfer model, it is possible that mechanical hypersensitivity either in the presence or absence of joint swelling is accompanied by dysregulation of DRG miRNA signalling.

I have shown that macrophage-derived lipid mediator, MaR1, can ameliorate mechanical hypersensitivity and increased DRG macrophage presence following
serum transfer. Moreover, MaR1’s anti-inflammatory and anti-nociceptive properties were observed in-vitro, in cultured macrophages and TRPV1-sensitive DRG neurons, respectively. Studies have shown that in T-cell populations MaR1 can exert anti-inflammatory action via miRNA signalling and associated modification of immune cell cytokine expression (Jin et al., 2018). As I have also observed in MaR1 induced reduction in pro-inflammatory cytokine signalling in macrophages it is worth investigating if, similar to Jin et al., (2018), miRNA mediated mechanisms are also involved in macrophage mediated pro-inflammatory signalling.

The aims of the present chapter were to:

- Examine miRNA expression profiles in lumbar DRG at timepoints where mechanical hypersensitivity occurs either in the presence (Day 5) or absence (Day 25) of joint swelling, following K/BxN serum transfer, in order to identify possible associated dysregulation of miRNA expression.
- Assess in cultured peritoneal macrophages challenged with LPS the expression of miRNAs previously associated with pro-nociceptive (miR-21-5p) and pro-inflammatory (miR-155) signalling, as well as miRs shown to be deregulated in K/BxN DRG.
- Investigate ability of MaR1 treatment to alter pro-inflammatory miR-155 signalling in cultured peritoneal macrophages
- Assess in cultured DRG neurons exposed to capsaicin, the ability of MaR1 treatment to alter expression of miRNAs previously associated with pro-nociceptive (miR-21-5p) and pro-inflammatory (miR-155) signalling, as well as miRs shown to be deregulated in K/BxN DRG.
5.2 Materials and Methods

5.2.1 Animals

All experiments were performed on adult male C57BL/6 mice (Envigo, UK), aged between 10-12 weeks old and weighing approximately 25g. All mice were kept in the same living conditions as outlined in section 2.2.1. For experiments utilizing K/BxN serum transfer model, mice were administered either K/BxN or non-arthritogenic control serum using the same protocol as outlined in section 2.2.2

5.2.2 miRNA extraction and Real time-qPCR

5.2.2.1 miRNA extraction from DRG tissue

At day 5 and day 25 following serum transfer, mice were deeply anaesthetised with sodium pentobarbital (60mg/300µl/mouse i.p.) (Euthasol; Merial). L3, 4 and L5 dorsal root ganglia were quickly dissected bilaterally, immediately snap frozen and stored at -80°C for later processing. After thawing tissue, the sample were homogenised using a pipette. Thereafter, RNA was extracted by resuspension of sample in lysis buffer (0.5% 2-Mercaptoethanol; 50% Thiocyanic acid, compound with guanidine (1:1)) (Invitrogen) followed by acid phenol extraction. Small RNA-enriched fractions were then isolated from total RNA using mirVana miRNA Isolation Kit (Invitrogen). After purification, RNA was eluted using RNase-free water. Purity and concentration of RNA samples were then estimated using Nano-Drop ND-100 Spectrophotometer (ThermoFisher Scientific); establishing that nucleic acid samples were sufficiently pure for analysis if 260/280 nucleic acid ratio was above 1.8.

After concentration of small RNA samples was determined, each small RNA sample was diluted to 5 ng/µl using nuclease-free water, and cDNA synthesised using the miRCURY LNA™ Universal cDNA Synthesis kit II (Qiagen) according to manufacturer’s protocol. Samples were taken as sufficiently pure for analysis if 260/280 nucleic acid ratio was above 1.8.
5.2.2.2 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reactions (RT qPCR) for all miRNAs were amplified with a LightCycler 480 (Roche) using ExiLENT SYBR® Green master mix (Qiagen). Using microRNA LNA protocol (Qiagen), Lightcycler 480 was programmed as follows: 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 10 s and annealing at 60°C for 1 min at a ramp rate 1.6°C/s.

All primers were purchased from Qiagen and noted in Table 5.1. Samples were run in duplicate, then averaged across a single sample. The relative quantities of miRNA were calculated using the 2−ΔΔCT method, where CT represents the threshold cycle, and normalised to artificial spiked-in controls; uniSp4 and UniSp6 (Qiagen).

<table>
<thead>
<tr>
<th>ID-miRBase Version 19.0</th>
<th>Mature sequence 5’-3’</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmu-miR-21a-5p</td>
<td>UACUUAUCAGACUGAUGUUGA</td>
<td>MIMAT0000530</td>
</tr>
<tr>
<td>mmu-miR-155-5p</td>
<td>UUAAUGCUAUUGUGAUAGGGGU</td>
<td>MIMAT0000165</td>
</tr>
<tr>
<td>mmu-miR-706</td>
<td>AGAGAAACCCUGUCUCAAAAAA</td>
<td>MIMAT0003496</td>
</tr>
<tr>
<td>mmu-miR-709</td>
<td>GGAGGCAGAGGCAGGAGGA</td>
<td>MIMAT0003499</td>
</tr>
</tbody>
</table>

Table 5.1: Sequences of miRNAs analysed used during real-time PCR. miRNA sequences were provided by Qiagen.
5.2.3 miRNA microarray and in-silico gene prediction analysis

5.2.3.1 Small RNA preparation for miRNA microarray
On days 5 and 25 following serum transfer, when preparing small RNA for microarray analysis L4 and L5 DRGs were freshly dissected bilaterally and briefly washed in PBS, before being snap frozen in liquid nitrogen and stored at -80°C. DRG tissue samples were then thawed and small RNA extracted using mirVana miRNA Isolation Kit (ThermoFisher). After isolation of small RNA isolation, RNA concentration was calculated for each sample using Bioanalyzer to determine suitability of samples for microarray analysis. Subsequently, miRNA microarray analysis was carried out on DRG-tissue derived small RNA samples using ‘GeneChip™ miRNA 4.0 Array’ (ThermoFisher).

5.2.3.2 miRNA microarray data analysis
Pre-processed data generated from ‘GeneChip™ miRNA 4.0 Array’ were processed and analysed using Transcriptome Analysis Console Software (eTAC 4.0; ThermoFisher). On submission of pre-processed CEL data files, TAC 4.0 software was used to produce data displaying all significant expression changes of known murine miRNAs (3195 genes) between our groups; control serum treated day 5 (C5), K/BxN serum treated day 5 (K5), control serum treated day 25 (C25) and K/BxN serum treated day 25 (K25). Our desired threshold for screening only dysregulated miRNAs were fold changes >2 with P-value <0.05. After which, TAC 4.0 software was used to generate analyses between groups include: Principal component analysis, volcano plots, hierarchical clustering, and Venn diagrams. TAC 4.0 assigned miRNA ID’s were cross referenced against NetAffx database (ThermoFisher) to provide identification of dysregulated miRNAs.

5.2.3.3 In-silico prediction of miRNA gene targets
miRNA gene targets were predicted based on miRNA prediction database results five distinct resources: TargetScan, microcosm (miRanda), DIANA-microT, miRDB and miRTarBase. These tools utilise a combination of machine-learning techniques, interaction energy analysis and statistical tests to minimize false positives, in addition
to evidence from published experimental data confirming expression of gene targets. miRTarBase is the only manually constructed database, which contains solely experimentally proven gene sets.

Custom made Python scripts were created to integrate and analyse the data obtained from above mentioned database sources and genes were extracted which were seen in multiple miRNA databases. From inputted gene targets, functions within the python script returned the number of database hits for each gene.

5.2.3.4 Data mining for shared miRNA gene targets

For all 15 dysregulated miRNAs in K/BxN Day 25 DRGs compared to Control Day 25, respective targets were collected using miRDB (Version 5.0). miRDB database was used as this was the only miRNA database which contained data for all 15 dysregulated miRNAs.

Starting with initial miRDB-assigned predicted gene targets for each of the 15 dysregulated miRNAs, custom made python scripts were created to integrate and analyse the data. This created a single dataframe containing information from every miRNA search displaying ['miRNA Name'], ['Gene Symbol'] and ['Target Score'] for each predicted gene.

All returned predicted targets in miRDB were assigned target prediction scores between 50 – 100 by the database. These scores were assigned by a computational target prediction algorithm. The higher the score, the more confidence miRDB has in validity of the prediction. The authors of miRDB software recommend that predicted targets with prediction score > 80 are the most likely binding targets [http://mirdb.org/faq.html#How_to_interpret_the_target_prediction_score; accessed May 2020]. Therefore, only genes with prediction scores of 80 and above were taken forward for subsequent analyses.

A function was then created, returning the number of shared predicted genes between each of the 15 dysregulated miRNAs. Shared genes could then be searched and identified within the database. These data were visualised using the python library NetworkX (NetworkX developers) to create a network graph, where nodes represent dysregulated miRNAs and edges connecting nodes represent shared predicted gene
targets from miRDB search. Darker connections indicate a greater number of shared predicted gene targets.

5.2.4 Primary Culture of Peritoneal Macrophages

Peritoneal macrophage cultures were prepared using the exact methods specified in section 4.2.2. In brief, macrophages collected via lavage of the peritoneal cavity and plated in macrophage medium on a 12 well plate; 1 x10⁶ cells per well. Cells were then placed in an incubator to adhere overnight at 37°C.

For experiments assessing effect of varying LPS concentrations on mRNA and miRNA expression, macrophages were then incubated for 3 hr with 1, 10 or 100 ng/ml lipopolysaccharide from Escherichia coli (O111:B4; Sigma-Aldrich) (LPS) in macrophage medium. Controls were incubated in macrophage medium alone.

For subsequent experiments assessing effects of MaR1 on miRNA expression, following 100 ng/ml LPS for 3 hr, either 3 ng/ml MaR1 or macrophage medium vehicle was added to cells for an additional 5 hours. From samples containing 1x10⁶ cells/well, total and small RNA were isolated as previously described in section 4.2.2.

Small RNA samples were then diluted to 5 ng/µl using nuclease-free water and cDNA synthesised using the miRCURY LNA™ Universal cDNA Synthesis kit II (Qiagen) according to manufacturer’s protocol. miRNA expression levels were then detected by quantitative polymerase chain reaction (RT qPCR).

mRNA expression levels were then detected by quantitative polymerase chain reaction (RT qPCR).

5.2.5 Preparation of dorsal root ganglia neuronal cell cultures

From male C57BL/6 mice, all DRG were dissected aseptically and placed in Hibernate A (Gibco; Thermo-Fisher) supplemented with 0.5 mM L-Glutamine (Gibco; Thermo-Fisher) and 2% B-27 Supplement (50X) (Gibco; Thermo-Fisher). Dorsal root ganglia were then dissociated in 2 mg/ml papain in Hank’s balanced salt solution (HBSS) for
30 min and placed in incubator (37°C and 5% CO₂). After incubation, cell suspension was centrifuged 800 rpm for 1 minute. Supernatant is then removed and replaced with 2.5 mg/ml collagenase in HBSS. Cells are then incubated for a further 30 min at 37°C. After incubation, cell suspension is centrifuged 800 rpm for 1 minute. Cells were then triturated and resuspended in Neurobasal A (Gibco; Thermo-Fisher) supplemented with 0.5 mM L-Glutamine (Gibco; Thermo-Fisher) and 2% B-27 Supplement (50X) (Gibco; Thermo-Fisher).

For qPCR experiments neuronal cells were plated on 12-well plates, with 10,000 cells/well. Cell were then placed in an incubator to adhere overnight at 37°C. For subsequent experiments assessing effects of MaR1 on mRNA and miRNA expression, following 1μM capsaicin exposure for 3 hr, either 3 ng/ml MaR1 or macrophage medium vehicle was added to neurons for an additional 5 hours. Culture media were then removed, with cell lysates obtained using lysis buffer provided by mirVana miRNA Isolation Kit (0.5% 2-Mercaptoethanol; 50% Thiocyanic acid, compound with guanidine (1:1)) (Invitrogen) followed by acid phenol extraction. Total and small RNA were isolated as previously described in section 2.2.6 and 4.2.3.

5.2.6 Data and Statistics

All data are presented as means ±SEM. For all experiments, differences between means are considered as statistically significant when P<0.05. All statistical analysis was conducted using GraphPad Prism (v8.0.0; GraphPad Software, USA). Multiple comparison of data used one-way ANOVA followed by post-hoc Tukey test, except for experiments assessing LPS concentration dependant changes to miRNA expression where post-hoc Dunnett test was used to comparing all results directly to control group.

For in silico analysis of miRNA expression all relevant statistical testing was carried out using Transcriptome Analysis Console Software (TAC 4.0; ThermoFisher) as outlined above.
5.3 Results

5.3.1 Dysregulation of miRNA expression in K/BxN serum transfer arthritis

Although miRNAs have been implicated in modulating clinical disease progression in K/BxN-induced arthritic disease progression (Bluml et al., 2011), little is known regarding which miRNAs are altered following passive immunization, at timepoints when mechanical hypersensitivity occurs either associated or dissociated from joint swelling. Therefore, I explored the possibility that distinct miRNA expression profiles may be seen in the DRG following K/BxN serum transfer; during hyperalgesia associated with peak joint swelling (day 5) and when persistent hyperalgesia is observed in the absence of joint swelling (day 25).

To investigate miRNA expression in DRG following K/BxN serum transfer, I utilised a Genechip miRNA array allowing for the analysis of 3195 known murine small RNAs in samples derived from lumbar DRG tissue. As expected, control serum treated mice displayed very similar miRNA profiles at both 5 and 25 days after serum transfer (Figure 5.3A), with only 1 differentially expressed small non-coding RNA detected. These results indicate that control serum administration has very little effect on miRNA expression over the time course of our experiment. Interestingly, 5 days after K/BxN serum transfer, from all analysed small RNAs I observed differential expression of only four: three upregulated and one downregulated small RNA following K/BxN serum transfer (Figure 5.3A, B). Whereas, 25 days after serum transfer, 25 small RNAs were shown to be differentially expressed; of which 20 small RNAs were upregulated and 5 small RNAs downregulated in K/BxN DRG as compared to same day controls (Figure 5.3A, B). Interestingly, of the 28 differentially expressed small RNAs at either 5 or 25 days after K/BxN serum transfer, relative to same day controls, only miR-190a-3p was shown to be upregulated in both groups relative to same day controls, with the majority of DRG miRNA expression changes exclusive to K/BxN animals 25 days after serum transfer (Figure 5.3B).

This evidence indicates differing patterns of miRNA expression in DRG after K/BxN serum transfer, with largely different miRNAs associated with persistent hyperalgesia in the presence or absence of joint swelling.
Figure 5.3: miRNA array reveals greatest differential miRNA expression in lumbar DRG 25 days after K/BxN serum transfer

(A) GeneChip™ miRNA array analyses displaying the number of separate differentially regulated miRNAs in lumbar DRG both 5 days (K5) and 25 days (K25) after K/BxN serum transfer as compared to controls and day 5 and 25; C5 and C25 respectively. From total number of differentially expressed miRNAs between two groups (grey), data is subdivided into number of downregulated (green) and upregulated (red) genes. (B) Venn diagram displaying number of differentially expressed miRNAs in DRG both 5 days (red) and 25 days (blue) after K/BxN serum transfer, as compared to same day controls. Number of miRNAs differentially expressed at both timepoints shown in purple intersection.
5.3.2 Dysregulation of miR-706 and miR-709 during persistent joint pain dissociated from joint swelling

Thus far our findings highlight that after K/BxN serum transfer, the most significant small RNA dysregulation occurs in DRG at timepoints where persistent pain is observed in the absence of joint swelling (Day 25). Therefore, I next looked to further clarify differences in miRNA expression in the DRG at day 25 after serum transfer. Based on data derived from our miRNA microarray, I identified dysregulation of 15 known miRNAs in DRG 25 days after K/BxN serum transfer; 10 miRNAs downregulated and 5 upregulated miRNAs respective to same day controls (Figure 5.3A). Although 25 days after K/BxN serum transfer all 15 miRNAs displayed at least 2-fold change as compared to controls, miR-706 and miR-709 were shown to be the most dysregulated. miR-709 was observed at 10 times lower levels in K/BxN DRG 25 days after serum transfer, whereas relative to same day controls, an 8-fold increase in miR-706 expression was seen in K/BxN DRG 25 days after serum transfer (Figure 5.4A).

It is hypothesised that miRNAs have the ability to work together for combinatorial modulation of gene expression (Cursons et al., 2018). Therefore, I next looked to assess the relationship between our prospective dysregulated miRNAs, using in-silico analysis of predicted miRNA gene targets. MiRDB-assigned predicted gene targets for each dysregulated miRNA were compared and a network analysis created highlighting shared predicted gene targets between miRNAs (Figure 5.4B). These analyses highlighted few commonly predicted genes between our miRNAs, with the unsurprising exception of miR-3473b and miR3473e (Figure 5.4B) which also share very similar gene sequences. These results indicate that if any combinatorial modulation of gene expression were to occur in K/BxN serum transfer model it would most likely be between miR-3473b and miR3473e, both of which were downregulated in DRG 25 days following serum transfer.

In order to validate the findings and subsequent analysis from the GeneChip miRNA array, qPCR was performed on separate samples derived from lumbar DRG tissue (L4 and L5) extracted 25 days after either K/BxN or control serum transfer. Unlike our microarray findings, from qPCR experiments I did not observe changes in expression of either miR-3473b (1.42 ±0.12) or miR-3473e (1.75 ±0.27), relative to respective
controls (1.95 ±0.36; 1.96 ±0.26) 25 days after K/BxN serum transfer (Figure 5.4C, D). However, comparable to our miRNA array findings, I observed upregulation of miR-706 (1.43 ±0.13) and concurrent downregulation of miR-709 (0.69 ±0.05) in DRG 25 days after K/BxN serum transfer when compared to respective controls (0.24 ±0.02; 1.42 ±0.10). Interestingly, when I conducted subsequent in-silico analysis assessing predicted miRNA gene targets I found that the gene encoding FGFR1, a protein present in the DRG known to induce mechanical hypersensitivity (Andres et al., 2013), may be a possible target miR-709.

Together these results suggest dysregulation of miR-706 and miR-709 in the DRG, is an observation unique to timepoints in K/BxN serum transfer model where persistent mechanical hypersensitivity is observed in the absence of overt joint swelling. miRNA-dependent modulation may provide a possible mechanism for this heightened nociceptive signalling to be explored in the future.
Figure 5.4: Dysregulation of miR-706 and miR-709 observed in lumbar DRG after K/BxN serum transfer at timepoints where heightened nociceptive responses are observed in the absence of joint swelling

(A) GeneChip™ miRNA Array analyses displaying lumbar DRG expression of identified dysregulated miRNAs 25 days after K/BxN serum transfer as compared to same day controls. (B) Network analysis of miRNAs either upregulated (red node) or downregulated (blue node) in DRG 25 days after K/BxN serum transfer. Edges connecting miRNAs represent the strength of transcriptional relationship, as defined by number of shared predicted gene targets taken from miRDB database. Darker connections indicate a greater number of shared predicted gene targets. (C-F) qPCR validation of miRNA expression in DRG 25 days after K/BxN serum transfer as compared to same day controls. * P<0.05, Student’s t-test. Data are expressed as mean ± SEM; n = 8-10 mice per group
5.3.3 Pro-inflammatory miR-155 expression is increased in peritoneal macrophages following exposure to high concentrations of LPS

miRNAs are post-transcriptional molecules which can regulate mechanisms associated with both inflammatory and nociceptive signalling leading to modulation of cytokine production (Chen et al., 2020). Our lab has shown that following nerve injury upregulation of miR-21-5p occurs in the DRG. Moreover, I previously have observed dysregulation of miR-706 and miR-709 in the K/BxN DRG, at timepoints associated with hind paw allodynia. Macrophages, present in the DRG, are known to communicate with neurons to maintain chronic inflammatory and nociceptive states (Simeoli et al., 2017). Therefore, I investigated if following challenge with pro-inflammatory LPS, altered miRNA signalling is observed in peritoneal macrophages.

Firstly, using qPCR I assessed expression of miR-706 and miR-709, both shown to be dysregulated in DRG 25 days after K/BxN serum transfer. No significant change was seen at all LPS concentrations tested (1-100ng/ml). Although there was a trend towards greater miR-706 expression at greater LPS concentrations (100ng/ml LPS: 3.94 ± 0.85) as compared to controls (1.29 ± 0.12) (Figure 5.5A). Likewise, miR-709 expression in peritoneal macrophages was not significantly altered at any concentration of LPS tested (Figure 5.5B). miR-21-5p expression was also unchanged in peritoneal macrophages following challenge with LPS at any concentration (Figure 5.5C). However following LPS stimulation at 10 ng/ml (15.94 ± 3.70) and 100ng/ml (15.64 ± 1.95), there was increased expression of pro-inflammatory regulator miR-155 compared to controls (1.17 ± 0.06) (Figure 5.5D). This change in miR-155 expression was not observed when LPS challenge was given at lower 1ng/ml concentration.

RT-qPCR experiments can be used to effectively quantify the levels of specific markers within a sample. The quantification of such markers is dependent upon effective detection of signal. All miRNAs investigated thus far are known to be present in DRG. However, as the DRG contains several different cell types, these miRNAs may not be native to macrophages. Therefore, for each of the miRNAs assessed, I investigated the average number of PCR cycles needed to detect a signal in our peritoneal macrophage derived samples. Interestingly, miR-706, miR-709 and miR-21-5p were all detected at levels below certainty real sample detection, indicating these miRs may not be present in peritoneal macrophages. Little, however, is known...
regarding miR-706 and miR-709 cell type specificity in the DRG. In contrast, miR-155 was present well above the reliably detectable threshold (30.0 ± 0.4 Average Ct Value) (Figure 5.5E). Therefore, our results indicate that expression of macrophage-derived pro-inflammatory signalling marker miR-155 is upregulated in peritoneal macrophages challenged with higher concentrations of LPS.

5.3.4 MaR1 attenuates pro-inflammatory miR-155 signalling in cultured macrophages following challenge with LPS

There is a marked increase in pro-inflammatory miR-155 signalling in macrophage cultures following challenge with LPS. MaR1 is known to skew bone-derived macrophages away from a M1 pro-inflammatory phenotype towards a M2 pro-resolution phenotype. Using qPCR, I show that in LPS challenged macrophages, MaR1 treatment also led to a decrease in expression of pro-inflammatory miR-155 (Figure 5.6).
Figure 5.5: Increased pro-inflammatory miR-155 expression in macrophages challenged with high concentrations of lipopolysaccharide

Cultured peritoneal macrophages incubated with or without varying concentrations of lipopolysaccharide (LPS) (1, 10, 100 ng/ml for 3 hr). (A-D) Quantification of miRNAs associated with heightened inflammatory and nociceptive states assessed by qPCR. *P < 0.05, **P < 0.01 compared with medium only controls; one-way ANOVA, post hoc Dunnett. Data are mean ±SEM; n = 4 experiments (E) Average PCR cycle number at which a detectable signal is observed from macrophage samples for all miRNAs and unisp6 control primers. Maximum number of 40 PCR cycles, with high uncertainty of real sample detection above 35 PCR cycles. Data are mean ±SEM; n = 4 experiments
Figure 5.6: MaR1 reduces expression of pro-inflammatory miR-155 in macrophages challenged with lipopolysaccharide

Cultured peritoneal macrophages incubated with or without lipopolysaccharide (LPS) (100 ng/ml for 3 hr) followed by MaR1 (3ng/ml) or vehicle for 5 hr. Quantification of mRNA expression for pro-inflammatory miRNA marker miR-155 was assessed by qPCR. ***P < 0.01 compared with medium/vehicle controls, # P < 0.05; one-way ANOVA, post hoc Tukey. Data are mean ±SEM; n = 5 experiments
5.3.5 Capsaicin-induced upregulation of miR-706 and miR-21 is unaffected by MaR1 treatment in TRPV1-sensitive DRG neurons

In response to inflammation and injury, alteration in miRNA expression can occur in association with heightened nociceptive and inflammatory signalling from DRG primary neurons.

Hindpaw allodynia in the absence of joint pain is associated with dysregulation of miRNA in DRG, following K/BxN serum transfer. Moreover, previous work from our lab highlights that in the DRG miRNA-mediated neuroimmune signalling occurs following activation of TRPV1-sensitive sensory neurons in culture (Simeoli et al., 2017). MaR1 treatment reduces capsaicin-induced intracellular calcium responses in TRPV1-sensitive DRG neurons (Figure 4.8). Therefore, I next investigated, using qPCR, whether MaR1 can attenuate expression of miRNAs following capsaicin exposure.

Following capsaicin exposure cultured DRG neurons exhibited greater expression of miR-706 (3.19 ± 0.51), as compared to cells exposed to medium only (1.04 ± 0.02) (Figure 5.7A). However, no change was observed in miR-709 expression between capsaicin and medium treated control (Cap Veh: 0.64 ± 0.12; Medium Veh: 1.01 ± 0.00) (Figure 5.7B). As expected, neuronal derived miR-21-5p expression was also elevated in DRG neurons following capsaicin exposure (Cap Veh: 3.10 ± 0.39; Medium Veh: 1.12 ± 0.01) (Figure 5.7C). No significant change was observed in pro-inflammatory miR-155 in DRG following capsaicin exposure (Cap Veh: 2.47 ± 0.97; Medium Veh: 1.15 ± 0.10) (Figure 5.7D). Interestingly, MaR1 had no significant effect on capsaicin-induced miRNA expression of miR-706 or miR-21-5p. Moreover, in unchallenged neurons, MaR1 did not influence expression of miR-706, miR-709, miR 21-5p or miR-155 (Figure 5.7A-D).

As miR-155 is known to be primarily immune-cell derived, it is unsurprising that assessment of PCR cycle number required for a detectable signal indicated that miR-155 is detected at levels below certainty of real sample detection in DRG neurons (39.00 ± 0.60 Average Ct Cycle) (Figure 5.7E).

Our results show MaR1 to be ineffective at modulating TRPV1-mediated upregulation of neuronal miRNAs. These results indicate that anti-nociceptive action of MaR1 on DRG neurons is unlikely to be mediated via modulation of miRNA expression.
Figure 5.7: MaR1 treatment does not affect capsaicin induced upregulation of miR-706 and miR-21 in TRPV1 expressing DRG neurons

Cultured DRG neurons incubated with or without capsaicin (Cap) (1µM for 3 hr) followed by MaR1 (3ng/ml) or vehicle for 5 hr. (A-D) Quantification of miRNAs associated with heightened inflammatory and nociceptive states assessed by qPCR. *P < 0.05, **P < 0.01 compared with medium/vehicle controls; one-way ANOVA, post hoc Tukey. Data are mean ±SEM; n = 4 experiments (E) Average PCR cycle number at which a detectable signal is observed from DRG neuron samples for all miRNAs and unisp6 control primers. Maximum number of 40 PCR cycles, with high uncertainty of real sample detection above 35 PCR cycles. Data are mean ±SEM; n = 4 experiments
5.4 Discussion

In the present chapter, I provide evidence that alongside mechanical hyperalgesia in the K/BxN serum transfer model of inflammatory arthritis there is a dysregulation of miRNAs in the DRG, with most prominent miRNA differential expression, when mechanical hyperalgesia presents in the absence of joint swelling; characterised by an up-regulation of miR-706 and down-regulation of miR-709 in the K/BxN DRG. In peritoneal macrophages challenged with LPS, although little evidence of miR-706 nor miR-709 expression is found, I observed LPS-induced increases in pro-inflammatory miR-155 expression which were attenuated by MaR1 treatment. In dissociated DRG neurons, exposure to capsaicin induced an upregulation of pro-nociceptive miR-21, as well as promoting increased miR-706 expression. This capsaicin-mediated increase in miR-21 and miR-706 expression, in the DRG, was not altered following MaR1 treatment indicating that the anti-nociceptive action of MaR1 is not mediated by modulation of neuronal-derived miRNA. Instead, MaR1-mediated attenuation of pro-inflammatory macrophage miRNA signalling likely diminishes macrophage-mediated pro-inflammatory mediator release to neurons, thereby reducing subsequent nociceptive neuronal sensitization.

Regulation of nociceptive and inflammatory signalling are both known to occur via miRNA-mediated mechanisms. In neurons, miRNA signalling can modulate nociceptive signalling via regulation of downstream intracellular genes targets, as well as directly sensitizing nociceptors (Park et al., 2014). Whereas, miRNA-mediated modulation of immune cell gene expression regulates the initiation, spread and eventual resolution of inflammation (Tahamtan et al., 2018).

In the K/BxN serum transfer model I have previously demonstrated that during arthritic joint swelling persistent mechanical hyperalgesia occurs which outlasts joint swelling. Following passive immunization, studies observe there to be dysregulation of over 100 genes in samples collectively obtained from peripheral blood leukocytes, ankle tissue, and synovial fluid of IgG immunized mice as compared to non-arthritic controls; with 17 genes consistently dysregulated during initiation (day 3), peak (day 7) and resolution phase (day 18) of joint swelling (Wang et al., 2017). Moreover, 200 miRNAs were identified to target these 17 consistently dysregulated genes (Wang et al., 2017). Specifically, in the K/BxN DRG, I discovered differential expression of 28 miRNAs with
the vast majority of miRNA dysregulation occurring when hyperalgesia persists in the absence of joint swelling. As I have established that there is heightened inflammatory and nociceptive signalling in the DRG at the timepoint, these findings suggest that mechanisms which are related to hyperalgesia in the absence of joint swelling may involve dysregulation of miRNAs in the peripheral nervous system. As demonstrated in chapter 2, increased pro-inflammatory macrophage presence in K/BxN DRG is observed when hyperalgesia presents in absence of joint swelling. As our experiments analysed all tissue types within the DRG, it is possible that this dysregulation of specific miRNAs in K/BxN DRG is mediated by these newly present pro-inflammatory macrophages. However, this hypothesis requires further investigation assessing miRNA profiles of isolated DRG macrophages following passive immunization when hyperalgesia is observed in the absence of joint swelling.

Co-expressed miRNAs have the ability to regulate gene expression in a combinatorial manner (Cursons et al., 2018). However, network analysis of shared gene targets between our dysregulated K/BxN miRNAs indicates very little shared targeting in the K/BxN DRG when hyperalgesia presents in the absence of joint swelling. miRNAs are known to have target multiple genes. Therefore, in the K/BxN DRG, rather than miRNAs acting in concert to target a single gene, individual miRNAs may act on multiple functionally related target genes. Therefore, instead of assessing combinatorial targeting effects between miRNAs, alternatively, a future experimental approach may be to conduct a pathway analysis of each dysregulated miRNA’s gene targets. In this manner, I can help elucidate if miRNA signalling in the K/BxN DRG could target a specific metabolic pathway and provide a greater understanding of the possible role K/BxN DRG miRNA dysregulation plays relating to concurrent persistent hyperalgesia dissociated from joint swelling.

Following passive immunization where hyperalgesia outlasts joint swelling, of the 24 dysregulated miRNAs observed miR-706 and miR-709 were the most altered in the DRG. Although no studies have directly assessed the role of miR-706 in models of pain or inflammation, miR-706 has recently been shown to promote cell death via the targeting of anti-apoptotic caspase activity and apoptosis inhibitor 1 (CAAP1) (Wang et al., 2020a). It has been reported that miR-706-5p affects the expression of cell division cycle associated 4 gene (Cdca4), a gene important for mitotic cell division. Moreover, the authors identified miR-706-5p as targeting the IL-17 gene (Al-Ghezi et
IL-17 has been shown to be pro-nociceptive and sensitize joint nociceptors facilitating mechanical allodynia. In the AIA model, neutralization of IL-17 is antinociceptive (Richter et al., 2012). However, in the K/BxN DRG I observe an increase in miR-706 in K/BxN DRG when hyperalgesia occurs without joint swelling. Although traditionally shown to dampen expression of mRNA binding targets, miRNAs are also known on occasion to increase expression of target genes (Tahamtan et al., 2018, Vasudevan et al., 2007). Further experimentation is required to elucidate the role of miR-706 and possible effect on IL-17 expression in K/BxN DRG.

Alongside upregulation of miR-706, I observe a downregulation of miR-709 in the K/BxN DRG when hyperalgesia occurs in the absence of joint swelling. miRNA-targeting databases, miRDB and TargetScan, identify FGFR1 to be a possible gene target for miR-709. FGFR1 is a cell surface receptor expressed on neurons in lumbar dorsal root ganglia and is activated by the wound-healing factor, basic fibroblast growth factor (bFGF) (Andres et al., 2013). Studies show that bFGF binding to FGFR1 induces phosphorylation of Erk1/2 on cultured nociceptive neurons and subsequently increases Na⁺1.8 channel current density in DRG neurons (Andres et al., 2013). Moreover, when injected intradermally bFGF induces mechanical hypersensitivity highlighting the pro-nociceptive ability of FGFR1-mediated signalling. In the adjuvant-induced arthritis model increased levels of bFGF are observed in inflamed joints (Qu et al., 1994). Taken together with our findings, a downregulation of miR-709 in the K/BxN DRG may result in an exacerbation of FGFR1 expression in nociceptive neurons. Greater FGFR1 receptor availability for bFGF ligands may lead to bFGF-induced neuronal sensitization in K/BxN DRG contributing to maintained nociceptive states. This hypothesis requires validation, by first identifying if in the K/BxN DRG downregulation of miR-709 occurs concomitant to upregulation of FGFR1 expression. Moreover, it is necessary that the FGFR1 ligand, bFGF, is also observed in the K/BxN DRG in order to form a mechanistic link underpinning FGFR1 receptor activation on neurons and possible nociceptor sensitization.

The DRG is a mixed cell population consisting of both neurons and macrophages. Pro-inflammatory miRNA-mediated macrophage signalling to nociceptive neurons in the DRG facilitates the maintenance of chronic pain states (Simeoli et al., 2017). In peritoneal macrophages challenged with LPS, I did not observe miR-706 or miR-709 at acceptably detectable levels indicating that any alterations in miR-706 and miR-709
observed in K/BxN DRG are unlikely a result of macrophage-mediated mechanisms. However, in opposition, a study has suggested that miR-709 targets GSK-3β from peritoneal macrophages exposed to LPS (100ng/ml) (Li et al., 2016). The authors suggest miR-709 acts as a negative regulator of macrophage inflammatory responses by suppressing the production of pro-inflammatory cytokines such as TNFα, IL-6 and IL-1β (Li et al., 2016). Compared to the findings by Li et al. (2016), when assessing relative expression of miR-709 following LPS challenge, I observe a similar trend of dose dependant increased miR-709 expression in peritoneal macrophages challenged with LPS albeit at extremely low levels, below what I determined as a certainty for signal detection. Although both experiments utilized 100ng/ml LPS concentrations, Li et al. (2016) incubated peritoneal macrophages in LPS for 12 hours as opposed to our 3-hour incubation. Therefore, it is possible that this greater incubation time allowed for miR-709 expression to occur above detectable levels, however the authors do not state their criteria for determining real sample detection.

miR-155 is a key regulator of the inflammatory response in macrophages (O'connell et al., 2007). I observe following pro-inflammatory LPS exposure, peritoneal macrophages exhibit increased miR-155 expression. In agreement, bone marrow derived macrophages polarised towards a M1 phenotype using LPS and IFN-γ also demonstrate upregulation of miR-155 (Zhang et al., 2013). Ship1 and Socs1 are known miR-155 gene targets, whose expression levels are greater in miR-155−/− -derived macrophages (Tan et al., 2015). It has been proposed that miR-155 acts by dampening Ship1 and Socs1 mediated- PI3K-AKT signalling, which ultimately leads to an exacerbation of NF-κB activity and increased production of pro-inflammatory cytokines including IL-1β, IL-6 and TNFα (Tan et al., 2015). I observe that LPS-induced miR-155 expression is attenuated by treatment with the pro-resolving lipid mediator MaR1. In cultured bone-marrow derived macrophages, LPS stimulation induced evident NF-κB p65 nuclear translocation which can be significantly attenuated by co-incubation with MaR1 (10nM) (Yang et al., 2019). Moreover, in a model of inflammatory colitis, MaR1 has shown efficacy in reducing proinflammatory mediator release and reducing NF-B activation (Marcon et al., 2013). In addition, human THP-1 monocyte/macrophages stimulated with LPS, another DHA-derived lipid mediator, Resolvin D5, has also been shown to inhibit production of pro-inflammatory IL-6 via attenuation of NF-κB signalling indicating that reduction of immune-mediated NF-κB
signalling represents a shared anti-inflammatory mechanism between SPMs (Chun et al., 2020).

Taken together, these findings suggest that MaR1 exhibits anti-inflammatory action in macrophages by dampening pro-inflammatory miR-155 expression, which in turn reduces phosphorylation and nuclear translocation of NF-kB p65. Inhibition of macrophage intracellular miR-155 mediated mechanisms likely underpin MaR1-associated dulling of pro-inflammatory mediator expression, previously observed in chapter 4. Maresin-1 likely initiates miR-155 inhibitory action via cell surface receptor binding. As LGR6, has been identified as the MaR1 receptor, this hypothesis could be verified by assessing if, in LPS-challenged peritoneal macrophages, MaR1-mediated inhibition of MiR-155 expression is perturbed by LGR6-silencing.

miR-155 is identified as enriched within immune cell populations under inflammatory conditions. However, specifically within sensory neurons of the DRG, I do not observe detectable levels of miR-155. Although the attenuation of miR-155 signalling is known to reduce neuroinflammation and nociceptive signalling, these actions are attributed to immune-mediated miR expression (Tan et al., 2015). Therefore, in conjunction with our results, this indicates that although miR-155 expression is known to exacerbate nociceptive signalling in pain models, this is likely due to modulation of immune-mediated pro-inflammatory signalling, increased pro-inflammatory mediator release and subsequent neuronal sensitization, rather than direct neuronal-mediated miR-155 modulation in response to inflammatory insult.

I observe upregulated miR-706 in the K/BxN DRG during states of persistent hyperalgesia in the absence of joint swelling. However, currently little is known regarding the role of miR-706 in nociceptive signalling. I observe in dissociated DRG neurons exposed to capsaicin increased miR-706 expression. With little miR-706 expression in cultured macrophages, collectively these results suggest that miR-706 upregulation in the K/BxN DRG may be neuronal-mediated. Following passive immunization, concomitant to miR-706 upregulation in the DRG, persistent hyperalgesia is observed in the absence of joint swelling. Future experiments could consider if a functional association occurs between miR-706 in the K/BxN DRG and persistent nociceptive signalling.
In agreement with previous studies by Simeoli et al. (2017), I demonstrate an increased expression of miR-21 from TRPV1 sensitive sensory neurons exposed to capsaicin. However, this capsaicin-induced miR-21 expression is not attenuated by MaR1 indicating that anti-nociceptive action of MaR1 on DRG neurons may not be mediated via modulation of neuronal miRNA expression. Studies show following nociceptor activation, neuronal-derived miR-21 containing exosomes can be internalised by macrophages, promoting a M1 macrophage phenotype and associated pro-inflammatory mediator expression (Simeoli et al., 2017). We observe little miR-21 expression from peritoneal macrophages in culture. Therefore, rather than being macrophage-initiated, neuronal-derived miR-21 signalling may be necessary to enable pro-inflammatory macrophage signalling. With the knowledge that MaR1 diminishes pro-inflammatory macrophage signalling and neuronal-derived miR-21 induces a pro-inflammatory M1 macrophage phenotype, future experiments could investigate if pro-inflammatory signalling from peritoneal macrophages transfected with miR-21 mimic is attenuated by MaR1 treatment.

However, evidence suggests that MiR-21 immune-mediated signalling may not necessarily always promote pro-inflammatory states. Alternatively in naïve CD4+ T-cells, following MaR1 treatment, there is an induction of miR-21 expression which promotes differentiation of naïve T cells into Treg cells with an anti-inflammatory phenotype (Jin et al., 2018). Together these findings highlight that the function and role of miR-21, and indeed MaR1-mediated miR-21 modulation, is complex and likely varies dependent on cell populations and condition. In cultured macrophages, although I have not detected changes of miR-21 I have observed that MaR1 treatment reduces miR-155 expression in LPS-challenged macrophages. Similarly, Jin et al. (2018) observe MaR1-mediated inhibition of pro-inflammatory miR-155 and miR-146 in T-cells. These findings indicate that, as opposed to miR-21 regulation, inhibition of miR-155 expression represents a consistent mechanism by which MaR1 reduces pro-inflammatory intracellular signalling from immune cells.
5.4.1 Chapter Key Findings

In the present chapter I provide evidence that:

- Following passive immunization with K/BxN serum, dysregulation of miRNAs occurs in the K/BxN DRG which is most notable at timepoints when hind paw hyperalgesia persists beyond resolution of joint swelling and is characterised by:
  o Up-regulation of miR-706 expression
  o Down-regulation of miR-709 expression
- Increased expression of pro-inflammatory miR-155 is observed in peritoneal macrophages challenged with LPS, which is attenuated by treatment with MaR1
- Capsaicin-induced increased intracellular expression of miR-706 and miR-21-5p in TRPV1 sensitive dissociated DRG neurons is unaffected by MaR1 treatment

These data suggest that dysregulation of miR-706 and miR-709 signalling in the periphery may be associated with maintained hyperalgesia following passive immunization. MaR1 is known to have anti-nociceptive and anti-inflammatory properties acting on neurons and macrophages, respectively. Our data indicate that MaR1-mediated attenuation of pro-inflammatory macrophage signalling occurs via modulation of intracellular pro-inflammatory miR-155 signalling. Whereas MaR1-mediated direct anti-nociceptive actions in neurons unlikely involves modulation of neuronal miRNA signalling mechanisms.
Chapter 6:
General Discussion
6.1 Summary of experimental findings

The aim of the studies presented in this thesis were to investigate and explore the underlying inflammatory and nociceptive mechanisms contributing to arthritic joint pain occurring either in the presence, or absence, of overt joint swelling in order to identify possible novel therapeutic targets against RA-related joint pain. To this end, I utilized the mouse K/BxN serum-transfer model of inflammatory arthritis, as well as conducting in-vitro experimentation using dissociated DRG neurons and peritoneal macrophage cultures.

In the K/BxN serum transfer model, behavioural studies were conducted to ascertain the time course of transient joint swelling and persistent hind paw hyperalgesia following passive transfer. Thereafter, cellular and molecular markers associated with pain and inflammation were assessed both peripherally in lumbar DRG and centrally in the spinal cord. In addition, in the K/BxN DRG, I identified specialised pro-resolving mediator lipid (SPM) profiles and miRNA profiles, at timepoints where mechanical hyperalgesia occurs either in the presence or absence of joint swelling. Based on metabolipidomic profiling of lumbar DRG, I assessed the ability of restorative SPM treatment to modulate nocifensive behaviour, as well as pro-inflammatory and pro-resolution neuro-immune signalling in the DRG (PNS) and dorsal horn spinal cord (CNS). Moreover, through in-vitro studies, I further investigated the ability of lipid mediator treatment to modulate pro-nociceptive and pro-inflammatory signalling in dissociated DRG neurons and peritoneal macrophage cultures, respectively. The key findings of this thesis are:

1. Following passive immunization with K/BxN serum, mechanical hypersensitivity alongside joint swelling, is associated with a greater inflammatory response from peptidergic DRG neurons and increased superficial laminae dorsal horn cFos activation.

2. Persistent hyperalgesia that outlasts joint swelling occurs concomitant to greater macrophage presence in K/BxN DRG

3. Mechanical hyperalgesia alongside joint swelling is associated with greater pro-inflammatory PGD\textsubscript{2} levels in the K/BxN DRG. While persistent hyperalgesia in the absence of joint swelling occurs concurrent with reduced pro-resolution MaR1 levels in the K/BxN DRG.
4. Repeated systemic treatment with MaR1 either during or post-joint swelling leads to sustained reversal of hyperalgesia, reduced pro-inflammatory macrophage presence in lumbar DRG (PNS) and reduced dorsal horn microglial activation (CNS).

5. When applied to peritoneal macrophages challenged with LPS, MaR1 reduced pro-inflammatory cytokine (TNFα & NOS2) expression and miR-155, with no impact on anti-inflammatory signalling.

6. MaR1 application inhibits neuronal activation of DRG neurons exposed to capsaicin in a dose dependant manner, with MaR1’s action dependant on GPCR signalling.

7. Following passive immunization, dysregulation of miRNAs occurs in K/BxN DRG, where hyperalgesia in the absence of joint swelling is associated with up-regulation of miR-706 expression and down-regulation of miR-709 expression.

8. Following capsaicin exposure, DRG neurons exhibit increased miR-706 and miR-21 expression, which is unaffected by MaR1 treatment.

Together, evidence within this thesis demonstrates that in inflammatory arthritis mechanical hyperalgesia which outlasts joint swelling is associated with increased pro-inflammatory macrophage presence, miRNA dysregulation and reduced pro-resolution MaR1 levels in the DRG. Repeated, but not acute, systemic MaR1 treatment causes long-lasting anti-hyperalgesic actions, likely via reduction of macrophage infiltration in the periphery, modulation of inflammatory macrophage phenotype in the DRG and subsequently reduced pro-nociceptive neuro-immune communication in the spinal cord. In-vitro studies highlight the ability of MaR1 to reduce pro-inflammatory cytokine and miRNA signalling from macrophages, in addition to exerting direct anti-nociceptive action on sensory neurons, by inhibiting nociceptor activation. Further investigation elucidating the intracellular mechanisms by which MaR1 exerts anti-inflammatory and anti-nociceptive action in neurons and immune cells may lead to novel approaches for rheumatoid arthritis pain treatment.

6.2 Nociception and inflammation in arthritis-associated joint pain

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterised by chronic inflammation, joint destruction and chronic pain. Clinical symptoms associated with
rheumatoid arthritis can currently be controlled by biologic or nonbiologic disease-modifying antirheumatic drug treatment (DMARDs) (Mcwilliams and Walsh, 2017). These DMARDs often show efficacy in reducing joint inflammation, structural damage and intense pain related to RA flares. Although, during acute flares of the disease pain intensity is strongly associated with inflammation severity, prior to and following inflammatory disease suppression, pain and joint inflammation are often weakly associated, with RA patients still experiencing lingering unmanaged pain when joint swelling and inflammation are pharmacologically reduced (Taylor et al., 2010).

In an attempt to also treat RA-associated pain, DMARDs can be administered in conjunction with non-steroidal anti-inflammatory drugs (NSAIDs). However, prolonged NSAID use in the treatment of pain associated with RA is not a viable long-term therapy (Walsh and Mcwilliams, 2014), with chronic NSAID use showing limited efficacy in alleviating chronic pain as well as undesired side effects including increased risk of peptic ulcer disease (Russell, 2001). Similarly, alternative RA-associated pain therapies, such as tumour necrosis factor (TNF) blockers, also have serious health consequences increasing risk of upper respiratory tract infection (Alldred, 2001).

Therefore, an urgent need exists to discover novel effective therapeutics for the treatment of persistent pain in RA which occurs while joint inflammation is controlled. To this aim, a greater understanding is required of the complex mechanisms driving RA-associated pain.

For this purpose, I utilised the mouse K/BxN serum transfer model of inflammatory arthritis, whereby persistent nocifensive behaviours outlast transient joint swelling, recapitulating the dissociation between pain and inflammation seen in RA patients and allowing for the investigation of nociceptive signalling mechanisms, in both the PNS and CNS, which occur either in the presence or absence of joint swelling. In Chapter 2, in line with previous studies I demonstrate that following passive immunization, mechanical hyperalgesia develops which persists beyond the resolution of clinical signs of arthritis. Moreover, in line with clinical observations, although the instance of mechanical hyperalgesia is consistent following serum transfer, I do not observe clear correlation with inflammation severity. Additionally, I also confirm previous observations of increased microgliosis in the dorsal horn of the spinal cord from K/BxN serum treated mice, which occurs concomitant to hind paw hyperalgesia either in the presence or absence of joint swelling (Christianson et al., 2010).
Although cell bodies of peripheral nociceptors reside in the DRG, prior to our investigations, few studies had assessed alterations in peripheral nociceptive signalling away from the joint, in the K/BxN DRG. When hyperalgesia occurs concomitant to joint swelling, expectedly I provide evidence for increased CGRP expression in the K/BxN DRG. These results are indicative of peripheral sensitization of joint nociceptors, likely due to the presence of pro-inflammatory mediators present proximal to nociceptor terminals of the inflamed joint. Evidence suggests that immune complex (IC) formation of collagen type II protein complexes (CII-ICs) directly activates DRG neurons (Bersellini Farinotti et al., 2019) and requires the presence of neuronal Fc gamma receptors. Fc gamma receptors are also the target receptors for K/BxN-G6PI immune complexes. These immune complexes accumulate in the inflamed joint following serum transfer indicating that, following passive immunization, IC formation in the K/BxN joint also directly activates nociceptors. Moreover, studies have also highlighted that, following serum transfer, in the inflamed arthritic paws there are greater levels of pro-inflammatory lipid bioactive lipid mediators such as PGD$_2$, PGE$_2$ and LTB$_4$ (Norling et al., 2016). These pro-inflammatory lipid mediators also likely contribute to increased nerve terminal excitability and peripheral sensitization of sensory neurons. Together these findings indicate that following serum transfer, while there is significant joint swelling, several mechanisms are at play in the joint contributing to peripheral sensitization of nociceptors and subsequent increased neuronal activation and CGRP expression in the DRG (Figure 6.1).

It is not only input from nociceptive terminals at the joint which may contribute to hyperalgesia in association with joint swelling. Notably, lipid mediator profiling of K/BxN DRG demonstrated that hyperalgesia observed alongside joint swelling is coupled with increased PGD$_2$ levels in the DRG, away from the site of swelling where cell bodies of sensory neurons innervating the inflamed joint are present. PGD$_2$ is known to be pro-nociceptive, with the presence of PGD$_2$ receptors, DP1 and DP2, observed in the vast majority of DRG neurons (Sousa-Valente et al., 2018, Ebersberger et al., 2011). Therefore, I can hypothesise that increased PGD$_2$ in the K/BxN DRG contributes to the peripheral sensitization of nociceptors and hyperalgesia observed during joint swelling. Interestingly, I observe that persistent mechanical hyperalgesia, when outlasting joint swelling, no longer features elevated PGD$_2$ levels in the K/BxN DRG. These findings are in agreement with studies demonstrating that following passive immunization pharmacological blockade of COX1/2 is only an
effective analgesic target when hyperalgesia occurs alongside joint swelling, but ineffective against hyperalgesia which outlasts joint swelling (Park et al., 2016, Christianson et al., 2010). These results indicate that as joint swelling subsides, alternate PGD$_2$-independent DRG mechanisms contribute to maintained nociceptive signalling and persistent mechanical hypersensitivity. Collectively, our work and others highlight that following serum transfer peripheral sensitization of sensory neurons mediated by pro-inflammatory and pro-nociceptive mechanisms at both the joint and K/BxN DRG likely contribute to hyperalgesia which presents alongside joint swelling. C-fibres which convey nociceptive information and facilitate mechanical allodynia terminate in the superficial laminae of the spinal cord (Peirs et al., 2020). Therefore, with increased peripheral sensory neuron activation at this timepoint, it is unsurprising that I concomitantly observe increased microglial activation, centrally, in the K/BxN dorsal horn spinal cord. Other groups reported increased microgliosis when mechanical hypersensitivity presents alongside joint swelling (Christianson et al., 2010). These spinal cord microglia are likely activated in response to peripheral noxious input, facilitating pro-inflammatory neuro-immune signalling in the spinal cord, central sensitization and further maintenance of nociceptive signalling.

Weeks following serum transfer, monocyte/macrophages which had previously infiltrated the K/BxN joint, undergo a phenotypic switch from proinflammatory (M1) to pro-resolution (M2) phenotype (Misharin et al., 2014), likely leading to reduced pro-inflammatory mediator release to nociceptor terminals in the joint and diminished facilitation of nociceptor sensitization. However, at timepoints where mechanical hyperalgesia outlasts joint swelling our studies identify, away from the joint, increased presence of pro-inflammatory (M1) macrophages in the K/BxN DRG. These M1 macrophages likely produce pro-inflammatory mediators known to sensitize sensory neurons, such as IL-1β, TNFα and IL-6 (Pinho-Ribeiro et al., 2017, Chen et al., 2020). Moreover, studies have confirmed IL1R, IL-6R and TNFR1/2, the respective receptors for IL-1β, TNFα and IL-6, to be present on DRG neurons (Mailhot et al., 2020, Inglis et al., 2005, Von Banchet et al., 2005). Together with our results, this suggests that, following passive immunization, increased pro-inflammatory macrophage-mediated signalling in the DRG contributes to heightened nociceptive states in the absence of joint swelling (Figure 6.1). Our findings in the K/BxN DRG, provide insight into these observations in the CNS, which are likely facilitated by increased pro-inflammatory
neuro-immune communication in the DRG, subsequently increasing noxious input from peripheral sensory neurons to second order neurons, facilitating associated microgliosis in the superficial laminae. However, I cannot exclude the hypothesis that, following serum transfer, inflammatory states are associated with an increase in systemic cytokines, which can cross the blood-brain barrier and directly activate microglia in the CNS. In the CIA model, prior to clinical signs of arthritis mechanical hypersensitivity is associated with central sensitization, which is dependent upon microglial-mediated pro-inflammatory signalling in the spinal cord (Nieto et al., 2016). Although, within this thesis I have not investigated mechanisms of central sensitization in the K/BxN model, when hyperalgesia presents in the absence of joint swelling, it is probable that microglial-mediated pro-inflammatory signalling also occurs in the K/BxN spinal cord.

In addition to exacerbated pro-inflammatory neuro-immune communication in the K/BxN DRG, when hyperalgesia outlasts joint swelling, lipid mediator profiling highlighted that aberrant pro-resolution mechanisms also occur in the K/BxN DRG, with lower levels of pro-resolution lipid mediator MaR1. MaR1 is a macrophage-derived SPM known to have anti-nociceptive and anti-inflammatory effects on neurons and macrophages, respectively (Serhan et al., 2012, Dalli et al., 2013). MaR1 modulates macrophage functions through binding with high affinity to LGR6 receptors promoting phagocytosis and efferocytosis (Chiang et al., 2019). In addition, in human-derived macrophages MaR1 treatment is known to cause a phenotypic swift away from pro-inflammatory M1 phenotype, instead favouring an M2 pro-resolution state (Dalli et al., 2013). Similarly, in peritoneal macrophages challenged with LPS, I observe that MaR1 treatment induces a reduction in expression of proinflammatory cytokines TNFα and Nos-2, as well as reducing pro-inflammatory miR-155 expression. These findings appear to be mechanistically linked, with studies identifying macrophage-mediated miR-155 expression to induce NF-κB activity and increased production of pro-inflammatory cytokines including IL-1β, IL-6 and TNFα (Tan et al., 2015). Moreover, MaR1 treatment was shown to diminish LPS-induced nuclear translocation of NF-κB p65, and related pro-inflammatory cytokine release from bone-marrow derived macrophages, (Yang et al., 2019). Together, with our findings, this indicates that MaR1 exhibits anti-inflammatory action in macrophages by dampening pro-inflammatory miR-155 expression, which in turn reduces phosphorylation and nuclear translocation.
of NF-kB p65 and subsequent expression of pro-inflammatory cytokines known to sensitize nociceptors.

Liquid chromatography-mass spectrometry analysis of human macrophages by Serhan et al. (2012) has shown that MaR1 can be produced endogenously by macrophages. In the K/BxN serum transfer model, I demonstrate that repeated systemic administration of MaR1 leads to long-lasting recovery of hind paw hyperalgesia, in conjunction with significant reductions in pro-inflammatory macrophage presence in the K/BxN DRG. Our findings from cultured peritoneal macrophages indicate that MaR1 can directly reduce pro-inflammatory macrophage signalling. Therefore, it can be hypothesised that in the K/BxN DRG following MaR1 treatment, the SPM can bind receptors present on the surface of pro-inflammatory macrophages in the DRG, leading to modulation of miR-155 mediated intracellular signalling and ultimately facilitating a reduction of pro-inflammatory cytokine production. Subsequently, MaR1-mediated reduction of pro-inflammatory DRG macrophage signalling may result in decreased sensitization of K/BxN DRG neurons, reducing nociceptive signalling and causing the observed recovery from mechanical hyperalgesia.

In addition to anti-inflammatory macrophage-mediated mechanisms, MaR1 exhibits direct anti-nociceptive action on sensory neurons. Specifically, studies in dissociated DRG neurons demonstrate that MaR1 has an inhibitory effect on capsaicin-induced TRPV1 currents, in a dose-dependent manner (Serhan et al., 2012). Similarly, in DRG neurons exposed to capsaicin. Although I observe a dose-dependent attenuation of TRPV1-mediated calcium influx in dissociated neuronal cultures, evidence for MaR1’s direct action on neurons could be strengthened by experiments using pure neuronal cultures to mitigate all possibility of MaR1 acting via alternate cell types to influence neuronal activity. MaR1-mediated attenuation of neuronal activation in capsaicin-sensitive neurons required GPCR_{i/o} signalling mechanisms; likely via binding to recently established MaR1 GPCR receptor, LGR6 (Chiang et al., 2019). Although Evidence for the

In the K/BxN DRG, I observed significant dysregulation of miRNA when mechanical hyperalgesia occurred in the absence of joint swelling; characterised by upregulation of miR-706 and down-regulation of miR-709. Moreover, in-vitro experiments demonstrated increases in miR-706 expression in TRPV1^{+} DRG neurons exposed to
capsaicin. As cultured macrophages display little miR-706 expression, taken together these results create the possibility that miR-706 upregulation in the K/BxN DRG is neuronal-mediated and engaged in maintaining nociceptive states in the DRG. However, as MaR1 is ineffective at attenuating increased miR-706 expression from capsaicin-sensitive sensory neurons, it can be hypothesised that the anti-nociceptive action of MaR1 on DRG neurons observed in culture is unlikely mediated via modulation of neuronal miRNA expression. Therefore, I theorise that, in the K/BxN DRG, as opposed to directly attenuating neuronal nociceptive miRNA mechanisms, anti-nociceptive action of MaR1 is more likely dependent on aforementioned modulation of pro-inflammatory immune-mediated miRNA signalling mechanisms.

In models of neuropathic and inflammatory pain, MaR1 displays antinociceptive action following systemic and intrathecal injections (Serhan et al., 2012, Fattori et al., 2019). In the tibia fracture model of peri-operative pain, intrathecal MaR1 elicited acute short-lived analgesic effects within hours indicative of direct attenuation of nociceptive neuronal signalling (Yang et al., 2019). However, following serum transfer, I do not observe acute anti-hyperalgesic effects of systemic MaR1 treatment. Together, this indicates that delivery method of MaR1 plays an important role determining the extent of the SPM’s anti-nociceptive capabilities. Whereby, high local MaR1 levels in close proximity to neurons, achievable via intrathecal administration, is likely required for MaR1 to exhibit acute direct effects on neuronal nociceptive firing. Therefore, it is more likely that following passive immunization long-lasting anti-hyperalgesic effects of repeated systemic MaR1 treatment can be attributed to modulation of macrophage-mediated pro-inflammatory neuro-immune interactions in the DRG.

Importantly, however, modulatory actions on neuro-immune signalling by MaR1 are not exclusive to the periphery. In models of neuropathic pain, MaR1 is shown to attenuate dorsal horn microglial activation and pro-inflammatory cytokine levels in the spina cord following injury (Gao et al., 2018). Similarly, following serum transfer, I observe a reduction in dorsal horn microglial activation in the K/BxN spinal cord after repeated systemic MaR1 treatment. It is possible that reduced spinal cord microglial activation is a reflection of MaR1-mediated reductions in nociceptive input to the spinal cord from sensory neurons. However, it is also feasible that MaR1 is acting directly on spinal cord microglia to reduce pro-inflammatory neuro-immune signalling, leading to a diminished central sensitization and reduced pain states following serum transfer.
Future studies may look to assess the impact of MaR1 treatment on centrally mediated nociceptive mechanisms in the K/BxN serum transfer model.
Figure 6.1: Schematic summarizing peripheral and central neuro-immune mechanisms facilitating heightened nociceptive signalling in K/BxN serum transfer arthritis

During arthritic joint swelling, infiltrating immune cells in the K/BxN joint produce pro-inflammatory mediators which can sensitize joint nociceptors and lead to increases in neuronal activation. At this timepoint, in the K/BxN DRG, there is also greater levels of pro-nociceptive PGD$_2$ which can also contribute to peripheral sensitization and neuronal activation via action on DP1/2 receptors present on DRG neuronal cell bodies. In response to this neuronal activation, cell bodies of nociceptors increase expression of the neuropeptide calcitonin gene related peptide (CGRP), whereas in the dorsal horn spinal cord, peripheral nociceptive activity induces activation of microglia and associated pro-nociceptive neuro-immune communication. Post-joint swelling, reduced MaR1 levels are observed in the K/BxN DRG. As opposed to in the joint, where a greater number of pro-resolution M2 macrophages are observed, in the DRG a greater number of pro-inflammatory M1 macrophages are present compared to control. Pro-inflammatory macrophages can release pro-inflammatory cytokines, via intracellular miR-155 and NF-κB mediated signalling mechanisms, which bind receptors present in the DRG resulting in the sensitization of peripheral nociceptors. These pro-inflammatory mechanisms can be perturbed by pro-resolution MaR1 treatment. Post joint swelling, miR-706 expression is increased in DRG neurons, however the role of miR-706 in nociceptive signalling is currently unclear. As joint swelling subsides and mechanical hypersensitivity persists, greater spinal cord microglial activation is still observed, likely now mediated by pro-nociceptive and pro-inflammatory macrophage-neuron signalling in the K/BxN DRG.
6.3 Translational Relevance of Findings

Pain associated with rheumatoid arthritis (RA) is commonly described by patients as the most debilitating feature of the condition (Mcwilliams and Walsh, 2017). Clinical studies have demonstrated, utilizing the painDETECT questionnaire and through surveying of pain intensity in RA patients that over 50% of patients reported significant pain levels, even while medicated with DMARD therapies controlling clinical disease activity (Ahmed et al., 2014). These findings highlight that, despite control of inflammation with DMARDs, many RA patients may be sensitized to pain. Similarly, in the K/BxN serum transfer model, I observe hyperalgesia which outlasts the resolution of joint swelling. Moreover, although following passive immunization hyperalgesia presents absolutely, in our pre-clinical studies I observed no clear correlation between severity of joint swelling and intensity of mechanical hyperalgesia. Similarly, clinician assessments of RA patients indicate a discordance between inflammation and patient-reported pain, whereby pain intensity is only weakly correlated with clinical markers of inflammation (Khan et al., 2012, Purabdollah et al., 2017).

In the K/BxN serum transfer model, hyperalgesia in the presence of joint swelling is associated with increased pro-inflammatory mediators in the inflamed joint, likely sensitizing nociceptor terminals of the joint and heightening nociceptive signalling. Similarly, in the human condition, numerous pro-inflammatory mediators with the ability to sensitize peripheral nociceptors, such as IL-1β, IL-6 and TNFα, have been identified in the RA synovium or synovial fluids (Brennan and Mcinnes, 2008). Using intradermal capsaicin and pin-prick model, there is evidence of enhanced hyperalgesia in RA patients when compared to controls (Morris et al., 1997). However, it is difficult to elucidate the direct contributions of cytokines to RA pain due to the limitations of human experimentation.

In addition to enhanced pro-inflammatory cytokines, RA patients are also shown to exhibit altered levels of bioactive lipid mediators in synovial fluid. Norling et al. (2016) show the presence of pro-inflammatory and pro-nociceptive PGD₂ and PGE₂ in synovial fluid of RA patients. Similarly in the K/BxN joint, prostaglandin levels are elevated during joint swelling (Norling et al., 2016). I identify, in the K/BxN DRG, greater PGD₂ levels when hyperalgesia occurs alongside joint swelling. It would be
intriguing to see how these findings directly compare to the human condition; however, no studies currently have assessed bioactive lipid mediator levels in the human DRG.

Following serum transfer, when hyperalgesia is observed in the absence of joint swelling, I find lower levels of MaR1 in the K/BxN DRG. MaR1 is identified in human synovial fluid samples from RA patients, albeit at comparatively lower levels than other SPMs such as RvD5 (Giera et al., 2012). Moreover, quantification of MaR1 levels in human blood and synovial fluid samples showed MaR1 levels were higher in individuals with inactive RA and lower in active RA, compared to healthy control subjects suggesting that the decline of MaR1 in RA patients may play a vital role in the conversion of the inactive phase into the active phase. Our preclinical studies demonstrate that MaR1 treatment modulates pro-inflammatory macrophage signalling in macrophages of K/BxN DRG; as well as reducing pro-inflammatory miR-155 expression in cultured peritoneal macrophages. Similarly, Jin et al. (2019) observe in CD4⁺ T-cells derived from RA patients that MaR1 treatment leads to facilitation of anti-inflammatory signalling and attenuation of miR-155 expression. Additionally, in RA patients, miR-155 has been found overexpressed in macrophages in synovial fluid and synovial tissue is association with low levels of anti-inflammatory mediators (Kurowska-Stolarska et al., 2011). Collectively with our results, these findings highlight that in both humans and rodents, aberrant MaR1 production underpins pro-inflammatory immune states associated with RA, with MaR1 treatment diminishing immune-mediated pro-inflammatory signalling via modulation of miR-155 signalling cascades.

In addition, our pre-clinical studies highlight that MaR1 can attenuate nociceptive activation of capsaicin-sensitive DRG neurons. Functional evidence demonstrates the presence of capsaicin sensitive TRPV1 channels in human DRG neurons. Whereby, capsaicin activation of cultured human DRG neurons induced action potentials and increased intracellular Ca²⁺ levels (Baumann et al., 1996, Haberberger et al., 2019). Collectively, these findings raise the possibility that MaR1 may also demonstrate anti-nociceptive action in humans, similarly inhibiting nociceptive activation of sensory neurons. In summary, the work presented in this thesis offers significant translational relevance to pain in RA, providing innovative avenues for the treatment of the human condition.
6.4 Limitations of Work

From the current work presented in this thesis there are several limitations requiring further discussion. To better understand mechanisms related to RA-associated pain I utilized the pre-clinical K/BxN serum transfer mouse model of inflammatory arthritis in which days following passive immunization, clinical signs of arthritis develop. The K/BxN serum transfer model shares several similar features to RA of which pain dissociated from joint swelling was most valuable for our investigations.

Disease development in the K/BxN serum transfer model is wholly dependent on G6PI-immune complex formation. This is unlikely the case in the human condition where low prevalence of antibodies to G6PI is seen in RA patients (Matsumoto et al., 2003). Moreover, following passive transfer of K/BxN serum, disease progression further relies on Fc receptor-mediated activation of innate immune cells by IgG complexes; without involvement of T-cells and B-cells of the adaptive immune system. Conversely, RA disease progression involves complex signalling mechanisms from both innate and adaptive immune systems (Mcinnes and Schett, 2011). This highlights that our pre-clinical model may not represent mechanisms of the adaptive immune system which may be critical for the human disease. However, no current pre-clinical model, recapitulates all aspects of human RA, whereby even within RA patient groups there is heterogeneity of the condition (Koenders and Van Den Berg, 2015). Therefore, irrespective of the various differences to human RA, the pre-clinical K/BxN serum transfer model still provides a useful tool for the study of nociception, either associated or dissociated from joint swelling.

Prior to experimental design, power calculations were not performed to establish the appropriate sample size for investigation, instead sample was decided based on prior published studies utilizing similar experimental designs. In future, statistical power calculations should be used to better estimate the appropriate sample size. This process will help to ensure that lack of significance between experimental groups is not due to within group variation caused by underpowered sample.

For the assessment of clinical signs of arthritis, a visual scoring system was used. This technique is frequently utilized, by ourselves and other groups, to infer the intensity and presence of joint inflammation (Monach et al., 2007, Norling et al., 2016). Following passive immunization, in agreement with previous studies, I demonstrate
clinical signs of arthritis to be transient, returning to baseline 2 weeks post serum transfer (Park et al., 2016). However, after resolution of joint swelling, I have not determined if concomitantly there is cessation of pro-inflammatory mediator release in the joint. Although this hypothesis is implied by studies demonstrating, alongside resolution of joint swelling, clearance of neutrophils in the previously inflamed joint and a phenotypic switch of joint macrophages to an anti-inflammatory M2 phenotype (Misharin et al., 2014). Future studies could promptly address this query, assessing levels of pro-inflammatory and pro-resolution mediators in the K/BxN joint when swelling has resolved, as compared to non-arthritic controls.

When conducting flow cytometry analysis of macrophages in the K/BxN DRG, I utilized markers against CD11b and CD206 to determine M1 (CD11b+CD206−) and M2 (CD11b−CD206+) macrophage phenotype. These markers have been effectively used in past studies identifying DRG macrophage phenotype (Simeoli et al., 2017). However, there is opposition to the hypothesis that macrophages exist in a binary M1/M2 state, with increasing understanding that there are several sub-states of macrophage activation and function (Mosser and Edwards, 2008). Although using markers for CD11b and CD206 provides insight into macrophage phenotype in the K/BxN DRG, further assessment of numerous alternate cytokine and genetic markers associated with pro-inflammatory (M1) and anti-inflammatory (M2) macrophage signalling would provide even greater insight into immune signalling in the K/BxN DRG.

To better understand potential effects of MaR1 on pro-inflammatory and pro-resolution macrophage signalling I induced a pro-inflammatory state in cultured peritoneal macrophages exposed to LPS. Although LPS is a well-established activator of monocytes and macrophages inducing pro-inflammatory macrophage signalling, the method is also acknowledged as representing an extreme of the pro-inflammatory spectrum (Mosser and Edwards, 2008). Therefore, it is unlikely that LPS-challenged peritoneal macrophages form a direct representation of macrophage signalling in the K/BxN DRG. This implies that, although likely sharing mechanistic similarities, in-vitro results highlighting the anti-inflammatory abilities of MaR1 may not be directly analogous to MaR1’s in-vivo action on macrophages of the K/BxN DRG. This issue could be addressed by repeating qPCR results from peritoneal macrophages, instead assessing MaR1 effects on inflammatory signalling in K/BxN DRG-derived
Macrophages are less readily available in DRG than peritoneum and therefore samples will need to be pooled from more animals to retrieve the necessary amount of macrophage RNA content for experimentation. In addition, this experiment will incur significantly higher costs in comparison to peritoneal macrophage cultures due to greater cost of K/BxN serum in comparison to LPS.

When investigating miRNA signalling in the K/BxN DRG, I utilized miRNA microarray techniques which identified dysregulation of miR-706 and miR-709 in the K/BxN DRG when hyperalgesia outlasts joint swelling. However, the sensory ganglia represents a mixed cell population with cell bodies of sensory neurons, satellite glial cells, dendritic cells, macrophages, and endothelial cells (Watkins and Maier, 2002). Our analysis does not allow for observed miRNA dysregulation to be attributed to a single, or multiple, cell types in the DRG. Therefore, this leaves our understanding of the role miR signalling plays in nociceptive signalling in the K/BxN DRG somewhat incomplete. Future studies could look to address this issue by conducting single cell RNA sequencing in K/BxN DRG when hyperalgesia outlasts joint swelling; most importantly discretely assessing neuronal and macrophage cell populations.

6.5 Future Directions

The data which is presented in this thesis provides an important step forward in the understanding the mechanisms underpinning heightened nociceptive states in both the presence and absence of joint swelling. However, with more time, further investigations could have been carried out. Future research directions to further elucidate the mechanisms underlying rheumatoid arthritis-associated joint pain are as follows:

1. **Investigate whether altered neuro-immune communication occurs in brain regions associated with nociceptive signalling following K/BxN serum transfer.** The majority of studies which utilize the K/BxN serum transfer model, including those of this thesis, focus primarily on either the joint, DRG or spinal cord with very little investigation of the brain. With hyperalgesia observed both in the presence and absence of joint swelling following serum transfer, an avenue for future studies may be to investigate, using immunohistochemical analysis, changes in microglial activation (using Iba1/ P-p38 as markers) and
neuronal activation (p-ERK as a marker) in the pain related brain regions such as periaqueductal grey (PAG), thalamus and somatosensory cortex, either in presence or absence of joint swelling. These experiments, in combination with current knowledge, would help form a more complete understanding of how neuro-immune communication in the PNS and CNS contribute to heightened nociceptive states in the K/BxN serum transfer model.

2. **Assess modulation of bioactive lipid mediator profiles in the dorsal horn spinal cord following K/BxN serum transfer.** Within this thesis I have assessed bioactive lipid mediator profiles in K/BxN DRG, where cell bodies of nociceptors reside. However, the action and production of lipid mediators is not confined to the peripheral nervous system. With this view, using LC/MS based lipid mediator profiling, future studies may look to address if in the dorsal horn spinal cord, where nociceptors synapse with second order neurons, there is altered bioactive lipid mediator signalling at timepoints where mechanical hyperalgesia occurs either in the presence or absence of joint swelling. These studies will provide insight into the role SPMs play in regulating inflammatory and nociceptive signalling in the CNS following serum transfer.

3. **Assess the ability of MaR1 to attenuate spinal cord microglial-mediated pro-inflammatory signalling.** I demonstrate that repeated systemic treatment of MaR1 results in attenuation of pro-inflammatory macrophage signalling in the K/BxN DRG. However, as treatment is systemic MaR1 can also act on immune cells in the CNS; namely microglia which are shown to be elevated in the K/BxN dorsal horn alongside hyperalgesia. Moreover, I have demonstrated that MaR1 treatment reduces microglial activation following passive immunization. MaR1 may act directly on glial cells in the CNS to reduce pro-inflammatory signalling to proximal spinal cord neurons. Future *in-vitro* studies could look to assess, using qPCR techniques, if pro-inflammatory and anti-inflammatory mediator expression is altered by MaR1 treatment in isolated activated microglia cultures.
4. **Examine the role of LGR6-mediated MaR1 signalling mechanisms in modulating nociceptive and inflammatory signalling.** Recently, the MaR1 receptor has been identified as the GPCR, LGR6, known to be present on macrophages (Chiang et al., 2019). Following serum transfer, I observe MaR1 mediated anti-hyperalgesic action alongside reduced pro-inflammatory macrophage presence in the K/BxN DRG. Future experiments could assess if following passive immunization these changes occur in a LGR6 dependant manner, by pharmacological inhibition of LGR6 or genetic LGR6 knockout prior to MaR1 treatment. *In-vitro* experiments, using qPCR techniques, could also assess if blockade of LGR6 signalling attenuates MaR1 mediated inhibition of pro-inflammatory TNFα, iNOS and miR-155 expression in LPS-challenged peritoneal macrophages. Additionally, it is yet to be proven that LGR6 is present on sensory neurons. Therefore, future experiments could investigate the presence of LGR6 on sensory neurons using immunohistochemistry. Subsequently, future experiments may then assess, in dissociated DRG neurons, MaR1-mediated inhibition of TRPV1⁺ neuronal activation is disrupted by inhibition of LGR6 function.

5. **Investigate if miR-706, and their respective gene targets, play a role in the maintenance of nociceptive states in the absence of joint swelling following serum transfer.** Work within this thesis has demonstrated an up-regulation of miR-706 in K/BxN DRG whilst hyperalgesia occurs in the absence of joint swelling. Future in-silico studies could build upon these findings to identify miR-706 gene targets, with subsequent pathway analysis identifying possible metabolic pathways related to nociceptive and inflammatory signalling. I also observe that miR-706 is also elevated in TRPV1⁺ DRG neurons following capsaicin exposure. Following serum transfer, future in-vivo studies may look to assess if silencing of miR-706 production is associated with alterations to mechanical hypersensitivity in the absence of joint swelling. These studies could provide evidence of a functional association between miR-706 and persistent nociceptive signalling in the K/BxN DRG.
6.6 Conclusions

In conclusion, findings presented within this thesis have demonstrated that in the K/BxN serum transfer model of inflammatory arthritis, there are distinct neuro-immune signalling mechanisms relating to mechanical hyperalgesia either in the presence or absence of joint swelling. Although in the CNS mechanical hyperalgesia is consistently associated with dorsal horn spinal cord microgliosis, in the K/BxN DRG, where cell bodies of nociceptors reside, uniquely in the absence of joint swelling, persistent hyperalgesia is associated with increased pro-inflammatory macrophage presence, increased miRNA dysregulation and reduced pro-resolution MaR1 levels. Supplementation of MaR1 led to long-lasting anti-hyperalgesic actions, likely via reduction of pro-inflammatory macrophage signalling to neurons in the DRG and dampening of nociceptive signalling. Furthermore, through in-vitro studies, I have demonstrated the ability of MaR1 to reduce pro-inflammatory miRNA and cytokine expression from macrophages, as well as directly inhibiting capsaicin-sensitive sensory neuron activation. Further studies are needed to clarify the molecular mechanisms by which MaR1 exerts anti-inflammatory and anti-nociceptive action and influences neuro-immune communication both peripherally in the DRG and centrally in the spinal cord.

Collectively, the work within this thesis provides significant advancement and improved understanding of the critical role neuro-immune interactions play in maintaining persistent nociceptive states associated with pain in RA. Future developments in the field, investigating pro-resolution treatment strategies, may lead to much needed novel therapeutics against arthritis joint pain.
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