

Studies on Macrophage Migration in Pathological Contexts

A thesis submitted to the University of East Anglia
for the degree of Doctor of Philosophy

By

Matthew Ian Yates

University of East Anglia

School of Biological Sciences

Norwich

September 2014

© This copy of the thesis has been supplied on the condition that anyone who consults it is understood to recognise that its copyright rests with the author and that no quotation from the thesis, nor any information derived therefrom may be published without the author's prior, written consent.

Abstract

Macrophages play key roles following nerve injury releasing cytokines and chemokines thought to be involved in the development and maintenance of neuropathic pain. ATP is a transporter of chemical energy, but can also act as an extracellular signalling molecule that signals through purinergic receptors. ATP, probably released from damaged neurons, can promote the migration of macrophages and microglia to the site of injury, and can be responsible for the onset of neuropathic pain. Purinergic signalling via ATP and other nucleotides is now well established and increasing evidence suggests that ATP could play important roles in pathophysiology.

The neuroprotective neurotrophin brain derived neurotrophic factor (BDNF) inhibited ATP-induced invasion of macrophages through an extracellular matrix. The well-characterised Sigma-1 Receptor chaperone, previously implicated in BDNF function, was shown to be an important overall regulator of macrophage invasion, and to be potentially implicated in BDNF suppression of ATP-induced invasion. BDNF may thus have a neuroprotective role to inhibit further macrophage recruitment to the site of injury.

Matrix metalloproteinases (MMPs) are capable of remodelling the extracellular matrix and the activation of inflammatory mediators. Exploration of MMP responses to extracellular ATP stimulation in a macrophage cell line revealed that MMPs -8, -12, and -13 as well as TIMP-2 steady state mRNA levels were significantly up-regulated. ATP, ATP can alter matrix remodelling.

Macrophage recruitment is a significant player in atherosclerosis and may be modulated by Apolipoprotein E genotype. N3-polyunsaturated fatty acids (obtained from dietary fish oils) have been implicated in cardiovascular protection. Using a high fat diet model the effects of dietary fish oil supplementation and Apolipoprotein E subtype were explored in ex vivo primary macrophages. Unexpectedly, a fish oil supplemented diet led increased macrophage migration speed and reduced TIMP-2 mRNA levels, suggesting that fish oil supplements play complex roles in atherosclerotic-related scenarios.

Acknowledgements

First and foremost, I must express my gratitude to my primary supervisor, Jelena Gavrilovic, for giving me the opportunity to study in her lab, and of course for her outstanding support and advice throughout the course of this project. I would also like to thank my other supervisors, including Mette Mogensen and Mohammed Hajihosseini for their contributions to this project. I would also like to thank my industrial partners, Pfizer, for inviting me to their PhD forum days and contribution to the beginning of my project.

I would like to acknowledge the members of the Gavrilovic Lab, past and present, for welcoming me into the BMRC and teaching me numerous techniques. Thank you to Dr Jon Howe, who taught me the invasion assay technique and generally being a source of entertainment during my first year, and to Dr Damon Bevan, whose teaching of Taqman techniques proved vital to this thesis, also to Dr Megan Murray, for being a PhD student to aspire to. I believe that I should offer special thanks to my fellow PhD student, Matthew Mayhew, for sharing the ups and downs, daily turmoil, stress and occasional elations of a promising result with me, and for being a healthy source of competition during the last four years. Dr Matthew Fenech and Dr Jeremy Turner also deserve my recognition, for their insightful comments during lab meetings.

I express my gratitude to the members of Mette Mogensen's lab; Deborah Goldspink, Jonathan Gadsby and James Perkins for being a source of knowledge, antibodies, other lab consumables and entertainment.

I would also like to offer my thanks to Dr Paul Thomas, for his seemingly endless knowledge of microscope techniques, and for his help in setting up microscopes and imaging techniques.

My appreciations also go to all the people who helped make Norwich a wonderful place to be, all of which would be too numerous to name – but Dr Frederick Wandschneider (for all of the coffee) and Dr Rachel de Ferrars (as my Norwich constant) deserve special thanks, along with Manuel Schär (too many reasons to name them all) and Emily Warner (beer hour). Thanks to all of my excellent housemates – Rachel, Fred, Manu, Stefan, Emily and Sara for putting up with my loud alarms and music. Thanks to my band – Andy, Neil, Gav,

and Emily, for allowing me to make a lot of noise with you. I would also like to thank all of the people who have helped along the way – Colette, Niraj, Laura, Basti, Andrea, Kelly and anyone else I've forgotten to name.

Sophie Millar deserves much love and gratitude for inspiring me, giving me motivation, emotional support and forcing me to work, and without whom, I think I would have probably gone insane in this last year.

Lastly, I would like to mention the overwhelming amount of emotional support from my family – Mum, Dad, Grandad and Rob for always keeping me going when everything seemed tough and for always being sympathetic of my pursuit of science, and also again to Rob for deflating my ego every once in a while when it wasn't. I hope I've made you all proud.

List of Abbreviations

2D	– Two-Dimensional
3D	– Three-Dimensional
ACSF	– Artificial Cerebral Spinal Fluid
ADP	– Adenosine Diphosphate
AMPA	– α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApoE	– Apolipoprotein E
ASIC	– Acid Sensing Ion Channel
ATP	– Adenosine Triphosphate
BBG	– Brilliant Blue G
BDNF	– Brain Derived Neurotrophic Factor
BIP	– Binding Immunoglobulin Protein
BMM	– Bone Marrow-derived Macrophage
CaMKII	– Calcium/Calmodulin dependent Protein Kinase II
CCI	– Chronic Constriction Injury
CCL	– C-C Chemokine Ligand
CCR	– C-C Chemokine Receptor
cDNA	– Complementary Deoxyribonucleic Acid
CFA	– Complete Freund's Adjuvant
CM	– Conditioned Media
CNP	– Chronic Nonmalignant Pain
CNS	– Central Nervous System
COX	– Cyclooxygenase
CSF	– Cerebral Spinal Fluid
C _T	– Cycle Threshold
CXCL	– C-X-C Chemokine Ligand
CXCR	– C-X-C Chemokine Receptor

DAMP – Damage Associated Molecular Pattern

DHA – Docosahexaenoic Acid

DHEA – Didehydroepiandrosterone

DMEM – Dulbecco’s Modified Eagle Medium

DNA – Deoxyribonucleic Acid

DRG – Dorsal Root Ganglion

DTG - Ditolylguanidine

EAE – Experimental Autoimmune Encephalomyelitis

ECM – Extracellular Matrix

EPA – Eicosapentaenoic Acid

ER – Endoplasmic Reticulum

ERK – Extracellular Signal Related Kinase

FGF – Fibroblast Growth Factor

FN - Fibronectin

FO – Fish Oil

GABA – gamma-Aminobutyric Acid

GFAP – Glial Fibrillary Acidic Protein

GLAST – Glutamate-Aspartate Transporter

GPCR – G Protein Coupled Receptor

GSK – Glycogen Synthase Kinase

GTP – Guanidine Triphosphate

HDL – High Density Lipoprotein

HFD – High Fat Diet

HIV – Human Immunodeficiency Virus

IBA1 – Ionised Calcium Binding Adapter 1

ICAM – Intracellular Adhesion Molecule

IFN - Interferon

IgG – Immunoglobulin G

IL – Interleukin

IPAG - 1-(4-Iodophenyl)-3-(2-Adamantyl)Guanidine

JNK – c-Jun N-terminal Kinase

KC – Keratinocyte-derived Chemokine

KCC2 – Potassium Chloride Transporter Member 5

LDL – Low Density Lipoprotein

LPS – Lipopolysaccharide

LTP – Long Term Potentiation

MAM – Mitochondrion Associated Membrane

MAP – Mitogen Activated Protein

MAPK – Mitogen Activated Protein Kinase

MCP – Monocyte Chemoattractant Protein

MCSF – Macrophage Colony Stimulating Factor

MDD – Major Depressive Disorder

MEK – Mitogen Activated Protein Kinase Kinase

mEPSCs – Miniature Excitatory Pro-Synaptic Currents

MG - Matrigel™

MHC – Major Histocompatibility Complex

MIP – Macrophage Inflammatory Protein

miRNA – Micro Ribonucleic Acid

MMP – Matrix Metalloproteinase

mRNA – Messenger Ribonucleic Acid

MS – Multiple Sclerosis

MT-MMP – Membrane Type Matrix Metalloproteinase

NFAT – Nuclear Factor of Activated T-cells

NGF – Nerve Growth Factor

NMDA – N-methyl-D-Aspartate

NMDG – N-methyl –D-Glucamine

NO – Nitric Oxide

OxLDL – Oxidised Low Density Lipoprotein

PAF – Platelet Activating Factor

PAFR – Platelet Activating Factor Receptor

PAMP – Pathogen Associated Molecular Pattern

PCR – Polymerase Chain Reaction

PDGF – Platelet Derived Growth Factor

PGE₂ – Prostaglandin E2

PI3K – Phosphoinositide 3 Kinase

PKC – Protein Kinase C

PLA₂ – Phospholipase A2

PLC – Phospholipase C

PNI – Partial Sciatic Nerve Injury

PNS – Peripheral Nervous System

PPADS – Pyridoxalphosphate-6-Azophenyl-2',4'-Disulfonic Acid

PREG - Pregnenolone

PROG - Progesterone

PUFA – Polyunsaturated Fatty Acid

qRT-PCR – Quantitative Real Time Polymerase Chain Reaction

RNA – Ribonucleic Acid

ROS – Reactive Oxygen Species

RVM – Rostral Ventromedial Medulla

S1-R – Sigma-1 Receptor

SCI – Spinal Cord Injury

SEM – Standard Error Mean

siRNA – Short Interfering Ribonucleic Acid

SMC – Smooth Muscle Cell

SNL – Sciatic Nerve Ligation

STZ - Streptozotocin

TGF – Tissue Growth Factor

TIMP – Tissue Inhibitor of Metalloproteinases

TLR – Toll Like Receptor

TNF – Tissue Necrosis Factor

TrkA – Tyrosine Kinase Receptor A

TrkB – Tyrosine Kinase Receptor B

TRP – Transient Receptor Potential

TRPV – Vanilloid Receptor Related Transient Receptor Potential

UDP – Uridine Diphosphate

UTP – Uridine Triphosphate

VCAM – Vascular Cell Adhesion Molecule

VEGF – Vascular Endothelial Growth Factor

VLDL – Very Low Density Lipoprotein

VSMC – Vascular Smooth Muscle Cell

WT – Wild Type

List of Figures

Figure 1.1	Structure of a neuron.....	2
Figure 1.2	A diagram to show the structure of a nerve.....	4
Table 1.1	A table to show the basic properties of the neuron fibre and their specialities.....	7
Figure 1.3	A diagram to explore common methods of producing animal models of neuropathic pain.....	14
Table 1.2	A table to show the MMPs and their ECM substrates, along with their activators.....	37
Figure 2.1	siRNA sequences as supplied by Dharmacon.....	56
Figure 2.2	A full list of antibodies used in Western Blot experiments.....	60
Figure 2.3	A diagram to show the steps of the inverted invasion assay.....	62
Figure 3.1	ATP significantly increases Raw 264.7 invasion through a Matrigel™/Fibronectin matrix.....	69
Figure 3.2	ATP significantly increases BMM invasion through a Matrigel™/Fibronectin Matrix.....	70
Figure 3.3	Invasion of Raw 264.7 through a Matrigel™/Fibronectin Matrix after pre-treatment with P2 antagonists.....	72
Figure 3.4	BDNF attenuates ATP induced invasion in Raw 264.7 cells through a Matrigel™/Fibronectin matrix.....	74
Figure 3.5	BDNF attenuates ATP induced invasion in Bone Marrow-derived Macrophages cells through a Matrigel™/Fibronectin matrix.....	75
Figure 3.6	The effects of the Sigma receptor agonist, DTG on the invasion of Raw 264.7 cells through a Matrigel™/Fibronectin matrix.....	77
Figure 3.7a	DTG suppresses migration.....	79
Figure 3.7b	DTG Supresses migration.....	81

Figure 3.8	The effects of the Sigma receptor antagonist, IPAG on the invasion of Raw 264.7 cells through a Matrigel™/Fibronectin matrix.....	83
Figure 3.9	The effects of Sigma receptor agonism, by DTG, and antagonism, by IPAG on the invasion of BMM through a Matrigel™/Fibronectin matrix.....	84
Figure 3.10	p38 is activated after 15 minutes stimulation with ATP, and repressed with ATP+BDNF stimulation.....	85
Figure 3.11	BDNF abrogates ATP-induced ERK1/2 phosphorylation after 60 minutes of stimulation.....	87
Figure 3.12	Western blot to determine Sigma-1 receptor expression in Raw 264.7 cells.....	88
Figure 3.13	Investigating the effect of a Sigma-1 knockdown using siRNAs on the invasion of Raw 264.7 through a Matrigel™/Fibronectin matrix towards ATP and BDNF.....	90
Figure 4.1	A table to show the expression of MMPs in response to 24hr stimulation with either ATP, Bradykinin or LPS in relation to the unstimulated control.....	100
Figure 4.2	A heat map to show the response of MMP expression in response to stimulation with either ATP or LPS in relation to the control.....	101
Figure 4.3	MMP-8, MMP-13 and TIMP-2 show a significantly altered level of expression in Raw 264.7 when stimulated with ATP, and/or Bradykinin and/or LPS.....	103
Figure 4.4a	MMP expression levels in Raw 264.7 cells after 24 hours treatment with either 10µM ATP, 1mM Bradykinin or 100ng/ml LPS.....	104
Figure 4.4b	MMP expression levels in Raw 264.7 cells after 24 hours treatment with either 10µM ATP, 1mM Bradykinin or 100ng/ml LPS.....	105
Figure 4.5	A table to show the expression of MMPs over the course of a 48hr stimulation with either 10µM ATP, 1mM ATP or 100ng/ml LPS in relation to the control.....	107
Figure 4.6	A heat map to show the response of MMP expression in response to stimulation with either ATP or LPS in relation to the control.....	108

Figure 4.7	Expression of MMP-8 over 48 hours.....	110
Figure 4.8	Expression of MMP-8 in response to stimuli over time.....	110
Figure 4.9	Expression of MMP-10 over 48 hours.....	111
Figure 4.10	Expression of MMP-10 in response to stimuli over time.....	112
Figure 4.11	Expression of MMP-12 over 48 hours.....	113
Figure 4.12	Expression of MMP-12 in response to stimuli over time.....	114
Figure 4.13	Expression of MMP-13 over 48 hours.....	115
Figure 4.14	Expression of MMP-13 in response to stimuli over time.....	116
Figure 4.15	Expression of TIMP-2 over 48 hours.....	117
Figure 4.16	Expression of TIMP-2 in response to stimuli over time.....	118
Figure 4.17	The effects of 8hr stimulation of Raw 264.7 cells with ATP and BDNF.....	119
Figure 5.1	A graph to show the average migration speed ($\mu\text{m/hr}$) of primary murine macrophages derived from mice with a humanised ApoE3 or ApoE4 knock-in.....	131
Figure 5.2	Expression of MMP-13 in primary murine macrophages.....	133
Figure 5.3	MMP-13 expression in comparison to either diet or ApoE subtype.....	134
Figure 5.4	Expression of MMP-15 in primary murine macrophages.....	136
Figure 5.5	Expression of MMP-15 in comparison to either diet or ApoE subtype.....	138
Figure 5.6	Expression of TIMP-2 in primary murine macrophages.....	140
Figure 5.7	Expression of TIMP-2 in comparison to either diet or ApoE subtype.....	142
Figure 6.1	What happens in response to nerve injury in macrophages.....	153

Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
List of Abbreviations	v
List of Figures	x
1.1. The Nervous System	3
1.1.1. Nociception	6
1.2. Neuropathic Pain	11
1.2.1. Neuropathic Pain	11
1.2.2. Animal Models of Neuropathic Pain	14
1.2.3. Treatment of Neuropathic Pain.	15
1.3. Cell Migration.....	18
1.4. Microglia and Macrophages.	19
1.4.1. Microglia.	19
1.4.2. Macrophages.....	20
1.5. ATP as an Extracellular Signalling Molecule.....	22
1.5.1. P1 Receptors	23
1.5.2. P2X Receptors	24
1.5.2.1. P2X4 Receptor.....	24
1.5.2.2. P2X7 Receptor.....	25
1.5.3. P2Y Receptors	26
1.5.3.1. P2Y6 Receptor.....	27
1.5.3.2. P2Y12 Receptor.....	27
1.6. The Response of Microglia and Macrophages to Damaged Neurons.....	29
1.6.1. Platelet Activating Factor	29
1.6.2. Neurotrophins.....	30
1.6.2.1. Nerve Growth Factor	30
1.6.2.2. Brain Derived Neurotrophic Factor.....	31
1.6.3. Cytokines and Chemokines	32
1.6.4. Sigma Receptors.....	33
1.7. Matrix Metalloproteinases	36
1.7.1. Structure and Activity	36
1.7.2. Tissue Inhibitors of Matrix Metalloproteinases.....	39
1.7.3. Matrix Metalloproteinases in Migration.....	40

1.7.4.	Matrix Metalloproteinases in Macrophages and Microglia.....	41
1.8.	Atherosclerosis.....	43
1.8.1.	Pathogenesis of Atherosclerosis	43
1.8.2.	Macrophages in Atherosclerosis.....	44
1.8.3.	Matrix Metalloproteinases in Atherosclerosis.....	46
1.8.4.	Apolipoprotein E	47
1.8.5.	Dietary Prevention of Atherosclerosis	48
1.9.	Hypothesis and Aims.....	50
2.1.	Cell Culture.....	52
2.1.1.	RAW 264.7	52
2.1.2.	Isolation and culture of Bone Marrow-derived Macrophages (BMMs).	52
2.1.3.	Culturing L-929 cells for conditioned media.....	53
2.2.	Humanised ApoE Knock-in mice	53
2.2	Cell Viability Testing.....	54
2.3.	Macrophage Treatments	54
2.3.1.	ATP	54
2.3.2.	BDNF	54
2.3.3.	LPS.....	55
2.3.4.	Bradykinin	55
2.3.5.	Interleukin-10.....	55
2.3.6.	MCP-1.....	55
2.3.7.	PPADs	55
2.3.8.	Suramin	56
2.3.9.	BBG.....	56
2.3.10.	DTG.....	56
2.3.11.	IPAG.....	56
2.3.12.	siRNA.....	57
2.4.	Total RNA Purification.....	58
2.5.	Reverse Transcription	58
2.6.	Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)	59
2.7.	Protein Extraction	59
2.8.	Western Blotting.....	60
2.8.1.	Protein Quantification	60
2.8.2.	Preparation of Protein Samples.....	60

2.8.3. Protein resolution by SDS-PAGE	60
2.8.4. Semi-dry transfer	61
2.8.5. Immunodetection of Proteins.....	61
2.9. Cell Migration and Time-Lapse Microscopy.....	62
2.10. Inverted Invasion Assay	62
2.11. Immunocytochemistry	64
2.12. Statistical Analysis.....	65
3.1 Introduction	67
3.1.1. Macrophage Migration in Neuropathic Pain.	67
3.1.2. Purinergic Signalling.....	67
3.1.3. BDNF	68
3.1.4. Sigma-1 receptor.....	68
3.2. Hypothesis and Aims.....	69
3.3. Results.....	70
3.3.1. ATP acts as a chemoattractant to Raw 264.7 cells and primary Bone Marrow-derived Macrophages in a Matrigel™/Fibronectin matrix.	70
3.3.2. Brain-Derived Neurotrophic Factor can attenuate ATP induced invasion in Raw 264.7 cells and BMMs.....	75
.....	77
3.3.3. The potential involvement of Sigma Receptors in Macrophage Migration towards ATP, and the subsequent attenuation of invasion by BDNF.	78
3.3.4. Elucidating mechanisms behind ATP-induced invasion and the attenuation of migration via BDNF, and Sigma-1 receptor agonism.....	87
3.3.5. Investigating the effects of Sigma-1 receptor on the invasion and signalling of Raw 264.7 macrophages.....	90
3.4. Discussion.....	93
4.1. Introduction	98
4.1.1. Matrix metalloproteinases and their inhibitors.....	98
4.1.2. MMPs in cell migration.	98
4.1.3. MMPs in Neuropathic Pain.	99
4.2. Hypothesis and Aims	100
4.3. Results	101
4.3.1. MMP expression is altered after 24 hours stimulation with ATP, Bradykinin and LPS.	101
4.3.2. MMP expression in response to stimuli over time.	108
4.3.3. The Effects of BDNF on MMP Expression.	127

4.4. Discussion.....	129
5.1. Introduction	134
5.1.1. ApoE in Atherosclerosis.	134
5.1.3. Fish Oils.	135
5.1.4. MMPs.....	136
5.2. Hypothesis and Aims.....	137
5.3. Results.....	138
5.3.1. The Effect of ApoE subtype and a fish oil-supplemented high fat diet on macrophage migration.....	138
5.3.2. The Effect of ApoE subtype and a fish oil-supplemented high fat diet on macrophage production of MMPs.....	140
5.4. Discussion.....	154
5.4.1. The Effect of Stimulation on migration and MMP expression of Primary Macrophages.	154
5.4.2. The Effect of diet on macrophage migration and MMP expression.....	155
5.4.3. The Effect of ApoE subtype on macrophage migration and MMP expression.....	158
6.1. BDNF abrogates ATP-induced invasion.....	161
6.2. Role of Sigma-1 receptor in macrophage migration.....	164
6.3. ATP can induce MMP and TIMP expression.	167
6.3.1. BDNF may attenuate MMP expression.....	169
6.4. A Fish Oil supplemented diet leads to increased macrophage migration and a reduction in TIMP-2 expression.	169
6.5. Future Work.....	172
6.5.1. BDNF abrogates ATP-induced invasion.....	172
6.5.2. Sigma receptor studies.	172
6.5.3. ATP-induced MMP expression.....	173
6.5.4. Dietary Fish Oils in Atherosclerosis.....	173
6.6. Overall Conclusions.....	174

Chapter 1: Introduction

1.1. The Nervous System

The nervous system in the body is made up of two interconnected sections, the Central Nervous System (CNS) comprising the brain and the spinal cord and the Peripheral Nervous System (PNS) which comprises all other nerves.

The PNS consists of two major types of neurons to help communicate what is happening in the body to the CNS and to respond to signals from the CNS: sensory neurons and motor neurons. Sensory neurons detect stimuli such as touch, taste, light, heat, cold and pain. Motor neurons stimulate muscles in the body to respond to certain stimuli resulting in muscle contraction and subsequent movement. Both sensory neurons and motor neurons are essential for all complex organisms.

The PNS is more readily exposed to chemical and environmental stimuli whereas in a healthy individual the CNS is protected by the blood brain barrier, the skull and the spine. However, the general structure of individual neurons is rather similar throughout the body, and do not vary greatly until they are observed at a molecular level – where the types of receptors expressed, neurotransmitters and other properties can be seen. All neurons have certain features, such as the cell body, the axon, and dendrites (Figure 1.1).

The axon, along which the action potential is transmitted, extends from the neuronal cell body towards the axon terminal or nerve ending. The axon can vary greatly in length, (up to a metre in the sciatic nerve), and axon thickness can vary between only 0.2 μ m to 20 μ m in diameter. To aid speed of transmission of the action potential, some axons have insulation in the form of a myelin sheath, comprising the Schwann cell plasma membrane which wraps around the axon in the PNS (with the oligodendrocyte performing a similar function in the CNS). However, the entire axon is not wrapped in Schwann cells and there are points at which the axon is unmyelinated and where the action potential has to be regenerated – these points are called the nodes of Ranvier. At the end of the axon, the neuron branches out into dendrites and a synapse is formed with other neurons or muscle.

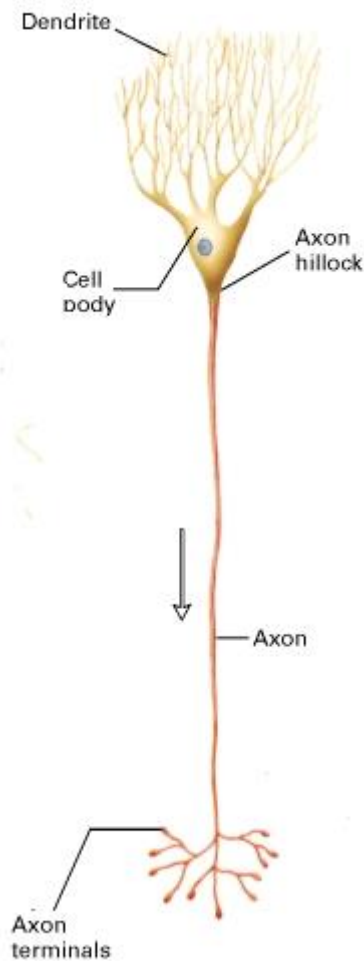


Figure 1.1. This diagram shows a myelinated nerve cell. The action potential is generated in the soma at the axon hillock (where the axon starts) and travels along the axon resulting in a synapse at the axon terminal dendrites. The arrow stipulates in which direction the action potential flows. Image adapted from Lodish et al., 2000.

At this point, the neuron may make connections with many other neurons (eg in the brain) and the synapse will cause the generation of an action potential in those neurons, so a single action potential can, if necessary, trigger action potentials in 100 neurons. Dendrites are found at the terminal of the neuron, so the axon is able to receive and send as many signals as possible. (Kandel, 2000).

Neurons are segregated spatially, for example sensory neuron cell bodies are located in the dorsal root ganglia, whereas the autonomic ganglion contains autonomic neurons, and the ventral horn of the spinal cord or the brain stem contain the cell bodies of motor neurons (Topp and Boyd, 2006).

PNS nerves have a very complex structure to allow insulation from other axons and to provide some protection to each axon. This is primarily one by organization of the peripheral nerves and association with 3 different layers of connective tissues. The three layers of connective tissue are called the endoneurium, the perineurium, and the epineurium, and each surround the axon to form a nerve (Topp and Boyd, 2006).

In more detail, the endoneurium is where the axon is associated with Schwann cells, where the myelin insulation is caused by the plasma membrane of the Schwann cell, when wrapped several times around the axon. An unmyelinated axon is still associated with a Schwann cell, but the Schwann cell does not wrap around the cell tightly, and does not provide the axon with several layers of myelin plasma membrane. The Schwann cell is then surrounded by extracellular matrix components including type IV collagen, laminin, heparan sulphate proteoglycan and fibronectin – which form the basal lamina. The axons are separated by types I and II collagen fibrils, macrophages and mast cells, with fibroblasts and endoneurial fluid (Topp and Boyd, 2006).

Several of these endoneurium-axon complexes are then bundled together by the perineurium, which is a dense connective tissue. The perineurium consists of around 15 layers of perineurial cells, with types I and II collagen fibres and elastic fibres, which provide the perineurium with mechanical strength. Perineurial cells are linked together by tight junctions, with the most inner of the perineurial cells forming a blood-nerve barrier by forming a diffusion barrier – thus controlling the endoneurial environment and forming a peripheral version of the blood brain barrier. The perineurium forms a nerve fascicle (Topp and Boyd, 2006).

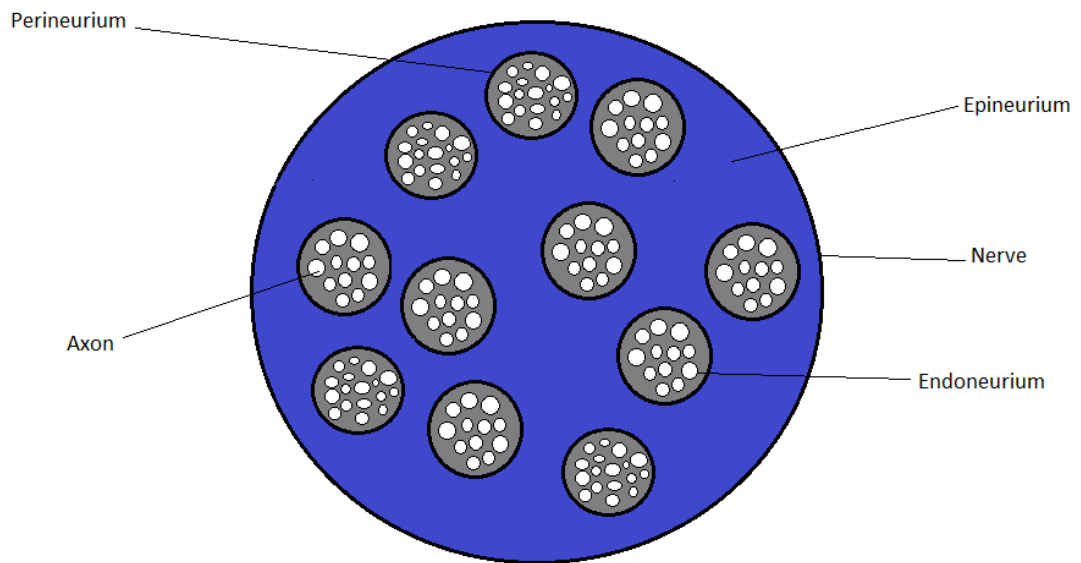


Figure 1.2. A diagram to show the structure of a nerve, adapted from Topp and Boyd, 2006. Axons are surrounded by the endoneurium, which are bundled together and thus several are then bound by the perineurium. These perineurial fascicles are then bound together by the epineurium to form a nerve.

Several nerve fascicles are then joined and held together by the epineurium to form a nerve. The epineurium can be divided into inter- and epi-fascicular regions, the epifascicular region is involved with Wallerian degeneration (the process by which neurons degenerate) of axons after injury, whereas the interfascicular region is attached to the perineurium, but loosely, as to allow for the sliding of each individual fascicle independently (Topp and Boyd, 2006).

1.1.1. Nociception

Nociception, the process whereby pain is perceived involves the detection of a noxious stimulus, such as intense heat, by primary sensory neurons. Even though pain is unpleasant and can be distressing, it is an essential process which is used as a mechanism to avoid harmful stimuli which could cause harm to the organism. We detect pain using a specific type of primary sensory neuron called a nociceptor (Julius and Basbaum, 2001). Nociceptors have certain characteristics which distinguish them from other neurons, such as the fact that they can only be activated by certain stimuli – intense heat, physical pressure, but that they are not activated by other normal stimuli such as light pressure, or

mild temperature changes. Nociceptors are also considered to be polymodal, and are either of the A δ or C class of sensory afferent fibre.

Sensory afferent fibres are grouped into 4 classes according to their myelination and diameter: A α , A β , A δ or C. However, it is acknowledged that A δ and C fibres are mainly used for nociceptive purposes. There are three main classes of nociception and pain, thermal nociception – fibres are activated by extreme temperatures – above 45°C or below 5°C. In thermal nociception, it is the thinly myelinated, small diameter A δ fibres which detect the stimulus. Mechanical nociception is a measure of intense pressure, and is also detected by the A δ fibres, which conduct quickly at 5-30 m/s. Polymodal nociceptors are activated by intense temperature – both hot and cold, and by chemical (such as acid or capsaicin) and mechanical stimuli. These nociceptors have C fibres, which are of small diameter but are unmyelinated, conducting slowly, usually less than 1 m/s (See Table 1.1 for summary) (Kandel, 2000, Lee, 2005)

The different fibres are said to be responsible for the perception of “first” and “second” pain, with “first” pain being the sharp fast pain at the beginning of an injury, such as stubbing your toe, whereas “second” pain would be the burning sensation afterwards. This is explained by the conduction speeds of the different nociceptor fibres, so the “first” pain would be caused by A δ fibres as they conduct the quickest, and “second” pain would be caused by C fibres (Kandel, 2000, Julius and Basbaum, 2001).

Many nociceptor receptors have been identified and classified. For example, it has been found that there are at least 2 sub-classes of C-fibre – those which express the peptide neurotransmitter substance P, and expresses TrkA (tyrosine kinase receptor which binds to nerve growth factor (NGF)), and those which do not express these proteins, but can be labelled with α -D-galactosyl-binding lectin IB₄, and express P2X₃ receptor, which is a specific type of ATP gated ion channel. (Julius and Basbaum, 2001).

It is thought that epigenetic modification makes a significant contribution to pain variability – rat models have been bred to show high autonomy and low autonomy – they share a common genetic background but have been bred to have high and low sensitivity

to pain. This is expressed in both spontaneous pain behaviour and the neuroma model of neuropathic pain. Pain is normally mediated by A δ and C fibres, with A β fibres signalling touch. It is thought that after nerve injury, A β fibres could also signal pain. A β afferents could be accountable for heritable differences in pain behaviour (Nitzan-Luques et al., 2011).

FIBRE TYPE	STRUCTURAL PROPERTIES	FUNCTION
AA AND AB	Large diameter Myelinated Conduction: 5-30 m/s	Proprioception (non nociception, i.e. light touch)
AΔ	Medium diameter Lightly myelinated Conduction: 5-30 m/s	Nociception - mechanical, thermal and chemical. Type I fibres have a 53°C temperature threshold, Type II have a 43°C threshold.
C	Small diameter Unmyelinated Conduction: less than 1 m/s	Nociception – mechanical, thermal and chemical, has a temperature threshold of 43°C but detects both hot and cold temperatures, also innocuous temperatures

Table 1.1. This table shows the basic properties of the neuron fibre and their specialities. Adapted from Kandel et al 2000, and Julius & Basbaum, 2001

Transient receptor potential (TRP) ion channels are expressed in many cell types and are important in sensation because they can act as molecular sensors, and they are implicated in nociception and pain, in particular, the vanilloid receptor related TRPs (TRPVs). For

example, it has been found that TRPV1 is activated by capsaicin (an ingredient of chilli peppers which causes pain and irritation), stimulating primary sensory afferents (nociceptors). TRPV1 was found to be highly expressed in small sensory neurons and is considered to be poly modal since it is activated not only by capsaicin, but also by noxious heat at temperatures higher than 43°C, and by acid, endogenous lipids and lipoxygenase products (Lee, 2005).

TRPV1 is also implicated in inflammation, as inflammatory agents such as bradykinin (a pain causing substance in the body) and histamine can either sensitize or activate the receptor, and it is thought that TRPV1 plays an important role in inflammatory pain (Endres-Becker et al., 2007). Bradykinin is able to activate sensory neurons via the PLA₂ and lipoxygenase pathway, and it also sensitizes neurons to heat, or in the case of TRPV1, heat and capsaicin (Lee, 2005).

TRPV2 is a homolog of TRPV1, which has structural similarity with TRPV1, but it is not sensitive to acid or capsaicin, which was unsuspected, TRPV2 does retain the ability to respond to heat over 52°C. And it is also expressed in other cells than sensory neurons. It has also been shown to be sensitive to membrane stretch and shock, so a role as a mechanic nociceptor could also be considered. Other homologs of TRPV1 – TRPV3 and 4 are also sensors for heat, although the threshold for activation is much lower (31°C for TRPV3 and 24°C for TRPV4), which would suggest that these are not nociceptors (Lee, 2005).

TRPA1 is a cold channel, and therefore activated by cold stimuli at less than 17°C, and is expressed in small sensory neurons, it is also co-localised with TRPV1 (which was explored earlier, is activated by heat) – this can explain why noxious cold can be detected as heat, and the experience is called “paradoxical heat”. TRPA1 is also activated by many other substances such as cinnamaldehyde, gingerol and mustard oil (Lee, 2005, Bautista et al., 2006). It has also been found that stimulation of phospholipid C activates TRPA1, as well as stimulation of G-protein coupled receptors. TRPA1 was also found to be highly expressed within sensory hair bundles in the inner ear – this, along with the fact that

siRNAs can silence TRPA1 and reduce hair cell mechanotransduction currents suggests that the receptor may also play a role in mechanotransduction.

ASIC or acid sensing ion channel is activated by extracellular acid, and is distributed widely in sensory neurons. It also has a role in nociception, in inflammatory pain as extracellular acid is one of the major noxious stimuli, ASIC expression can be mediated by non-steroidal anti-inflammatory drugs (NSAIDs), which further implicates the role of the receptor in nociception (Lee, 2005).

By looking at different receptors which are expressed by different sensory neurons, it is possible to observe why different sensory neurons have different functions. For example, type I and type II A δ fibres have different thresholds to temperature, this could merely be because type I fibres express TRPV1 as a heat receptor, and type II fibres express TRPV2, which has a higher temperature threshold.

1.2. Neuropathic Pain

1.2.1. Neuropathic Pain

Neuropathic Pain is a pathology of pain and can be described as “pain resulting from disease or damage of the peripheral or central nervous system” (Scadding, 2004). Neuropathic pain serves no useful purpose. There are many causes of neuropathic pain – such as trauma and tumours, but conditions including diabetes, multiple sclerosis, HIV and vitamin B12 deficiency can contribute to a higher risk factor for developing the condition (Scadding, 2004).

Neuropathic pain can be characterised by two conditions; hyperalgesia and allodynia. Hyperalgesia is a condition where there is an increased intensity of pain produced by a noxious stimulus and can lead to a delayed perception of pain and painful after-sensations. Allodynia is when an individual experiences low intensity mechanical stimuli can cause pain (so a low intensity stimulus such as someone brushing the skin can cause pain). Allodynia is a prominent marker of nerve-injury evoked pain syndromes (Chaplan et al., 1997, Scadding, 2004, Sandkuhler, 2009).

Individuals suffering from neuropathic pain may suffer from the delayed onset of pain, and an example of this would be central post-stroke pain, where a patient has suffers from pain several months and possibly years after the initial stroke. The pain felt by a sufferer of neuropathic pain is different from normal nociceptive pain (as are the mechanisms by which it is produced). Neuropathic pain is usually a burning, raw and stabbing sensation and can also be affected by fatigue and emotion, and there can be sensory impairment (Scadding, 2004, Baron et al., 2010).

A basic pathophysiology of neuropathic pain is as follows; novel ectopic impulse generators develop at several points in the PNS as a result of damage, and inflammatory cells such as macrophages and leukocytes release a variety of chemicals including noradrenalin, bradykinin, ATP, BDNF, histamines and serotonin which work to sensitize nociceptors to increase nerve activity. As a result of these changes the threshold to activate nociceptors is lowered causing peripheral sensitization. Connections are formed between injured and uninjured nerve fibres leading to cross-excitation – meaning that non-painful stimuli can induce pain. Synaptic plasticity can occur in the spinal cord, which

can lead to possibly irreversible abnormal neuron connectivity (Scadding, 2004, Pasero, 2004).

Ectopic nerve activity causes the sensation of ongoing spontaneous pain and shooting pain, however, there is no stimulus that causes this and the pain felt is of no use. The activation of A δ and C-fibres have high activation thresholds, because they indicate tissue damage, however, in neuropathic pain states this is dramatically changed in both injured and surrounding uninjured neurons. This could be due to increased expression of voltage gated sodium channels, observed as increased mRNA transcripts correlating with ectopic nerve activity and neuropathic pain (Hains et al., 2004, Baron et al., 2010). Further evidence in support of this view is provided by the chronic pain states experienced by people with paroxysmal extreme pain disorder and erythromelalgia – both of these disorders are hereditary and are caused by mutations of SCN9A – a gene which encodes the Nav1.7 voltage gated sodium channel (Hains et al., 2004, Baron et al., 2010).

It is also possible that K⁺ channels such as Nav1.8 and Nav1.9 are involved in neuropathic pain by contributing to changes that are seen in membrane excitability of nociceptive nerves. The potassium channel is highlighted as a possible therapeutic target for neuropathic pain because it is involved in pain modulation and can suppress nociceptive neurons by hyperpolarisation (Lee, 2005, Baron et al., 2010, Zhao et al., 2010).

Several different receptors are dysregulated in neuropathic pain, such as TRPV1 – which is down-regulated on injured neurons, and as activation thresholds of such receptors can be altered, if the threshold was lowered to below 38°C, then affected receptors would produce ectopic action potentials constantly. In addition, abnormal function or expression of the cold receptors such as TMRM8 could lead to cold allodynia (Baron et al., 2010). Other cell types also undergo changes – for example it is thought that microglial expression of receptors such as P2X4 increase in chronic pain conditions (Horvath and DeLeo, 2009) (see later).

Central sensitisation is thought not to be caused by damage to the CNS itself, but is a consequence of repeated ectopic nerve activity and related to neurotrophic factors such as BDNF. Central sensitisation is often associated with reduced activation thresholds in dorsal horn neurons in response to noxious stimuli and also increased receptive field of

DRG neurons, thus inducing a secondary hyperalgesia and allodynia (Coderre and Melzack, 1992, Jin et al., 2003, Baron et al., 2010, Biggs et al., 2010).

CNS involvement can occur when excitatory amino acids and neuropeptides are released from peripheral afferent fibres; this can lead to post synaptic changes in 2nd order sensory neurons (those in the dorsal grey column or sensory nuclei) which can include the increased expression of sodium channels and phosphorylation of neurotransmitters such as NMDA and AMPA. Such changes act to lower the activation thresholds so that both A δ and A β fibres can activate second order neurons. This neuronal hypersensitivity leads to increased pain from non-noxious stimuli such as light touch (Coderre and Melzack, 1992, Jin et al., 2003, Baron et al., 2010).

Several mechanisms can contribute to ectopic nerve activity and central sensitization, which is one of the reasons why it is so difficult to treat neuropathic pain in patients. For example, after peripheral nerve injury or lesion, inflammatory processes will occur, thus causing activated macrophages to migrate into the afflicted area and release pro-inflammatory cytokines including tumour necrosis factor α (TNF α). In the CNS microglia can release immune modulators which can maintain neuropathic pain, thus contributing to peripheral sensitisation (Baron et al., 2010). There is also a loss of inhibitory GABAergic neurons (neurons which produce GABA), which can contribute to neuropathic pain, through disinhibition mechanisms after a Cl⁻ shift – GABA is excitatory and depolarizing when chloride ions flow out of the cell (Zhou et al., 2011).

The mechanisms by which neuropathic pain is initiated are multifaceted and complex and as such there are several different areas to explore in research. Some proposed mechanisms include increased nociceptor excitability through increased glutamate input to sensory neurons (Baron et al., 2010), decreased GABA inhibition in dorsal horn neurons (Zhou et al., 2011), and phosphorylation of NMDA (Woolf and Mannion, 1999) amongst others.

1.2.2. Animal Models of Neuropathic Pain

There are several animal models that are commonly used to study neuropathic pain, and these methods can themselves be grouped into various categories. These include central pain models – where pain is the result of CNS pathology; peripheral nerve injury models – where nerve injury results in hyperalgesia and allodynia and disease-induced models of neuropathic pain (Honoré et al., 2010). An overview of the most commonly used animal models of neuropathic pain of relevance to studies on macrophages in this thesis is outlined in this section.

Spinal Cord Injury (SCI) is a central pain model of neuropathic pain, which often causes spontaneous and evoked pain. SCI is usually induced by contusion in which a compression of the spinal cord takes place at T8. Weight dropping is another common method of producing SCI – in which a weight is allowed to drop on the exposed spinal cord at T12-T13. SCI results in hypersensitivity to light mechanical stimuli and allodynia within 1 day, and severe paraplegia (Jaggi et al., 2011, Colleoni and Sacerdote, 2010).

Partial nerve lesion models are peripheral nerve injury models, and are amongst the most common models of neuropathic pain in use, these include spinal nerve ligation (SNL), chronic constriction injury (CCI) and partial sciatic nerve injury (PNI). SNL is performed by ligating unilaterally and tightly the spinal nerves at L5 and/or L6. SNL is considered to be a model of neuropathic pain with relevant damage and the advantage of having injured and intact spinal segments (Kim and Chung, 1997, Colleoni and Sacerdote, 2010, Jaggi et al., 2011).

CCI of the sciatic nerve results in a similar neuropathy to injuries in man. CCI is easily reproducible as it is formed by three loose ligations that are tied around the common sciatic nerve at the mid-thigh level, resulting in the axotomisation of many of the axons. The neuropathy produced lasts for up to 7 weeks, and mechanical and thermal hyperalgesia (Colleoni and Sacerdote, 2010, Jaggi et al., 2011).

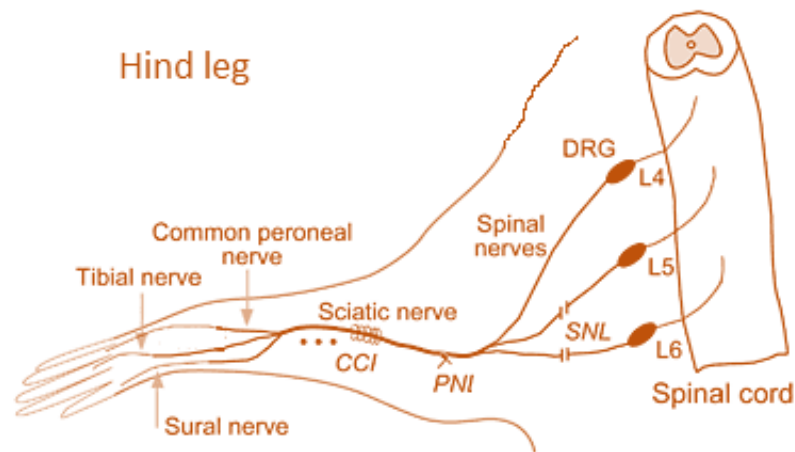


Figure 1.3. A diagram to explore common methods of producing animal models of neuropathic pain, including SNL, PNI and CCI, as demonstrated in the hind leg of the animal. Adapted from (Ueda and Rashid, 2003) and (Suter et al., 2011)

PNI is a common animal model for neuropathic pain, performed by tying a tight ligature around the diameter of the sciatic nerve (Seltzer et al., 1990, Malmberg and Basbaum, 1998). Nerve injured animals appear healthy and well groomed, but nonetheless, there is a marked decrease in the thermal and mechanical pain thresholds, indicating hyperalgesia, which persists for at least 6 weeks (Colleoni and Sacerdote, 2010).

1.2.3. Treatment of Neuropathic Pain.

Neuropathic pain is a particularly difficult condition to treat because there are so many mechanisms in which can cause neuropathic pain to arise, and so it is important to try and treat the mechanisms and thus cure neuropathic pain. This aside, there are some treatments available for neuropathic pain, even though they may not be fully effective, with a pain reduction of 30% (measured by questionnaires) considered a successful treatment.

One of the problems with treating neuropathic pain is that there are many different types of neuropathic pain, and even though many of the symptoms are the same, this would indicate that the mechanisms differ and therefore treatments will vary. Most drug trials have taken place with patients with either painful polyneuropathy or postherpetic neuralgia (Attal et al., 2006).

In patients with painful polyneuropathy, several drugs have been trialled, including antidepressants, antiepileptics and opioids. Tricyclic antidepressants such as amitriptyline, clomipramine and imipramine inhibit the presynaptic reuptake of both noradrenaline and serotonin, and block adrenergic, cholinergic and sodium channels (Abdi et al., 1998, Jefferies, 2010).

Tricyclic antidepressants have shown moderate efficacy as a treatment in trials, whereas selective serotonin reuptake inhibitors give clinically insufficient pain relief – although serotonin-noradrenaline reuptake inhibitors (venlafaxine, duloxetine) are moderately effective (Attal et al., 2006). However, the success of tricyclic antidepressants depends on the type of neuropathic pain, as they have been found to be ineffective against neuropathic cancer pain, HIV neuropathy and phantom limb pain (Micó et al., 2006, Jefferies, 2010).

Opioid treatment is thought to have a consistent efficacy in the treatment of neuropathic pain, however, due to their addictive nature; they are mainly reserved for second- or third-line drugs. The opioid levorphanol has been found to produce dose-dependent pain relief in patients with peripheral or central neuropathic pain (Finnerup et al., 2010).

The opioids oxycodone and tramadol also produce positive effects; both drugs are μ -opioid receptor agonists, with Tramadol also being able to inhibit the reuptake of noradrenaline or serotonin. Tramadol is associated with a quick onset of pain relief and has a low abuse potential (Attal et al., 2006, Jefferies, 2010).

The antiepileptic carbamazepine (a voltage-gated sodium blocker) had significant effects for patients with diabetic painful polyneuropathy. Oxycarbazepine (voltage gated sodium and calcium channel blocker) and Lamotrigine (a presynaptic voltage gated sodium channel inhibitor – which inhibits the presynaptic release of excitatory neurotransmitters) also had a significant effect on pain reduction. Another antiepileptic Topiramate, which blocks sodium channels and also inhibits glutamate release by acting on AMPA and kainate receptors – which are glutamate receptor ion channels (Dingledine et al., 1999), failed to have a significant effect in patients with diabetic painful polyneuropathy (Attal et al., 2006, Jefferies, 2010).

The best antiepileptics to date for treating neuropathies are gabalin, gabapentin and pregabalin. These drugs are thought to work by binding to the $\alpha_2\delta_1$ subunit of voltage dependant calcium channels, thus giving a reduced release of presynaptic transmitters. Pregabalin has analgesic, anxiolytic (anti-anxiety) and anticonvulsant properties. It has no known drug interactions and has little metabolic steps before it is excreted which increases the predictability of drug action. It has shown to be consistent in reducing pain in patients with either painful diabetic neuropathy or postherpatic neuralgia with doses ranging from 150-600mg/day, and gabalin has shown the same effects. One problem with the initial pregabalin trials is that non-responders to gabapentin were excluded from the trial and the drug functions through the same mechanism resulting in possible bias of trial results (Abdi et al., 1998, Wodarski et al., 2009, Attal et al., 2010, Semel et al., 2010).

1.3. Cell Migration

The ability of cells to migrate is a fundamental biological function, which is known to contribute to immune defence, morphogenesis and wound healing, it is also essential for tumour formation and metastasis.

In 2D, cell migration begins with polarisation in response to an extracellular signal due to modifications in the cytoskeleton that leads to the formation of lamellipodia at the leading edge, and a uropod at the rear. The leading edge is characterised by actin polymerisation and the clustering of integrins, forming focal adhesions with the ECM substrate. Focal adhesions disassemble at the trailing edge as the cell moves forward (Horwitz and Parsons, 1999, Verollet et al., 2011).

Cells attach to and move across the ECM mainly through the integrin family of cell surface receptors (Ref). Proteases of different classes are involved in the direct cleavage on integrin extra-cellular domains and in the proteolysis of the ECM, sometimes revealing pro-migratory cryptic sites (Stringa et al., 2000). In the migrating cell, there are bundles of microtubules that form a network within the cytoskeleton, the role of microtubules are complex and include determination of direction of migration (Ridley et al., 2003, Verollet et al., 2011, Vicente-Manzanares and Horwitz, 2011).

At the rear of the cell, the uropod is categorised by retraction, where all of the focal adhesions are disassembled. This is a process that is led by the GTPase, dynamin, and myosin light chain phosphatase, which prevents the phosphorylation of myosin II, which transmits a force along actin filaments to adhesion sites. Together, these processes help the cell to propel itself forward (Horwitz and Parsons, 1999, Ridley et al., 2003).

In macrophages, structures known as podosomes are formed which consist of F-actin rich structures surrounded by integrins; these podosomes are focused at the leading edge of the cell. The degradation of the ECM is dependent on matrix metalloproteinases (see chapter 1.7), with the degradation of fibronectin and collagen I being dependent partially on the presence of MMP-14 which is a crucial element in both the migration and chemotaxis of macrophages (Verollet et al., 2011).

1.4. Microglia and Macrophages.

1.4.1. Microglia.

Microglia have often been described as the macrophages of the nervous system and characterised by their role in immune function and the nervous system. They are located in nervous tissue and have roles including phagocytosis, cytokine secretion and antigen presentation to help protect neurons against infection and injury. Microglia have also been shown to play a role in development and are involved in physiological conditions such as neuronal function, synaptogenesis and developmental apoptosis. For example, it has been shown that nerve growth factor (NGF) (secreted by microglia in retinal tissue) is essential for developmental apoptosis in retina (Lobov et al., 2005, Bessis et al., 2007). Microglia secrete brain derived neurotrophic factor (BDNF) implicating these cells indirectly in synaptic function as BDNF directly regulates synaptic properties in the spinal cord (Coull et al., 2005, Bessis et al., 2007).

Microglia make up around 5-10% of glial cells in the CNS. In the resting state microglia have small soma and thin branched processes and act as sensors to changes in normal conditions which could threaten homeostasis, such as infection, ischemia or trauma. When microglia are activated by these stimuli, they undergo a series of changes in morphology, gene expression and function. Several genes are upregulated following activation including genes which control cell surface receptors such as CD11b (integrin α M) and CD18 (integrin β 2). Activated microglia also express MHC complexes (Tsuda et al., 2005).

During development, microglia infiltrate central nervous tissue at a very early age and form a meshed network of cells with the ability to detect and react to changes in the local environment. They have been shown to be able to secrete a number of cytokines shown to be implicated in neuronal function, and it is thought that the majority of neuronal functions are likely to be regulated by microglia (Bessis et al., 2007).

Glial cells and specifically microglia are of interest in a neuropathic pain context due to their underlying roles in synaptogenesis, the immune system and their activation in response to nerve injury. Rather than remaining in a dormant state in the healthy CNS, microglia are known to be constantly surveying the area to detect and respond to any

stimuli which pose a threat to physiological homeostasis (Jasmin et al., 2010). There is a growing body of evidence that suggests that the enhanced output of dorsal horn nociceptive neurons may be at least partially as a result of glia-neuron interactions (Scholz and Woolf, 2007, Beggs and Salter, 2010), and perhaps these have increasing importance in the development and maintenance of neuropathic pain.

Microglia and astrocytes show increased activation after either PNS or CNS injury, with the morphology of microglia changing from a resting state (cells have a small soma with long and thin processes), to an active state, (large soma and amoeboid shape, with short and thick processes). The activation of microglia can be demonstrated by staining with Iba1 or p38 as these proteins are expressed upon activation (Ito et al., 1998, Wodarski et al., 2009, Clark et al., 2010, Toda et al., 2011).

Further evidence that microglia could be involved in the onset of neuropathic pain is found after CCI. After CCI, the release of several cytokines and neurotrophins ensues; this has an effect on the primary afferent neurons and increases the excitability of the neurons (Lu et al., 2009, Wodarski et al., 2009, Biggs et al., 2010). However, this will be further explored later.

1.4.2. Macrophages

Macrophages are phagocytic cells that play an essential role in both innate and acquired immunity. First described by Ilya Mechnikov in 1893, they are able to respond to a variety of environmental cues by changing their phenotypes and physiologies (Vereyken et al., 2011). Macrophages have many specialised functions, which include phagocytosis, motility and diverse gene expression which are specialised to their role in immunity (Gordon, 2007). Macrophages are referred to as professional phagocytes and are extremely efficient at internalizing particles and pathogens (Aderem and Underhill, 1999).

Macrophages originate from bone marrow precursors and are present in most, if not all tissues and differentiate from circulating peripheral blood mononuclear cells which migrate into tissues as part of their steady state activities, or in response to stimuli, such as inflammation (Mosser and Edwards, 2008).

Macrophages respond to several endogenous stimuli (for example, IFN γ , TNF α , IL-10, IL-17, ATP) which are released or synthesised directly after injury or infection which in turn lead to the activation of macrophages (Mosser and Edwards, 2008). Macrophages are able to respond to numerous self and non-self molecules due to the expression of pattern recognition receptors on the cell surface, which are able to recognise pathogen associated molecular patterns (PAMPs). Amongst the receptors that macrophages express, there are complement receptors, integrins, Fc receptors, lectins and scavenger receptors, and Toll-like receptors.

Classically activated macrophages are referred to as M1 and alternatively activated as M2 macrophages. Typically, M1 macrophages release IL-1 β , IL-6 and TNF α , along with TGF α and TGF β , causing tissue damage, whereas M2 macrophages are anti-inflammatory producing IL-10 (Eming et al., 2007) and actively promote proliferation and tissue repair (Mills, 2012). However, it is thought that there are more subtypes of macrophages as they are known to have wide roles in a range of processes (for example, wound healing and regulating the immune response), although there is some difficulty in determining how to characterise them (Mosser and Edwards, 2008).

As macrophages are present in most tissues and in the circulating blood, they can migrate into the site of injury after peripheral nerve injury, and play a part in the onset and maintenance of chronic pain conditions (reviewed in Clark et al 2013).

1.5. ATP as an Extracellular Signalling Molecule.

In 1972, Burnstock proposed new roles for nucleotides as neurotransmitters after it was shown that ATP was able to act as a transmitter in guinea pig inhibitory nerves, despite it being well known that intracellular ATP is the key to free energy. P2 receptors were discovered and now these purinoceptors are divided into 2 families – ionotropic P2 receptors (P2X) and metabotropic receptors (P2Y). Nucleotides can leak and be released from all cells including damaged neurons, and can be involved in cell to cell communication in both normal physiological processes and pathophysiological processes, especially during trauma (Burnstock, 1972, Burnstock, 2007).

Initial opposition to the idea came from the fact that ATP was first recognised for its many roles in intracellular biochemical processes – and the intuition that a simple molecule such as ATP would be unlikely to be used as an extracellular messenger – given that it is so ubiquitous, even though enzymes to break down extracellular ATP were known to exist (Burnstock, 1972, Burnstock, 2007).

Purinergic receptors were first described in 1976, and later 2 receptor subtypes were defined, P1 receptors – which are activated by adenosine, and P2 receptors, activated by ATP/ADP. P2 receptors were later discovered to have 2 subtypes – P2X – a family of ligand gates ion channel receptors and P2Y – a family of G-protein coupled receptors. There are known to be 7 P2X subtypes, and 8 P2Y receptors (Burnstock, 1972, Burnstock, 1976, Burnstock, 2007).

Glial cells are thought to be implicated in the so called “purinergic nervous system”. Astrocytes express many types of P2X and P2Y receptors and can release ATP in response to stimuli, or spontaneously. It is possible for astrocytes to communicate with neurons at synapses, microglia and also vasculature. Microglia also express P2X and P2Y receptors and are thought to play an important role in pathological conditions in the brain, including chronic pain (Jin et al., 2003, Nasu-Tada et al., 2005, Trang et al., 2009, Tsuda et al., 2008, Inoue and Tsuda, 2009, Beggs and Salter, 2010, Inoue and Tsuda, 2012, Trang et al., 2012).

1.5.1. P1 Receptors

There are four different P1 receptors, P1A1, P1A2A, P1A2B and P1A3, all of which are members of the rhodopsin like family of GPCRs. P1 receptors have been shown to couple to adenylyate cyclase (enzymes which can convert ATP to cAMP) (Hanoune and Defer, 2001), and P1A2B has also previously been shown to act through $G_{\beta/11}$ to regulate activity of PLC in humans. Null mice for each of the P1 receptors (excluding P1A2B) have been bred, although none of these receptor subtypes appear to play a crucial role in development. However, the overexpression of both P1A1 and P1A3 has been shown to have cardioprotective effects (Burnstock, 2007, Idzko et al., 2014).

P1 receptors are activated by adenosine, and there are a number of subtype selective agonists which have been activated, with most of them closely resembling the structure of adenosine. Methylxantines such as caffeine and theophylline are weak P1 receptor antagonists (caffeine's mental stimulation and alertness effect is due to the blocking of P1 receptors and inhibiting the action of adenosine) (Burnstock, 2007).

Microglia express P1A1 receptors, along with P1A2A and P1A3 receptors, stimulation of the P1A1 and P1A2A receptors has previously been shown to cause activation and proliferation, although in some cases, it has also been shown to lead to apoptosis, depending on the environment (Hasko and Cronstein, 2013).

Stimulation of the P1A2A receptor can lead to the upregulation of cyclooxygenase-2 (COX-2) and the release of the pain-related molecule Prostaglandin E_2 , which could be indicative of a pro-inflammatory role. However, by-products of COX-2 could be responsible for the resolve of inflammation – suggesting that this may indeed be a neuroprotective role. Stimulation of P1A2A can also lead to the synthesis and release of NGF (Abbracchio and Ceruti, 2007, Hasko and Cronstein, 2013).

Adenosine stimulation of microglia has been shown to inhibit the production of IL-1 β and MMP-12 in infiltrating macrophages, and up-regulating IL-1, IL-6 and IL-12 in infiltrating cells, suggesting a pro-inflammatory phenotype. However, several studies have shown the ability of Adenosine to alleviate neuropathic pain behaviours, and to act as an analgesic (Belfrage et al., 1995, Guieu et al., 1996, Tsutsui et al., 2004, Haskó et al., 2005, Loram et al., 2009, Yamaoka et al., 2013).

1.5.2. P2X Receptors

P2X receptors are thought to be the first purinergic receptors to appear in evolution, and have a highly conserved structure (Burnstock, 2007). P2X receptors have three subunits, which each possess two transmembrane domains, one of which is involved in ion channel gating, and one which is involved in lining the ion pore. P2X receptor N- and C- terminals have binding motifs for protein kinases, and the receptor itself has a large extracellular loop which contains 10 conserved cysteine residues that are involved in the formation of a series of disulphide bridges. Within the P2X receptor, there is also a hydrophobic region close to the pore which may be involved in receptor modulation by cations, and an ATP binding site. Despite the structure and function of P2X receptors being highly conserved, sequence homology is about 30% (Burnstock, 2007, Verkhratsky and Burnstock, 2014).

There are seven genes which encode for P2X subunits, with the genes for P2X4 and P2X7 genes being located at the tip of chromosome 12. P2X4 and P2X7 receptors are closely related in terms of sequence homology (de Rivero Vaccari et al., 2012). Both P2X4 and P2X7 receptors are expressed in both macrophages and microglia.

1.5.2.1. P2X4 Receptor

The P2X4 receptor is one of the most predominant receptor subtypes expressed on immune and neural cells, and has been shown to be activated by ATP, but not by the structural homolog α,β -meATP. P2X4 receptor is known for being potentiated by ivermectin, which can increase the effect of activation of P2X4. P2X4 acts as a cation-selective channel when exposed to ATP for short amounts of time, however, when the exposure to ATP is longer, the channel can become larger and permeable to larger cations (Bernier et al., 2012). P2X4 desensitization has been described as intermediate, although the recovery time is quick. P2X4 is insensitive to blockade by the antagonists suramin and PPADs, and in fact, these antagonists appear to increase currents evoked by activation of the P2X4 receptor (Burnstock, 2007, de Rivero Vaccari et al., 2012).

P2X4 receptors have been reported to be involved in chronic pain conditions, in 2003 Tsuda *et al*, reported that P2X4 receptors were markedly upregulated after injury to the L5 spinal nerve in rats, and that P2X4 receptors were co-localised with microglia, but not astrocytes or neurons. It was observed that treatment with antisense oligodeoxynucleotides targeting P2X4 receptors resulted in a marked recovery in paw

withdrawal threshold tests – suggesting that P2X4 receptors are essential for mechanical tactile allodynia. Spinal administration of ATP stimulated microglia in normal rats also produces tactile allodynia dependent on P2X4 receptor. It was demonstrated that tactile allodynia can be reversed by the addition of TNP-ATP, a P2X antagonist – thus showing that P2X4 stimulation of microglia is sufficient to produce tactile allodynia (Tsuda et al., 2003).

Signalling through the p38-MAPK pathway in spinal microglia is also implicated in producing allodynia, nerve injury leads to the phosphorylation/activation of p38 MAPK. Inhibition of p38 MAPK leads to the suppression of allodynia, suggesting that it is involved in generating or maintaining allodynia and neuropathic pain (Jin et al., 2003, Tsuda et al., 2005). Trang *et al* in 2009 demonstrated that activation of P2X4 receptors evoked an increase in the activation of p38-MAPK activation – which leads to the synthesis and release of BDNF (Trang et al., 2009).

P2X4 receptors are upregulated after spinal nerve injury, for which Lyn has been reported as a critical signalling molecule. The fibronectin-integrin system is thought to play an important role in the upregulation of P2X4 receptors after spinal nerve injury – *in vivo* studies have shown that after spinal nerve injury the level of fibronectin protein is increased after 3-7 days, this is at the same time point that P2X4 receptors are seen to be upregulated. Also in primary cultured microglial cells, fibronectin was seen to increase the level of P2X4 receptors. Fibronectin has also been injected intrathecally in rats, which results in an upregulation of P2X4 receptors and tactile allodynia (Nasu-Tada et al., 2005, Tsuda et al., 2008).

It has been shown that the deletion of P2X4 in mice results in the abrogation of several immune responses, including a significant reduction in inflammasome activation. This leads to decreased IL-1 β , and a reduction in the infiltration of macrophages, along with other innate immune cells into the site of injury, after SCI (de Rivero Vaccari et al., 2012).

1.5.2.2. P2X7 Receptor

The P2X7 receptor is an important and unique member of the P2X receptor family. P2X7 was first cloned from monocytes and microglia – the P2X7 receptor is, like the P2X4

receptor, one of the most predominant receptor subtypes expressed on immune and neural cells, it is mainly expressed in peripheral macrophages and microglia. P2X7 is different from the other purinergic receptors, in that it is activated only by high concentrations of ATP (>100 μ M), and upon prolonged exposure, forms a large pore which is permeable to large dyes such as YO-PRO-1, the prolonged opening of this pore can lead to cell death. Prolonged activation of P2X7 can also lead to membrane blebbing and the release of inflammatory cytokines (Burnstock, 2007, He et al., 2012).

P2X7 receptors are also expected to be involved in neuropathic pain. P2X7 is upregulated in the dorsal horn after peripheral nerve injury in microglia, but not neurons or astrocytes, suggesting that P2X7 receptors mediates activation of microglia in chronic pain conditions (He et al., 2012).

Recently, structurally unrelated P2X7 agonists have been shown to reduce tactile allodynia in 3 different models of neuropathic pain (Tsuda et al., 2010). It has also been found that both selective, and non-selective P2X7 antagonists can lead to significantly reduced carrageenan-induced mechanical hyperalgesia (Teixeira et al., 2010).

He *et al* (2012) showed that CCI induced mechanical allodynia was reduced after treatment with the P2X7 receptor antagonist Brilliant Blue G (BBG), although not completely rescued. BBG was also successfully partially reducing thermal hypersensitivity (He et al., 2012).

1.5.3. P2Y Receptors

P2Y receptors differ from P2X receptors as they are linked to GPCRs and also differ in their selectivity to nucleotides, for example, some are selective to uracil nucleotides, whereas others are activated by adenine nucleotides (Ben Yebdri et al., 2009). As GPCRs, the P2Y receptors have a structure with an extracellular N terminus, seven transmembrane motifs and an intracellular C terminus. There is very little sequence homology in P2Y receptors which may explain their differences in the specificity of ligands (Burnstock, 2007).

P2Y receptors have different mechanisms of action depending on the G protein they couple too. P2Y1, P2Y2, P2Y4 and P2Y6 all bind to G_{q/11}, and activate phospholipase (PLC) – and releasing intracellular stores of Ca²⁺. P2Y12, P2Y13 and P2Y14 inhibit the adenylate

cyclase and therefore reduce cAMP levels by coupling with G_i . It is known that P2Y₁₁ can act as part of both of these signalling pathways (Ralevic and Burnstock, 1998).

1.5.3.1. P2Y₆ Receptor

P2Y₆ receptors are selective for UDP, and have a wide tissue distribution and can be found in the brain, heart, lungs, spleen, intestines and macrophages. P2Y₆ is slow to desensitize after activation, and acts by increasing activation of PLC and PKC, which leads to the formation of inositol-1,4,5-triphosphate (IP₃) and thus mobilization of intracellular calcium stores (Mamedova et al., 2004, Yu et al., 2013). It is also thought that P2Y₆ activation can lead to increased IL-8 production. (Bar et al., 2008). It has previously been shown that the antagonism of P2Y₆ can lead to the abrogation of inflammatory responses, suggesting that P2Y₆ may have a role in inflammation (Uratsuji et al., 2012). In microglia, the activation of P2Y₆ by UDP has been identified as a mediator of microglial phagocytosis (Koizumi et al., 2007).

P2Y receptors have a role in chronic pain conditions, P2Y receptors are metabotropic and are G-protein coupled receptor linked (Burnstock et al., 2011). It has recently been found that microglia express P2Y₆, and after peripheral nerve injury the expression of P2Y₆ mRNA is increased in a parallel time course with the development of tactile allodynia. Stimulation of the P2Y₆ receptor with UDP was found to facilitate microglial phagocytosis (Koizumi et al., 2007, Tsuda et al., 2010).

Recently, P2Y₆ receptor antagonists, along with P2Y₁₁ antagonists, were found to significantly reduce tactile allodynia in SNL rats. The SNL was also found to increase the expression of P2Y₆ for up to 21 days after injury. Thus demonstrating that P2Y₆ is indeed expressed in spinal microglia, and are upregulated in response to nerve injury – which may suggest that these receptors play a role in the development and or maintenance of neuropathic pain conditions (Barragan-Iglesias et al., 2014).

1.5.3.2. P2Y₁₂ Receptor

P2Y₁₂ is activated primarily by ADP, but can also be activated by ATP. P2Y₁₂ is heavily expressed in platelets, and is the target of the antiplatelet drug cloprigel, as there is a critical role for P2Y₁₂ in platelet activation (Wallentin, 2009, Liverani et al., 2014). P2Y₁₂ is also found in the brain and glial cells, and in smooth muscle cells. It is thought that P2Y₁₂

receptor can indeed regulate microglial activation, as P2Y₁₂ knockout mice possess microglia that are unable to polarize, migrate or even extend processes towards sites of trauma, thus suggesting that P2Y₁₂ is essential in migration of microglia towards the site of injury (Haynes et al., 2006). It is known that P2Y₁₂ has a number of effectors including PI3K and PLC, but the signal transduction downstream of P2Y₁₂ is not yet fully understood (Irina et al., 2008).

P2Y₁₂ receptors have a more understood role in neuropathic pain – it is known that the P2Y₁₂ receptor regulates a number of effectors including PI3K, adenylate cyclase, Phospholipase C (PLC), by signalling through G_i (Guidetti et al., 2008). Irino *et al* (2008) found that Akt activation downstream of PI3K and activation of the PLC pathway was necessary for microglia migration in response to an ADP chemoattractant (Irina et al., 2008).

P2Y₁₂ receptors have been found to be upregulated in the spinal cord ipsilateral to nerve injury, and tactile allodynia can be prevented after treatment with the P2Y₁₂ receptor antagonists MRS2395 and AR-C69931-MX. Knockout mice lacking P2Y₁₂ receptor display impaired tactile allodynia after peripheral nerve injury whilst basal levels of mechanical pain sensitivity do not change. It was also found that the intrathecal administration of AR-C69931-MX and oral administration of a P2Y₁₂ blocker – clopidogrel to injured rats can alleviate tactile allodynia. Taken together, these results suggest that P2Y₁₂ is important in the pathophysiology of neuropathic pain, and that blocking the actions of the receptor could be a potential treatment for chronic pain conditions (Tozaki-Saitoh et al., 2008).

The actions of P2Y₁₂ receptor have also been shown to be important in the migration of microglial cells. In response to ATP or ADP, microglia were found to display microspike processes and lamellipodia, which can suggest that P2Y₁₂ is responsible for mediating process extension in microglia – an initial process in migration of microglia. Inhibitors of PI3K and PLC were found to block ATP/ADP mediated process extension. Interestingly, it was also observed that ATP treatment increases the amount of active β 1 integrin in a time dependent manner, which is abrogated when P2Y₁₂ is blocked with AR-C69931-MX, suggesting that ATP can increase β 1 integrin mediated migration through P2Y₁₂ (Ohsawa et al., 2010).

1.6. The Response of Microglia and Macrophages to Damaged Neurons.

After peripheral nerve injury, or trauma to either the peripheral or central nervous system, it is possible for chronic pain conditions to arise. As explored previously, the mechanisms by which these conditions arise are complex and as of yet, not fully understood. However, recently focus has shifted from neuron-neuron interactions to microglia-neuron interactions to determine how neuropathic pain can develop.

After damage to the peripheral nerves, there is a significant activation of microglia in the dorsal horn, this is followed by increased proliferation and expression of proteins such as CD4 and CD14. It is thought that chemokines, macrophage colony stimulating factor, interleukins, ATP and neurotrophins could all contribute to the onset of neuropathic pain, and be involved in neuron-microglia signalling (Tsuda et al., 2005). Similarly, the activation and subsequent migration of macrophages into the site of injury is thought to be important (Scholz and Woolf, 2007, Ristoiu, 2013).

1.6.1. Platelet Activating Factor

There is accumulating evidence that platelet activating factor (PAF), and its receptor (PAFR) play a role in the mediation of pain signalling. PAF is a phospholipid mediator that is known to regulate the functions of a variety of cells in peripheral tissue and the nervous system – for example PAF is known to increase calcium concentration in dorsal root ganglion neurons, inhibit cAMP production, and activate the p38-MAPK pathway (Ma et al., 2010, Okubo et al., 2012, Pethő and Reeh, 2012).

It is known that an injection of PAF into the periphery enhances pain sensitivity in animals, however, when a structurally related but inactive protein, lyso-PAF, is injected, there is no response. Yet, PAFR antagonists have been found to produce anti-nociceptive effects in inflammatory pain models, and *in vivo*, PAFR deficient mice show a reduction in p38-MAPK signalling in DRG neurons following peripheral nerve injury (Tsuda et al., 2011).

Whereas wild type mice develop tactile allodynia after nerve injury, it was found that PAFR-deficient mice developed a reduced allodynia, and in WT mice, the expression of PAFR mRNA was increased in both the DRG and in macrophages, thus peripheral nerve

injury may cause the transcriptional upregulation of PAFR in macrophages and the DRG. It was also found that PAFR deficient mice demonstrated a marked suppression of the upregulation of TNF α and IL-1 β – suggesting that the PAF/PAFR system could contribute to tactile allodynia via regulation of TNF α and IL-1 β in macrophages in the injured DRG (Tsuda et al., 2011). There is also evidence to suggest that P2X receptors are involved in neuropathic pain, as P2X inhibitors were found to suppress PAF-induced tactile allodynia.

1.6.2. Neurotrophins.

Neurotrophins are a part of a family of closely related proteins; they are known to control survival and development of neurons, and also to influence the function of all neurons, and maintain neurons in adulthood. There are four major neurotrophic factors; Nerve Growth Factor (NGF), Brain Derived Neurotrophic Factor (BDNF), Neurotrophin-3 and Neurotrophin-4, which are all structurally related, however, sometimes Glial Derived Neurotrophic Factor and Ciliary Neurotrophic Factor are referred to as neurotrophins (Skaper, 2012).

Neurotrophins signal through one or more of the three members of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases – named TrkA, TrkB and TrkC, additionally, neurotrophins can signal through the p75 receptor. This section will focus on NGF and BDNF.

1.6.2.1. Nerve Growth Factor

Nerve Growth Factor was first defined and characterised by Rita Levi-Montalcini and Stanley Cohen throughout the 1950s and is now well characterised. Nerve growth factor binds to TrkA, and induces autophosphorylation of TrkA, which in turn can lead to the activation of various signalling pathways, including the PI3K/Akt pathway, MAPK/ERK pathway and the PLC γ pathway (Hong et al., 2012).

In the context of neuropathic pain, there are many contradictory reports in the literature, with many studies suggesting NGF be used as a treatment to alleviate neuropathic pain, whereas some literature suggests that NGF is indeed an agonist of neuropathic pain, and that anti-NGF functional antibodies could be used as a treatment.

NGF is produced as a mediator in some pain states, and can modulate the expression of several pain related transmitters, ion channels and receptors, including substance P. Some studies have demonstrated that the systemic administration of NGF can lead to the development of thermal hyperalgesia and allodynia in rats (Lewin et al., 1994). Leading on from this, Woolf et al in 1996, displayed that an anti-NGF functional antibody could indeed reverse hyperalgesia after CFA induced development of neuropathic pain. It was also found that anti-NGF antibodies could reverse established pain in the Chung SNL model of neuropathic pain (Woolf, 1996, Wild et al., 2007).

However, it has also been found that intrathecal administration of NGF has been found to restore the effectiveness of opioid treatment after CCI (Cirillo et al., 2010) and peripheral administration of NGF has been found to be beneficial in trials in patients with sensory neuropathy. Also, NGF treatments in rats have shown that after CCI, there was markedly reduced activity of microglia and astrocytes, including reduced production of inflammatory mediators (Cirillo et al., 2010).

NGF treated rats also showed a higher tolerance to Von Frey filaments (testing for mechanical hyperalgesia) and the plantar test (testing for thermal hyperalgesia), thus NGF produced a partial recovery of neuropathic pain behaviour (Cirillo et al., 2010).

1.6.2.2. Brain Derived Neurotrophic Factor

BDNF acts through its cognate receptor TrkB, which leads to the phosphorylation of PLC γ , leading to the formation of DAG and IP $_3$ and the subsequent release of intracellular calcium. BDNF is thought to be key molecule in the signalling pathway for microglia-neuron interaction and consequently, a molecule of interest concerning the onset of neuropathic pain (Coull et al., 2003, Trang et al., 2009).

BDNF has roles in cell survival, differentiation and cell migration in the CNS. BDNF has previously been linked with neuropathic pain, with studies demonstrating that after peripheral nerve injury, the expression of BDNF is increased (Coull et al., 2005, Zhang et al., 2012). BDNF is also able to disinhibit neurons in lamina I of the dorsal horn – a group of nociceptive neurons. For pain hypersensitivity to occur in lamina I neurons, there is first a change in the modal specificity of the output neurons which happens after a disruption

of chloride homeostasis – BDNF causes a rise in intracellular $[Cl^-]$, which suppresses GABA inhibition (Coull et al., 2003, Trang et al., 2009, Beggs and Salter, 2010).

Damaged afferent neurons show spontaneous activity and release additional cytokines and neurotrophins such as ATP and BDNF, which can exert long term effects on dorsal horn excitability (Lu et al., 2009, Biggs et al., 2010). 10-20 days of CCI of the sciatic nerve can produce a characteristic electrophysiological signature - i.e. a signature of change in the synaptic excitation of five different electrophysiologically defined neuronal phenotypes (described as tonic, delay, irregular, phasic and transient) in the dorsal horn of rats, the signature is described at the percentage of each type of neuron firing in each of the central, vertical and radial sections of the substantia gelatinosa. The appearance of this electrophysiological phenotype as described by Lu *et al*, 2009 is said to coincide with the onset of mechanical allodynia and hyperalgesia – tell-tale signs of neuropathic pain. Lu *et al*, 2009 also showed that exposure to BDNF can produce a similar electrophysiological signature to that shown in CCI and also *in vitro* (Lu et al., 2009).

Biggs *et al*, 2010 argue that BDNF may be solely responsible for the development of central sensitisation, bringing about the critical step transferring information from activated microglia to dorsal horn neurons, and thus developing neuropathic pain. Evidence for this is that BDNF and CCI seem to invoke similar electrophysiological injury signatures in the different defined neuronal phenotypes explained, but IL-1 β , a cytokine thought to be involved in neuropathic pain, does not produce this injury signature. Also, BDNF and CCI have similar effects on miniature Excitatory Pro-Synaptic Currents (mEPSCs) in relay neurons, and BDNF from activated microglia seem to promote dorsal horn excitability, which is attenuated when BDNF is sequestered by TrkBd5 (Lu et al., 2009, Biggs et al., 2010).

1.6.3. Cytokines and Chemokines

Cytokines are a large, diverse group of small proteins which can act as signalling molecules at small concentrations and work to regulate inflammation and can normally regulate growth and survival, along with differentiation. Cytokines can be grouped into having pro- (IL-6, TNF α) or anti- inflammatory (TGF β , IL-10) effects. Chemokines are chemotactic

cytokines, and have a generic function to induce cell migration, inducing the migration of immune cells to various locations where they are required (Ramesh et al., 2013).

Chemokines and their receptors are widely expressed in the immune and nervous systems. Chemokines are small secreted proteins which act through GPCRs, and have a central role in the normal inflammatory response, however, there is increasing evidence that chemokines, along with other cytokines can influence chronic pain conditions (White et al., 2007, Ramesh et al., 2013).

In particular, it has been shown that CCL2 (MCP-1), CCL3 (MIP-1 α) and CCL5 (RANTES) secreted by activated T cells, can produce a dose-dependent tactile allodynia, through a direct action of chemokines onto neurons (Oh et al., 2001). Chemokines have also been found to excite neurons from the DRG in culture, and promote the release of pain related neurotransmitters Substance P, and calcitonin gene-related peptide (Sun et al., 2006). It is thought that the mechanism behind this involves the excitation of TRPV1 and TRPA1 (as described earlier), as CCR1 and CCR2 activation can also excite TRPV1 and TRPA1 through PLC γ and PKC mediated events (White et al., 2007).

Interestingly, MCP-1 administration has been found to excite and influence action potentials in a group of DRG neurons overexpressing CCR2 as a response to neuropathic pain, suggesting that the production of MCP-1 can function as a neurotransmitter in the DRG neurons and that MCP-1 production by macrophages and microglia at the site of injury could contribute to the onset of neuropathic pain. It appears that chemokines could play a key role in the onset of chronic pain as they serve to regulate inflammatory events, but can act simultaneously upon neurons in the DRG (Abbadie, 2005, White et al., 2005, Sun et al., 2006, White et al., 2007, Miller et al., 2008, Jung et al., 2009).

1.6.4. Sigma Receptors

Sigma receptors was originally thought to be a subtype of opioid receptors and were initially described in the CNS by Martin et al in 1976, but were later found to be a different pharmacological entity (Martin et al., 1976, Su et al., 1988a, Su et al., 1988b). Outside the brain, Sigma receptors were found in a number of other tissues including the kidneys, liver, testes and intestine. Quirion and colleagues proposed the classification of two subtypes of sigma receptor based on the affinity of ligands such as (+)-pentazocine and

dextromethorphan binding, with Sigma-1 receptor showing a high affinity, and Sigma-2 receptor showing low affinity – although there are nonselective ligands, such as 1,3-Di-(2-tolyl)guanidine (DTG) and haloperidol which show similar affinity to both sigma receptor (Quirion et al., 1992, Maurice and Lockhart, 1997).

The exact functional role of the sigma receptor remains unclear still, with studies suggesting that they have a role in antipsychotic activity, prevention of amnesia, modulation of neurotensin, inhibition of re-uptake of noradrenalin, the modulation of cell surface signalling molecules and the chaperoning and maturation of BDNF (Levant and Nemeroff, 1990, Maurice and Lockhart, 1997, Su et al., 2010, Hashimoto, 2013). However, Itzhak (1989) demonstrated that binding to sigma-1 receptor is regulated by $G_{i/o}$ (Itzhak, 1989, Maurice and Lockhart, 1997).

Sigma-1 Receptor was cloned in 1996, it is a 223 residue transmembrane protein associated with the endoplasmic reticulum, specifically at the endoplasmic reticulum – mitochondria interface. Sigma-1 receptor is able to regulate the stability of IP3 receptors and calcium signalling between the endoplasmic reticulum and the mitochondria along with ion channel firing, neurotransmitter release, inflammation, cellular differentiation and neuron survival (Su et al., 2010, Hashimoto, 2013). There have been no other known proteins which share sequence homology with Sigma-1 receptor (Su et al., 2010).

In the dormant state of Sigma-1, the receptor forms a complex with the chaperone, Binding immunoglobulin Protein (BiP). However, it is thought that when cells undergo stress, or through certain stimulation, sigma-1 receptors can dissociate from BiP, and become activated. Activated sigma-1 receptors are able to translocate to the endoplasmic reticulum network and to the plasma membrane in order to modulate signalling through cell-surface receptors, possibly including sodium channels such as Nav 1.8, or ion channels, along with other receptors such as TrkB (Su et al., 2010). Sigma-1 receptor also plays a role in the release of BDNF: when the effect of Sigma-1 receptor knockdown was explored, it was found that the level of secreted mature BDNF was significantly reduced, although no effect was observed on intracellular levels of BDNF (Hashimoto, 2013). Furthermore, Yagasaki and colleagues found that imipramine can enhance BDNF-induced TrkB signalling through PLC γ , but when cells were treated with BD1047 (a sigma-1 antagonist), the

potentiation of PLC γ activation through TrkB was attenuated, suggesting that Sigma-1 may be able to modulate signalling through TrkB (Yagasaki et al., 2006).

The sigma receptor modulation of microglial activation has previously been explored, and it was observed that Sigma-1 receptors play a role in several aspects in microglial function. Sigma-1 was found to regulate the function of microglia by suppressing the release of intracellular calcium – although calcium independent regulation of microglial activation was also reported. Altogether, it was reported that the antagonism of Sigma-1 receptors with DTG was found to suppress membrane ruffling, inflammatory response (measured by observing production of TNF α , IL-10 and NO) and migration in microglia (Hall et al., 2009). It is thought that because microglial migration is a calcium dependent process and requires an influx of calcium, that DTG, or the antagonism of sigma-1 may inhibit the action of purinergic receptors.

Sigma-1 receptor has also been implicated in the onset and maintenance of chronic pain conditions. It has been found that the activation of spinal sigma-1 receptors resulted in both thermal and mechanical hyperalgesia, along with the phosphorylation of spinal ERK. The antagonism of sigma-1 receptor was reported to lead to the attenuation of pain behaviours (de la Puente et al., 2009, Roh et al., 2011, Moon et al., 2013). A study by Romero *et al*, tested the effects of a novel sigma-1 receptor antagonist on neuropathic pain, it was found that the antagonism of sigma-1 receptor abrogated ectopic nerve activity to the same level as pregabalin , along with alleviating chronic pain-like behaviour in formalin-induced neuropathic pain, capsaicin-induced hypersensitivity and SNL (Romero et al., 2012).

In addition, it was found that sigma-1 receptor has a role in chemotherapy (Paclitaxel)-induced neuropathic pain. In sigma-1 receptor knockout mice, and with the treatment of sigma-1 receptor antagonists, paclitaxel failed to induce the phosphorylation of ERK in the spinal cord, but not in wild type mice. It was also found that sigma-1 knockout mice did not develop cold allodynia or mechanical allodynia (Nieto et al., 2012, Zamanillo et al., 2013). Taken together, these studies suggest that sigma-1 receptor may play a crucial role in the development of chronic pain conditions, and antagonism of sigma-1 could be a potential treatment for patients with neuropathic pain.

1.7. Matrix Metalloproteinases

1.7.1. Structure and Activity

There are five subfamilies of proteinases – serine proteinases, cysteine proteinases, aspartic proteinases, threonine proteinases and finally metalloproteinases. Whilst both cysteine proteinases and aspartic proteinases are mainly active intracellularly, serine proteinases act extracellularly to cleave surrounding ECM components amongst other targets. Metalloproteinases can be further subdivided into families including matrix metalloproteinases (MMPs), ADAMs (a disintegrin and metalloproteinase), and the ADAMTSs (ADAM with a thrombospondin motif) (Ugalde et al., 2010).

MMPs are zinc dependent endopeptidases known for their ability to degrade extracellular matrix components, they also play a role in processing bioactive molecules such as chemokines and cytokines. There are 23 human MMPs, which are either secreted into the extracellular milieu or anchored onto the cell membrane (Birkedal-Hansen et al., 1993, Parks et al., 2004). MMPs can be further divided into groups depending on which ECM component they display the highest affinity with; there are gelatinases (MMP-2/MMP-9), collagenases (MMP-1, MMP-8, MMP-13, MMP-18) and stromelysins (MMP-3, MMP-10, MMP-11), matrilysins (MMP-7, MMP26), membrane-type MMPs (MT-MMPs; MMP-14, MMP-15, MMP-16, MMP-17, MMP-24 and MMP-25), as well as currently uncategorised MMPs (Wang et al., 2014) (See Table 1.2).

Traditionally, matrix metalloproteinases were thought to be involved in the remodelling of the ECM and allowing cells to migrate in both normal and pathological conditions (Murphy and Gavrilovic, 1999). Whilst this has later been shown in several processes, for example in migration of vascular smooth muscle cells (Newby, 2006) and macrophage migration in inflammatory conditions (Gong et al., 2008), along with general cell migration (Itoh, 2006), it is thought that MMPs also have roles in controlling cell behaviour and functions, which includes roles in adipocyte differentiation, both pro- and anti-inflammatory effects, platelet aggregation and apoptosis (McCawley and Matrisian, 2001, Murphy and Nagase, 2008).

FAMILY	NAME	OTHER NAMES	SUBSTRATE	ACTIVATED BY
COLLAGENASES	MMP-1	Collagenase-1	Type-I, II, III, VIII, and X Fibrillar Collagens.	MMP-3, MMP-10, Plasmin
	MMP-8	Collagenase-2, Neutrophil collagenase	Type I, II, and III Fibrillar Collagens. Mouse CXCL5	MMP-3, MMP-10, Plasmin
	MMP-13	Collagenase-3	Type I, II, III, IV, IX, X, XI Collagens, Fibronectin, Laminin, Gelatin	Plasmin, MMP-2, MMP-3, MMP-10, MMP-14, MMP-15
GELATINASES	MMP-2	Gelatinase A	Gelatin, Fibronectin, Type I and IV Collagen, CCL7, CXCL12	MMP-1, MMP-7, MMP-13, MMP-14, MMP-16, MMP-17, MMP-25
	MMP-9	Gelatinase B	Gelatin, Latent VEGF, Fibrin,	Plasmin, MMP-2, MMP-3, MMP-13
MATRILYSINS	MMP-7	Matrilysin	Type IV Collagen, Fibronectin, Nidogen, Elastin, Laminin, Fibrin, Fibrinogen	Plasmin, MMP-3
	MMP-26	Matrilysin-2	Type IV Collagen, Gelatin, Fibronectin, Fibrin, Fibrinogen	MMP-26
STROMELYSIN	MMP-3	Stromelysin-1	Type IV Collagen, Fibronectin, Aggrecan, Nidogen, Laminin, E-Cadherin, Latent TGF- β	Plasmin, Elastase
	MMP-10	Stromelysin-2	Type IV Collagen, Fibronectin, Aggrecan, Nidogen	Plasmin, Elastase
	MMP-11	Stromelysin-3	α 2 macroglobulin, type VI collagen	Furin

	MMP-19		Type IV Collagen, Fibronectin, Aggrecan,	Trypsin
MEMBRANE TYPE	MMP-14	MT1-MMP	Type-I, II and III Collagen, Fibronectin, Vitronectin, Laminin, Aggrecan, ProMMP2, Fibrin	Plasmin, Furin
	MMP-15	MT2-MMP	Proteoglycans	-
	MMP-16	MT3-MMP	Type III collagen, Syndecan-1	-
	MMP-17	MT4-MMP	Gelatin, Fibrin, Fibrinogen	-
	MMP-25	MT5-MMP	Fibronectin, Proteoglycan, Gelatin	-
	MMP-26	MT6-MMP	Type IV Collagen, Gelatin, Fibronectin	-
UNCLASSIFIED	MMP-12	Metalloelastase	Type IV Collagen, Fibronectin, Aggrecan, Elastin	Plasmin
	MMP-20	Enamelysin	Aggrecan	-
	MMP-23	CA-MMP	Gelatin	-
	MMP-27	-	-	-
	MMP-28	Epilysin	Casein	-

Table 1.2. A table to show the MMPs and their ECM substrates, along with their activators. The information from which this table was derived was adapted from (Parks et al., 2004), (Nagase et al., 2006) (Murphy and Nagase, 2008) and (Grobewska et al., 2012).

The structure of MMPs varies, as do their substrates and functions, although they do share some structural homology. A matrix metalloproteinase typically consists of a conserved prodomain of around 80 residues in length, and a catalytic metalloprotease domain, which consists of 170 amino acids. Most MMPs (not MMPs -7, -23 or -26) contain a proline rich hinge region which varies in length, and a carboxy-terminal hemopexin domain, which acts to mediate interactions between the MMP and its substrate (Nagase et al., 2006).

In the inactive enzyme the prodomain interacts with the Zn^{2+} atom in the catalytic domain of the MMP and confers a level of control onto the MMP as the prodomain has to be cleaved (often by a serine protease or another MMP) for the enzyme to be activated (Parks et al., 2004).

1.7.2. Tissue Inhibitors of Matrix Metalloproteinases

The activity of metalloproteinases is strictly regulated at several levels, including regulation of transcription by growth factors and cytokines, zymogen activation and also by inhibition by their endogenous inhibitors, the tissue inhibitors of matrix metalloproteinases, TIMPs (Brew and Nagase, 2010). There are 4 TIMPs in total, which are 184-194 amino acids in length, and formed of two domains, which each have three conserved disulphide bonds. The N terminal of TIMPs is conserved and responsible for folding, and chelating the Zn^{2+} ion in the active site of MMPs, thus blocking the activity of MMPs, however, the C-terminal of TIMPs can vary in length and is responsible for the differing specificity of TIMPs (Bourboulia and Stetler-Stevenson, 2010, Perez-Sayans Garcia et al., 2012, Khokha et al., 2013).

TIMP-2 and TIMP-3 are known to inhibit all MMPs, TIMP-4 can inhibit most MMPs, but TIMP-1 displays a weak affinity for the MT-MMPs (MMP-14, MMP-16, MMP-19 and MMP-24). TIMP-3 has further differences with the other TIMPs, as it displays a high affinity for members of the ADAM and ADAMTS families, and is able to bind to the extracellular matrix, whereas the other TIMPs are soluble (Nagase et al., 2006).

TIMPs can also be important in the activation of MMP-2 and MMP-9, which are secreted in their inactive zymogen form of proMMP-2 and proMMP-9. In latent forms of the MMP, there is a cleavable propeptide inserted into the catalytic site of the MMP which must be removed so that the MMP can be active. These proMMPs bind to TIMPs in between the C-terminal of the TIMP, and the heamopexin domain of the proMMP, and are activated (Murphy, 2011). For example, MT1-MMP activates proMMP-2 in a process which involved a complex of TIMP-2 and MT1-MMP binding proMMP-2 (Bernardo and Fridman, 2003).

It is thought that TIMPs have other biological activities, including the promotion of proliferation, synaptic plasticity and both pro-and anti-apoptotic activities, (Perez-Sayans Garcia et al., 2012), although these activities remain controversial..

1.7.3. Matrix Metalloproteinases in Migration

Although it is possible for cell migration to be independent of MMPs, the expression of MMPs has been found to be crucial in the migration of several cell types, in this section, an overview of the role of MMPs in cell migration will be presented.

In the intima of rat carotid artery, there are no smooth muscle cells, however, after injury; it is observed that smooth muscle cells migrate into the carotid artery – during which both of the gelatinases – MMP-2 and MMP-9 are found to be upregulated. The broad spectrum inhibition of MMPs using GM-6001 was found to almost completely ablate the migration of smooth muscle cells into the carotid artery for up to 4 days after injury – thus showing that smooth muscle cell migration is dependent on MMP activity (Bendeck et al., 1994, Bendeck et al., 1996).

In the context of ovarian cancer, a highly metastatic disease, it was demonstrated that MMP expression was up-regulated, and thought to be involved in the invasion and metastasis of ovarian cancer cells. It was found that in ovarian cancer cells, there was an upregulation of MMP activation induced by lysophosphatidic acid, in particular MT1-MMP and MMP-2, leading to an enhanced invasive phenotype of ovarian cancer cells. This suggests that the expression of MMP-2 and MT1-MMP are responsible for invasion as a direct result of increased hydrolysis of matrix components (Fishman et al., 2001).

The effect of MMP expression in tumour invasion and metastasis has been widely researched, as the constant degradation and remodelling of the ECM by MMPs is required to allow for invasion of cells. In a lung carcinoma cell line, it was found that the overexpression of MT1-MMP resulted in the increased survival of cancerous cells and the increased formation of metastatic nodules when the cells were inoculated into mouse tail vein. The dependence of cell migration on MMPs was also demonstrated when MMP-1 was suppressed in malignant melanoma cells, which lead to the inhibition of cells into a Matrigel™ matrix (Nabeshima et al., 2002).

Furthermore, the dependence of MMPs in migration has been shown in leukocytes. Tester *et al*, demonstrated that MMP-8 was required for the neutrophil response to LPS, and loss of MMP-8 led to a marked reduction in chemotaxis towards LPS (Tester et al., 2007). MMP-8 has also been linked to leukocyte rolling and adhesion on vascular endothelium in response to atherosclerosis, suggesting that it is required for the normal monocyte response and important in the migration of monocytes into the vessel wall towards MCP-1 (Laxton et al., 2009).

1.7.4. Matrix Metalloproteinases in Macrophages and Microglia

Rapid cell migration is an important feature of all cells involved in the immune system, and when they are required, both cell types have to move through complex and diverse environments in 3D. As the process of cell migration has been shown to be dependent on MMPs in various cell types, it is thought that MMPs may play an important role in the migration and invasion in both macrophages and microglia.

In vivo, it has been found that MMPs are involved in the invasion of macrophages – and it has been found that MMP expression is upregulated in macrophages during infiltration, and GM-6001 and MMP-2 knockout in asthmatic mice can reduce the influx of macrophages into the bronchoalveolar lavage, and result in the loss of normal chemotactic ability in these cells. This leads to an accumulation of macrophages and other inflammatory cells in the lung parenchyma which can in turn lead to airway hyper-responsiveness (Corry et al., 2002, Verollet et al., 2011).

In vitro, MMPs have also been shown to be involved in the migration and invasion of macrophages. In 2013, Murray *et al*, found that MMP-10 was at least partially responsible for macrophage migration – in cells transfected with siRNA targeting MMP-10, cell migration speed reduced approximately three-fold. It was also observed that bone marrow derived macrophages from MMP-10^{-/-} mice migrated over a 2D surface of fibronectin significantly slower than WT mice. It was also demonstrated that BMMs from MMP-10^{-/-} mice resulted in a significantly reduced level of invasion in a Matrigel™/Fibronectin matrix (Murray et al., 2013).

Microglia have been found to express MMPs, and secrete MMP-3 in response to activation. MMP-2 and MMP-9 are also expressed by microglia and are thought to contribute to the

formation of the glia limitans – a thin barrier of astrocyte foot processes which has many functions – including the prevention of over migration by both glial cells and neurons. MMPs are thought to play roles in tissue repair, synaptic plasticity and the formation of memories (Konnecke and Bechmann, 2013).

However, MMPs -8 and -9 may be involved in the breakdown of the blood brain barrier in pathological conditions (Vandenbroucke et al., 2012, Konnecke and Bechmann, 2013). MMP-9 is further expected to be involved in the bioavailability of cytokines and the generation of autoimmune epitopes in some pathological conditions, and it is thought that the inhibition of MMPs could be beneficial as a therapy in autoimmune conditions (Konnecke and Bechmann, 2013).

It is also thought that MMPs could be involved in neuropathic pain – a study by Kawasaki *et al* suggests that both gelatinases are involved in early and late phase development of chronic pain. It was found that MMP-9 is rapidly upregulated in injured spinal dorsal root ganglion primary sensory neurons. MMP-2 however, was found to have a delayed response in DRG satellite cells and spinal astrocytes (Kawasaki *et al*, 2008). It was discovered that inhibition of the MMP-9 inhibits the early phases of neuropathic pain, whereas MMP-2 inhibits the late phase – suggesting that the MMPs have different roles in chronic pain. Further studies show that both MMPs activate interleukin-1 β which could play a role in chronic pain, but also MMP-9 activates microglia, and MMP-2 activates astrocytes (Kawasaki et al., 2008).

MMPs have also been reported to be upregulated following spinal cord injury in macrophages within the spinal cord lesion – with MMP-1 being upregulated in these cells. There was also found to be a short induction of MMP-2 expression in these macrophages, with both MMP-9 and MMP-12 being upregulated at a later phase, with expression levels peaking at 24 days after injury. There was little reported TIMP activity (Buss et al., 2007). These studies put forward a compelling argument which suggests that MMP activity may indeed be involved in the onset and maintenance of chronic pain conditions.

1.8. Atherosclerosis

1.8.1. Pathogenesis of Atherosclerosis

Atherosclerosis was first described by Felix Marchand in 1904, defining the hardening of arteries with a fatty substance and Nikolai Anichkov first demonstrated the role of cholesterol alone in the development of atherosclerosis using a rabbit model in 1913 (Konstantinov et al., 2006). At the time the research was somewhat dismissed as Anichkov could not replicate the results in rats, as they are carnivorous and are better suited to high levels of cholesterol.

In the western world, atherosclerosis is the underlying cause of around 50% of all deaths, and is characterised by a fatty occlusion in the arterial lumen. In 1977, Ross *et al* (Ross et al., 1977) set forth the 'response to injury' hypothesis, which infers that atherosclerosis is a response to injury. More recently, atherosclerosis is thought to be an inflammatory disorder (Hansson, 2005).

The 'response to injury' hypothesis states that injury to the vessel wall leads to the desquamation of the endothelial cells. There are potentially many sources of injury to the vessel wall – such as chronic hyperlipidaemia, infection, mechanical trauma (such as increased shear stress due to high blood pressure), or due to chemical factors such as homocysteine and oxLDL (Ross et al., 1977). After this initial injury there is dysfunction of the endothelium which can lead to the trapping of lipoprotein and the appearance of adhesive glycoproteins on the surface of the endothelial cells lining the artery – this leads to the adherence of monocytes and lymphocytes and the subsequent migration into the vessel wall (Ross, 1993).

The migrating monocytes differentiate into macrophages and move further into the intima towards oxidized LDL (oxLDL), and MCP-1. The macrophages are then able to contribute to the recruitment of more macrophages by synthesizing and secreting more MCP-1, along with cytokines such as IL-6 and IL-8 (Tedgui and Mallat, 2006). The macrophages in the intima then act to engulf the atherogenic lipoproteins present and are unable to process them, thus the macrophages become laden with fatty lipoproteins and become foam cells (Falk, 2006). The accumulation of foam cells along with leukocytes denotes the formation of the fatty streak (Ross, 1993). Continued cell migration into the fatty streak and the

accumulation these cells leads to the development of more advanced atherosclerotic lesions. Meanwhile, the migrating and proliferating smooth muscle cells form a connective tissue matrix which consists of collagens, proteoglycans and elastins (Ross, 1993).

As a consequence of the continued accumulation and proliferation of the cells in the fatty streak, the lumen becomes convoluted. In advanced atherosclerotic lesions there are alternating layers of smooth muscle cells and foam cells, which build up and eventually lead to the partial, or in extreme cases, complete occlusion of the vessel (Ross, 1993, Randolph, 2014).

As the rupture of the plaque can lead to many complications, including stroke and myocardial infarction, plaque stabilization is an issue. After formation of the atherosclerotic plaque, the structure consists of a fibrous cap enclosing the central core of lipids and usually necrotic debris, surrounded by foam cells. Whilst the fibrous cap provides the stability, the core (or atheroma) is soft and thrombogenic, and so the integrity of the fibrous cap is important (van der Wal and Becker, 1999).

Factors that are of importance in the stabilization of the plaque include matrix synthesis and the amount of smooth muscle cells present in the plaque, along with the thickness of the fibrous cap. Destabilising factors include the amount of lipids present and inflammation, along with the presence of proteases, such as MMPs, which can breakdown the matrix synthesised by smooth muscle cells. It is these aspects which are in constant balance and ultimately determine if a plaque is indeed vulnerable to rupture (van der Wal and Becker, 1999). However, the role of MMPs in atherosclerosis has yet to be determined, and will be further explored in this thesis.

1.8.2. Macrophages in Atherosclerosis

There is no step in the development of the fatty streak and the atherosclerotic plaque that does not involve the participation of macrophages and their precursor monocytes. Monocytes infiltrate the vessel wall where they differentiate into macrophages – this continued accumulation of macrophages depends on macrophage colony stimulating factor and granulocyte colony stimulating factor. Whilst inside the vessel wall, macrophages secrete cytokines and chemokines, and metalloproteinases, along with

growth factors and other hydrolytic enzymes, which can contribute to the stabilisation of the plaque or, if apoptosis occurs, the necrotic atheroma (Ross, 1999).

During migration, macrophages degrade the ECM components that they need to as to get to their destination; this could possibly lead to some plaque destabilisation. Macrophages then act to phagocytose any cell debris and pathogens; however, in the context of atherosclerosis, macrophages also internalize oxLDL and become foam cells. Foam cells are able to re-export cholesterol. Macrophages are capable of producing both pro-inflammatory cytokines (IL-6, IL-10, TNF α), and anti-inflammatory cytokines (IL-10, TGF β) which can act to either promote or suppress the immune response in atherosclerosis. Macrophages can also facilitate tissue repair, and promote the proliferation and migration of smooth muscle cells into the intima to contribute to the fibrous cap (Johnson and Newby, 2009).

In mice, it has been recognised that there are two distinct subsets of circulating monocytes which have differing cell surface markers and chemokine receptor expression patterns – characterised by Ly6C (a glycoprotein), CCR2 and CX₃CR1 expression levels (Waldo et al., 2008, Johnson and Newby, 2009). It was found that the migration of monocytes into the atherosclerotic plaque depends on both CCL2 and CX₃CL1 (fractalkine) expression and that the inhibition of these pathways almost abolishes atherosclerosis (Combadière et al., 2008). However, monocytes that have a low expression of Ly6C show a circulating behaviour, and interact with the endothelium without migrating into the intima, thus showing a protective effect in atherosclerosis conditions (Johnson and Newby, 2009).

Johnson and Newby, 2008 outline that the role of macrophages in atherosclerosis can be summarised into a series of paradoxical roles. For example, macrophages can display an advantageous phenotype – producing granulocyte colony stimulating factor, IL-4 and IL-13 and thus exiting from lesions and participating in fibrosis and calcification – thus stabilizing the atherosclerotic plaque. On the other hand, macrophages can produce macrophage colony stimulating factor, TNF α , and IFN γ contributing to the inflammation and leading to further invasion into the plaque, phagocytosis and uptake of oxLDL, degradation of essential matrix and further cholesterol uptake – leading to the formation of foam cells. It has also been shown that the expression of CD14 correlates with complicated, advanced

lesions and plaque rupture (Johnson and Newby, 2009, Hermansson et al., 2014) . Therefore, it seems that the macrophage genotype and subsequent phenotype expressed largely contributes to the stability of the atherosclerotic plaque, and therefore the prognosis of the disease (Waldo et al., 2008).

1.8.3. Matrix Metalloproteinases in Atherosclerosis

MMPs are thought to play an important role in atherosclerosis, given their ability to remodel the extracellular matrix, MMPs can contribute to either the stabilisation or destabilisation of the atherosclerotic plaque. The degradation of ECM components is required for both the infiltration of monocytes into the intima, and the migration of smooth muscle cells (Watanabe and Ikeda, 2004, Bäck et al., 2010). MMP expression could lead to the stability of a plaque, or the destruction of the plaque – as plaque rupture results from the destruction of the intimal ECM (Newby, 2005). MMPs are also involved in the process of plaque angiogenesis, which can be a marker for a vulnerable plaque (Schäfers et al., 2010).

It has been found that MMPs are expressed in atherosclerotic plaques by both smooth muscle cells and macrophages/foam cells, and some MMPs – including MMP-1, MMP-2, MMP-3, MMP-7 along with MMP-9, MMP-10, MMP-11, and MMP-12 have all been shown to be upregulated in the plaque (Galis et al., 1994, Dollery et al., 1995, Ketelhuth and Bäck, 2011), the overexpression of MMPs is thought to promote plaque rupture (Lancelot et al., 2008). In macrophages, it is thought that MMP-9 is the most abundant MMP, and it has been shown to be upregulated after adhesion to ECM components such as collagen (the most abundant ECM component in the atherosclerotic plaque) and fibronectin. Other pro-inflammatory, pro-atherogenic cytokines such as TNF α can influence the upregulation of MMP-14, MMP-15 and MMP-16, and activate MMP-2 (Newby, 2005).

TIMP expression is also found to be upregulated, perhaps to attempt to limit the activity of MMPs – however MMPs are still active, as macrophage and foam cell conditioned media has been shown to degrade collagen from fibrous plaque caps *in vitro*. The MMPs secreted from macrophages can also lead to further activation of MMPs secreted by smooth muscle cells (Newby, 2005).

MMPs are of particular interest in the disruption of the arterial wall and the processes which lead up to the rupture of the plaque, especially those belonging to the collagenase family. It is suggested that the mechanism behind the increased expression of MMPs is driven by oxidative stress, although it is also inferred that thrombin and the plasminogen cascade may play a role in the activating of MMPs, the former in a positive feedback loop (Galis and Khatri, 2002). Recently, it has been found that MMP-12 overexpression leads to accelerated plaque development, whereas in MMP-12^{-/-} mice, plaque development is greatly reduced, and selective MMP-12 inhibitors have been shown to promote a stable plaque (Johnson et al., 2011, Scholtes et al., 2012). Whilst there are many studies which link the expression and activation of MMPs in atherosclerosis, and the promotion of either a stable or unstable plaque phenotype, there are still more studies required which address the role of specific MMPs and their interactions during atherosclerosis initiation.

1.8.4. Apolipoprotein E

Apolipoprotein E (ApoE) was discovered in the 1973 by Shore and Shore (Shore and Shore, 1973), it is a 34kDa protein consisting of 299 amino acids. ApoE was identified in triglyceride rich lipoproteins and was found to be induced in cholesterol feeding in both animal and human models. Plasma ApoE is synthesized primarily by the liver, although the brain is the second most common site of synthesis – astrocytes produce ApoE, but stressed neurons are also capable of synthesising the lipoprotein. Macrophages are also a site of synthesis of ApoE (Greenow et al., 2005, Mahley, 1988).

Upon its discovery, ApoE was reported to be a multifunctional protein involved in lipid transport and in cardiovascular disease (a common animal model for cardiovascular disease is ApoE knockout mice), and was shown to be a critical ligand in the plasma clearance of triglyceride, and cholesterol-rich lipoproteins – including chylomicron remnants, high density lipoprotein (HDL) subclasses, very low density-lipoproteins (VLDL) and intermediate density-lipoprotein (Curtiss, 2000, Cash et al., 2012, Mahley et al., 2009). ApoE is involved in cholesterol efflux from macrophage foam cells, uptake of lipoprotein particles in the liver, and the regulation of several immune and inflammatory responses (Tiwari et al., 2008).

Structurally, ApoE has two separate domains that are connected together via a hinge region. The first 191 residues make up the N-terminal domain, which contains the receptor binding region between residues 134 and 150 and Arg-174, this forms a four-helix antiparallel bundle. Residues 225-299 make up the C-terminal domain, which from residues 244-272 contains the lipid binding region (Hauser et al., 2011, Cudaback et al., 2011).

There are three common isoforms of ApoE, which are encoded by a gene on chromosome 19, these are known as E2, E3 and E4, which differ in frequency (Cudaback et al., 2011, Mahley et al., 2009). The E2 allele occurs in about 5-10% of individuals, and contains a cysteine residue at sites 112 and 158. The E3 allele is the most common allele, occurring in around 65-70% of individuals, E3 has a cysteine residue at site 112, and an arginine residue at site 158. The E4 allele contains an arginine residue at both 112 and 158, and occurs in around 15-20% of individuals. An individual's genotype can be homogeneous or heterogeneous. It is thought that the amino acid differences among the isoforms substantially affect their structures and roles in disease (Mahley et al., 2009, Kofler et al., 2012). This will be further explored in chapter 5.

1.8.5. Dietary Prevention of Atherosclerosis

There has been discussion about the link between diet and atherosclerosis since it was first discovered that there was a link between cholesterol and the formation of atherosclerosis. Currently there is a trend towards the rising obesity of the nation, and as such there is an increased interest in the effect of weight loss and the link between diet and cardiovascular disease (Parikh et al., 2005).

Currently, patients with an increased risk of cardiovascular disease are prescribed with statins (inhibitors in the cholesterol synthesis pathway), which can have adverse health effects and thus attention is now being given to natural products, including plant metabolites and by-products such as flavonoids, along with fish oils, as source of n-3 polyunsaturated fatty acids (PUFAs) (Salvamani et al., 2014). The nutritional modulation of normal immune function is also being explored, and it has been found that n-3PUFAs are able to reverse inflammation and act as anti-inflammatory agents (Grimble, 2007). It is

thought that a diet containing fish oils could be beneficial, and lead to a reduced risk of the development of atherosclerosis and cardiovascular disease.

It has previously been shown that n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are able to decrease the expression of adhesion molecules on both monocytes and endothelial cells, although the true functional value of this is unclear. However, Luu *et al*, demonstrated that monocytes from human donors receiving a fish oil supplement undergo a change which is directly linked to the reduced potential of these monocytes to induce an inflammatory response in the vessel wall. This may in part explain the beneficial effect that n-3 PUFAs play in reducing the risk of atherosclerosis. Due to the reduction of the expression of adhesion molecules on monocytes and endothelial cells induced by the fish oil supplemented diet, there would be less extravasion into the vessel wall, as monocytes would be unable to adhere to the vessel wall (Luu et al., 2007).

1.9. Hypothesis and Aims

This thesis shall explore the hypothesis that ATP is partly responsible for macrophage migration in pathological contexts. To explore this hypothesis, the following aims will be tested:

- Investigation of macrophage migration by neuropathic pain-relevant stimuli including ATP and BDNF
- To determine the expression of matrix metalloproteinases in macrophages in response to migration-modulating stimuli
- To determine the effects of dietary n3 fatty acids on macrophage migration *in vitro* and to explore if the expression of the ApoE3/4 subtype has an effect on the modulation of macrophage migration.

Chapter 2: Materials and Methods

2.1. Cell Culture

2.1.1. RAW 264.7

The Raw 264.7 macrophage cell line (TIB-71, ATCC) was grown using Dulbecco's Modified Eagle Medium (DMEM), with glucose at 4500mg/L (Invitrogen). This media was supplemented with 100 units/ml of Penicillin/Streptomycin antibiotics (Invitrogen) and 5mM L-Glutamine (Invitrogen), along with 10% (v/v) Fetal Calf Serum (FCS). Cells were passaged approximately every 2 days using an 18mm blade cell scraper (Fisher Scientific) and incubated at 37°C, and 5% CO₂ (v/v). Before experiments, Raw 264.7 were centrifuged at 1000 r.c.f for 5 minutes, counted and re-suspended at the density required.

2.1.2. Isolation and culture of Bone Marrow-derived Macrophages (BMMs).

The method of isolating bone marrow-derived macrophages was performed as previously described (Murray et al., 2013), adapted from (Leverrier and Ridley, 2001). The protocol is as follows:

C57/BL6 mice (age 2 months) were killed by schedule 1. Under sterile conditions, skin and tissue was cut away from the hind legs to isolate bone. The end of each bone were cut off, and the bone marrow was flushed out with macrophage medium (see below) using a 2ml syringe and 25-G needle. Live and dead cells were counted using trypan blue staining, and cells were centrifuged at 1000rcf for 5 minutes. The pellet was resuspended at 1x10⁶ cells/ml with macrophage medium and 10% (v/v) L-cell conditioned media. Where possible, 11ml at 1x10⁶ cells/ml were plated onto 10cm bacteriological petri dishes (Falcon, cat no. 351029). Cells were incubated at 37°C/5% CO₂ (v/v) for 3 days. After 3 days, the non-adherent population was collected and the plates gently rinsed with macrophage medium.

The non-adherent cells were counted again, and centrifuged at 1000rcf for 5 minutes, the pellet was resuspended at 1x10⁶ cells/ml in macrophage medium with 10% (v/v) L-cell conditioned media, and 11ml was plated onto bacteriological petri dishes and further incubated for 5-7 days at 37°C/5% CO₂ (v/v). The non-adherent population was discarded and adherent cells scraped for further study into fresh macrophage medium, constituting differentiated BMMs.

Macrophage Medium consisted of RPMI 1640 (GIBCO), 1% (v/v) Sodium Pyruvate (Invitrogen), 1% (v/v) Non-Essential Amino Acids (GIBCO), 100 units/ml of Penicillin/Streptomycin antibiotics (Invitrogen) and 5mM L-Glutamine (Invitrogen), and 10% (v/v) of each Fetal Calf Serum, and L-929 cell conditioned medium (a source of macrophage colony stimulating factor). 0.00002% (v/v) of β -mercaptoethanol was also added to the media.

To determine the purity of the resulting macrophage population, immunocytochemistry to display the macrophage marker F4/80 (following procedures outlined in 2.11) was performed. The purity of the macrophage population was found to be >90%, photographs of F4/80 staining are shown in figure 2.2.

2.1.3. Culturing L-929 cells for conditioned media.

L-929 were used as a source for M-CSF-1 (Leverrier and Ridley, 2001). Briefly, L-929 cells were thawed into non-vented tissue culture flasks (TPP, cat no. 90075) until confluent. Cells were passaged using trypsin EDTA and expanded as required. Cells were allowed to adhere for 24 hours and the flasks were locked to seal the flasks. Cells were allowed to condition media with CSF-1 for 2-3 weeks. Cells were checked and media was collected into 50ml Falcon tubes. Tubes were centrifuged at 1000rcf for 5 minutes to remove debris, supernatants were pooled together and filters with a 0.2 μ m single use filter and aliquoted into 5ml bijoux. Aliquots were stored at -20°C.

L-929 cells were grown in media consisting of High Glucose Dulbecco's Modified Eagle Medium (Invitrogen), 1% (v/v) Sodium Pyruvate and 100 units/ml of Penicillin/Streptomycin antibiotics (Invitrogen) and 5mM L-Glutamine (Invitrogen), and 10% (v/v) of Fetal Calf Serum.

2.2. Humanised ApoE Knock-in mice

Mice that were homozygous for either the human ApoE3 gene or the human ApoE4 gene in a targeted replacement of the endogenous murine ApoE gene (first described in (Sullivan et al., 1997) and (Knouff et al., 1999) respectively) were purchased from Taconic.

At 13-14 weeks, ApoE3 and ApoE4 mice were randomly selected to be fed a palletised high fat diet containing 45% of calories from fat (Research Diets, New Brunswick), or a high fat

diet containing supplements of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (30g EPA + DHA/kg at a 2:3 ratio) (Research Diets, New Brunswick). Mice were fed on these diets for 8 weeks before being sacrificed. The fish oil supplementation led to a daily intake of 1500mg EPA and 2250mg DHA.

2.2 Cell Viability Testing

The cell suspension tested was diluted 3:4 with Trypan blue – a vital dye solution. Dead cells take up the dye and appear blue under the microscope, whereas live cells remain unstained. The resulting cell suspension was measured using a haemocytometer and the numbers of both dead and live cells were counted, allowing a percentage viability to be calculated.

2.3. Macrophage Treatments

All experiments that were carried out in either BMMs or Raw 264.7 were performed using the relevant tissue culture medium described in 2.1, and in the presence of 0.2% (v/v) FCS, unless stated otherwise.

2.3.1. ATP

Adenosine 5'-triphosphate disodium salt hydrate (ATP; Sigma) purified from E.Coli, was dissolved to a 0.1M stock solution in ddH₂O for storage at -20°C until later use. This stock solution was later diluted to 10mM and then 10µM in the appropriate media and added to either the top of a Transwell® or onto adhered macrophages in a Nunclon™ coated MultiWell™ plate. Macrophages remained exposed to ATP for the duration of the experiment before imaging, RNA extraction or protein extraction.

2.3.2. BDNF

Endotoxin free lyophilised active human BDNF full length protein (expressed in E.coli; Abcam) was dissolved in ddH₂O to a concentration of 100µg/ml stock solution and stored at 20°C until later use. This stock solution was later diluted to 100ng/ml in the appropriate media and added to either the top of a Transwell® or onto adhered macrophages in a Nunclon™ coated MultiWell™ plate. Macrophages remained exposed to BDNF for the duration of the experiment before imaging, RNA extraction or protein extraction.

2.3.3. LPS

A 1mg/ml stock solution of Lipopolysaccharide (LPS) (purified from E.coli; R&D systems) was diluted into the respective media to a concentration of 100ng/ml was added into the top of a Transwell®, or onto adhered macrophages in a Nunclon™ coated MultiWell™ plate. Macrophages continued to be exposed to the LPS for the duration of the experiment.

2.3.4. Bradykinin

Bradykinin acetate salt (Sigma) was dissolved in 0.1M acetic acid to a stock concentration of 0.1M and stored at -20°C until later use. Bradykinin was then diluted to 1mM in the appropriate media and added onto adhered macrophages in a Nunclon™ coated MultiWell™ plate. The cells were exposed to Bradykinin throughout the experiment until RNA extraction.

2.3.5. Interleukin-10

Endotoxin free Recombinant murine Interleukin-10 (expressed in E.coli; Cell Signaling Technology) was diluted in PBS containing 0.2% (v/v) BSA to a concentration of 100µg/ml and stored at 4°C. This stock solution was later diluted to 100ng/ml in the appropriate media and added onto adhered macrophages in a Nunclon™ coated MultiWell™ plate. The cells remained exposed to IL-10 throughout the course of the experiment.

2.3.6. MCP-1

Endotoxin free recombinant murine MCP-1/CCL2 (expressed in E.coli; R&D systems) was diluted in PBS containing 0.2% (v/v) BSA to a concentration of 100µg/ml and stored at -20°C, this stock solution was later diluted to 100ng/ml in the appropriate media and added either to the top of a Transwell® or onto adhered macrophages in a Nunclon™ coated MultiWell™ plate. The cells remained exposed to MCP-1 throughout the course of the experiment.

2.3.7. PPADs

The selective P2 antagonist PPADs was dissolved in ddH₂O to a stock solution at 100mM. This stock solution was diluted to a working stock of 100nM which was added to the lower

well of a Transwell® for 1 hour to pre-treat the macrophages with this antagonist before being washed off.

2.3.8. Suramin

The P2 antagonist Suramin was dissolved in ddH₂O to a stock solution at 100mM. This stock solution was diluted to a working stock of 100nM which was added to the lower well of a Transwell® for 1 hour to pre-treat the macrophages with this antagonist before being washed off. Suramin is also capable of blocking calmodulin binding to recognition sites, and G-protein coupling to G protein coupled receptors (La Rocca et al., 1991). Suramin is actively currently used as a treatment for sleeping sickness, caused by trypanosomes, and has previously been investigated as a possible treatment for prostate cancer (La Rocca et al., 1991).

2.3.9. BBG

The selective P2X₄/P2X₇ antagonist PPADs was dissolved in ddH₂O to a stock solution at 30mM. This stock solution was diluted to a working stock of 30nM which was added to the lower well of a Transwell® for 1 hour to pre-treat the macrophages with this antagonist before being washed off.

2.3.10. DTG

The Sigma-1 receptor agonist, DTG (1,3-Di-(2-tolyl)guanidine; Tocris) was dissolved to a 25mM stock solution in DMSO and stored at -20°C. This stock solution was later diluted to 100µM in the appropriate media and added to either the bottom of a Transwell®, or onto adhered macrophages. DTG was exposed to the cells throughout the course of the experiment.

2.3.11. IPAG

The Sigma-1 receptor antagonist, IPAG (Tocris) was dissolved to 100mM in DMSO as a stock solution and stored at -20°C. This stock solution was later diluted to 30µM in the appropriate media and added to either the bottom of a Transwell®, or onto adhered macrophages. IPAG was exposed to the cells throughout the course of the experiment.

2.3.12. siRNA.

5nmol of lyophilised SMARTpool ON-TARGET siRNAs (Dharmacon) targeting the mouse gene for Sigma-1 receptor (OPRS1) was dissolved in 250µl siRNA suspension buffer (Fisher) to a concentration of 20µM, which was mixed by vortexing. The SMARTpool ON-TARGET siRNA and Allstars negative siRNA control scrambled siRNA conjugated with Alexa 488 (Qiagen) were stored at -20°C. The siRNAs were then diluted further to a working concentration of 50nM in the appropriate media containing 10% (v/v) FCS, and 60µl/ml of HiPerFect transfection reagent (Qiagen) was added into the mixture – which was mixed well and allowed to incubate for 5 minutes to allow transfection complexes to form.

Macrophages were seeded at a density of 0.5×10^6 cells/well in a 6 well plate, 24 hours prior to transfection. 400µl of the siRNA and HiPerFect mixture was added to each well in a ‘drop-wise’ manner, and remained on the cells for 30 minutes before an additional 2ml of culture media was added onto the cells. The samples were then incubated for a further 24 hours before being using in an inverted invasion assay, or being made quiescent to be lysed for use in a western blot experiment.

Table 2.1. siRNA sequences as supplied by Dharmacon.

	Sequence 5’-3’
SMARTpool ON-TARGET siRNA “Sigma-1 Receptor”.	GGCUUGAGCUUACCACCUA
	GAGAAGAAAUAGCGCAGCU
	GAAAGAGGGCACCACGAAA
	UAUUCAGACAGCAGCGUAU

2.4. Total RNA Purification

Total RNA was purified from macrophage cell lysates as part of the process of using the RNeasy Minikit (Qiagen), according to the manufacturer's instructions. Briefly, culture medium was aspirated from the adherent cells and washed with PBS, before being lysed with buffer RLT – a cell lysis buffer containing guanidium isothiocyanate and supplemented with 10% (v/v) β -mercaptoethanol. Cell lysates were further homogenised by trituration before being transferred to a gDNA eliminator column. After centrifugation, an equal volume of 70% (v/v) Ethanol was added to the sample to promote RNA precipitation and binding to the silica-gel based membrane of the RNeasy mini-spin column. The spin column was washed repeatedly using high salt buffers to further purify the RNA. RNA was then eluted in RNase-free water and stored at -20°C . RNA yield was measured using the Nanodrop 2000 spectrophotometer (Thermo Scientific) at 230nm, 260nm and 280nm wavelengths. RNA concentration was measured at 260nm and the ratios of absorbance at 260/280, and 260/230 were used to determine the quality of the RNA. Samples with a 260/280 ratio of over 1.8 were deemed to be of acceptable purity.

Later, in chapter 5, we used a Cells to C_T[™] kit (Ambion) to isolate RNA and reverse transcribe RNA in a one-step reaction. The kit was used in accordance with the manufacturer's instructions, Briefly, cell medium was removed and DNase I and a lysis solution was added to cells, this mixture was incubated at room temperature for 5 minutes, and then Stop solution was added, thus ceasing lysis. The lysate mixture was then added to an RT master mix and ran through a thermal cycle, the samples were then ready to be used for qRT-PCR. As this method does not allow for a standard curve, results were analysed using a $2^{-\Delta\Delta\text{Ct}}$ method.

2.5. Reverse Transcription

500ng-1 μg of isolated mRNA in a 15 μl volume (diluted with dH₂O) was reverse transcribed to complementary DNA (cDNA) using random primers (Invitrogen), dNTP mix (Bioline) and Maloney Murine Leukaemia Virus (M-MLV; Promega) reverse transcriptase in a 25 μl reaction according to the manufacturer's instructions. Briefly, 250ng/ml random primers were added to each sample, and heated to 70°C for 10 minutes to denature the secondary structure of the mRNA. Samples were immediately placed on ice to prevent the secondary structures from reforming and to allow the random primers to anneal. Then, 0.2mM dNTP

mix and 200units/ μg RNA M-MLV was added to the primed RNA, and reverse transcription was promoted by heating the samples to 42°C. Enzyme activity was stopped by destroying the enzyme with a step of heating to 70°C for 10 minutes. Samples were stored at -20°C until further use.

2.6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time RT-PCR reactions were performed using a 7500 Fast RT-PCR system (Applied Biosystems) according to the manufacturer's protocol. Briefly, five nanograms of cDNA was loaded into each well of an Applied Biosystems Taqman[®] plate, along with 8.33 μl of 2x qRT-PCR MasterMix (Applied Biosystems) and 100nM each of the forward and reverse primer, and 200nM of probe in a 25 μl reaction.

Standard Taqman[®] cycling conditions were used as follows, samples were heated to 95°C for 10 minutes to activate the Taq polymerase component of the MasterMix, which was followed by 40 cycles of 15 seconds melting at 95°C and 1 minute of annealing, extending and data collection.

The ribosomal 18S gene or ywhaz was used as an endogenous control to for normalisation, to account for differences in total cDNA in each sample, and the relative amount of target cDNA was determined using a standard curve, which for each reaction was prepared using a range from 0.625ng-20ng cDNA. Data is represented as a level of target mRNA relative to 18s, and as a fold change of the control condition value. As a quality control, all samples which were shown to express 18s or ywhaz at ± 1.5 CT values from the mean were excluded from the analysis.

2.7. Protein Extraction

Cell cultures were made quiescent at least 24 hours prior to stimulation and protein extraction. Cell cultures were then washed twice in ice-cold PBS before being lysed in Reverse Phase Protein Array (RPPA) buffer, containing; 1% (v/v) Triton X-100, 5mM HEPES, 150mM NaCl, 1.5mM MgCl₂, 1mM EGTA, 100mM NaF, 1mM Na₃VO₄, 10mM Na Phosphatase, 10% (v/v) Glycerol at pH 7.4, before using 1x PhosStop phosphatase inhibitor cocktail tablet (Roche) and 1x cOmplete ULTRA protease inhibitor tablet was added per 10ml to the buffer. Cells were incubated on ice in RPPA buffer for at least 20 minutes and

occasionally shaken. For further homogenisation, samples were scraped with individual 18mm blade cell scrapers before being transferred to an Eppendorf tube. All samples were centrifuged at 4°C at 13,000 r.p.m for 10 minutes and the supernatant transferred to a fresh Eppendorf tube. Samples were stored at -20°C until use.

2.8. Western Blotting

2.8.1. Protein Quantification

To measure the amount of protein in a lysate, a Coomassie plus kit (Pierce) was used according to the manufacturer's instructions. This kit is designed so that protein binds to a coomassie dye in the acidic environment provided by the reagent, resulting in a colour change from reddish-brown to blue, which can be measured by the absorbance at 595nm.

Briefly, protein standards of known concentrations ranging from 0.125mg/ml to 2mg/ml were prepared and 10µl of each plated in duplicate in a 96 well plate. The same volume of samples containing an unknown amount of protein were also plated in duplicate, then 240µl of coomassie plus reagent was added to each well and incubated for 15 minutes at room temperature. Absorbance at 595nm was then read in a spectrophotometer plate reader (Omega).

Protein concentration was then calculated by preparing a standard curve from the known protein samples.

2.8.2. Preparation of Protein Samples

RPPA extracted protein samples were diluted 5x with 5x reducing sample buffer (containing 0.625M Tris, 2% (v/v) SDS, 10% (v/v) Glycerol, 20% (v/v) β-mercaptoethanol and 2% (v/v) Bromophenyl blue). Samples were then denatured by boiling for 5 minutes at 95°C. The concentration of protein was typically standardised to 5-10µg per sample.

2.8.3. Protein resolution by SDS-PAGE

5-10µg protein was loaded into each well of a SDS-polyacrylamide gel (Usually 10% resolving gel with a 5% stacking gel) along with a well containing a Spectra Multicolour Broad Range Protein Ladder (10kDa – 260kDa; Pierce). Gels were run at constant current (30mA per gel) until proteins were deemed to be adequately resolved, using the protein ladder as a marker.

2.8.4. Semi-dry transfer

Polyvinylidene Fluoride (PVDF; BIO-RAD) membranes were activated by briefly soaking in 100% methanol for 5 minutes. Activated PVDF, and thick filter paper (BIO-RAD) were equilibrated in semi-dry transfer buffer. The resolving polyacrylamide gel was then sandwiched between thick filter paper and the PVDF membrane. Semi-dry transfer was performed using a Transblot SD Semi-Dry Transfer Cell (BIO-RAD), at 15V for 35 minutes.

2.8.5. Immunodetection of Proteins

Immunodetection of proteins was performed as described in (Baker et al., 2012). Typically, membranes were blocked for 3hr at room temperature in TBS-T containing 5% (v/v) BSA. Membranes were then incubated with the primary antibody diluted in TBS-T containing 5% (v/v) BSA overnight at 4°C. After incubation with the primary antibody, membranes were washed 5x over the course of 50 minutes in TBS-T, and then incubated with the secondary antibody (conjugated with HRP), diluted in TBS-T containing 5% (v/v) BSA. Membranes were further washed 5x over the course of 60 minutes in TBS-T before 5 minutes of incubation with in-house ECL solution, which reacts with the HRP enzyme bound to the membrane via the secondary antibody. The resulting chemiluminescence was measured by exposure to Amersham Hyperfilm ECL (GE Healthcare), a photosensitive film. The photosensitive film was then developed in an autoradiograph film processor (Xograph Healthcare Ltd). A full range of antibodies used for western blotting can be found in table 2.2.

Table 2.2. A full list of antibodies used in Western Blot experiments.

ANTIBODY	CONCENTRATION	SUPPLIER
RABBIT ANTI ERK1/2	1:1000	Cell Signaling Technology
RABBIT ANTI P-ERK1/2	1:1000	Cell Signaling Technology
RABBIT ANTI P38	1:1000	Cell Signaling Technology
RABBIT ANTI P-P38	1:1000	Cell Signaling Technology
RABBIT ANTI SIGMA-1 RECEPTOR	1:50	Abcam
GOAT ANTI-RABBIT-HRP	1:2000	Dako

2.9. Cell Migration and Time-Lapse Microscopy

Cell migration experiments using time-lapse microscopy was performed as described in (Murray et al., 2013). Briefly, 24 well plates were coated with Matrigel™ (BD Biosciences) and Fibronectin from bovine plasma (Sigma) at the concentration of 10µg/ml Matrigel, and 10ng/ml Fibronectin for experiments described in chapter 3. However, for experiments in chapter 5, 24 well plates were uncoated, and cells were exposed to the Nunclon™ surface of the multiwell plates. Macrophages were seeded into the wells at a density of 10×10^4 , and then incubated at 37°C, 5% (v/v) CO₂ and allowed to adhere for 3 hours. Treatments were administered as described.

Cells were imaged over the course of 17 hours at 10 minute intervals with a monochrome Axioplan CCD camera (Zeiss) attached to a widefield, Axiovert 200M inverted light microscope (Zeiss), which used Axiovision software. Cell migration velocity and distance was tracked and analysed using ImageJ software (NIH), and the 'Manual Tracking' plug-in (F. Corderlières, Institutue Curie, France), and the 'Chemotaxis and Migration Tool' available from Ibidi (Trapp and Horn, Ibidi GmbH).

2.10. Inverted Invasion Assay

A normal invasion assay involves the invasion of cells from the top of a thin gel matrix down to the underside of a solid membrane towards a chemoattractant. The invasion assay can also be inverted, which is slightly more complex to perform but ultimately allows for a different analysis of results which can be quantified (Hennigan et al., 1994, Kim et al., 2008, Murray et al., 2013).

In the inverted invasion assay, once the matrix plug is set, the Transwell is inverted and cells are seeded directly onto the underside of the polycarbonate filter membrane. Cells are allowed to adhere and then the Transwell is inverted once more, so that now it is the right way up, although cells are already on the bottom of the membrane. A chemoattractant is then added to the top of the matrix plug and cells are allowed to invade up through the matrix plug. Live cells can be imaged with Calcein AM and then the entire Transwell can be imaged using confocal optical sectioning, this allows us to view where cells have travelled to in the matrix plug, and allows for better quantification of results.

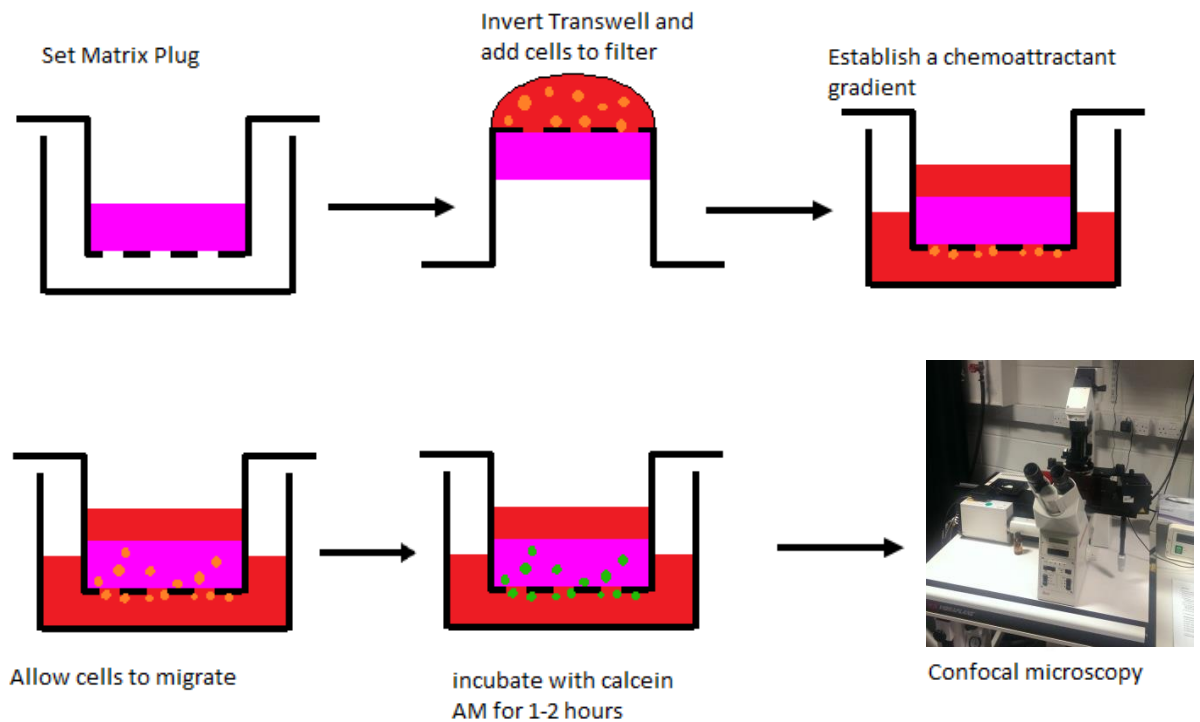


Figure 2.1. A diagram to show the steps of the inverted invasion assay. Once finished, a confocal microscope is used so it is possible to see how far the cells in question have travelled through the matrix.

Inverted invasion assays were performed to measure chemotaxis of cells towards different chemoattractants. Matrigel™ (BD Biosciences) was diluted with ice cold PBS to 4mg/ml, 100µl of the Matrigel solution was added to each Transwell® with 8µm pores (Corning) and allowed to set at 37°C for 1 hour. Transwells® were then inverted and 1×10^5 cells (Raw 264.7 or BMMs) in 100µl DMEM + 10% (v/v) Fetal Calf Serum were seeded directly onto the underside of the Transwell® filter. The Transwell® inserts were then covered with the base of a 24 well plate (Falcon) and cells were allowed to adhere for 3 hours at 37°C and 5% (v/v) CO₂. Once the cells adhered the Transwells® were turned once more and washed twice in wells containing 1ml of media in the lower well (DMEM + 0.2% (v/v) Fetal Calf Serum), before being placed in the lower well containing 1ml of DMEM + 0.2% (v/v) Fetal Calf Serum. 100µl of chemoattractant was then added to the upper side of the Transwell® - in identical medium to the lower well. The Transwells® were then incubated at 37°C + 5% (v/v) CO₂ for 48 hours to allow invasion of cells into the Matrigel™.

After invasion, cells were stained with 4mM Calcein AM and incubated for 1 hour before being imaged on a Leica TCS SP2 UV system confocal microscope at 40x magnification –

invasion can then be quantified. For each invasion Transwell®, 3 z-stacks were taken to cover 210µm through the Matrigel™ matrix, accounting to 15 images taken 15µm apart.

Invasion is demonstrated as a percentage of cells that have migrated over 30µm. To determine this, the 'Area Calculator' plug-in for Image J (NIH) was used to demonstrate the number of fluorescing pixels on each slice of the z-stack above a set threshold level of fluorescence. The number of pixels from all images above 30µm were then calculated as a percentage of the total number of fluorescing pixels in the z-stack.

2.11. Immunocytochemistry

Immunocytochemistry was carried out essentially as described in (Murray et al., 2013); roughly 40,000 cells were plated per 13mm glass coverslip (VWR) in 24 well plates and allowed to adhere for 1 hour before the addition of 500µl of the appropriate cell culture media was added. Cells were then incubated at 37°C, 5% (v/v) CO₂ overnight. Coverslips with then fixed in a 4% (v/v) paraformaldehyde solution and washed in PBS. Non-specific binding was blocked by incubating the cells for 1 hour in NGS, followed by incubation for 1 hour with a rat anti-F4/80 primary antibody diluted 1:1000 (AbD Serotec) without washing. Coverslips were then washed at least 3x with PBS to remove unbound primary antibody. Coverslips were then incubated with an anti-rat Alexa Flour-488 (Abcam) conjugated secondary antibody diluted at 1:1000 for 1 hour before washing 3x with PBS. Cells were then incubated with DAPI and washed quickly before mounting on microscope slides with Hydromount mounting solution. Example photographs of F4/80 staining are shown in Figure 2.2.

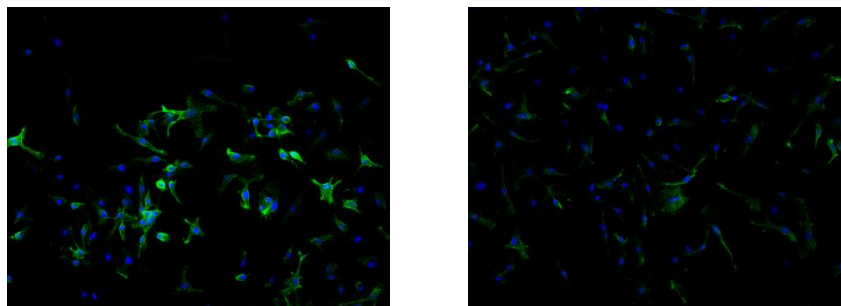


Figure 2.2. Photographs of BMM populations prepared using the method outlined in section 2.1.2, captured at x200 demonstrating the nuclear stain, DAPI (blue) and the macrophage marker F4/80 (Green).

2.12. Statistical Analysis

Unless otherwise stated, statistical analyses were a two-sample unequal variance two-tailed Students' t test and were performed using Microsoft Office Excel 2010. A p-value of less than 0.05 was deemed to be significant.

Chapter 3: ATP as a Chemoattractant to Macrophages

3.1 Introduction

3.1.1. Macrophage Migration in Neuropathic Pain.

Migration of cells is a basic biological function that contributes towards normal development and differentiation. Macrophages, neutrophils and microglia rely heavily on migration, as they are professional phagocytes (Krause, 2000). It is an essential role of these cells to migrate towards and engulf invading organisms such as bacteria, and apoptotic cells. Recent findings explore the importance of extracellular nucleotides such as ATP in the migration of professional phagocytes towards their intended targets (Corriden and Insel, 2012).

In chemotaxis – the directed migration of a cell towards a chemical cue, there are three distinct processes, motility, polarization and gradient sensing. Cell motility is driven by actin extension of microtubules which enable random and directed migration. Polarization is dictated by the recognition of extracellular clues which leads to the rearrangement of the cell cytoskeleton that promotes the formation of a leading edge of a cell and disintegration of the integrin formations at the rear of the cell (Iglesias and Devreotes, 2008).

Directional movement of cells towards an area can be regulated by ATP, ADP and adenosine, along with other nucleotides, (Corriden and Insel, 2012). After injury, ATP is released from all cells in millimolar concentrations, which can promote the release of other chemoattractant cytokines from surrounding cells, and thus amplify the chemoattractant signals that drive cell migration. However, there is currently debate over whether these nucleotides act as a “touch me” chemokinetic factor or a “find me” signal, thus promoting phagocytic activity (Elliott et al., 2009, Chekeni and Ravichandran, 2011).

3.1.2. Purinergic Signalling.

There are known to be 7 P2X subtypes, and 8 P2Y receptors (Burnstock, 1972, Burnstock, 1976, Burnstock, 2007). It is thought that purines can induce directed migration in microglia and macrophages towards sites of injury (Kurpius et al., 2007, Irino et al., 2008, Lambert et al., 2010). P2X4, P2X7, P2Y6 and P2Y12 receptors are thought to have links with microglial and macrophage migration.

3.1.3. BDNF

BDNF signals through its receptor TrkB, and can also signal through p75. BDNF is able to signal through MAPK, PI3K and PLC γ pathways, and is thought to have many neuroprotective effects. For example, BDNF has been found to be able to reverse synapse loss, improve learning and memory and normal cell signalling, and normalise gene expression in Alzheimer's disease in rats and primates (Nagahara et al., 2009). The administration of BDNF has also been shown to be able to improve cognitive function in aged animals.

BDNF has been shown to have neuroprotective effects by leading to the decrease of expression of pro-inflammatory cytokines such as TNF α , and leads to the increase of the expression anti-inflammatory cytokines such as IL-10. It has also been found that BDNF can protect against glutamate toxicity and ethanol-induced damage in neurons, and that BDNF enhances neural regeneration (Chen et al., 2013). However, it has also been found that BDNF may contribute to the formation of neuropathic pain (Beggs and Salter, 2010) by the disinhibition of lamina I neuron signalling.

Hetman *et al* suggested that the neuroprotective effects of BDNF were due to mechanisms downstream of ERK and PI3K (Hetman et al., 1999).

3.1.4. Sigma-1 receptor.

Sigma receptors are a 223 amino acid transmembrane protein (Su et al 2010) that regulates a variety of cellular functions, including IP 3 Ca $^{2+}$ signalling, ion channel firing, neurotransmitter release, differentiation, neuronal survival, and inflammation. In its native state on the endoplasmic reticulum, Sigma-1 receptor associates with the chaperone binding immunoglobulin protein (BiP) (Su et al 2010, Hashimoto 2013). It is thought that Sigma-1 can play a role in chaperoning proBDNF and misfolded proteins.

In 2009, Hall *et al* discovered that activation of Sigma receptor via the agonist DTG, suppressed many of the functions of primary microglial cells, such as morphology changes, migration, phagocytosis calcium influx and nitric oxide production. Gannon *et al*, 2001, also demonstrated that activation of Sigma-1 receptor inhibited nitric oxide production in response to LPS.

It is thought that the agonism of sigma-1 receptor could lead to the modulation of signalling through several cell surface receptors including sodium channels, TrkB and possibly purinergic receptors.

3.2. Hypothesis and Aims

This chapter shall explore the hypothesis that ATP can modulate the migration of macrophages in a context of neuropathic pain. In this chapter, the following aims will be investigated:

- ATP can act as a chemoattractant and thus be responsible for macrophage migration.
- To explore the mechanism behind ATP-induced migration of macrophages.
- To determine if other neuropathic pain related stimulants such as BDNF can influence macrophage migration, and to explore the mechanisms behind this.

3.3. Results

3.3.1. ATP acts as a chemoattractant to Raw 264.7 cells and primary Bone Marrow-derived Macrophages in a Matrigel™/Fibronectin matrix.

Using the inverted invasion assay technique, the chemoattractive ability of Adenosine 5'-Triphosphate (ATP) to Raw 264.7 macrophages was explored, in part to investigate the role of purinergic receptors in the migration of macrophages towards the site of peripheral nerve injury. 10µM ATP was chosen as a concentration as it is an ideal concentration for the activation of P2X4 receptor, which is of interest (Tsuda *et al*, 2006).

Raw 264.7 cells were allowed to invade into the Matrigel™ matrix, supplemented with 10µg/ml Fibronectin, towards 10µM ATP for 48 hours before being stained with Calcein-AM and imaged by confocal microscopy. It was found that 10µM ATP significantly increased invasion into the Matrigel™/Fibronectin matrix by approximately 2.5 fold, when compared to the control condition (Figure 3.1). Whilst the result shown is from one experiment, this result is representative of 22 separate experiments.

Whilst initially planning to confirm this result in microglial cells isolated from either spinal cord, or cortex tissues, isolating microglial cells proved not to yield the number of cells required and the focus shifted to investigate with primary bone-marrow derived macrophages. To confirm this result in primary macrophages, murine bone marrow-derived macrophages were differentiated from bone marrow stromal cells. Here we report that BMMs respond to ATP in a similar manner to Raw 264.7 cells. BMMs invaded into Matrigel™/Fibronectin over 48 hours, ATP increased the invasion of BMMs 2 fold when compared to the control condition (Figure 3.2). Figure 3.2 shows a result that is representative of 4 separate experiments.

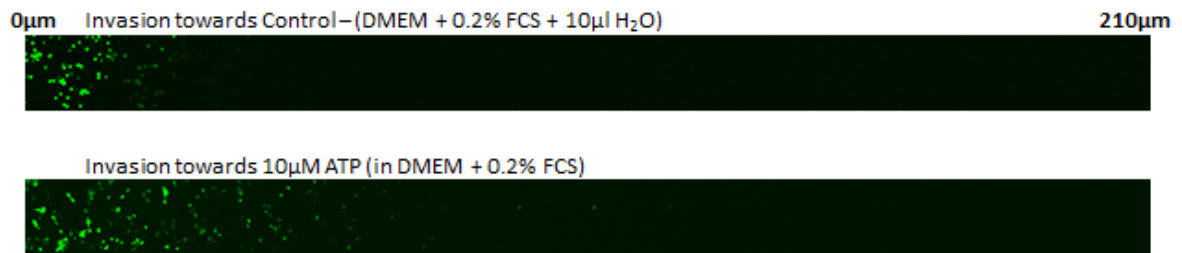
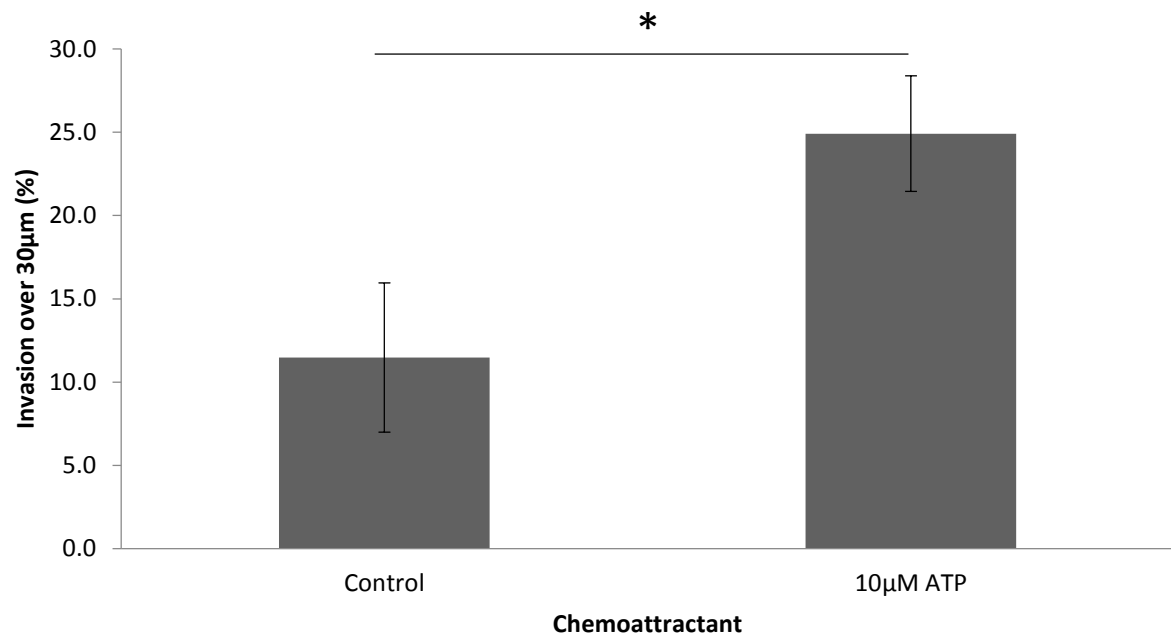


Figure 3.1 ATP significantly increases Raw 264.7 invasion through a Matrigel™/Fibronectin matrix. Raw 264.7 were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix, after which all live cells were stained with a Calciin-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each bar represents the mean \pm 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = $p \leq 0.05$.

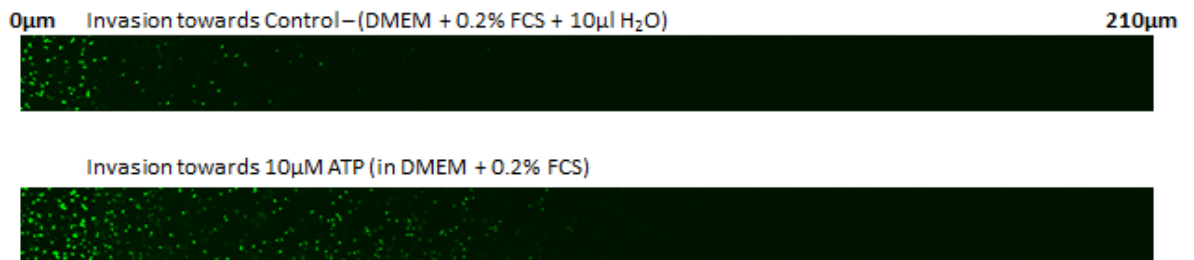
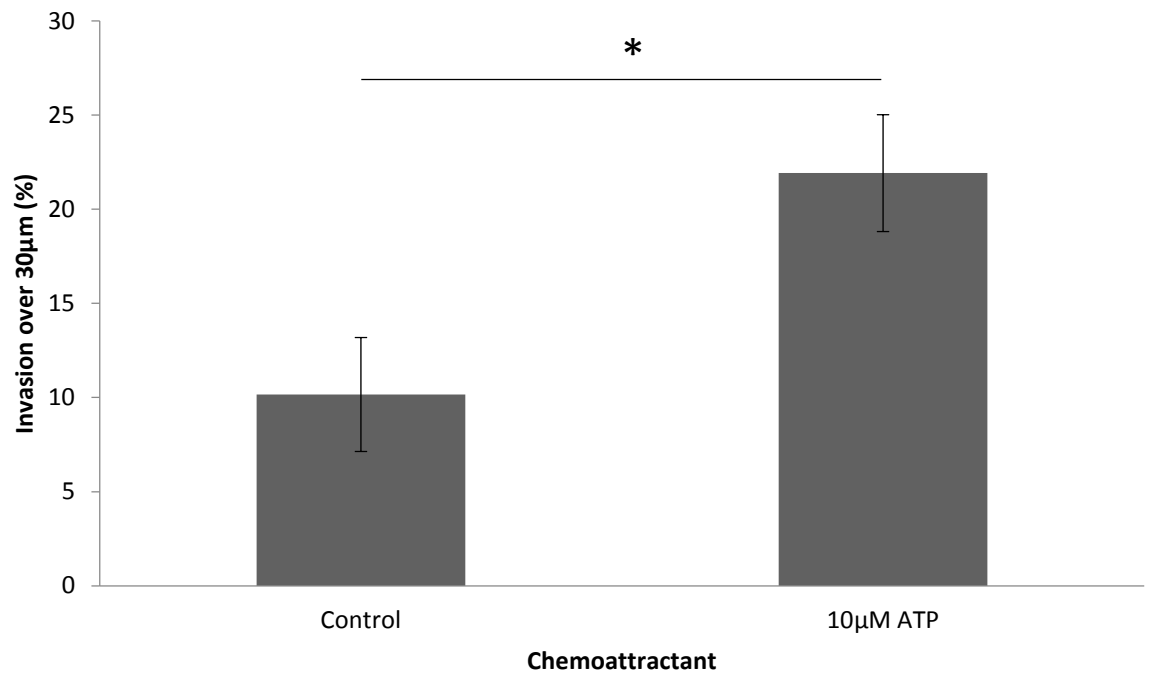


Figure 3.2. ATP significantly increases BMM invasion through a Matrigel™/Fibronectin Matrix. BMMs were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix, after which all live cells were stained with a Calcein-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each Bar represents the mean \pm 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = $p \leq 0.05$.

The next step of investigation was to determine the receptor responsible for ATP-induced invasion in Raw 264.7 and BMMs. As ATP acts through purinergic receptors, broad-spectrum pharmacological inhibitors of purinergic receptors, Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADs), and Suramin were used, along with the triphenylmethane dye, Coomassie Brilliant Blue G-250. PPADs is a non-selective inhibitor of purinergic channels P2X1, P2X2, P2X3, P2X5, P2Y2 and P2Y4 channels, Suramin is also a non-selective P2 antagonist, capable of blocking P2X1, P2X2, P2X3, P2X5, P2Y2, P2Y4 and P2Y6. Coomassie Brilliant Blue G-250 (BBG) is an inhibitor of the P2X7 receptor and a weak inhibitor of P2X4.

Using the inverted invasion assay, Raw 264.7 cells were pretreated with the pharmacological inhibitors for 10 minutes, before washing the cells and adding the chemoattractant to the assay. The Raw 264.7 were allowed 48 hours to invade into the Matrigel™/Fibronectin matrix before being imaged. The results, shown in Figure 3.3, demonstrate that all three P2 receptor antagonists reduce invasion towards 10µM ATP. In Figure 3.3, suramin is shown to have the most potent effect on invasion, dramatically reducing invasion of Raw 264.7 cells over 30µM 15-fold. BBG and PPADs are also shown to have an effect on ATP induced invasion, significantly reducing invasion approximately 2.4 fold, it should be noted that suramin was originally developed as an anti-microbial agent and can affect cells using other mechanisms such as antagonism of several GPCRs, and so its action may not entirely be limited to P2 receptor antagonism.

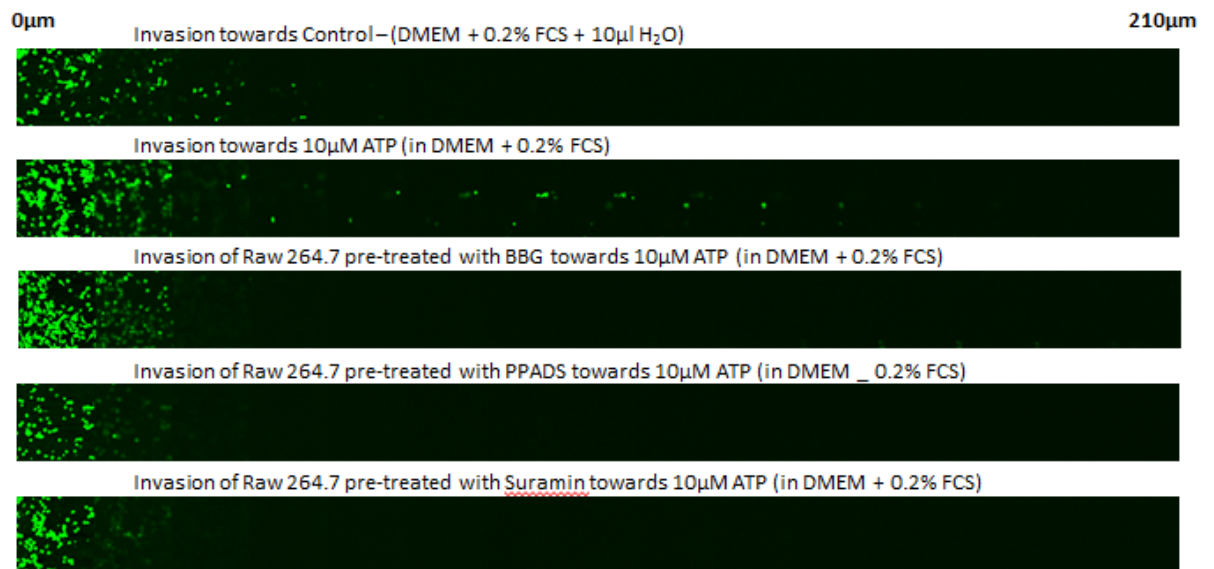
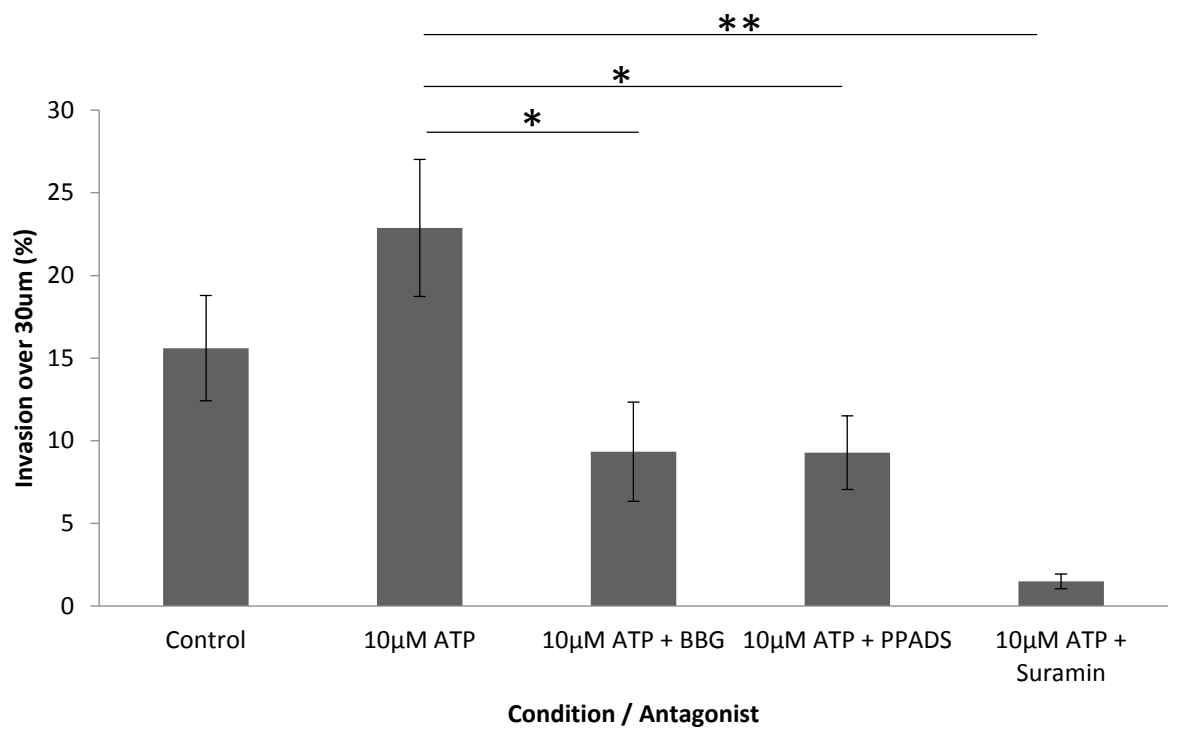


Figure 3.3. Invasion of Raw 264.7 through a Matrigel™/Fibronectin Matrix after pre-treatment with P2 antagonists. Raw 264.7 were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix after 10 minute pre-treatment with P2 receptor antagonists BBG, PPADS and Suramin. After 48hrs all live cells were stained with a Calcein-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each Bar represents the mean ± 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = $p \leq 0.05$, ** = $p \leq 0.01$.

3.3.2. Brain-Derived Neurotrophic Factor can attenuate ATP induced invasion in Raw 264.7 cells and BMMs.

In 2005, Coull *et al* discovered that ATP stimulation of microglia causes the release of Brain-Derived Neurotrophic Factor (BDNF), and that BDNF was a crucial signalling molecule between microglial cells and neurons. It was shown that ATP-stimulated microglia signal to lamina 1 neurons, which, through disruption of the transmembrane anion gradient may be responsible for tactile allodynia. Upon the blockade of BDNF signalling, tactile allodynia was reversed. Later, Trang *et al* (2009), further elucidated that ATP-induced release of BDNF in microglia was through P2X4 mediated activation of the p38-MAPK pathway.

These findings mused the possibility that BDNF may impact upon macrophage invasion, therefore we determined the effects of the combination of ATP and BDNF on the migration of macrophages. The results in Figure 3.4 show that 10 μ M ATP significantly increases from the control condition (by nearly 4-fold), whereas BDNF alone has no effect when compared to the control condition. Interestingly, ATP in combination with BDNF does not differ significantly from the control, but when compared to ATP alone, displays a significant 1.8 fold reduction in invasion. Figure 3.4 shows a representative of 11 separate experiments.

To confirm the result in primary macrophages, we exchanged Raw 264.7 cells for BMMs, and repeated the experiments. Figure 3.5 shows that unsurprisingly, BMMs respond in a similar manner. 10 μ M ATP significantly increases invasion over 30 μ m compared to the control condition (from 20.8% in the control condition to 47.8% ATP), BDNF alone has no effect, but, when compared to ATP alone the combination of ATP+BDNF significantly reduces invasion by 1.65-fold. In this experiment, 100ng/ml of the TLR4 agonist Lipopolysaccharide (LPS) was added to act as a positive control – which leads to a significant increase compared to the control condition.

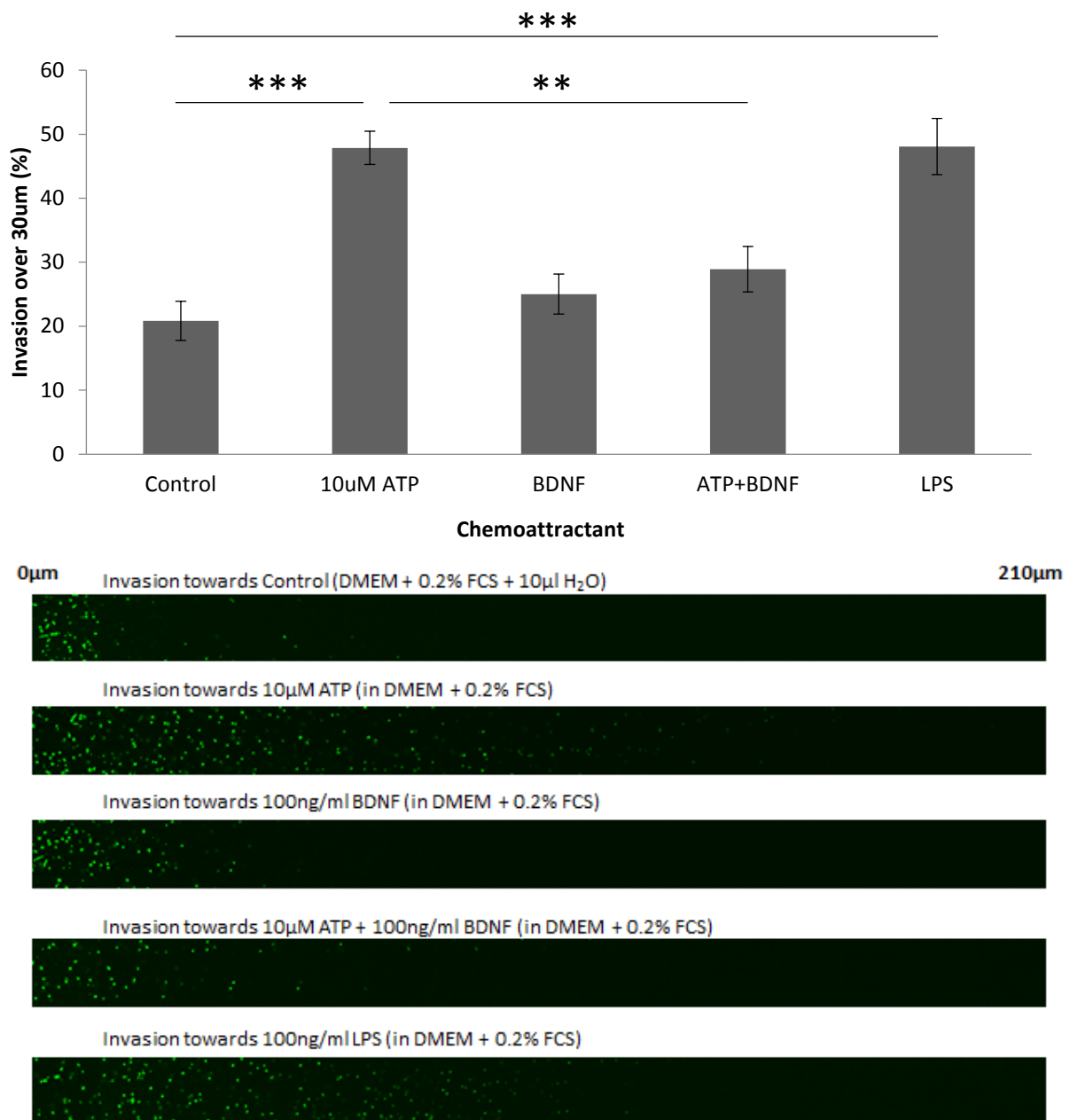


Figure 3.5. BDNF attenuates ATP induced invasion in Bone Marrow-derived Macrophages cells through a Matrigel™/Fibronectin matrix. BMMs were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix towards 10µM ATP, 100ng/ml BDNF or the combination of ATP + BDNF, in this experiment, 100ng/ml LPS was also included as a positive control. Afterwards all live cells were stained with a Calciin-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each bar represents the mean ± 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.

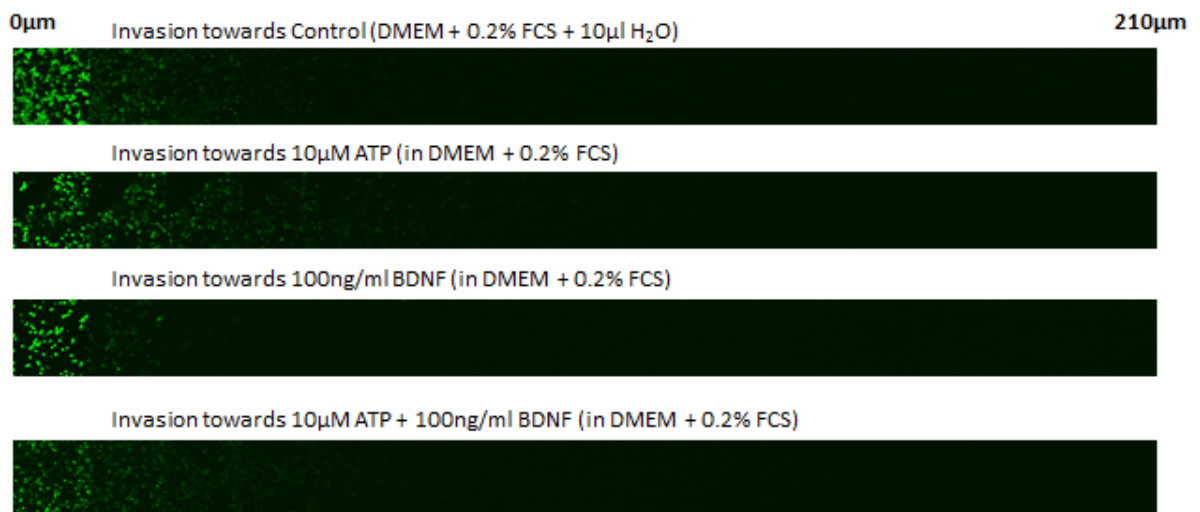
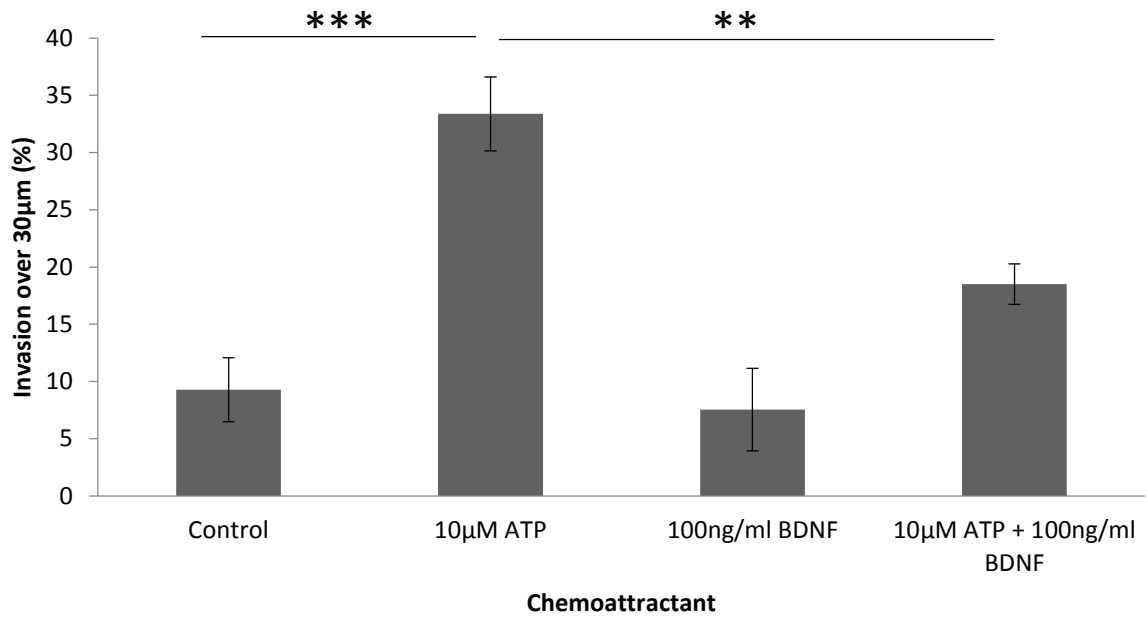


Figure 3.4. BDNF attenuates ATP induced invasion in Raw 264.7 cells through a Matrigel™/Fibronectin matrix. Raw 264.7 were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix towards 10µM ATP, 100ng/ml BDNF or the combination of ATP + BDNF. Afterwards all live cells were stained with a Calcein-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each bar represents the mean ± 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001.

3.3.3. The potential involvement of Sigma Receptors in Macrophage Migration towards ATP, and the subsequent attenuation of invasion by BDNF.

The next steps of investigation involved elucidating the mechanisms by which BDNF attenuates ATP induced invasion in both Raw 264.7 cells and primary bone marrow-derived macrophages, during which, Sigma-1 receptor became of interest to our investigation.

Due to the findings that sigma-1 can suppress a range of microglial activities, and its link with BDNF processing, we investigated the effect of agonism and antagonism of the Sigma-1 receptor using pharmacological methods.

The effect of the Sigma-1 receptor agonist, DTG on the invasion of Raw 264.7 cells was explored in Figure 3.6. As previously characterised in figure 3.4, ATP is shown again to significantly increase invasion of Raw 264.7 cells, and the combination of ATP + BDNF shows an abrogation of invasion. Upon treatment with 100 μ M DTG, there was no change in the amount of invasion on the control or with BDNF when cells were also treated with DTG. However, the amount of invasion was markedly less with cells in the ATP + DTG condition – with invasion at 33.3% over 30 μ m in ATP alone conditions – reduced 16-fold with ATP + DTG. Also, invasion was further reduced in ATP + BDNF conditions – from 18.5% invasion in ATP + BDNF, reduced by 4-fold when these cells were treated with DTG.

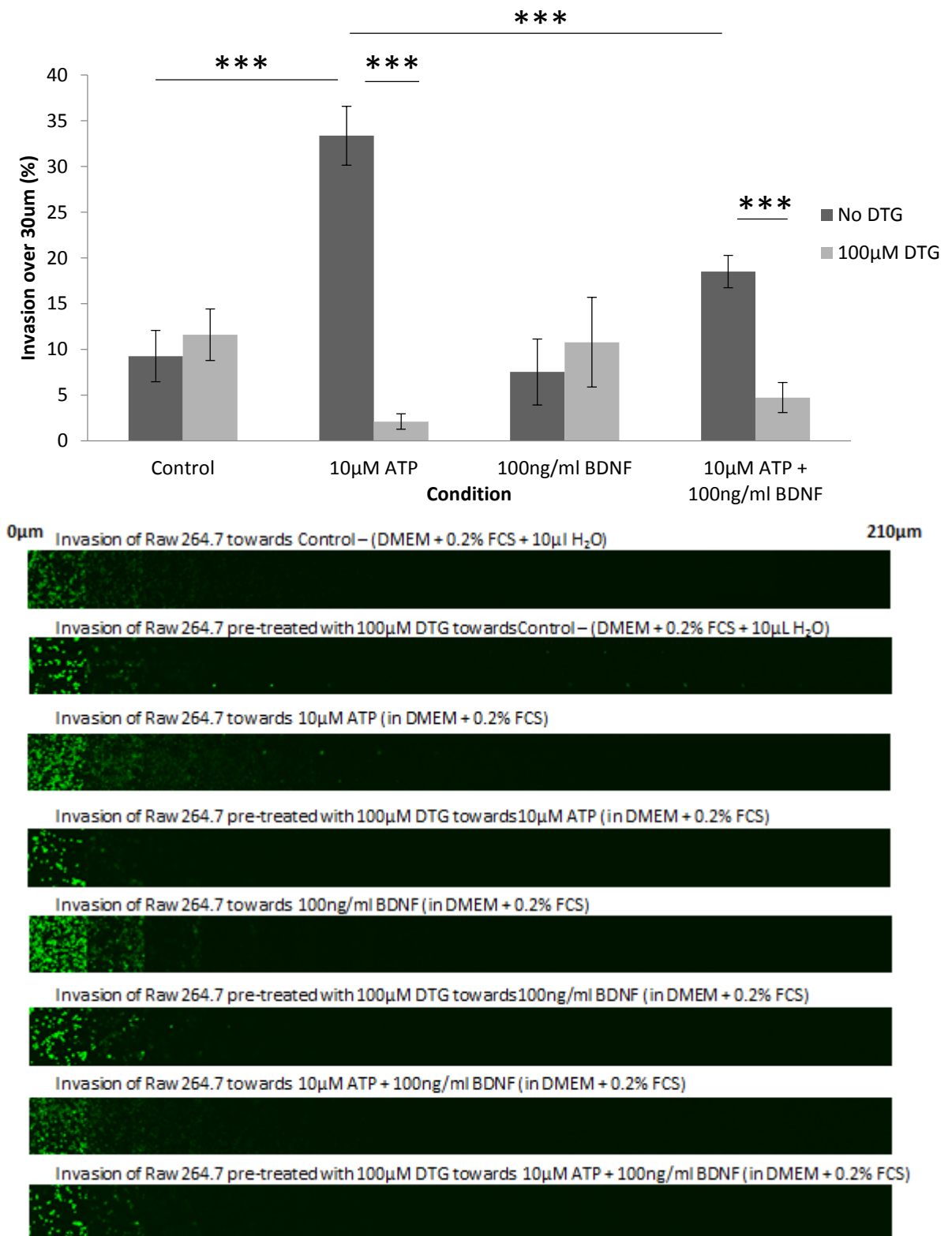


Figure 3.6. The effects of the Sigma receptor agonist, DTG on the invasion of Raw 264.7 cells through a Matrigel™/Fibronectin matrix. Raw 264.7 cells were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix towards 10µM ATP, 100ng/ml BDNF or the combination of ATP + BDNF, with or without the addition of 100µM DTG. Afterwards all live cells were stained with a Calcein-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each bar represents the mean ± 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

The effect of DTG on the random migration of Raw 264.7 was also explored to determine if Sigma-1 receptor was also involved in 2D migration. Raw 264.7 were cultured in 2D on a thin coating of Matrigel™ and Fibronectin for 3 hours before addition of the tested stimulants and subsequent time-lapse microscopy was performed. Phase-contrast images were captured every 10 minutes for 17 hours and cells were later tracked using the Manual Tracking plugin for Image J (NIH).

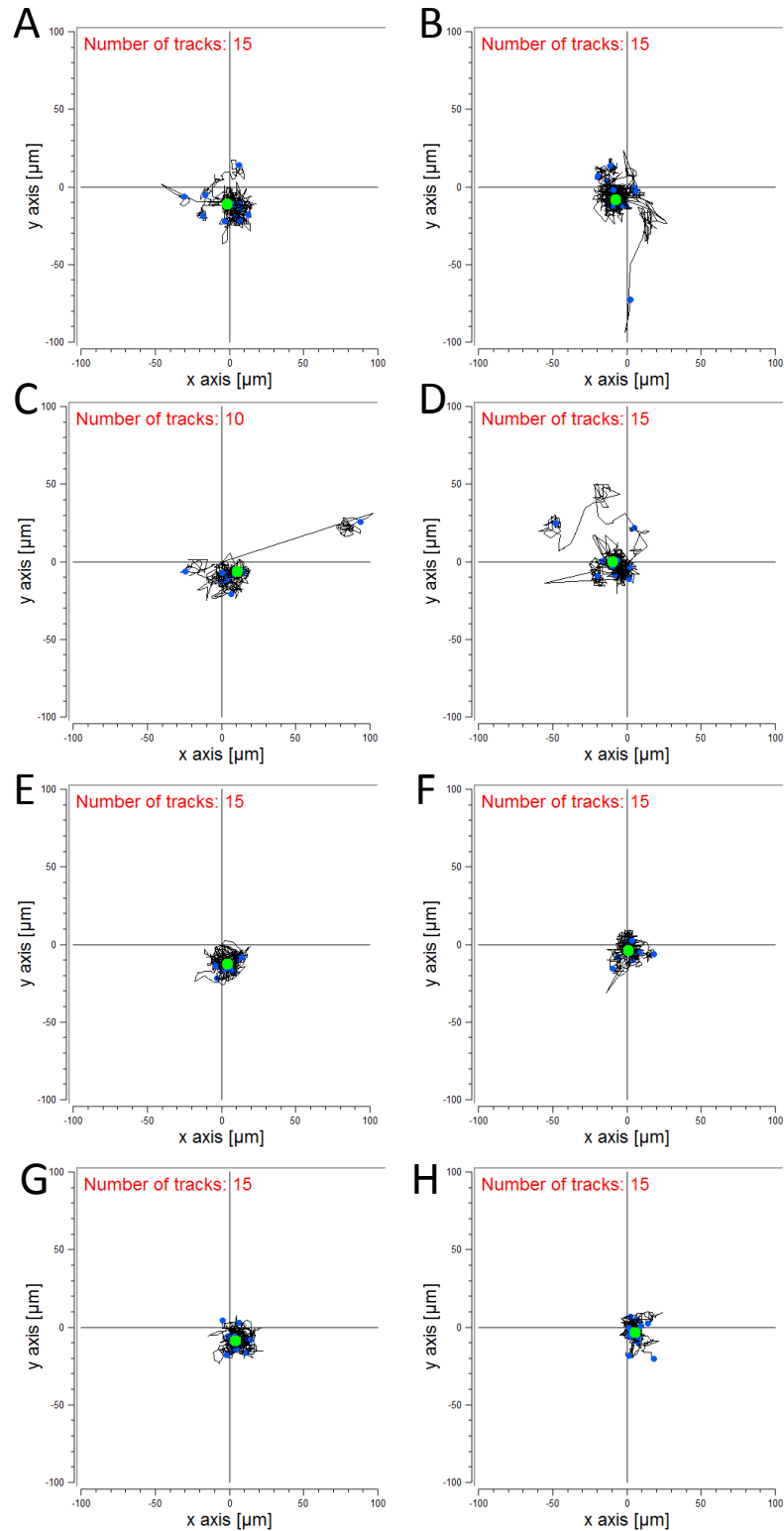


Figure 3.7 DTG suppresses migration. Raw 264.7 cells were plated in wells of a 24 well plate at a concentration of 1×10^5 /well and stimulated with either (A) Control, (B) $10 \mu\text{M}$ ATP, (C) 100 ng/ml BDNF, (D) $10 \mu\text{M}$ ATP + 100 ng/ml BDNF, (E) $100 \mu\text{M}$ DTG, (F) $100 \mu\text{M}$ DTG + $10 \mu\text{M}$ ATP, (G) $100 \mu\text{M}$ DTG + 100 ng/ml BDNF or (H) $100 \mu\text{M}$ DTG + $10 \mu\text{M}$ ATP + 100 ng/ml BDNF. Cells were observed migrating over a period of 17 hours at 37°C and $5\% \text{ CO}_2$ and tracked using the Manual Tracking plugin software and ImageJ. $N=15$ tracks per condition

Figure 3.8 shows that the average speed of randomly migrating Raw 264.7 cells was significantly reduced when cells were treated with DTG, although it should be noted that migration speeds are quite low. Figure 3.7 A and E show the trajectories of these 2 conditions which reflect this observation. 10 μ M ATP was shown to increase random migration speed of cells from 10.8 μ m/hr to 15.1 μ m/hr, again 100 μ M DTG was shown able to suppress migration significantly (Figure 3.7 tracks B and F) down to 7.7 μ m/hr. Interestingly, the combination of ATP + BDNF was found to increase random migration speed from the control (from 10.8 μ m/hr to 17.1 μ m/hr – Figure 3.7 tracks A and D), whilst DTG reduced migration speed of this condition significantly. DTG was shown to have no effect on the speed of BDNF stimulated cells. When cells are exposed to DTG, they appear to move around on the spot without migrating to a different point, hence decreasing the Euclidean distance. This data is representative of 3 separate experiments.

The effect of antagonising the Sigma-1 Receptor was explored with the antagonist IPAG on the migration of Raw 264.7 cells through a Matrigel™ and Fibronectin matrix. Cells were treated to 30 μ M IPAG in the lower well for one hour prior to the chemoattractant being added. After 48hrs invasion, it was observed that treatment with IPAG significantly increased the amount of invasion over 30 μ m in the control condition - leading to a four-fold increase in the amount of invasion. As seen previously in Raw 267.4 cells, 10 μ M ATP was also found to have an increase in the amount of invasion, and IPAG treatment had no further effect. IPAG was also shown to significantly increase the invasion in cells attracted to 100ng/ml BDNF by 3-fold. Interestingly, an increase in invasion was seen when ATP + BDNF attracted cells were treated with IPAG, although this observation was not significant, there was a definite trend towards the rescue of invasion, similar to levels seen with ATP attraction alone (a 4.7 fold increase in invasion over 30 μ m, $p=0.07$). This is representative of 3 experiments, IPAG at this concentration had no effect on cell viability.

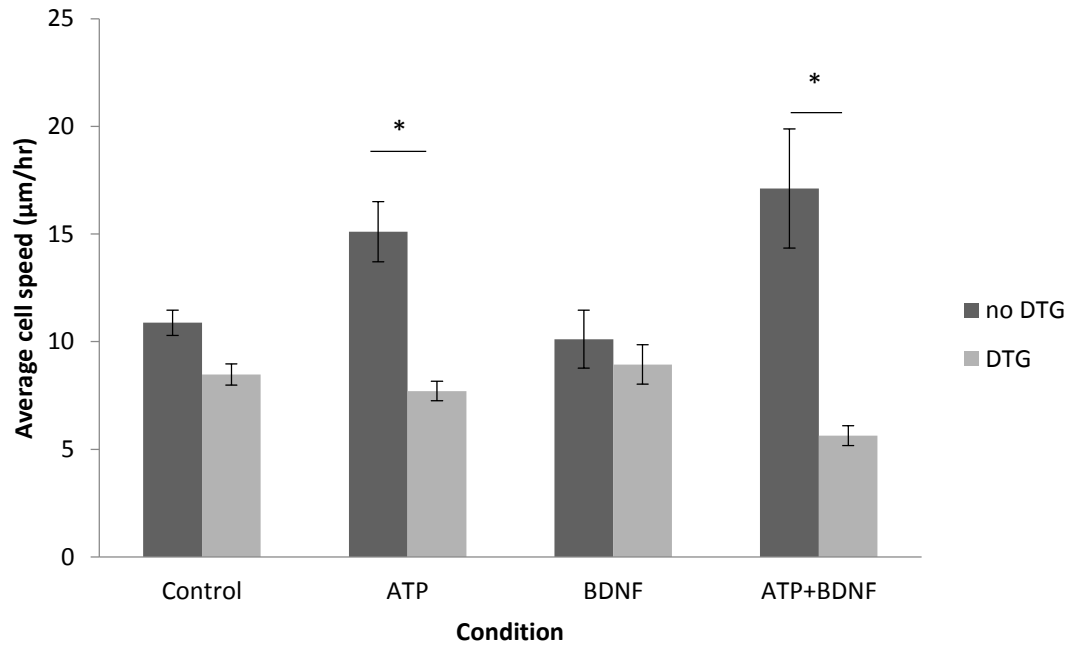


Figure 3.8 DTG supresses migration. Raw 264.7 cells were plated in wells of a 24 well plate at a concentration of 1×10^4 cells/well and stimulated with $10 \mu\text{M}$ ATP, 100ng/ml BDNF and a combination of ATP and BDNF, with or without $100 \mu\text{M}$ DTG. Cells were observed migrating over a period of 17 hours at 37°C and $5\% \text{CO}_2$. Cells were tracked using the Manual Tracking plugin software and ImageJ. $N=15$ tracks per condition. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

The next step of inquiry was to characterise the effects of Sigma-1 antagonism and agonism on invasion of primary bone marrow-derived macrophages. In BMMs cells exposed to IPAG invade into the Matrigel™/Fibronectin matrix significantly 2.5-fold more than in the control condition (Figure 3.10). DTG also significantly decreased invasion as shown previously. Invasion over 30µm in ATP + BDNF conditions again showed a significant decrease when compared to ATP alone (2.2-fold). There was no change in invasion towards ATP + BDNF when cells were treated with DTG, but interestingly, when cells were treated with IPAG, invasion towards ATP + BDNF was significantly increased 2-fold, thus potentially rescuing the BDNF effect in this condition. Figure 3.10 shows results representative of 3 separate experiments.

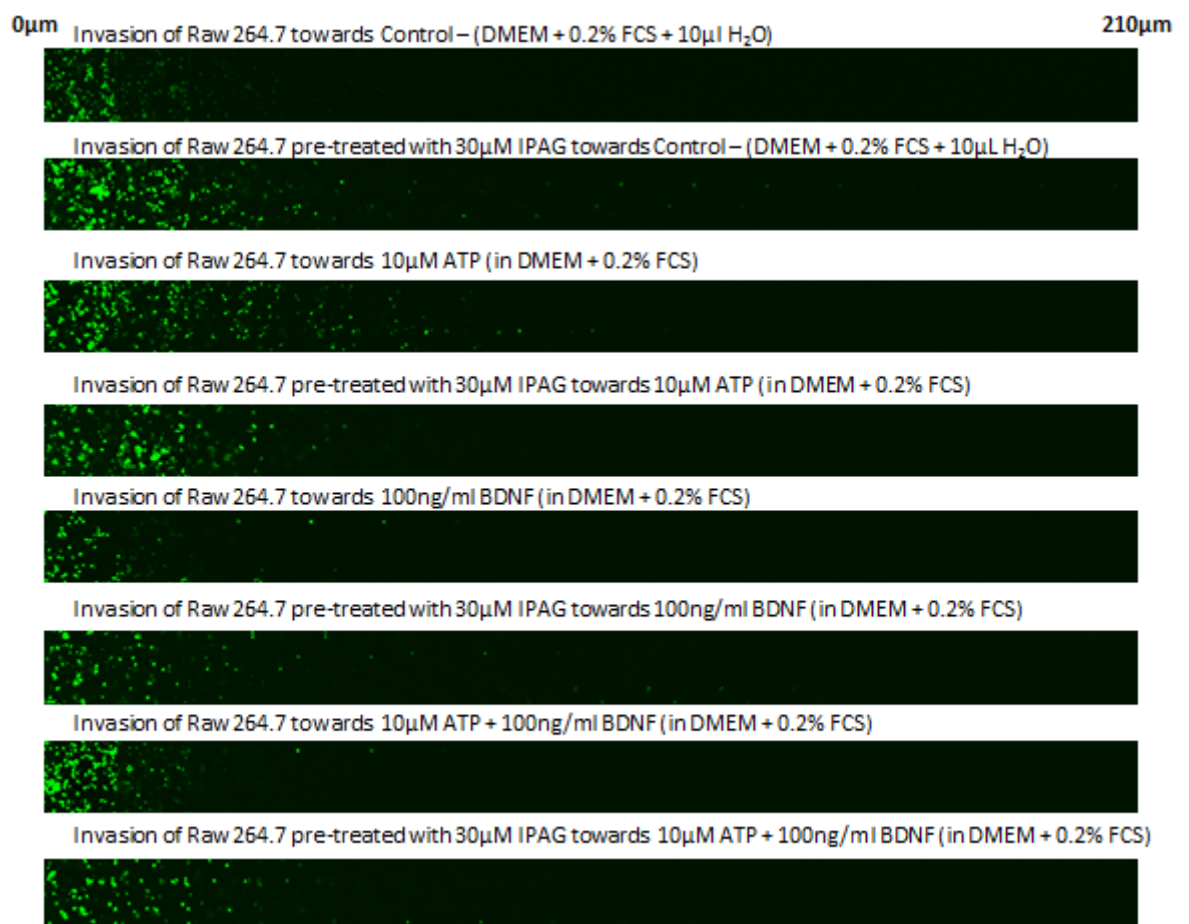
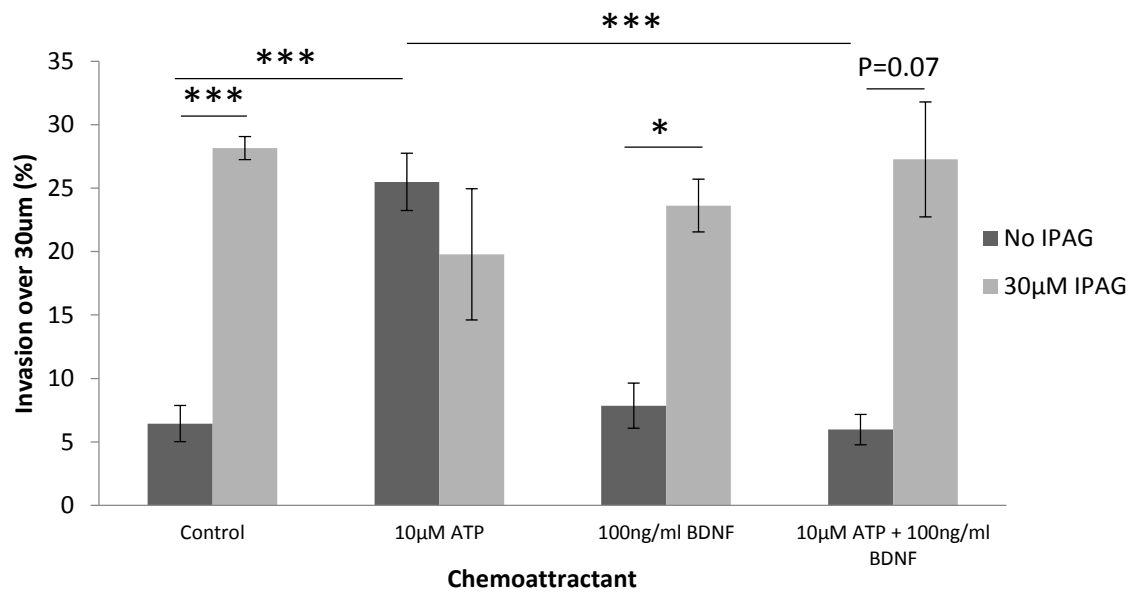


Figure 3.9. The effects of the Sigma receptor antagonist, IPAG on the invasion of Raw 264.7 cells through a Matrigel™/Fibronectin matrix. Raw 264.7 cells were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix towards 10µM ATP, 100ng/ml BDNF or the combination of ATP + BDNF, with or without the addition of 30µM IPAG. Afterwards all live cells were stained with a Calcein-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each bar represents the mean ± 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

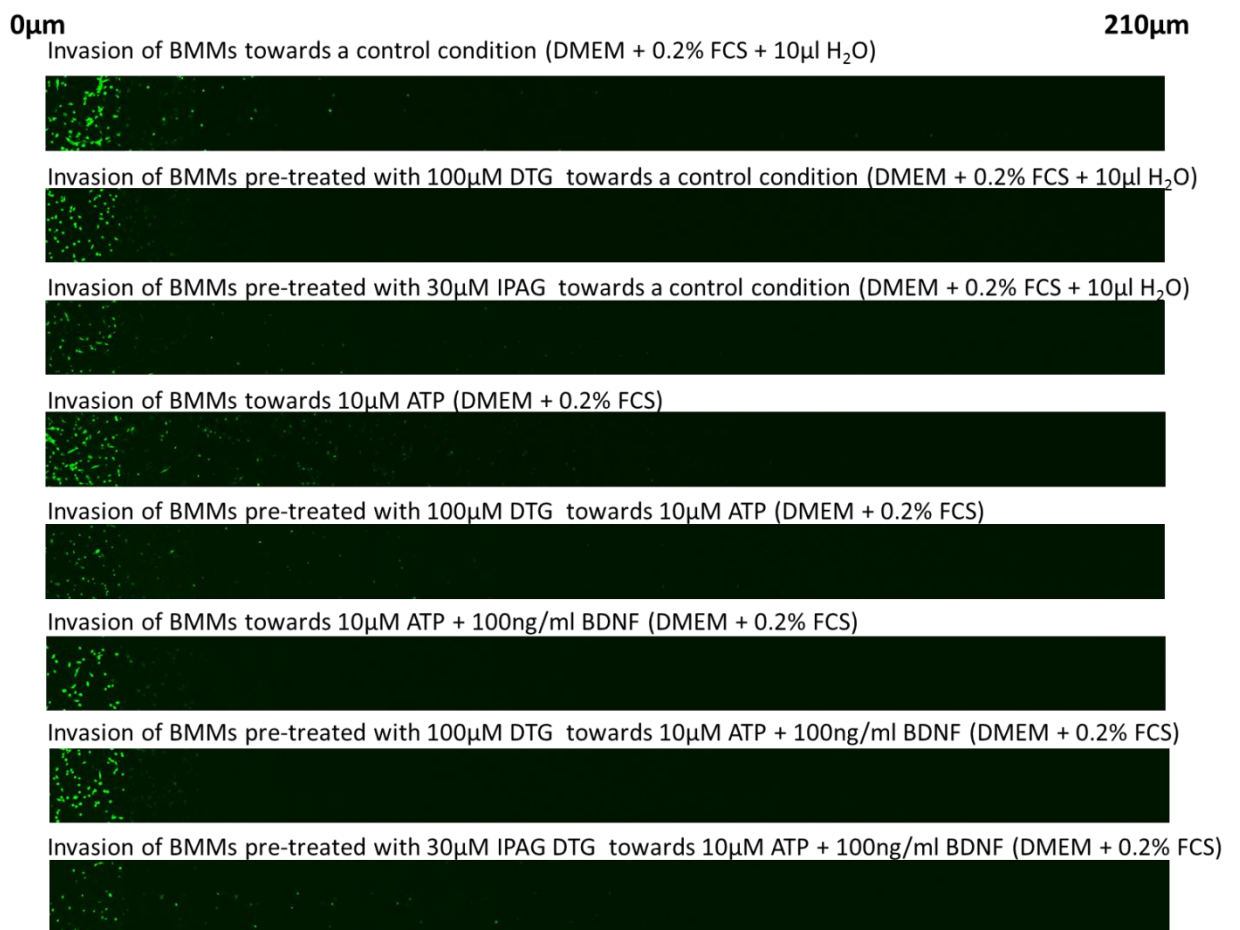
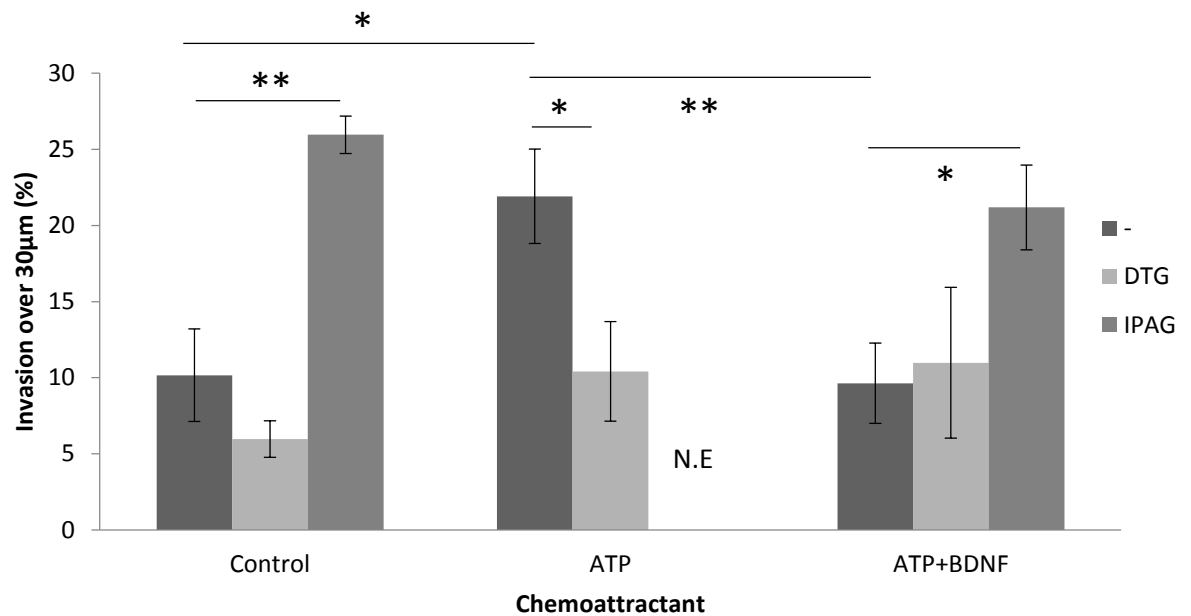


Figure 3.10. The effects of Sigma receptor agonism, by DTG, and antagonism, by IPAG on the invasion of BMM through a Matrigel™/Fibronectin matrix. BMMs were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix towards 10µM ATP, 100ng/ml BDNF or the combination of ATP + BDNF, with or without the pre-treatment of either 100µM DTG or 30µM IPAG. Afterwards all live cells were stained with a Calcein-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each bar represents the mean ± 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001. N.E = not tested.

3.3.4. Elucidating mechanisms behind ATP-induced invasion and the attenuation of migration via BDNF, and Sigma-1 receptor agonism.

It was of interest to explore the cell signalling mechanisms behind ATP-induced invasion, and BDNF attenuation of ATP-induced invasion, initially focusing on the p38-MAPK pathways, which was previously implicated in ATP signalling (Trang et al., 2009).

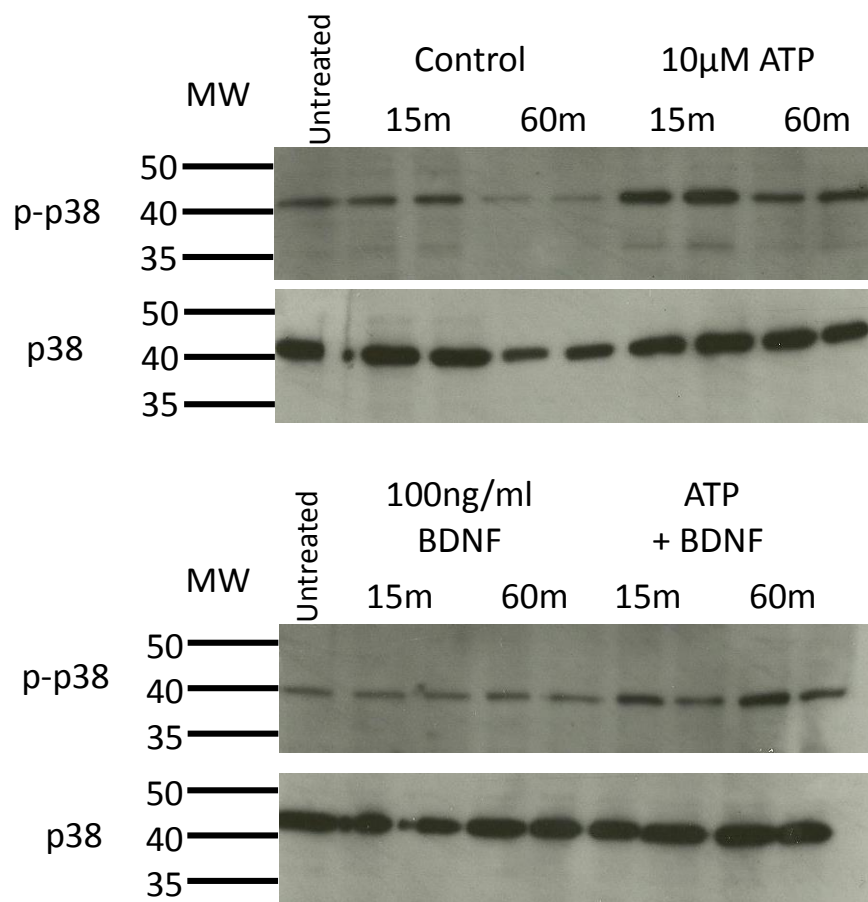


Figure 3.11. p38 is activated after 15 minutes stimulation with ATP, and repressed with ATP+BDNF stimulation. Activation of p38 was explored using western blotting methods. Here we show the total p38 (expected size 41 kDa), and activated phosphorylated form p-p38 in response to stimulation with 10 μ M ATP, 100ng/ml BDNF or a combination of ATP + BDNF for either 5, 15, 30 or 60 minutes before cell lysis and protein extraction. This figure has been determined from 1 gel, n=1.

Raw 264.7 cells were grown until confluent then made quiescent in serum free growth media. Cells were then treated with stimulants including 10 μ M ATP, 100ng/ml BDNF or a combination of ATP + BDNF. Control conditions received a media change, whereas the untreated control did not receive a media change. Cells were then lysed as described on chapter 2.

At 15 minutes of ATP stimulation, an increase of phosphorylation was seen when compared to the untreated control, and the control conditions (Figure 3.11), at 60 minutes of ATP stimulation; the level of phosphorylation appears reduced, but not returned to control levels. BDNF stimulation does not appear to have much of an effect on phosphorylation levels at either 15 or 60 minutes. After 15 minutes stimulation, the phosphorylation in ATP+BDNF is markedly reduced from the level of phosphorylation resulting from ATP alone. However, at 60 minutes, the phosphorylation appears to be at the same level as ATP alone, suggesting a short term effect in cells treated with both ATP and BDNF.

Figure 3.12 explores the activation of ERK1/2 (expected size 42/44kDa, respectively) upstream of. In this blot although the exposure of the gel is weak, we see increased phosphorylation in the control condition at 30 minutes stimulation; however, this may be due to an increased level of total ERK1/2 (possibly due to an increased level of protein loaded into the gel). When stimulated with 10 μ M ATP, phosphorylation appears to be increased at 60 minutes, but at no time-point before this. In the combination of ATP + BDNF, there appears to be a very slight phosphorylation, which is sustained until 30 minutes, but reduced back to untreated control levels by 60 minutes – and thus markedly reduced when compared to the phosphorylation seen with ATP alone.

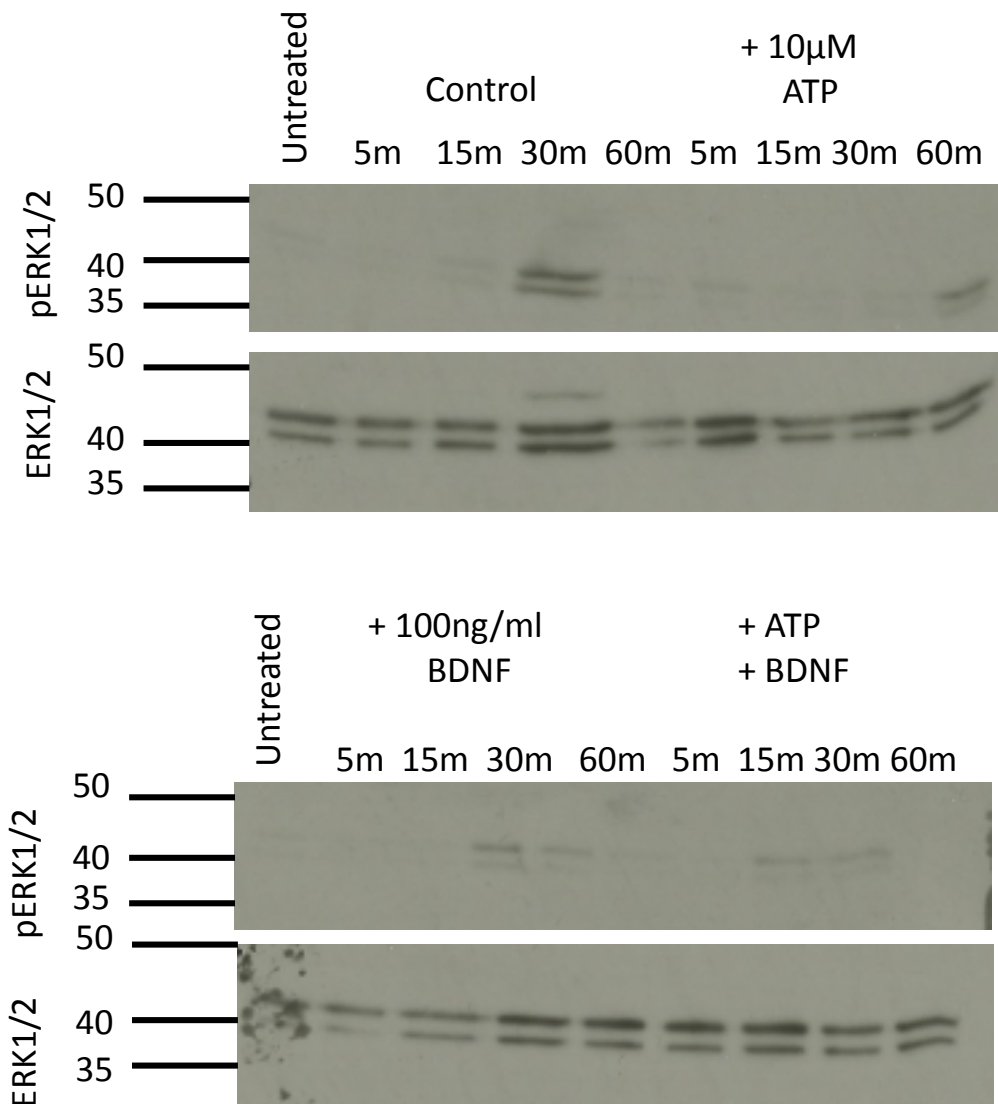


Figure 3.12. BDNF abrogates ATP-induced ERK1/2 phosphorylation after 60 minutes of stimulation. Activation of ERK1/2 was explored using western blotting methods. Here we show that the total ERK 1/2 (expected size 42/44 kDa respectively), and its activated form pERK1/2 in response to stimulation to 10µM, 100ng/ml BDNF or a combination of ATP + BDNF for either 5, 15, 30 or 60 minutes before cell lysis and protein extraction. **This figure is a representative of 1 gel. N=1.**

3.3.5. Investigating the effects of Sigma-1 receptor on the invasion and signalling of Raw 264.7 macrophages.

To further explore the effects of Sigma-1 receptor in macrophage invasion, the next step was to employ siRNAs targeting Sigma-1 to knock down the expression of the receptor. Raw 264.7 cells were transfected using a set of four siRNAs each targeting different regions of the OPR1 gene (sequences published in chapter 2), or siRNAs with a scrambled sequence.

After 48hrs exposure to the siRNAs, cell lysates were collected to ensure that the sigma-1 receptor protein levels were indeed reduced. Figure 3.13 shows the resulting blot from this experiment.

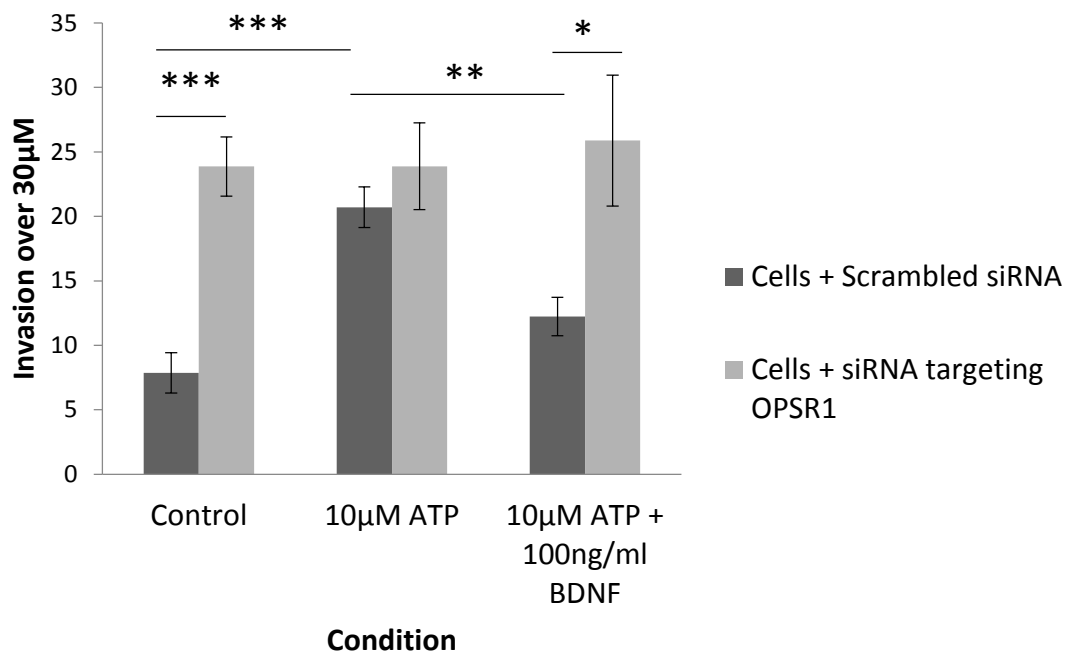


Figure 3.13. Western blot to determine Sigma-1 receptor expression in Raw 264.7 cells. After 48 hours treatment with siRNAs targeting the OPR1 gene, encoding Sigma-1 receptor, cell lysates were collected prior to Western blotting for sigma-1 receptor. Lane 1: Lysate from growing Raw 264.7 cells. Lane 2: Control, cells treated with transfection reagent alone. Lane 3, Scramble: cells treated with siRNAs with scrambled sequences. Lane 4, Knockdown: cells treated with siRNAs targeting OPR1, n=2 separate experiments.

In Figure 3.13, a strong signal in the positive control, from a lysate of exponentially growing Raw 264.7 cells was observed. Weaker signals in both the scramble and control conditions and thus it is difficult to draw firm conclusions but in the knockdown condition, there is a very weak signal for sigma-1 receptor suggesting possible silencing of sigma-1 receptor protein levels.

Using cells treated with siRNAs targeting sigma-1 receptor, and those with scrambled sequences, an inverted invasion assay was performed, using ATP and ATP + BDNF as chemoattractants. In the scramble conditions, the cells behave as previously seen, with cells attracted to ATP resulting in a 2.7-fold significant increase compared to the control condition. ATP + BDNF leads to a 1.6-fold significant decrease in invasion compared to ATP conditions, similar to data previously shown (Figure 3.14).

However, with cells transfected with siRNAs targeting OPR1, it was found that there was an overall increase in the amount of invasion when compared to those cells with scrambled siRNA. This resulted in a 3-fold increase between scramble control and knockdown control, and a rescue of the ATP + BDNF scramble condition, up 2.1-fold (Figure 3.14). This experiment is representative of 3 separate experiments.



Raw 264.7 transfected with Scrambled siRNA towards a control condition



Raw 264.7 transfected with siRNA targeting OPR1 towards control condition



Raw 264.7 transfected with Scrambled siRNA towards a 10µM ATP



Raw 264.7 transfected with siRNA targeting OPR1 towards 10µM ATP



Raw 264.7 transfected with Scrambled siRNA towards a 10µM ATP + 100ng/ml BDNF



Raw 264.7 transfected with siRNA targeting OPR1 towards 10µM ATP + 100ng/ml BDNF



Figure 3.14. Investigating the effect of a Sigma-1 knockdown using siRNAs on the invasion of Raw 264.7 through a Matrigel™/Fibronectin matrix towards ATP and BDNF. Cells were allowed 48 hours to invade into a Matrigel™/Fibronectin matrix towards 10µM ATP, 100ng/ml BDNF or the combination of ATP + BDNF, with the prior transfection of either Scramble siRNAs or siRNA targeting OPR1 (Sigma-1 receptor). Afterwards all live cells were stained with a Calcein-AM dye and Transwells™ were imaged using a Leica confocal microscope every 15µm through 210µm, a representative of images taken from each condition is shown beneath the graph. Invasion over 30µm was then quantified using ImageJ image analysis software. Each bar represents the mean ± 1 S.E.M. N = 6 (3 fields from 2 separate Transwells™). P value calculated by students' t-test * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.4. Discussion

In this chapter invasion and migration of macrophages in response to the extracellular nucleotide ATP was explored, along with invasion in response to BDNF and the combination of ATP and BDNF. Certain mechanisms which may underlie these observations were explored including the possible role of the Sigma-1 Receptor in macrophage migration.

It was found that ATP significantly increased invasion of Raw 264.7 cells and primary bone marrow-derived macrophages through a Matrigel™/Fibronectin matrix. This is supported by several other studies such as Lambert *et al*, 2010, who discovered that both adult and fetal human microglial migrated towards ATP in a Boyden chamber assay, and that human monocyte migration was enhanced with a low concentration of ATP (1-10µM). Ohsawa *et al*, 2010 observed microglia extending processes towards ATP in a Boyden chamber assay, and suggest that integrin-β1 may play an important role in the migration of microglia towards ATP (Ohsawa et al., 2010, Lambert et al., 2010). However, it has been suggested that ATP is released at the leading edge of neutrophils, and acts only as a chemokinetic factor, and guides circulating leukocytes to other chemoattractants (Chen et al., 2006).

Kronlage *et al*, (2010) showed that macrophage migration towards C5a depends on autocrine release of ATP and direct activation of relevant purinergic receptors resulted in membrane ruffling reminiscent of lamellipodial formation (Kronlage et al., 2010). However in a subsequent study these authors reported that ATP was not chemoattractive for murine peritoneal macrophages in 2D or 3D (Isfort et al., 2011).

Purinergic receptor antagonists BBG, Suramin and PPADs on ATP significantly decreased invasion towards 10µM ATP. These results suggest that macrophage migration towards ATP is dependent on a number of purinergic receptors – including P2X4, P2X7, P2Y2, P2Y6 and P2Y12. There is some supporting evidence in the literature, P2Y12 is thought to be responsible for microglial process extension, although it has also been suggested that P2Y6 and P2Y2 are also important in migration of microglia (Ohsawa et al., 2010, Koizumi et al., 2013, Kim et al., 2012). In addition, P2X4 and P2X7 receptors have also been suggested to be of importance in cell migration (Horvath and DeLeo, 2009, Beggs et al., 2012, Nasu-Tada et al., 2006). From this experiment it is not possible to determine which receptor is

responsible for ATP-induced invasion, but future studies exploiting specific purinergic receptor blockade, such as 5-BDBD (a P2X4 receptor antagonist) or A-438079 (a specific P2X7 antagonist) may prove insightful (He et al., 2007, Balázs et al., 2013) in addition to siRNA approaches.

It has been reported that P2X4 receptor activation by ATP results in the release of BDNF (Trang et al., 2009) and relating this to the scenario of neuropathic pain it was hypothesised in this thesis that macrophages at the site of injury will be exposed to both ATP and BDNF. It was found that the a combination of BDNF caused a repression in the level of ATP-induced invasion of both Raw 264.7 and BMMs and this apparently novel observation could suggest a feedback mechanism to prevent further macrophage recruitment. BDNF may thus be neuroprotective, since fewer macrophages migrating to the site of injury would limit the release of pro-inflammatory cytokines and chemokines, which trigger neuronal damage or apoptosis.

There are several reports to suggest that BDNF is neuroprotective. It has been reported that P2X7 receptors and BDNF, along with NMDA converge on the PI3K pathway at GSK3 in a PKC dependent manner and consequently lead to neuronal survival by reducing PI3K inhibition (Ortega et al., 2010). The ERK pathway has also been shown to play a role in BDNF neuroprotection, and activation of ERK was revealed to prevent cortical neuron apoptosis (Hetman et al., 1999, Almeida et al., 2005). Reduced levels of BDNF have also been linked to reduced neuroprotection in MS (Azoulay et al., 2008).

BDNF signals through TrkB, which then leads to the phosphorylation of PLC γ , and can also lead to the phosphorylation of MAPK and PI3K, causing a cascade of intracellular signalling and result in a range of activities. Once PLC γ is activated, the hydrolysis of phosphatidylinositol 4,5-bisphosphate occurs, thus producing diacylglycerol (DAG) and inositol 4,5-triphosphate (IP $_3$). DAG activates protein kinase C and IP $_3$ can release calcium from its intracellular stores (Yoshii and Constantine-Paton, 2010).

Signalling mechanisms underpinning the repression of ATP-induced invasion by BDNF revealed that BDNF treatment in combination with ATP suggest reduced activation of ERK 1/2 and p38 MAP kinases. ATP has been shown to invoke phosphorylation of ERK 1/2 and

p38 in various cell types previously (Trang et al., 2009, Tsuda et al., 2012, Inoue and Tsuda, 2009, Beggs and Salter, 2010).

Mizoguchi and colleagues previously found that BDNF increased intracellular calcium concentration through PLC γ activation and also sustained this increase in microglia, unlike ATP, which resulted in a transient increase in calcium levels. It is suggested that BDNF may have an unknown additional mechanism to activate microglia (Mizoguchi et al., 2014).

The effects of Sigma-1 receptor were also examined. Sigma-1 receptor has previously been found to suppress many functions of microglia (Hall et al., 2009) and also has links with BDNF. Sigma-1 Receptor has been found to be neuroprotective in some scenarios such as hypoxia and β -amyloid neurotoxicity (Ruscher et al., 2011). Fujimoto and colleagues in 2012 discovered that Sigma-1 agonism can potentiate post-translational processing of BDNF. It has also been proposed that Sigma-1 receptor is capable of modulating inter-organelle signalling, and thus altering reactions to certain extracellular signals (Fujimoto et al., 2012, Su et al., 2010).

The effects of the agonism of Sigma-1 receptors by DTG were investigated, the results of which were that DTG suppresses both Raw 264.7 cell invasion and migration, and can suppress the invasion of BMMs. These findings are similar to the findings of Hall *et al*, 2009, where it was found that sigma-1 agonism by 100 μ M DTG could reduce microglial migration. Antagonism of Sigma-1 by IPAG was also explored, it was observed that sigma-1 antagonism induced macrophage invasion into a Matrigel™/Fibronectin matrix, but did not enhance ATP-induced invasion. Interestingly, we also report that in BMMs, (and at least a trend in Raw 264.7 cells) IPAG was able to overcome the suppression of invasion observed with BDNF + ATP, suggesting a possibility that Sigma-1 receptor could be involved in BDNF abrogation of ATP-induced invasion. It should be noted however that IPAG treatment alone also induced Raw 264.7 cell invasion – this may be because IPAG has previously been shown to be invoke calcium signalling (Brimson *et al*, 2011).

The role of Sigma-1 receptor in invasion was further explored using a gene silencing approach. The knockdown of Sigma-1 receptor resulted in the increase of invasion of Raw 264.7, and was able to rescue the abrogation of ATP-induced invasion by BDNF. BDNF is known to signal through PLC γ , and sigma-1 receptor has been reported to be able to

modulate Trk B signalling through PLC γ , suggesting a potential mechanism of cross-talk between BDNF and Sigma-1 receptor (Yagasaki et al., 2006), see figure 3.15.

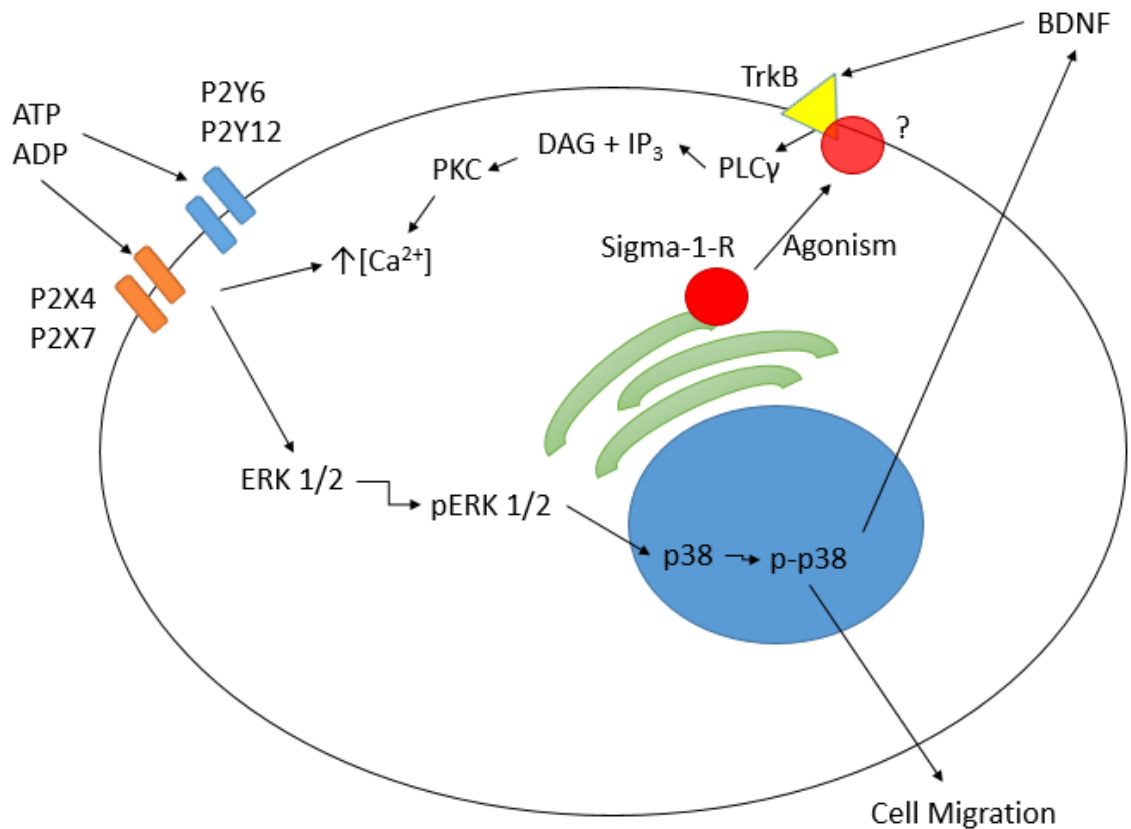


Figure 3.15. A Diagram to demonstrate the potential mechanism for ATP-induced invasion and BDNF attenuation. ATP, which is quickly broken down to ADP by apyrase, activated purinergic receptors and leads to activation of ERK1/2-p38 MAP kinase pathway, which in turn leads to increased cell migration. Trang et al, 2009 also observed a release in BDNF after activation of this pathway. BDNF acts through TrkB which leads to activation of PLC γ , leading to an increased intracellular calcium. In this chapter, it is speculated that Sigma-1 receptor is agonised and translocates to modulate TrkB signalling, which could lead to the abrogation of ATP-induced cell migration through currently unknown mechanisms.

Here, we report for the first time (to our knowledge) that BDNF is capable of reducing the invasion of macrophages through a mechanism linked to the activation of Sigma-1 receptor, however, the mechanism remains yet to be elucidated. Since microglia and possibly macrophages produce BDNF endogenously, one could speculate that the reduction in invasion would result in a reduction in recruitment of further microglia, could result in an overall reduction in further BDNF production. The reduction of BDNF produced would reduce the risk of the disinhibition of lamina 1 neurons and therefore a reduction in painful signalling.

Chapter 4: Exploring the Matrix Metalloproteinase response of Macrophages upon ATP stimulation.

4.1. Introduction

4.1.1. Matrix metalloproteinases and their inhibitors.

Matrix Metalloproteinases (MMPs) were thought to act primarily to catabolise components of the extracellular matrix and can thus be divided into groups dependent on their extracellular matrix specificity, including, collagenases, stromelysins, matrilysins and gelatinases, along with the membrane type MMPs (Parks et al., 2004, Gill and Parks, 2008).

MMPs have previously been associated with cell migration, having a role in cells to break down matrix components, and thus migrate through the cellular microenvironment. Recently, however, it has been found that MMPs can cleave a wide variety of extracellular, bioactive substances and regulate the activity of these proteins – including, but not limited to; the release of growth factors from the extracellular matrix or the cell membrane, the shedding of cell adhesion molecules, activation of other MMPs and cleavage of growth factor receptors (Parks et al., 2004, Nuttall et al., 2007, Gill and Parks, 2008, Wolf et al., 2013, Murray et al., 2013).

The function of MMPs *in vivo* depends on the balance between them and their inhibitors. Tissue Inhibitors of Metalloproteinases (TIMPs) are the endogenous inhibitors of MMPs, of which there are four members. TIMPs inhibit MMPs through interaction of the N-terminal domain of the TIMP, which co-ordinates with the Zn²⁺ containing catalytic site of the MMP, thus binding in a similar manner to the MMP and its intended substrate (Gill and Parks, 2008, Parks et al., 2004). However, there are other inhibitors of MMPs such as α 2-macroglobulin (an inhibitor of many proteinases), which is produced in the liver and found in circulating blood (Kessenbrock et al., 2010, Hopps and Caimi, 2012).

4.1.2. MMPs in cell migration.

Interstitial cell migration is a multistep process, consisting of pseudopod protrusion at the leading edge of the cell; integrin mediated adhesion of the cell to the extracellular matrix, cleavage of extracellular matrix by cell-surface expressed proteases, contraction of the cell body and then a rear retraction and translocation of the cell body (Wolf et al., 2013, Fisher et al., 2009). In mesenchymal derived cells, interstitial invasion is controlled by MMPs, in particular MMP-14 (Miyazawa et al., 2013, Wolf et al., 2013).

MMPs are also crucial in leukocyte migration – the process by which leukocytes adhere to endothelial cells by first rolling, then adhering, and finally extravasation through the vessel wall into the extracellular matrix. It has previously been found that monocytes and other peripheral blood leukocytes require the production of MMPs to move through basement membranes, with specific importance attributed to the role of the gelatinases – MMP-2 and MMP-9, which are capable of degrading specific proteins in the basement membrane (Robinson. S.C., 2002).

As macrophages are a key cell in the innate immune system, it is of fundamental importance that they have the ability to mobilise in response to external signals, such as cytokines and trauma, and therefore, MMPs may play a major role in the remodelling of the extracellular matrix to allow macrophages and circulating monocytes to follow these signals. Robinson *et al*, established that the exposure of peripheral blood monocytes to chemoattractants could indeed result in the increased expression of MMPs.

4.1.3. MMPs in Neuropathic Pain.

There appears to be an early and late phase response to spinal nerve ligation in DRG satellite cells, and MMP-9 has been seen to be swiftly upregulated (Kawasaki et al., 2008, Lakhan and Avramut, 2012, Tsuda et al., 2012, Chattopadhyay et al., 2007, Buss et al., 2007), followed by a late-phase response increase of MMP-2. Vereyken *et al*, 2011 argues that after peripheral nerve injury, the MMP response could lead to increased axonal damage (Vereyken et al., 2011). Bradykinin, implicated in pain responses, can modulate MMP expression in a number of cell types (Yoo et al., 2011) including macrophages (Abdulkhalek et al., 2012). In the CNS, BDNF expression has been correlated with that of MMP9 (Colak et al., 2012) and of interest is the recent observation that MMP9 can degrade the BDNF receptor, TrkB (Navaratna et al., 2013).

4.2. Hypothesis and Aims

Hypothesis: Since ATP induced the invasion of both primary macrophages and Raw 264.7 cells through a Matrigel™/Fibronectin matrix it is possible that MMPs may be regulated in this context.

Aims:

To explore if ATP could act by inducing the expression of MMPs and to determine the response of macrophages to other stimuli, including the inflammatory pain related molecule Bradykinin and BDNF, alongside the well-known MMP regulator, bacterial pathogen associated molecular pattern marker, LPS.

4.3. Results

4.3.1. MMP expression is altered after 24 hours stimulation with ATP, Bradykinin and LPS.

The expression of MMPs explored by Raw 264.7 cells was explored in response to stimulation with physiologically relevant concentrations of the extracellular signalling molecule ATP, the pain-associated molecule - Bradykinin, and the TLR4 agonist and pathogen associated molecule – LPS. Bradykinin was chosen to be explored due to its known role in inflammatory pain, and as such, it was intended to explore any similarities between the modulation of MMP expression between ATP and Bradykinin.

The expression of all matrix metalloproteinases and tissue inhibitors of metalloproteinases was investigated in response to either a control condition (serum-starved for 24hrs) or 24hr stimulation with 1mM ATP, 1mM Bradykinin or 100ng/ml LPS. A summary of MMP and TIMP expression change can be seen in Figure 4.1. As it was desired to focus on the expression levels of MMPs in more detail, and to focus primarily on genes which displayed a change in expression, figure 4.1 and 4.2 display the results of the only experiment. For reference, figure 4.2 displays the findings of this experiment as a $2^{\Delta C_T}$ change. Later it was desired to focus solely on the alterations of MMP expression in response to ATP, a “heat map” of MMP and TIMP expression in response to either ATP or LPS only was produced and can be found in the appendix.

In figure 4.1, it is highlighted that there is a significant increase in the levels of expression of MMP-8 in response to ATP and Bradykinin, whereas MMP-13 expression is significantly increased in response to stimulation with ATP and LPS. It is also evident that TIMP-2 is the only detectable TIMP in Raw 264.7 cells, and this expression is significantly increased in response to 1mM ATP stimulation. These findings are elucidated in further detail in figure 4.2.

Condition	1mM ATP	1mM Bradykinin	100ng/ml LPS
McolA	Not Expressed	Not Expressed	Not Expressed
McolB	Not Expressed	Not Expressed	Not Expressed
MMP-2	↔	Not Expressed	Not Expressed
MMP-3	Not Expressed	Not Expressed	Not Expressed
MMP-7	Not Expressed	Not Expressed	Not Expressed
MMP-8	↑*	↑*	↑
MMP-9	↔	↔	↔
MMP-10	↔	↔	↓
MMP-11	Not Expressed	Not Expressed	Not Expressed
MMP-12	↔	↔	↔
MMP-13	↑*	↑	↑*
MMP-14	↑	↑	↑
MMP-15	↔	↑	↑
MMP-16	Not Expressed	Not Expressed	Not Expressed
MMP-17	Not Expressed	Not Expressed	Not Expressed
MMP-19	↔	↔	↑
MMP-20	Not Expressed	Not Expressed	Not Expressed
MMP-21	↔	↔	↔
MMP-23	Not Expressed	Not Expressed	Not Expressed
MMP-24	Not Expressed	Not Expressed	Not Expressed
MMP-25	↔	↑	↑
MMP-28	↑	↑	↔
----	----	----	----
TIMP-1	Not Expressed	Not Expressed	Not Expressed
TIMP-2	↑*	↑	↔
TIMP-3	Not Expressed	Not Expressed	Not Expressed
TIMP-4	Not Expressed	Not Expressed	Not Expressed

Figure 4.1. A table to show the expression of MMPs in response to 24hr stimulation with either ATP, Bradykinin or LPS in relation to the unstimulated control. Gene expression of the murine family of MMPs was explored using Taqman gene expression assays in the Raw 264.7 cell line in triplicated samples. Any samples in which the cycle threshold (C_T) was below 40 or undetected are shown as “Not expressed”. In this table “↑” represents an increase in gene expression from the control, whereas “↓” represents a decrease in gene expression from the control, “↔” represents when gene expression remains unchanged from the control. “↑*” represents when the gene expression is significantly increased from the control condition. Significance was measured using Student’s t-test and significance assumes a p value of ≤ 0.05 , $n=3$.

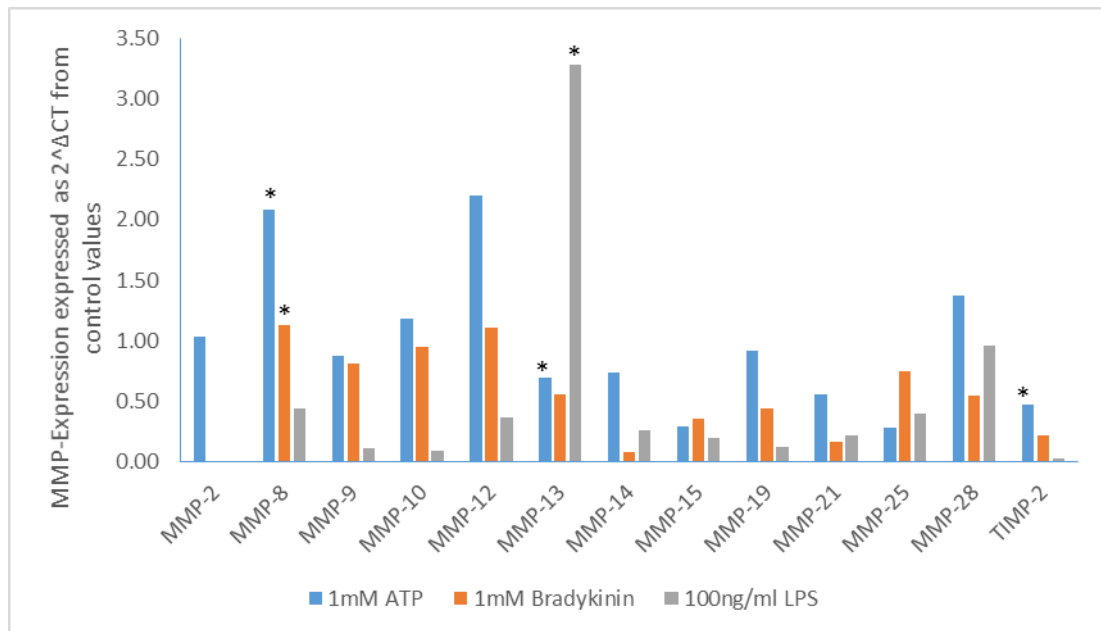


Figure 4.2. A graph to show the expression of MMPs in response to 24hr stimulation with either ATP, Bradykinin or LPS in relation to the unstimulated control. Gene expression of the murine family of MMPs was explored using Taqman gene expression assays in the Raw 264.7 cell line in triplicated samples in a graphical form, with raw C_T values expressed as $2^{-\Delta C_T}$. “*” represents when the gene expression is significantly increased from the control condition. Significance was measured using Student’s t-test and significance assumes a p value of ≤ 0.05 , n=3

Figure 4.3 explores the expression of the MMPs with a significant change in gene expression from the control, which consists of MMP-8, MMP-13 and TIMP-2. MMP-8 was found to be expressed in Raw 264.7 cells under control conditions with a C_T value of 33.9, whereas treatment for 24 hours with 1mM ATP significantly increased the expression of MMP-8 two-fold, and lowers the C_T value to 32.8. 24 hour treatment with Bradykinin was found to also significantly increase the relative level of MMP-8 almost five-fold. 24 hour treatment with LPS did not significantly alter the expression of MMP-8, but was found to slightly increase the relative level of expression.

MMP-13 was shown to be well expressed in Raw 264.7 cells in control conditions with a C_T value of 27.1, 24 hour treatment with 1mM ATP was found to significantly double the expression of MMP-13. After treatment with Bradykinin, there is a slight increase in the level of MMP-13 expression, although this is not significant. Treatment with LPS also leads

to a dramatic significant increase in the level of MMP-13 in Raw 264.7, leading to expression 80 times higher than that found in the control condition.

Tissue Inhibitor of Metalloproteinases – 2 was found to be the only TIMP to be expressed in Raw 264.7 cells. 24hr treatment with ATP was shown to increase the relative expression levels 2.5 fold. Bradykinin and LPS are shown not to have a significant effect on the expression of TIMP-2.

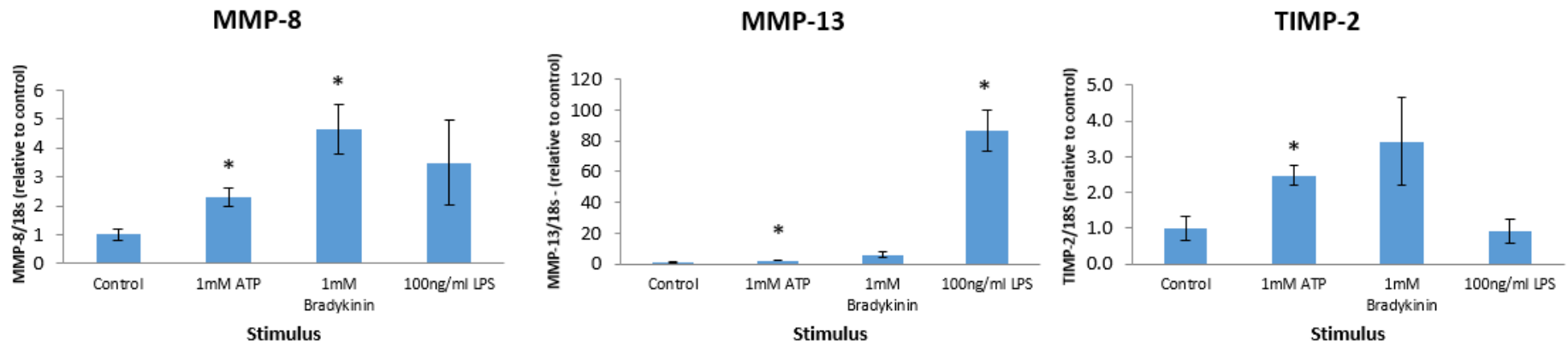


Figure 4.3. MMP-8, -13, and TIMP-2 show a significant altered level of expression in Raw 264.7 cells when stimulated with ATP, Bradykinin or LPS. This figure summarises the sections of significant data that was found when the expression of MMPs and TIMPs was explored in Raw 264.7 cells in response to stimulation. qRT-PCR was performed on cDNA extracted from stimulated Raw 264.7 cells after 24 hour stimulation with either 1mM ATP, 1mM Bradykinin or 100ng/ml LPS. Statistical significance was determined using Student's t-test. * = $p \leq 0.05$, $n=3$.

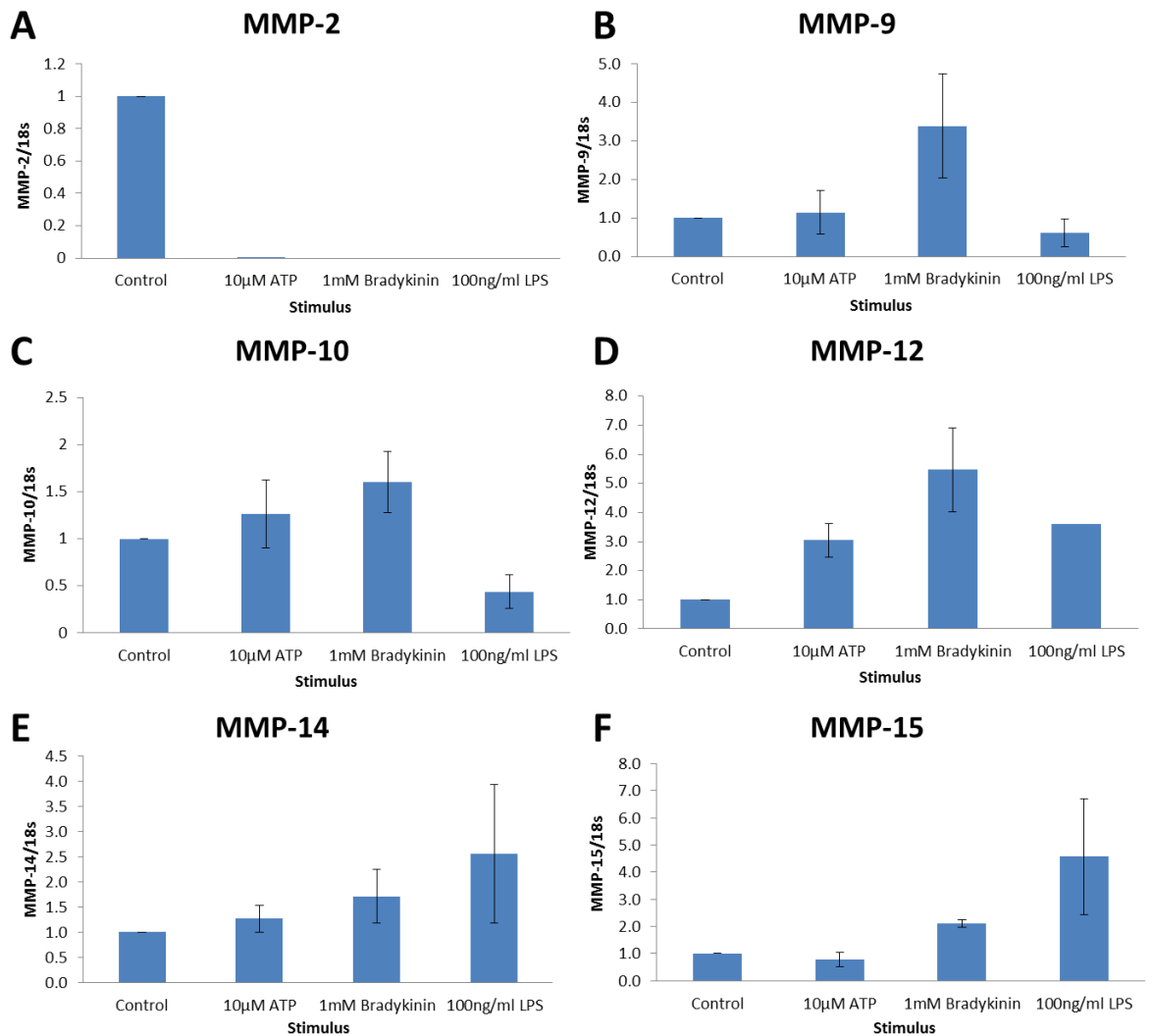


Figure 4.4. MMP expression levels in Raw 264.7 cells after 24 hours treatment with either 10µM ATP, 1mM Bradykinin or 100ng/ml LPS. Gene expression of the murine family of MMPs was explored using Taqman gene expression assays in the Raw 264.7 cell line in triplicated samples. This figure shows the gene expression changes for MMPs which were found to have insignificant changes regardless of treatment with ATP, LPS or Bradykinin. Statistical significance was measured using student's t-test, n=3.

Figures 4.4 and 4.5 demonstrate a graphical representation of the results of the gene expression screen demonstrated in Figure 4.1. Figure 4.4 shows that Raw 264.7 expression of MMP-2 decreases in expression with 24 hours treatment of all tested substances, to the point where it is no longer detectable. MMP-9 is shown to be unchanged by ATP or LPS treatments, but expression is found to rise by 3 fold with Bradykinin treatment. MMP-10 expression is relatively unchanged with ATP or Bradykinin treatment, although with LPS treatment, there appears to be a twofold decrease in expression when compared to the control.

Figure 4.4 D shows the expression levels of MMP-12 in Raw 264.7 cells, with treatment of ATP for 24 hours resulting in a threefold increase in the expression of MMP-12, similarly, treatment with Bradykinin appears to increase expression to 5x the level of the control condition. LPS also induces expression to a level similar to the level seen in ATP induction of MMP-12. Levels of MMP-14 seem to remain relatively unchanged after stimulation and

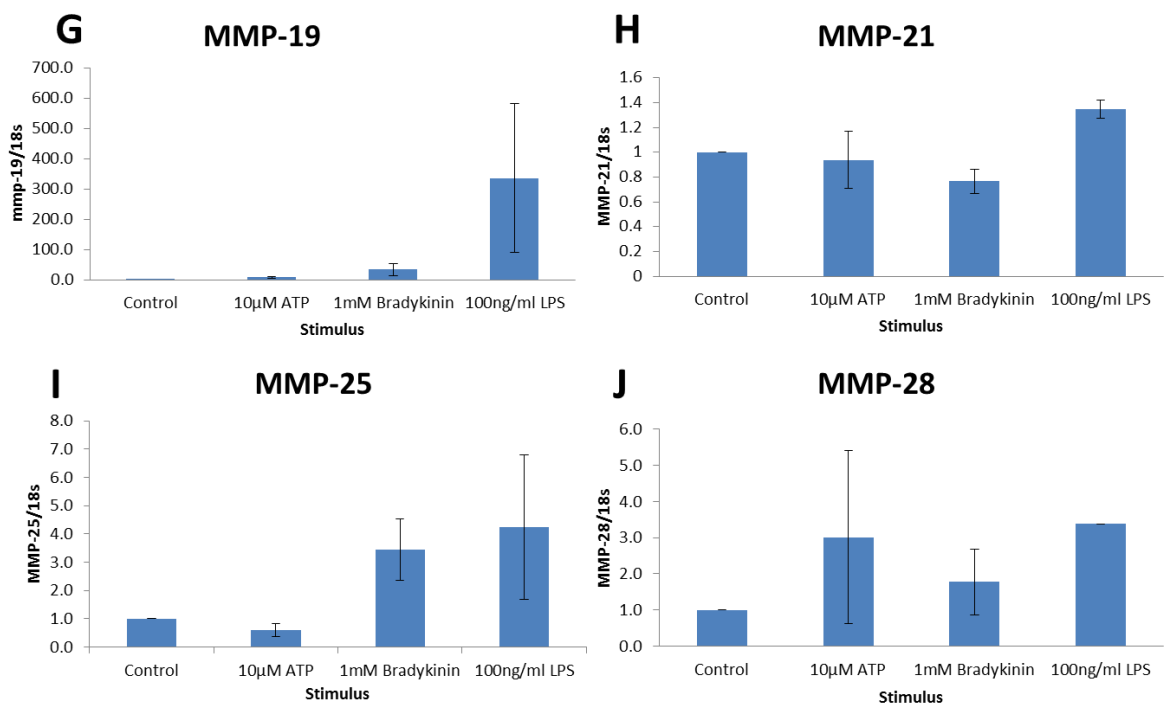


Figure 4.5. MMP expression levels in Raw 264.7 cells after 24 hours treatment with either 10µM ATP, 1mM Bradykinin or 100ng/ml LPS. Gene expression of the murine family of MMPs was explored using Taqman gene expression assays in the Raw 264.7 cell line in triplicated samples. This figure shows the gene expression changes for MMPs which were found to have insignificant changes regardless of treatment with ATP, LPS or Bradykinin. Statistical significance was measured using student's t-test, n=3.

with MMP-15, there is indeed a slight decrease in expression after treatment with ATP, although Bradykinin and LPS appear to slightly increase the expression of MMP-15.

Figure 4.5 displays the results of the remaining matrix metalloproteinases, where trends in modulation of expression were observed. MMP-19 is shown to be relatively unchanged after 24 hours treatment with either ATP or Bradykinin, but after treatment with LPS, displays a 400fold increase. MMP-21 expression is relatively unchanged in any condition. MMP-25 was shown to be increased threefold in both Bradykinin and LPS conditions. Finally, MMP-28 expression was potentially increased threefold with ATP and LPS treatment, and twofold with Bradykinin treatment.

4.3.2. MMP expression in response to stimuli over time.

To investigate in more detail those MMPs expressed at detectable levels, the expression of MMPs in Raw 264.7 in response to ATP was explored through a time-course.

Treatment with two concentrations of ATP, firstly at 10 μ M, included exploring potential effects of the activation of the P2X4 receptor, and 1mM ATP – to determine effects of the activation of the P2X7 receptor. LPS was also included as a positive control and to confirm findings as the effects of LPS stimulation on MMP expression is well characterised.

Figure 4.6 shows a table summary of the results of the MMP time-course investigation. . It is of interest to note that both MMP-14 and MMP-28 were found to be undetected, considering their expression in the previous experiment summarised in figures 4.1 – 4.4. There is an increase in the level of MMP-8 at 8hrs in the control condition¹, and an increase at both 4 and 8 hours with both 10 μ M ATP and 1mM ATP stimulation, where LPS provides a significant increase at both 24hrs and 48hrs after initial stimulation. MMP-10 was also found to be upregulated at 8 hours in both the control condition and after stimulation with 10 μ M ATP. MMP-12 was found to be upregulated at 4 hours after stimulation with ATP at either concentration and MMP-13 was found to be upregulated at 8 hours in the control,

¹ The control condition in these experiments is classed as a media change at the investigated time-point. As ATP can be released from all cells after stress (even as small as a media change), it is important to replicate these stress conditions.

4 hours with 10 μ M ATP and at 4 hours and 8 hours 1mM ATP. These results will be further analysed in figures 4.7 to 4.16.

Condition	Control				10 μ M ATP				1mM ATP				100ng/ml LPS			
	4	8	24	48	4	8	24	48	4	8	24	48	4	8	24	48
MMP-8	\leftrightarrow	\uparrow^*	\leftrightarrow	\leftrightarrow	\uparrow^*	\uparrow^*	\leftrightarrow	\leftrightarrow	\uparrow^*	\uparrow^*	\leftrightarrow	\uparrow	\uparrow	\uparrow	\uparrow^*	\uparrow^*
MMP-10	\leftrightarrow	\uparrow^*	\leftrightarrow	\uparrow	\uparrow	\uparrow^*	\leftrightarrow	\leftrightarrow	\leftrightarrow	\uparrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\uparrow	\uparrow
MMP-12	\leftrightarrow	\uparrow	\uparrow	\uparrow	\uparrow^*	\uparrow	\leftrightarrow	\leftrightarrow	\uparrow^*	\uparrow	\leftrightarrow	\leftrightarrow	\uparrow	\uparrow^*	\uparrow	\uparrow
MMP-13	\leftrightarrow	\uparrow^*	\leftrightarrow	\leftrightarrow	\uparrow^*	\leftrightarrow	\leftrightarrow	\downarrow	\uparrow^*	\uparrow^*	\leftrightarrow	\leftrightarrow	\uparrow	\uparrow	\uparrow	\uparrow
MMP-14	Not Expressed				Not Expressed				Not Expressed				Not Expressed			
MMP-19	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow	\uparrow
MMP-28	Not Expressed				Not Expressed				Not Expressed				Not Expressed			
TIMP-2	\leftrightarrow	\leftrightarrow	\leftrightarrow	\uparrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\downarrow	\uparrow	\leftrightarrow	\leftrightarrow	\leftrightarrow	\leftrightarrow

Figure 4.6. Expression of MMPs over the course of 48hr stimulation with either 10 μ M ATP, 1mM ATP or 100ng/ml LPS in relation to the control. Gene expression of the murine family of MMPs was explored using Taqman gene expression assays in the Raw 264.7 cell line in quadruplicate samples. Any samples in which the cycle threshold (C_T) was below 40 or undetected are shown as “Not expressed”. In this table “ \uparrow ” represents an increase in gene expression from the control, whereas “ \downarrow ” represents a decrease in gene expression from the control, “ \leftrightarrow ” gene expression remains unchanged from the control. “ \uparrow^* ” gene expression is significantly increased from the control condition. Significance was measured using Student’s t-test and significance assumes a p value of ≤ 0.05 , n=4.

Figures 4.7 and 4.8 explore how the expression of MMP-8 changes over time in response to ATP and LPS stimulation. After the initial screen shown previously, bradykinin was no longer explored, whilst ATP was explored at 2 different concentrations - 10 μ M and 1mM – to determine if there is any difference between the activation of P2X4, and other purinergic receptors with 10 μ M ATP, or the desensitization of many purinergic receptors and the activation of P2X7 by 1mM ATP. Both figures show MMP expression as a fold change when compared to the 'baseline expression' of the MMP in Control conditions after 4 hours after the media change. Figure 4.6 explores the expression of MMP-8 in response to stimulation over time, clustered to allow observation of changes caused by stimulant at a particular time-point, whereas 4.7 shows expression clustered per stimulant, to allow observation of the effect of time.

From Figure 4.7, it is shown that MMP-8 (Collagenase-2) is significantly upregulated after stimulation with ATP at both 10 μ M ATP and 1mM ATP at 4hrs from 1, to 48.61 and 18.27 respectively, indicating that ATP induces an increased transcription of MMP-8. LPS at 4hr stimulation leads to an insignificant rise in MMP-8 expression. ATP at both 10 μ M and 1mM concentrations leads to a further rise in expression of MMP-8 after 8 hours of stimulation, four-fold with 10 μ M ATP, and almost ten-fold with 1mM ATP.

After 24hrs, the significant rise in MMP-8 by ATP falls down to less than control levels – with a 2-fold decrease from control conditions seen at 24hrs stimulation with 10 μ M ATP. However, at 24hrs stimulation with LPS leads to a 12 fold increase in expression when compared to the control conditions. After 48 hours, MMP-8 expression is raised by stimulation with both 1mM ATP, and LPS.

Figure 4.8 demonstrates the changes that occur over time within groups of stimulation. In the control conditions (which at each time point represent a media change), lead to an 11-fold rise in MMP-8 expression at 8hrs, which falls to a 2-fold increase at 24hrs. 10 μ M ATP stimulation leads to an early rise in MMP-8 expression (48 fold the control levels at 4hr), which remains stable until 24hrs, when there is a significant decrease in MMP-8 expression to 1.2 fold control levels (at 4hrs).

1mM ATP expression over time shows an early rise in MMP-8 levels, compared to the control – with a 4hr level of 18-fold control levels, which peaks at 8hr at 108 fold. Similarly

to 10 μ M ATP, 1mM ATP stimulation leads to a fall in MMP-8 expression at 24hrs, but there is a slight increase seen at 48hrs, to almost 10 fold control levels. LPS, shows an insignificant rise in MMP-8 expression, until 48hrs, when there is a significant increase in MMP-8 expression, suggesting that LPS drives a late-phase response of MMP-8 expression.

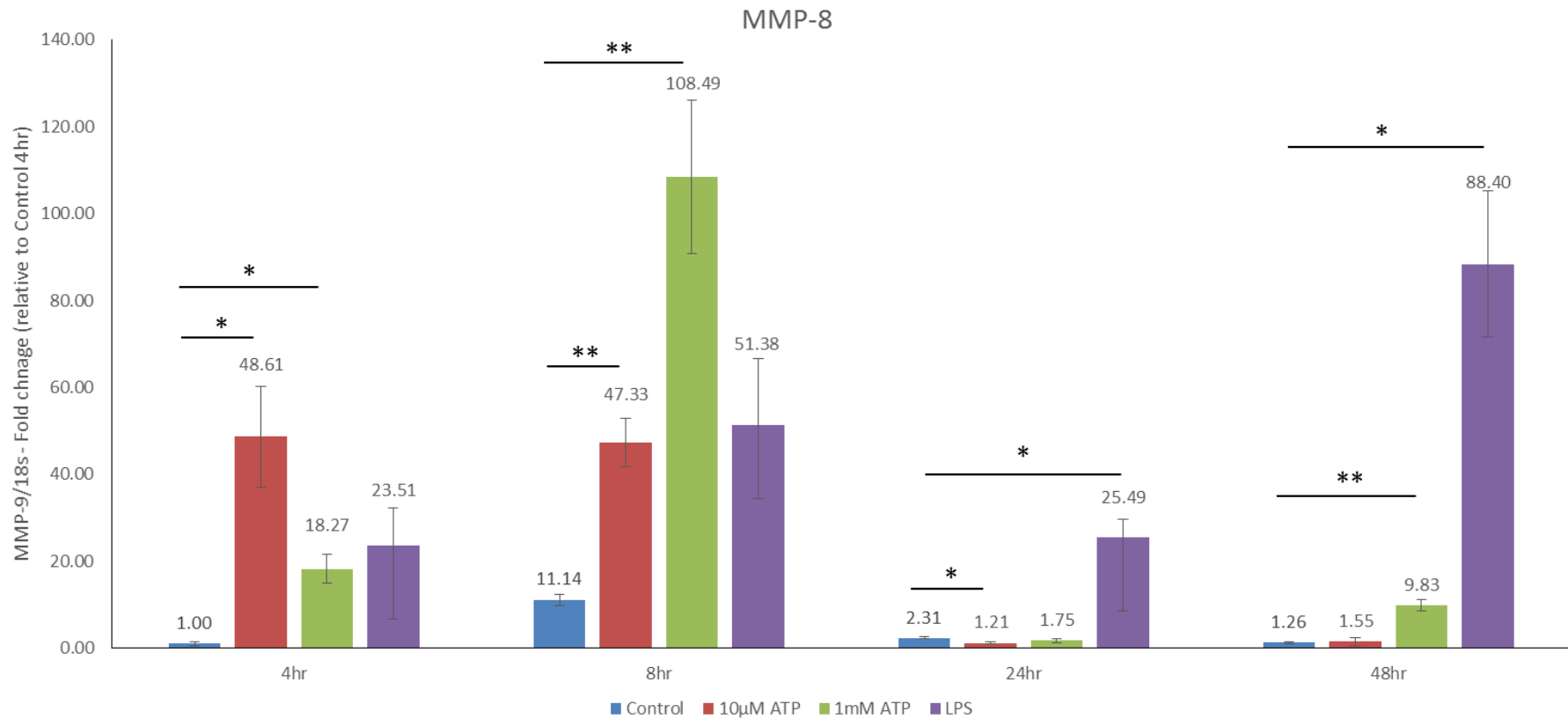


Figure 4.7. Expression of MMP-8 over 48 hours expressed in time-clusters. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS on the expression of MMP-8 were explored over 48 hours. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change when compared to the expression at 4 hours. Statistical significance was determined using Student's t-test, * = p<0.05. n=4.

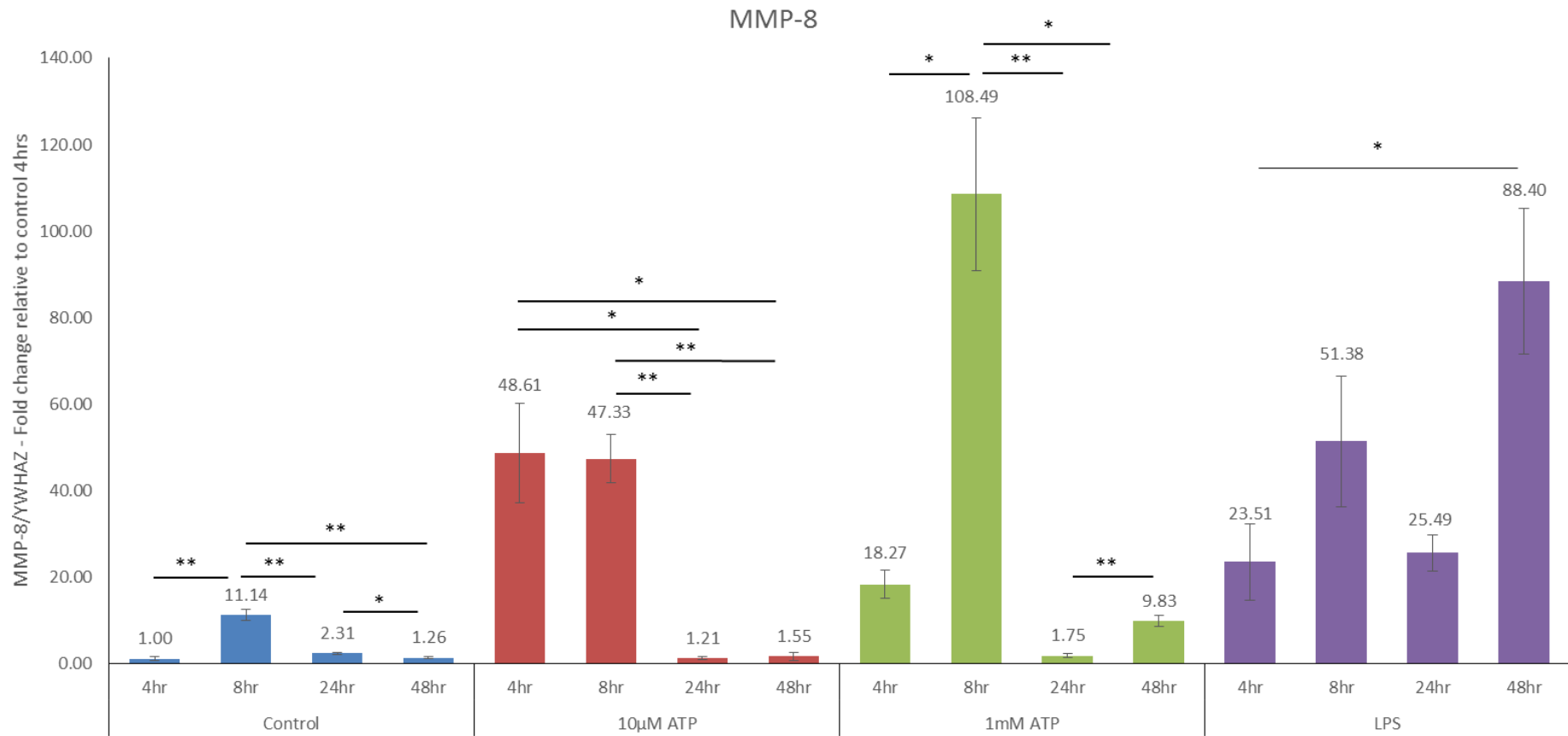


Figure 4.8. Expression of MMP-8 in response to stimuli over time. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS were explored, and MMP expression measured after 4hrs, 8hrs, 24hrs, and 48hrs. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change relevant to the unstimulated control level. Statistical significance was determined using Student's t-test, * = p<0.05.

Figures 4.9 and 4.10 demonstrate the response of MMP-10 expression in Raw 264.7 cells during the time-course. Whilst the majority of expression changes in response to stimuli appear insignificant, at 8 hours, it is possible to see a significant three-fold increase in the expression of MMP-10 after stimulation with 10 μ M ATP (but not 1mM ATP), although at other time points, expression remains relatively unchanged.

LPS leads to a trend increase in expression of MMP-10 at 24hrs and 48hrs (to 35 fold control levels at this time-point), again indicative of a late-phase response, however, neither of these changes were found to be significant.

Figure 4.10 displays MMP-10 changes in response to time per stimulant. Here, it is possible to observe a significant change between the control condition at 4 and 8 hours up two-fold, and then back down to baseline levels at 24 hours. Whilst 10 μ M ATP stimulates Raw 264.7 cells to express MMP-10 highly at 4 and 8 hours, after 24 hours there is a significant drop at 24 hours back to control baseline levels – suggesting an early phase response. A similar early phase response pattern can be seen after stimulation with 1mM ATP.

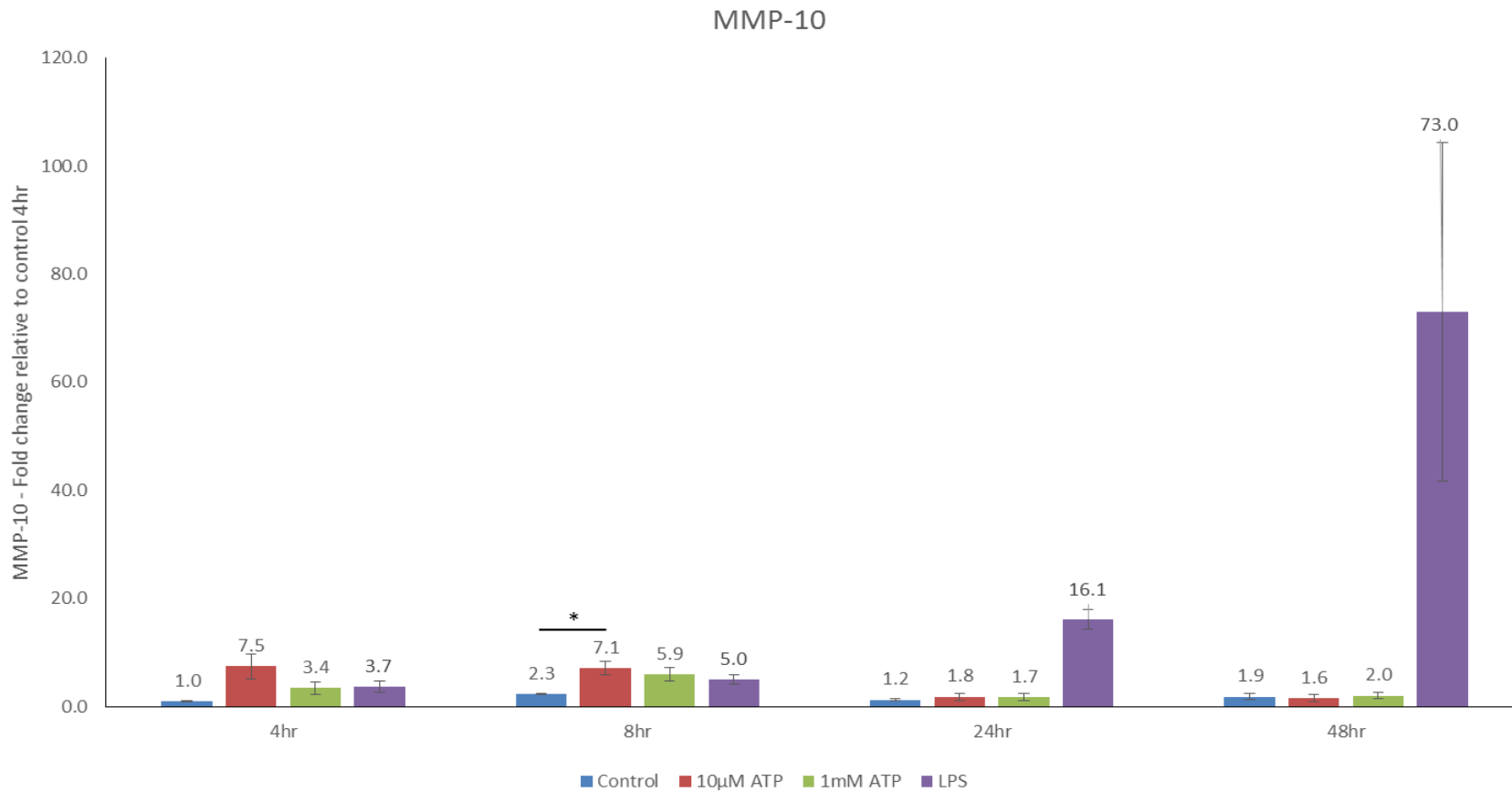


Figure 4.9. Expression of MMP-10 over 48 hours expressed in time-clusters. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS on the expression of MMP-10 were explored over 48 hours. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change when compared to the expression at 4 hours. Statistical significance was determined using Student's t-test, * = $p < 0.05$. $n = 4$.

MMP-10

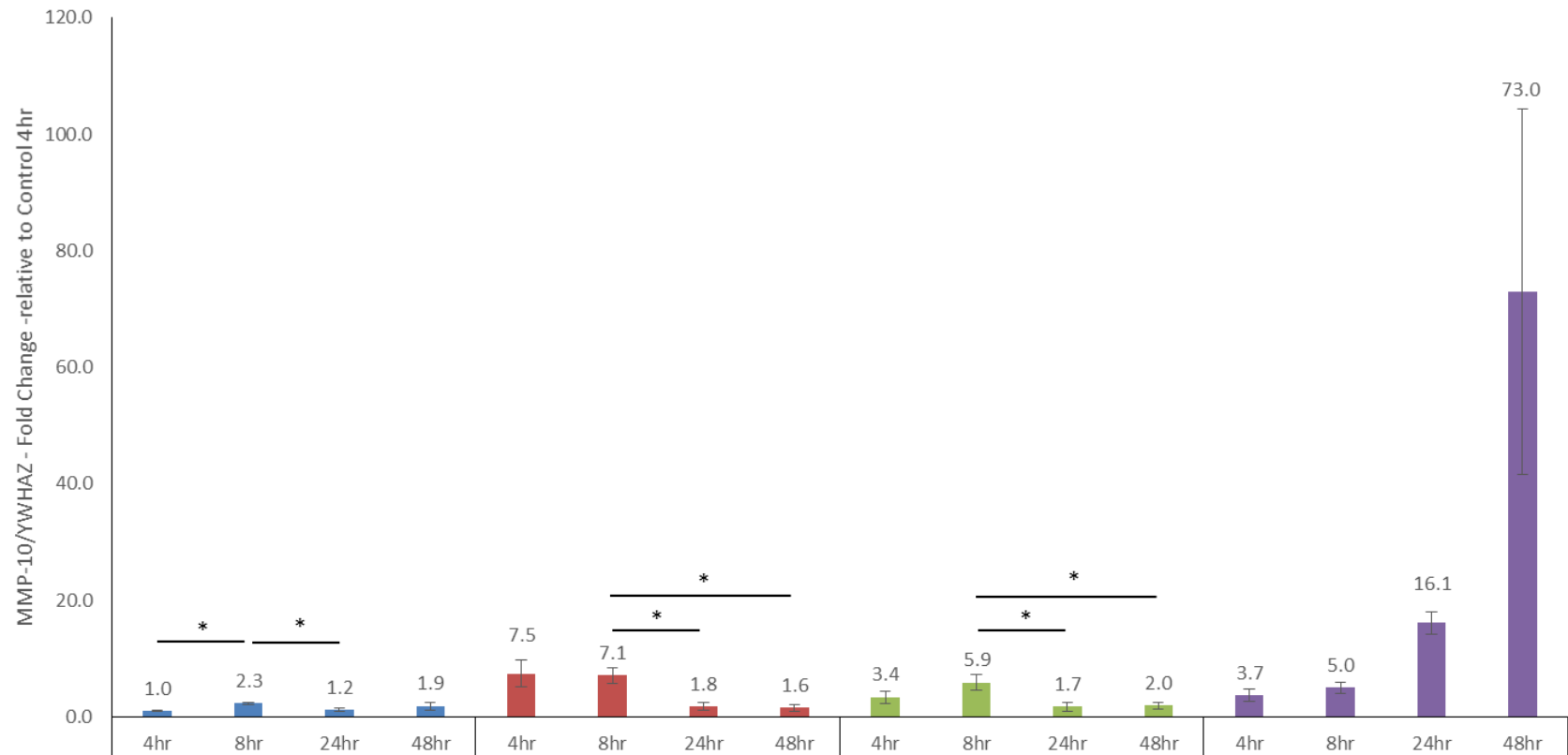


Figure 4.10. Expression of MMP-10 in response to stimuli over time. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS were explored, and MMP expression measured after 4hrs, 8hrs, 24hrs, and 48hrs. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change relevant to the unstimulated control level. Statistical significance was determined using Student's t-test, * = p<0.05.

The expression profile of MMP-12 was next explored, the results of which are shown in figures 4.11 and 4.12. Figure 4.11 demonstrates the effect of stimuli at multiple time-points, at the 4hr point, there is a significant increase from the control condition to 10 μ M ATP to 11.4-fold, and a similar increase after 1mM ATP stimulation to 6.2-fold control levels.

Throughout the time-course experiment, there is an increased expression of MMP-12 after LPS stimulation of Raw 264.7 cells, although this increase is more marked (but $p>0.05$) towards the late-stage of the time-course, at 8hrs, there is a significant increase at 8 hours when compared to the control value.

Figure 4.12 shows that there is relatively little change in MMP-12 expression levels in stimuli clusters dependent on time, although there is a slight increase after 8hrs stimulation with 1mM ATP. LPS mediated expression of MMP-12 rises dramatically towards the 48 mark, however, these results were not found to be significant.

MMP-12

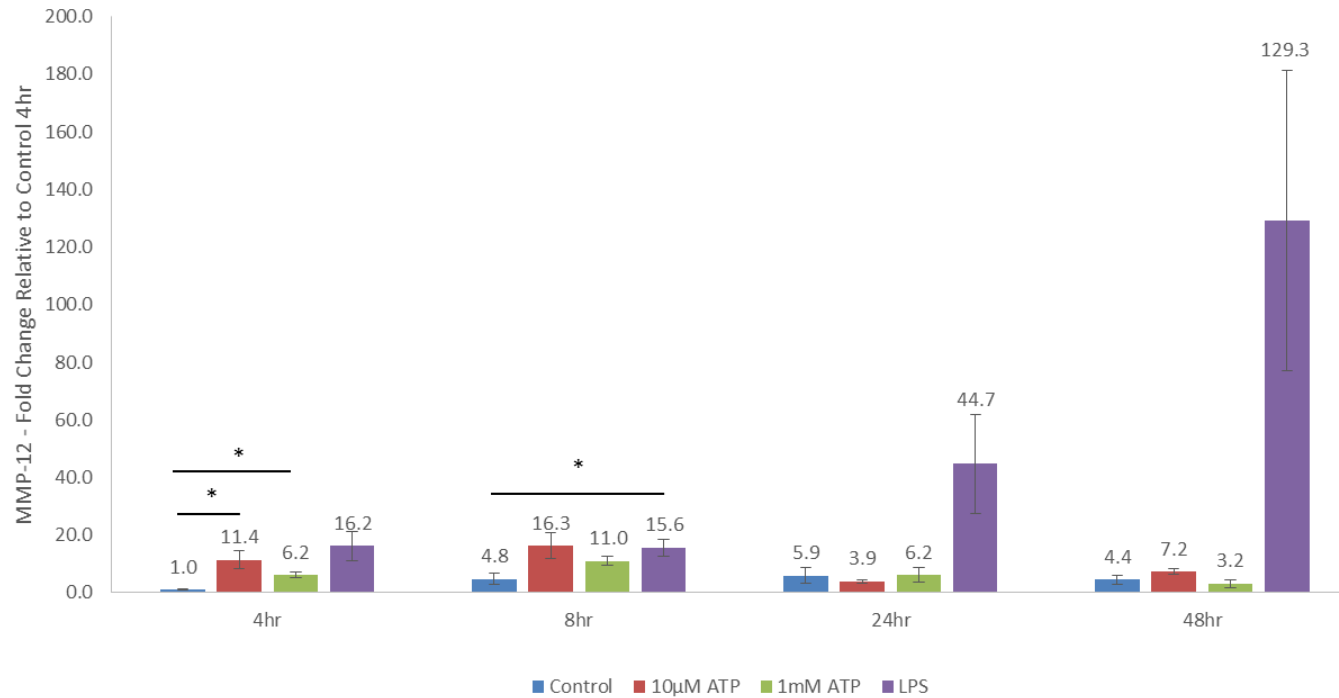


Figure 4.11. Expression of MMP-12 over 48 hours expressed in time-clusters. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS on the expression of MMP-12 were explored over 48 hours. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change when compared to the expression at 4 hours. Statistical significance was determined using Student's t-test, * = $p < 0.05$. $n = 4$.

MMP-12

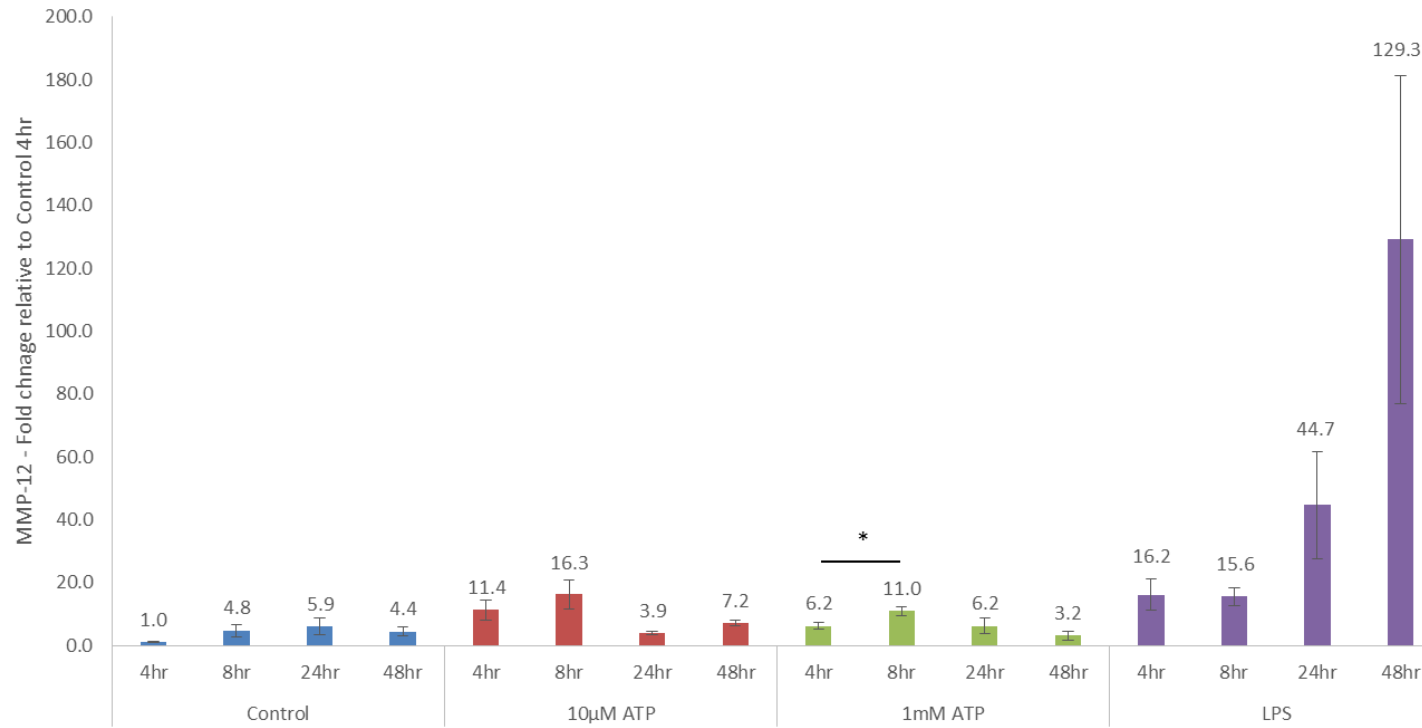


Figure 4.12. Expression of MMP-12 in response to stimuli over time. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS were explored, and MMP expression measured after 4hrs, 8hrs, 24hrs, and 48hrs. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change relevant to the unstimulated control level. Statistical significance was determined using Student's t-test, * = $p < 0.05$.

The expression of MMP-13 after stimulation in Raw 264.7 cells was next explored, the results of which are displayed in figures 4.13 and 4.14. In these figures, due to large changes in the expression of MMP-13 throughout the time-course period, the data is expressed on a logarithmic scale.

From figure 4.13, it is possible to see that at 4hrs, stimulation with ATP at either concentration tested leads to a significant increase in the expression of MMP-13. 10 μ M ATP leads to an increase in MMP-13 expression to 57.5-fold that of control levels, whereas 1mM ATP leads to a 75-fold increase in the expression of MMP-13. LPS is also shown to increase MMP-13 expression, however, this result was not found to be significant.

At 8hrs, the effect of MMP-13 expression induced by stimulation with 10 μ M ATP is lost, and falls back to control levels (however, figure 4.14 displays that at 8hrs, the control condition influences a significant increase in the expression of MMP-13 – possibly due to the stress exerted on cells by a media change). 1mM ATP is shown to retain a significant increase in the expression of MMP-13 at 8 hours. Similarly, LPS is shown to retain an increase in stimulation, but this is not significant until 24 hours – where LPS is 120-fold that of the control levels at this time-point. A further increase can be seen at 48 hours, however, error bars are large and thus the result was found to be not significant.

Figure 4.14 shows a range of changes seen over time in response to stimulation. From this graph, it is possible to see significant increases from the control at 4hrs at the 8hr time-point, which then significant falls at 24 hours. The expression profile of MMP-13 in response to 10 μ M ATP shows an early-phase response profile – whereby there is a significant down-regulation of MMP-13 expression at 24 and 48hr time-points, when compared to 4 and 8 hour time-points. This pattern can also be seen when Raw 264.7 cells are stimulated with 1mM ATP.

LPS is shown to lead to consistently high levels of expression, starting at 47-fold control levels at 4hours, there is an increase in the expression of MMP-13 in LPS stimulated cells at 24 hours, again, this suggests that LPS stimulation of Raw 264.7 cells leads to a later-stage MMP-response.

MMP-13

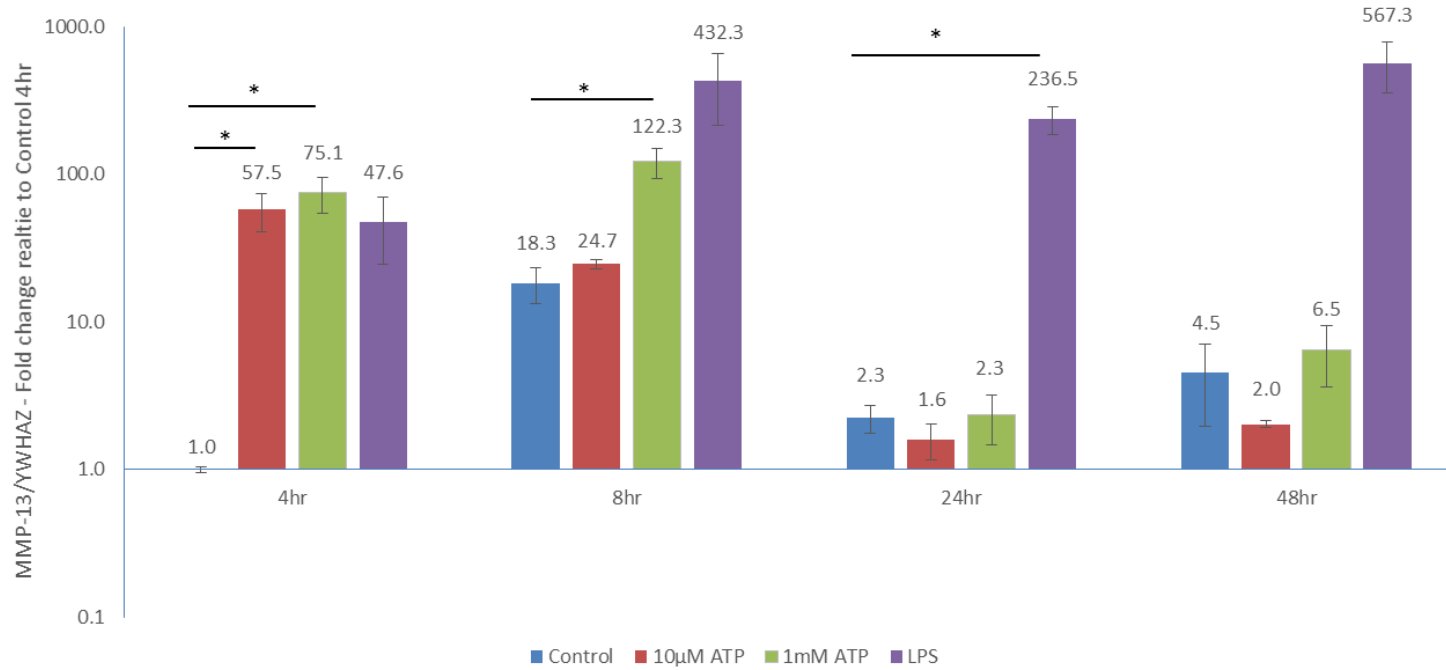


Figure 4.13. Expression of MMP-13 over 48 hours expressed in time-clusters. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS on the expression of MMP-13 were explored over 48 hours. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change when compared to the expression at 4 hours. Statistical significance was determined using Student's t-test, * = $p < 0.05$. $n = 4$.

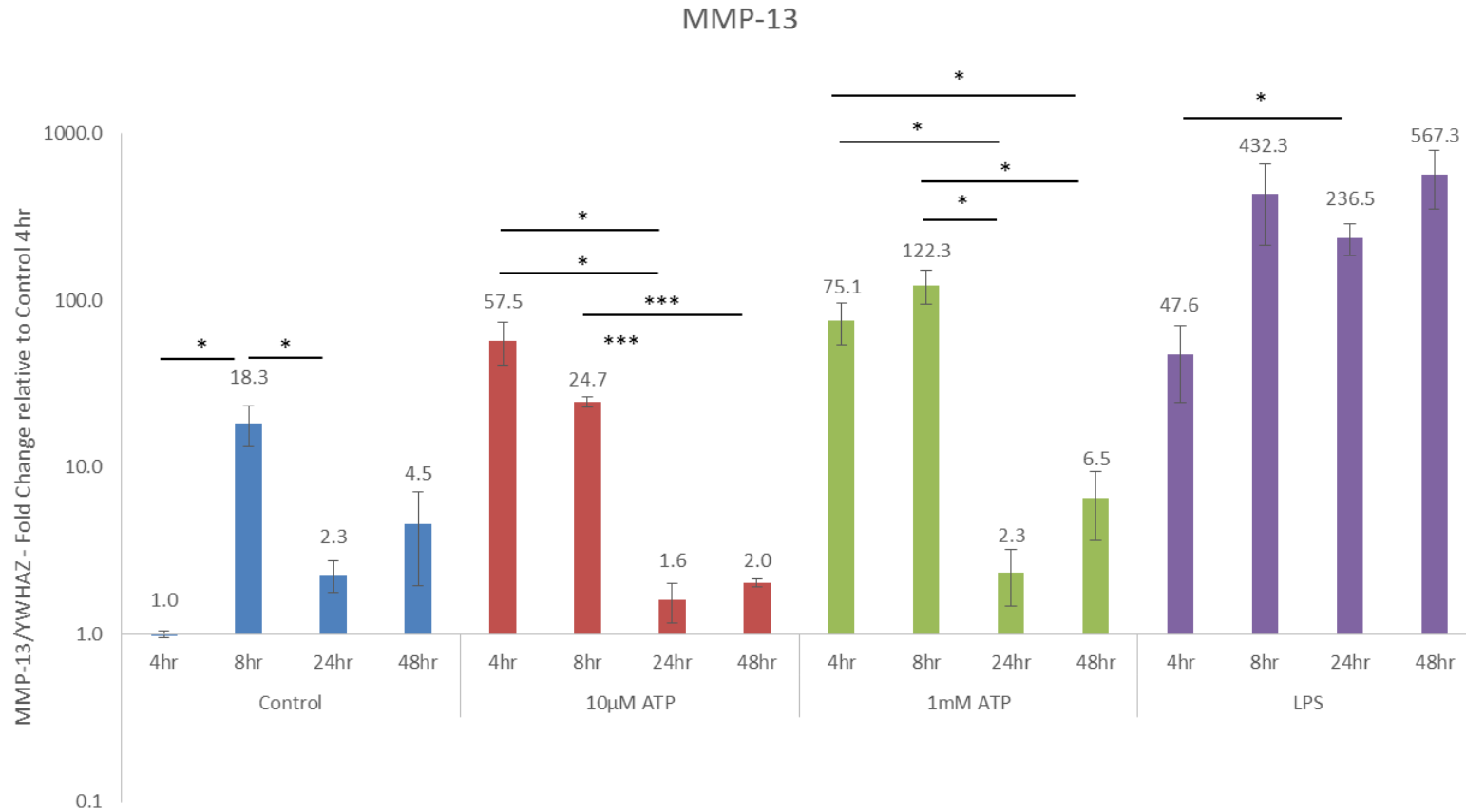


Figure 4.14. Expression of MMP-13 in response to stimuli over time. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS were explored, and MMP expression measured after 4hrs, 8hrs, 24hrs, and 48hrs. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change relevant to the unstimulated control level. Statistical significance was determined using Student's t-test, * = p<0.05, ***= p<0.001.

Next, the expression of TIMPs was explored, it was found from the previous screen that only TIMP-2 was expressed in Raw 264.7 cells. Figure 4.15 and figure 4.16 display the results of the TIMP-2 expression time-course.

Figure 4.15 demonstrates that the expression level of TIMP-2 remains largely unchanged in the early phases of the time-course, however, at 24 hours, in contrast to the result found in figure 4.1 and figure 4.2, it is possible to see a significant reduction from the control level of TIMP-2, to the level of TIMP-2 expression when cells are stimulated with 1mM ATP. Although general expression is found to rise at 48 hours, there is a notable significant decrease in the expression of TIMP-2 in 10 μ M ATP stimulated cells, and 1mM ATP stimulated cells from the control level.

Figure 4.16 demonstrates that in the control conditions, at 48hours, the expression of TIMP-2 is significantly induced 2.5 fold when compared to earlier expression levels. It is also shown that there is a rise in TIMP-2 expression after stimulation with 1mM ATP at 48 hours, when compared to the level seen at 24 hours.

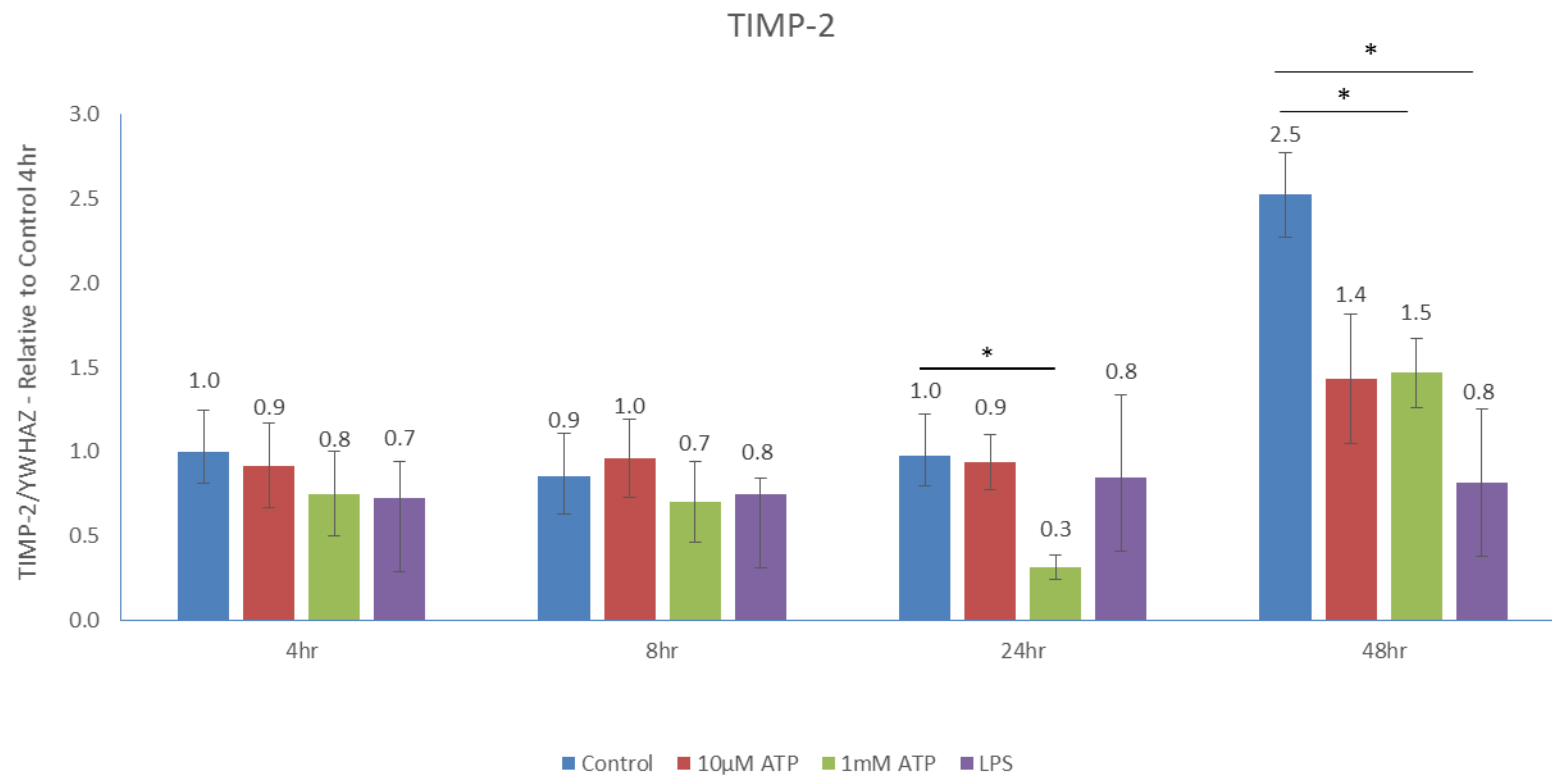


Figure 4.15. Expression of TIMP-2 over 48 hours expressed in time-clusters. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS on the expression of TIMP-2 were explored over 48 hours. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change when compared to the expression at 4 hours. Statistical significance was determined using Student's t-test, * = $p < 0.05$. $n = 4$.

TIMP-2

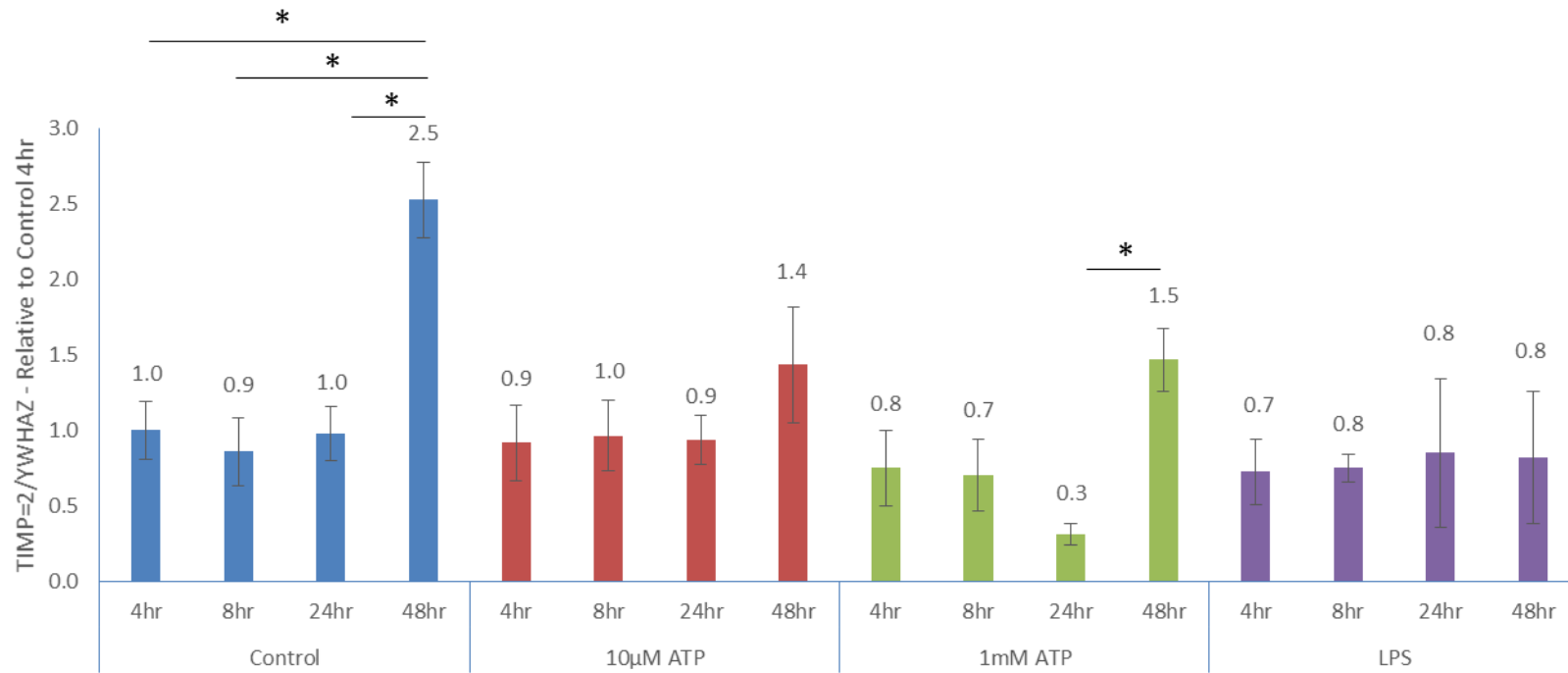


Figure 4.16. Expression of TIMP-2 in response to stimuli over time. The effects of an unstimulated control, 10µM ATP, 1mM ATP and 100ng/ml LPS were explored, and MMP expression measured after 4hrs, 8hrs, 24hrs, and 48hrs. MMP expression was normalised to the housekeeping gene YWHAZ, and is displayed as a fold change relevant to the unstimulated control level. Statistical significance was determined using Student's t-test, * = p<0.05, ***= p<0.001.

4.3.3. The Effects of BDNF on MMP Expression.

In an attempt to confirm the findings of the previous sections, and to also explore the effects of BDNF stimulation on Raw 264.7 macrophages, the expression of key MMPs MMP-8, MMP-12, MMP-13 and TIMP-2 were explored after 8 hours of treatment with 10 μ M ATP and 100ng/ml BDNF, in combination and alone in 3 separate experiments. The results of a representative experiment in each case (aside from MMP8 where amplification was only seen in one further experiment) can be seen in Figure 4.17.

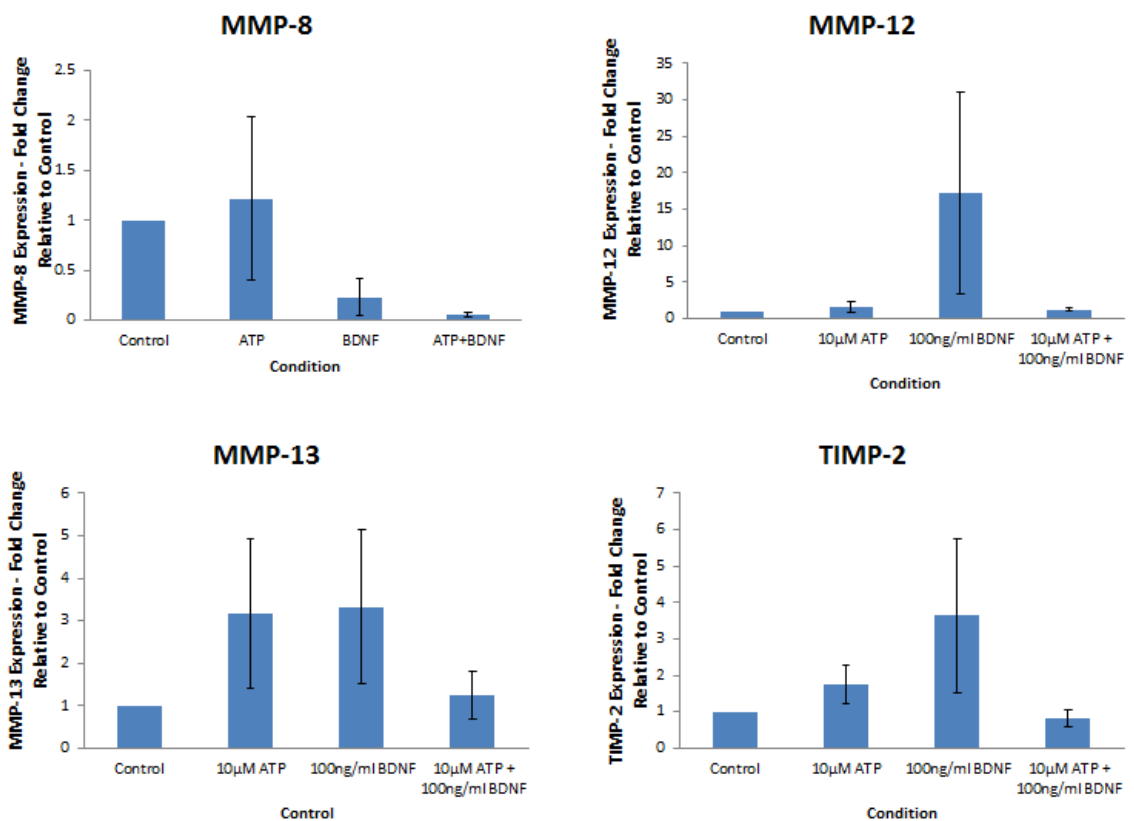


Figure 4.17 The effects of 8hr stimulation of Raw 264.7 cells with ATP and BDNF. Gene expression of the murine family of MMPs was explored using Taqman gene expression assays in the Raw 264.7 cell line in quadruplicated samples. In this figure, MMP-8, MMP-12, MMP-13 and TIMP-2 gene expression was explored. Statistical significance was measured using student's t-test, n=4.

MMP-8 expression was shown to have a decrease in expression after stimulation with BDNF, or the combination of ATP and BDNF. However, all changes were shown to be insignificant. MMP-12 expression remains unchanged after treatment with ATP, and the combination of ATP and BDNF, although with BDNF treatment there is an 18-fold increase in MMP-12 when compared to the control condition.

MMP-13 expression is shown to be upregulated 3-fold by both ATP and BDNF treatments, although this is not significant, but in combination, these stimulants appear to show no change in MMP-13 expression. TIMP-2 has a trend increase to 4-fold the levels of the control, although this was found to be insignificant.

4.4. Discussion

In this chapter, the expression levels of several MMPs in response to stimulation with ATP and LPS, including Bradykinin for the first part, were explored. A preliminary expression screen was performed to explore which MMPs were expressed in Raw 264.7 cells, and how ATP and LPS affected these levels. It was found that out of 12 expressed MMPs in Raw 264.7, only MMP-8, and -13, were regulated by stimulation with ATP and/or LPS.

The increase of MMPs upon stimulation with ATP could be responsible for the increased invasion seen in both Raw 264.7 and BMMs previously described in chapter 3. It would be ideal to measure the MMP expression of cells inside the invasion assay, however, to expose the cells to harsh treatment such as dispase could have an effect on MMP expression itself. However, measuring the MMP expression profile of cells inside the invasion assay should be considered in future studies.

We observed that the expression levels of MMP-8, MMP-13 and TIMP-2 were all significantly altered after 24 hours stimulation with 1mM ATP. MMP-8 is also known as collagenase-2, whereas MMP-13 is collagenase-3. TIMP-2 is an inhibitor of matrix metalloproteinases, but also has a wider role in the activation of other MMPs (Hernandez-Barrantes et al., 2002).

MMP-8 is known as a tumour suppressive gene which has a variety of substrates ranging from collagen to non-matrix proteins such as angiotensin and chemokines – such as interleukins 6 and 8. Laxton *et al*, 2009, showed that by knocking out MMP-8 reduced atherosclerosis in mice, and that MMP-8 promotes atherosclerosis by increasing leukocyte rolling and thus migration into the blood vessel wall (Laxton et al., 2009). Quillard *et al*, 2014 argues that MMP-8 may also be partially responsible for plaque rupture in atherosclerosis, as it serves to breakdown the wall of the atherosclerotic plaque. MMP-8 has also been shown to play a role as a negative regulator of tumour progression in breast cancer, by activating interleukins 6 and 8 (Quillard et al., 2014, Thirkettle et al., 2013).

MMP-8, along with MMP-12, is also thought to be involved in the regulation of inflammation due to its ability to cleave CXCL11 (Cox et al., 2008, Kessenbrock et al., 2010). CXCL11, also known as I-TAC, is a lymphocyte attracting chemokine, when cleaved at the

N-terminal there is an inactivation of the chemokine, but if cleaved again at the C-terminal by MMP-8, the chemokine is once more activated and is solubilised from the ECM (thus attracting lymphocytes).

Despite reports that MMP-8 can modulate leukocyte migration, there have been no previous reports that macrophage stimulation with ATP can lead to an increase in the levels of MMP-8. In this model, it is speculated that an increase in MMP-8 can lead to an increase in inflammation, and could, for example, exacerbate the inflammation after nerve injury or in atherosclerosis by increased processing of pro-inflammatory cytokines and chemokines.

MMP-13 is thought to be the main collagenase in mice, and is capable of hydrolysing many different collagen types, along with gelatin, aggrecan and Fibronectin, and MMP-13 is expressed in several tissues (Li et al., 2010, Waldman et al., 2010). MMP-13 is thought to also play a role in atherosclerosis and plaque rupture – predominating over MMP-8 as the functional collagenase in mouse atheroma (Quillard et al., 2014). It is also thought that collagenases such as MMP-8 and MMP-13 are important in facilitating nerve repair after peripheral injury as excess collagen is produced, which hinders the growth of new axons and thus limits regeneration attempts (Koopmans et al., 2009).

In this chapter, it is reported that after stimulation with ATP, there is indeed an increase in the expression of MMP-13 in Raw 264.7 macrophages. Previous studies have shown that in bovine articular osteoclasts, high doses of ATP elicited a two-fold increase in the expression of MMP-13 – a similar result to that shown in figure 4.3 (Waldman et al., 2010). It has also been shown that extracellular ATP enhances the invasion of prostate cancer cells *in vitro*, via a mechanism of activating Rho GTPase and upregulating MMP-13 (Zhang et al., 2010) - thus providing support for the hypothesis that ATP promotes invasion of macrophages through the increased expression of MMPs.

TIMP-2 is also upregulated after 24 hours stimulation with 1mM ATP, which could possibly suggest that the increased MMPs were simply being inhibited, and thus inactive in inducing invasion. However, TIMP-2 can also indirectly lead to an enhancement of pro-MMP-2 activation by presentation to MT1-MMP (MMP-14) facilitating processing of pro-MMP2

to its active form, thus supporting pericellular proteolysis and in fact, enhancing invasion (Hernandez-Barrantes et al., 2002, Parks et al., 2004).

The expression levels of these MMPs over time were also explored. These results do not strictly agree with the results from the previous screen of MMP expression (figures 4.1-4.4), in that neither MMP-14 and MMP-28 are undetected in the time-course experiment, and that the expression of MMP-8 and MMP-13 is no longer significantly upregulated at 24 hours when stimulated with 1mM ATP – although a significant increase is seen at an earlier time-point. Nonetheless, it is important to note that MMP-8 and MMP-13 are indeed expressed very highly in relation to the control conditions at earlier time-points, and thus be worthy of further investigation in ATP-induced invasion of macrophages.

In the time-course experiment performed, it was found that there are significant changes within the control condition over time. As the control condition represents a media change at the specific time-point, this can result in cellular stress which can include a release of intracellular ATP at small levels. Any changes in the control conditions may be due to the effects of the cellular stress of a media change. During the course of investigation, the measurement of ATP release was attempted using a bioluminescence assay for quantitative determination of ATP based on the mechanism of luciferase ATP requirement to produce light. However, these attempts were unsuccessful (data not shown).

It should also be noted that there are no significant expression level changes to TIMP-2 found in this time-course experiment.

MMP-10 is shown to be elevated when compared to the control at 8 hours when stimulated with 10 μ M ATP. MMP-10 is also known as stromelysin-2, and has previously been found to be essential for the migration and invasion of macrophages (Murray et al., 2013), and is also thought to contribute to monocyte migration and blood brain barrier disruption in cerebral adrenoleukodystrophy (Thibert et al., 2012).

MMP-12 (macrophage metalloelastase) expression levels were shown to be expressed at a significantly higher level than that seen in the control conditions after 4 hour stimulation with both high and low doses of ATP. MMP-12 has previously been shown to be essential for macrophage infiltration, with mutations leading to a lack of macrophage migration

(Haq et al., 2011). Taken together, this result provides further evidence that MMPs may be involved in ATP-induced invasion of macrophages.

ATP stimulation has previously been found to influence the activity of MMPs, for example, it has been found that the stimulation of BV2 microglial cells leads to an increase in the level of urokinase-type plasminogen activator (uPA), which is known to influence MMP activation. ATP induced uPA increase was found, in this system to lead to the upregulation of MMP-9 activity (Shin et al., 2010). ATP has previously been found to induce the migration of glial cells via activation of P2X7 receptor. In a study by Murphy and Lynch, the upregulation of MMP-9 produced in microglia by ATP was shown to be accompanied by a decrease in TIMP-1. It was found that ATP also induced the activation of Cathepsin B, which in turn degrades TIMP-1, thus inducing glial cell migration by an increase in MMP activation and a decrease in TIMP-1 activity (Murphy and Lynch., 2012).

The effects of BDNF on MMP expression observed were mixed with a trend towards reduced MMP8 expression (where BDNF was added with ATP) but enhanced MMP12 expression (where BDNF was added alone).

In conclusion, MMP expression is apparently regulated in Raw 264.7 macrophages by exogenous ATP and some of the trends observed may correlate with enhanced invasion. BDNF may suppress certain MMP expression but seems to be context-dependent. It would also be of interest to determine if the macrophage migration seen is MMP-dependent using broad spectrum MMP inhibitors such as GM-6001, and other specific MMP inhibitors – such as blocking antibodies to specific MMPs, before moving onto knockdown experiments using siRNAs. Overall further study is required since there were inconsistencies between experiments.

Chapter 5: An exploration into the effects of Apolipoprotein E subtype and a Fish Oil supplemented diet on primary macrophage migration.

5.1. Introduction

5.1.1. ApoE in Atherosclerosis.

Atherosclerosis is the major underlying cause of heart attacks and strokes, and thus is responsible for over 50% of all deaths in the western world. Although atherosclerosis is a multifactorial disease, it can be considered an inflammatory disease, caused by injury to the endothelial lining of the artery wall – thus leading to changes in the permeability in the arterial wall, increased expression of cell surface adhesion molecules and production of cytokines (Kleemann et al., 2008, Guns et al., 2010, Koupnova et al., 2012, Li et al., 2014b). The progression of atherosclerosis is fully outlined in chapter 1.

ApoE is thought to be potently anti-atherogenic, and it has been found that in humans and other animals that decreased expression of ApoE is associated with a pro-atherogenic lipoprotein profile and atherosclerotic disease (Mahley and Rall, 2000, Greenow et al., 2005, Curtiss, 2000). As mentioned earlier, ApoE knockout mice are prone to atherosclerosis and extremely hypercholesterolaemic compared to wild type mice – the hypercholesterolaemia is due to impaired clearance of lower density lipoproteins, with complex atherosclerotic lesions forming even if the ApoE null mice are fed a low fat diet. In ApoE knockout mice, atherosclerosis regresses when ApoE is restored (van den Elzen et al., 2005, Greenow et al., 2005, Brown et al., 2008).

Although ApoE is usually absent in normal vessels, its expression is high in atherosclerotic plaques (Greenow et al., 2005, Cudaback et al., 2011), where it is believed to be synthesized locally by resident macrophages. The anti-atherogenic role of ApoE is multifaceted, with ApoE playing a role in several processes, including (but not limited to): reverse cholesterol transport and hepatic uptake of lipoprotein particles, inhibition of LDL oxidation, anti-inflammatory activity, and the inhibition of T-lymphocyte activation and proliferation, platelet aggregation, and both smooth muscle cell and endothelial cell proliferation. ApoE has a key role in the regulation of overall lipoprotein metabolism and transport and thus is an important protein in anti-atherogenic function (Mahley et al., 2009, Mahley and Rall, 2000, Quillard et al., 2014).

The Framingham Heart Study (2004) showed that there is a connection between the ApoE genotype and the onset of carotid atherosclerosis, which was defined as intimal-medial

thickness (IMT). Whilst it was found that the ApoE2 homozygous genotype showed the lowest thickness, whereas ApoE3 displayed a slightly thicker IMT. Individuals with the ApoE4 genotype displayed the largest IMT, suggesting that ApoE4 is related to a higher risk of atherosclerosis, with ApoE2 having the lowest risk (Elosua et al., 2004).

Mahley et al, 2009 suggests that the structural changes in ApoE isoforms are due to the polymorphisms in the alleles are the cause of their altered function, thus causing differences between how the N- and C-terminals interact. The C-terminal domain of ApoE is predicted to form amphipathic α -helices that are responsible for lipid binding. ApoE2 and ApoE3 preferentially bind small, phospholipid rich HDL, whereas ApoE4 binds preferentially to large triglyceride rich VLDL (Mahley et al., 2009).

X-ray crystallography reveals differences in ApoE isoforms in the orientation of side chains and alterations in the rearrangements of salt bridges. In ApoE4, Arg-112 and Glu-109 form a salt bridge – this causes the Arg-61 side chain to extend away from the helix bundle, whereas in ApoE3, the side chain is buried. The orientation of Arg-61 in ApoE4 encourages domain interaction with Glu-255 in the lipid binding region, thus causing ApoE4 to have a more compact conformation than ApoE3. Domain interaction is a key structural property in all proteins, in ApoE4, this domain interaction could be important in many of its pathogenic effects (Mahley et al., 2009, Mahley and Rall, 2000).

5.1.3. Fish Oils.

There have been many epidemiological and observational studies on the effects of omega-3 polyunsaturated fatty acids (PUFAs) on cardiovascular disease outcomes. Most evidence of the benefits of omega-3 PUFAs were obtained from eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA). Humans get omega-3 PUFAs from fish oils, although the original source of omega-3 is marine microorganisms (Monk et al., 2013).

PUFAs are thought to be beneficial because they are known to reduce hepatic synthesis of triglycerides and increase hepatic fatty acid oxidation. Although total cholesterol levels are unaffected by the consumption of PUFAs, the concentrations change – LDL cholesterol concentrations tend to rise by 5-10%, and HDL cholesterol levels rise by 1-3%, Serum

triglycerol concentrations are found to decrease by 25-30% (Marai and Massalha, 2014, Bargut et al., 2014).

Low levels of cardiovascular disease in populations with high fish intake – such as those seen in Alaskan natives, Japanese, and Greenland Eskimos suggests that there may be a link between the consumption of fish and protection against cardiovascular disease (von Schacky et al., 1985). A large body of epidemiologic data from randomised control trails suggests that EPA and DHA relay a cardioprotective action. Thus, there is a case to propose that the intake of fish oils such as EPA and DHA are indeed beneficial to those at risk from cardiovascular disease (Calder, 2006, Grimble, 2007).

The current American Heart Association dietary guidelines recommend combined EPA and DHA at a dose of 1000mg/day, which equates around 2 oily fish meals per week, for the secondary prevention of coronary heart disease (Marai and Massalha, 2014). It is also thought that a high intake of fish oil may lower blood pressure in hypertensive patients, and be beneficial to patients with inflammatory diseases such as rheumatoid arthritis (Calder, 2001).

5.1.4. MMPs.

As explored in Chapter 1, MMPs are of particular interest in the disruption of the arterial wall and the processes which lead up to the rupture of the plaque, especially those belonging to the collagenase family. It is suggested that the mechanism behind the increased expression of MMPs is driven by oxidative stress, although it is also inferred that thrombin and the plasminogen cascade may play a role in the activating of MMPs, the former in a positive feedback loop (Galis and Khatri, 2002).

Recently, it has been found that MMP-12 overexpression leads to accelerated plaque development, whereas in MMP-12^{-/-} mice, plaque development is greatly reduced, and selective MMP-12 inhibitors have been shown to promote a stable plaque (Johnson et al., 2011, Scholtes et al., 2012). Whilst there are many studies which link the expression and activation of MMPs in atherosclerosis, and the promotion of either a stable or unstable plaque phenotype, exploration of dietary aspects remains limited.

5.2. Hypothesis and Aims.

Hypothesis: dietary n3-fatty acids can have effects on macrophage migration and MMP-expression which are revealed in *ex vivo* analysis.

Aims:

To determine the effect of the ApoE subtype, as well as the effect of the intake of fish oils on macrophage migration, in response to ATP, MCP-1 and Interleukin-10 (IL-10) in these cells. It is also an aim to determine the expression of certain MMPs in these primary macrophages from diet-fed mice and to explore how the treatments described previously can modulate MMP regulation.

5.3. Results

5.3.1. The Effect of ApoE subtype and a fish oil-supplemented high fat diet on macrophage migration.

The effect of ApoE subtype, diet and stimulation on the random migration of primary murine macrophages was explored. Primary murine macrophages were isolated from adult mice with a homozygous humanized ApoE3 or ApoE4, which were fed on either a High Fat Diet (HFD), or a High Fat Diet supplemented with Fish Oils EPA and DHA (HFD+FO). Bone marrow-derived macrophage precursors were differentiated *in vitro* in accordance to the protocol outlined in Chapter 2.1.2 and purity was determined by staining with F4/80, as outlined in Chapter 2.11.

After differentiation, the macrophages were cultured in 2D on plastic for 3 hours before addition of the tested stimulants and subsequent time-lapse microscopy was performed.

The average speed of macrophages from mice with the ApoE3 genotype and a HFD, the speed remained similar regardless of stimulation with MCP-1, ATP and MCP-1 + IL-10. It is interesting here to note that the macrophages taken from mice fed on a diet supplemented with fish oils travel around significantly faster (1.8-fold) than their counterparts fed on HFD alone. Again, macrophages from ApoE3 mice fed on a HFD+FO diet, macrophages migrated at a similar speed regardless of stimulation – although at around 42 $\mu\text{m/hr}$ – with the exception of macrophages stimulated with MCP-1, which migrated more slowly than untreated cells (not significant).

The migration speed of ApoE4-mouse derived macrophages was similar to that seen in the ApoE3 cells. Migration speeds for macrophages from mice in the ApoE4 + HFD diet range from 20 $\mu\text{m/hr}$ to 25 $\mu\text{m/hr}$, with no significant differences between the different stimulus conditions.

Fish Oil dietary supplementation led to a significant increase in macrophage migration speed when compared to ApoE4 + HFD diet cells. As in ApoE3 mouse derived cells, the effect of the fish oil diet was to increase macrophage migration speed by 2-fold in all conditions. In the MCP-1 condition, MCP-1 treatment was the only condition which also

demonstrated a difference between ApoE subtype – with the ApoE4 subtype + fish oil supplementation leading to a 1.3 fold increase compared to the condition for that of ApoE3 + Fish oil.

Since no effect was seen in random migration experiments, some preliminary data was obtained using chemotaxis assays, which shows that IL-10 was able to abrogate MCP-1 induced chemotaxis (See appendix). Unfortunately many cells died in the Ibidi chambers and thus further analysis was not possible.

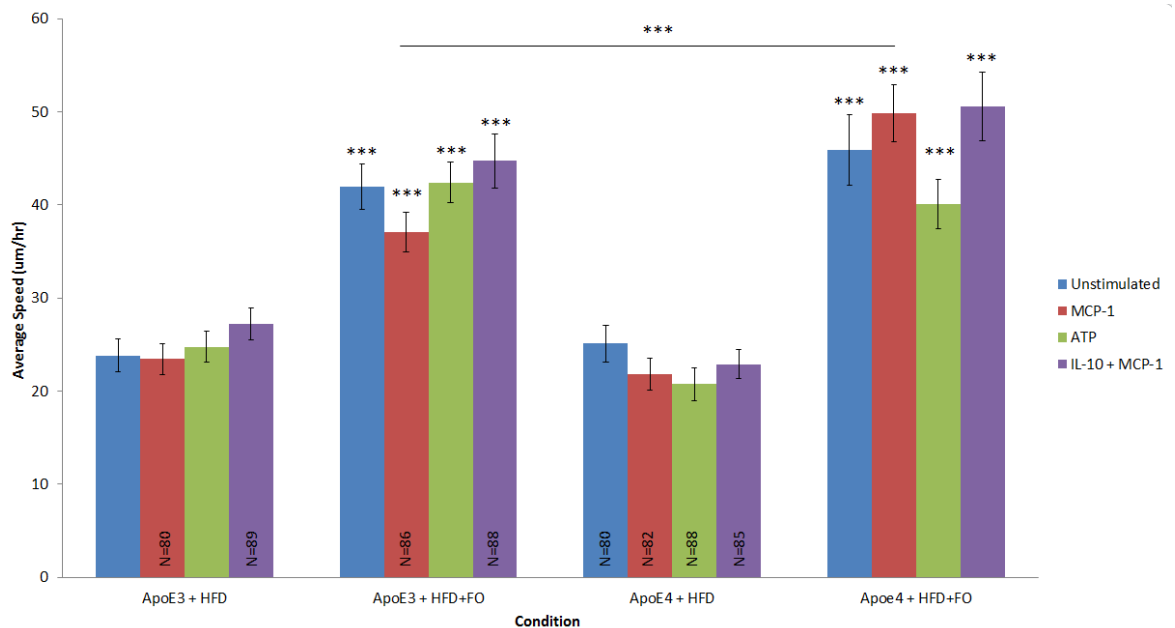


Figure 5.1. A graph to show the average migration speed ($\mu\text{m/hr}$) of primary murine macrophages derived from mice with a humanised ApoE3 or ApoE4 knock-in, fed on either a HFD or a HFD+FO diet (standard high fat diet supplemented with EPA and DHA fish oils). Macrophages were subjected to control conditions (blue bar), stimulation with 100ng/ml MCP-1 (red bar), stimulation with 10 μM ATP (green bar) and a combination of 100ng/ml Interleukin-10 and 100ng/ml MCP-1 (purple bar). This graph is the combination of the results from three separate experiments (with 3 mice in each), in which 10 individual cells from three separate wells were tracked, thus giving an n=90 cells in all conditions unless otherwise stated in the individual bars. Bars shown are mean \pm 1 S.E.M * = $p < 0.001$ when compared to the results from the control diet value from the same stimulation.**

5.3.2. The Effect of ApoE subtype and a fish oil-supplemented high fat diet on macrophage production of MMPs.

MMP expression of the isolated primary macrophages was measured and is reported in the following section. Figure 5.2 demonstrates the expression of MMP-13 in the primary macrophages explored in this chapter, after challenging with the stimulants MCP-1, ATP, IL-10 and a combination of MCP-1 and IL-10. There was a slight increase in the expression of MMP-13 in both ATP and IL-10 stimulated cells, although these are not significant (Figure 5.2.A). When the combination of MCP-1 and IL-10 is added to the ApoE3 HFD cells, there is a decrease in the expression of MMP-13 which is significantly less than the level when compared to stimulation of cells with just MCP-1.

The effects of stimulation on the expression of MMP-13 in ApoE4 HFD cells are demonstrated in Figure 5.2.B, no difference in the expression of MMP-13 after 8 hours stimulation was observed with MCP-1, ATP, IL-10 or the combination of MCP-1 and IL-10. In cells from ApoE3 mice fed on a HFD+FO diet, expression does increase to 1.5-fold when the cells are stimulated with ATP, IL-10 and the combination of MCP-1 and IL-10, but this does not reach significance. In figure 5.2.D, the expression of MMP-13 remains at the level of the control with all tested stimulants.

Reanalysis of the data in terms of diet for ApoE3 cells (Figure 5.3.A) revealed significant changes in the expression of MMP-13 in untreated, ATP- and MCP-1 treated cells, In ApoE4 cells (Figure 5.3.B), FO diet resulted in an apparent 5-fold suppression of MMP-13 expression in cells stimulated with MCP-1, ATP or IL-10.

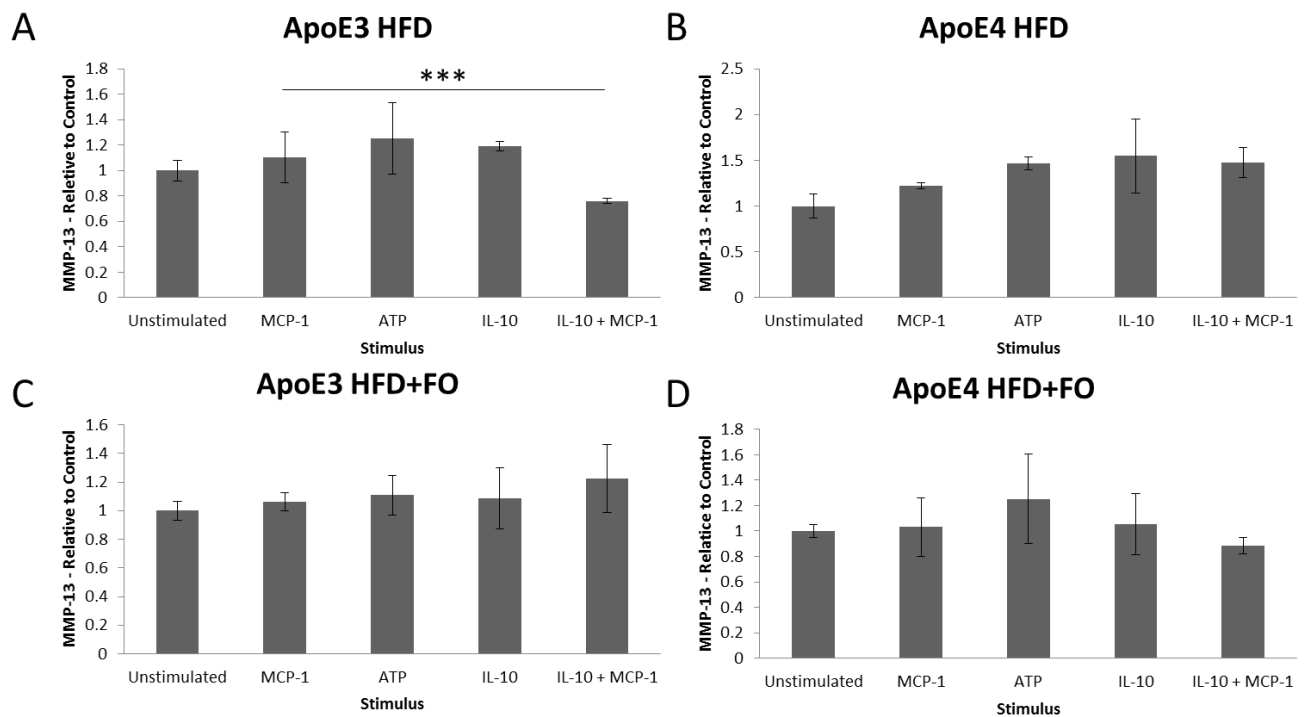


Figure 5.2. Expression of MMP-13 in primary murine macrophages. Graphs show expression of MMP-13 in either (A) Primary macrophages derived from mice with a humanised ApoE3 knock-in fed on a high fat diet (B) Primary macrophages derived from mice with a humanised ApoE4 knock-in fed on a high fat diet (C) Primary macrophages derived from mice with a humanised ApoE3 knock-in fed on a high fat diet supplemented with EPA and DHA, or (D) Primary macrophages derived from mice with a humanised ApoE4 knock-in fed on a high fat diet supplemented with EPA and DHA. MMP-13 expression was measured using Taqman on RNA derived using a cells to C_T© kit and analysed using the $2^{\Delta\Delta CT}$ method, results are normalised to 18s and displayed as a fold change relative to the control condition, these graphs represent the result of one experiment. n=4. Bars shown are mean \pm 1 S.E.M *** = p<0.001.

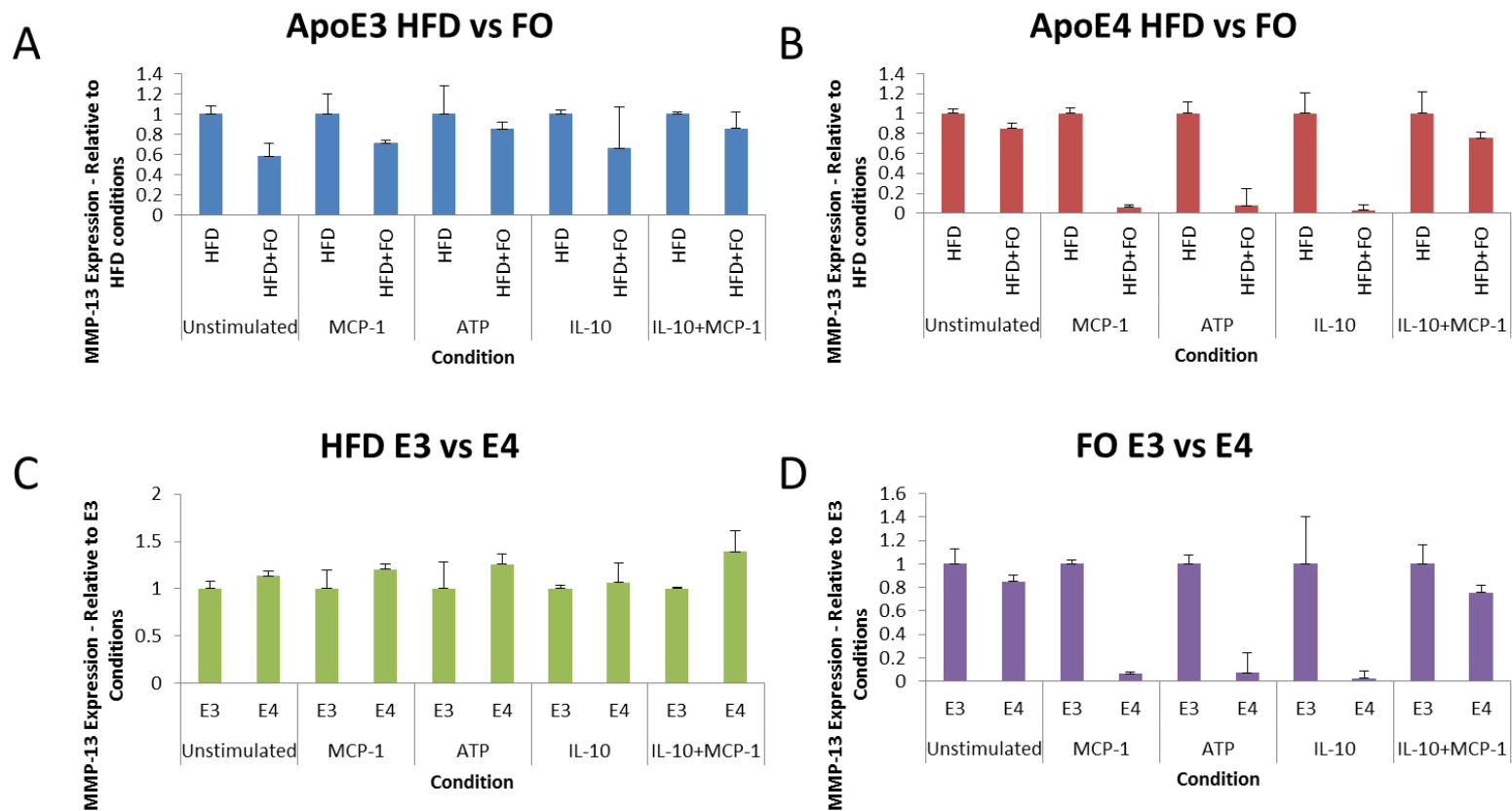


Figure 5.3. MMP-13 expression in comparison to either diet or ApoE subtype. (A) shows the comparison between MMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE3 knock in macrophages (B) shows the comparison between MMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE4 knock in macrophages. (C) shows the comparison between MMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet, whereas (D) shows the comparison between MMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet supplemented with fish oils. MMP-13 expression was measured using Taqman on RNA derived using a cells to C_T kit and analysed using the $2^{\Delta\Delta CT}$ method, results are normalised to 18s and displayed as a fold change relative to the high fat diet result in A and B, or to the E3 result in C and D, these graphs represent the result of one experiment. n=4 unless otherwise stated, bars shown are mean \pm 1 S.E.M.

Comparison of MMP-13 expression by ApoE subtype in the HFD fed mice revealed that there were no significant differences between the ApoE3 macrophages and the ApoE4 macrophages derived from mice fed on the HFD diet (Figure 5.3.C). The response of ApoE4 macrophages to ATP, MCP-1 or IL-10 were apparently more sensitive to the FO diet than ApoE3 cells (Figure 5.3.D). This contrasts with the observations on macrophages from mice on HFD alone (see above).

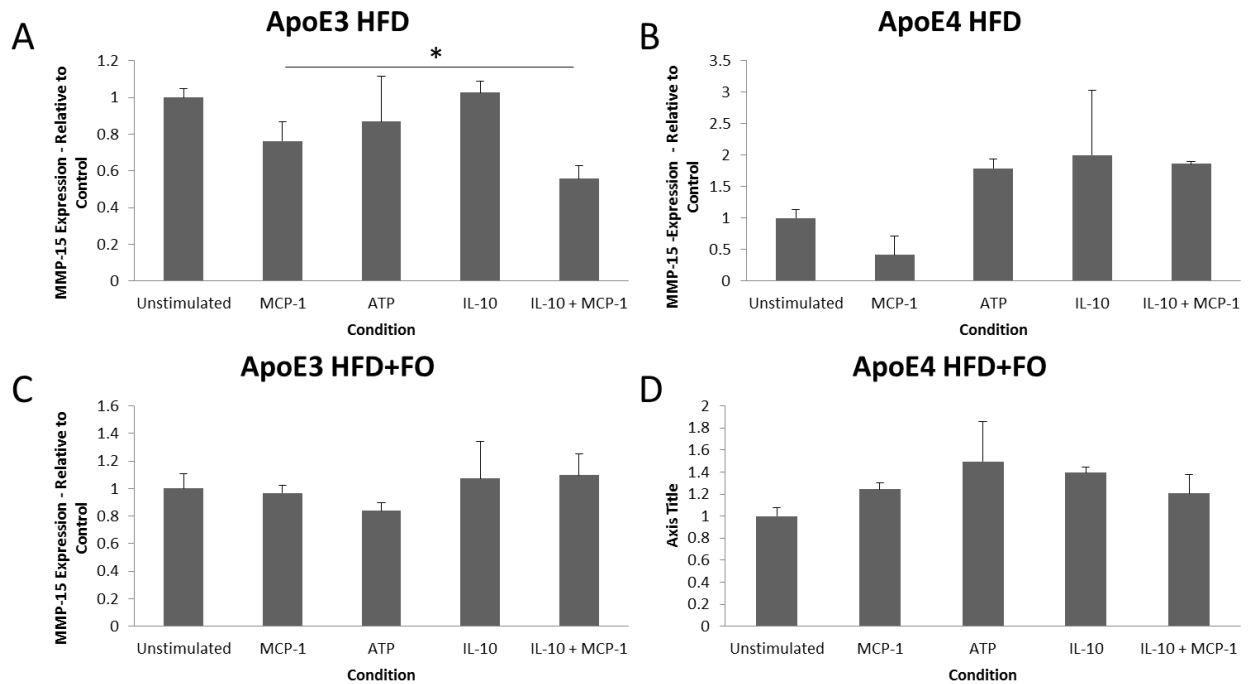


Figure 5.4. Expression of MMP-15 in primary murine macrophages. Graphs show expression of MMP-15 in either (A) Primary macrophages derived from mice with a humanised ApoE3 knock-in fed on a high fat diet (B) Primary macrophages derived from mice with a humanised ApoE4 knock-in fed on a high fat diet (C) Primary macrophages derived from mice with a humanised ApoE3 knock-in fed on a high fat diet supplemented with EPA and DHA, or (D) Primary macrophages derived from mice with a humanised ApoE4 knock-in fed on a high fat diet supplemented with EPA and DHA. MMP-15 expression was measured using Taqman on RNA derived using a cells to C_T kit and analysed using the $2^{-\Delta\Delta CT}$ method, results are normalised to the value for 18s and displayed as a fold change relative to the control condition, these graphs represent the result of one experiment. n=4 unless otherwise stated. Bars shown are mean \pm 1 S.E.M * = p<0.05.

Figure 5.4 shows the expression of MMP-15 in response to stimulation from all four sets of cells. MMP-15 is also known as MT2-MMP, and is responsible for cleavage of proteoglycans, it was chosen to be further investigated due to a moderately high expression seen in experiments performed in Chapter 4. Figure 5.4 A shows the response from the ApoE3 HFD macrophages. MMP-15 expression appears unchanged by MCP-1 or IL-10 treatment, but, in the combination of IL-10 + MCP-1, there is a 2-fold significant decrease when compared to MCP-1 treatment alone. Expression of MMP-15 in ApoE4 cells from mice fed on a HFD diet is largely unchanged in response to all treatments (Figure 5.4.B) thus indicating a potentially altered response of ApoE4 cells to MCP1+ IL10..

The expression of MMP-15 in ApoE3 cells derived from mice fed on a HFD + FO diet is shown in Figure 5.4.C. In this figure, although observations are not significant it is possible to see that after stimulation with MCP-1, MMP-15 expression drops to around 0.4 fold the control value, whereas in ATP, IL-10 and IL-10 + MCP-1, the expression of MMP-15 rises to around 1.8 fold the level of the control conditions.

In ApoE4 cells from mice fed on a HFD+FO, there appears to be a 1.5-fold increase in MMP-15 when cells were stimulated with MCP-1 and ATP. Stimulation with IL-10 and a combination of IL-10 + MCP-1 also results in a slight increase in expression of MMP-15 (1.5 fold and 1.3 fold control levels respectively), however, no changes were significant.

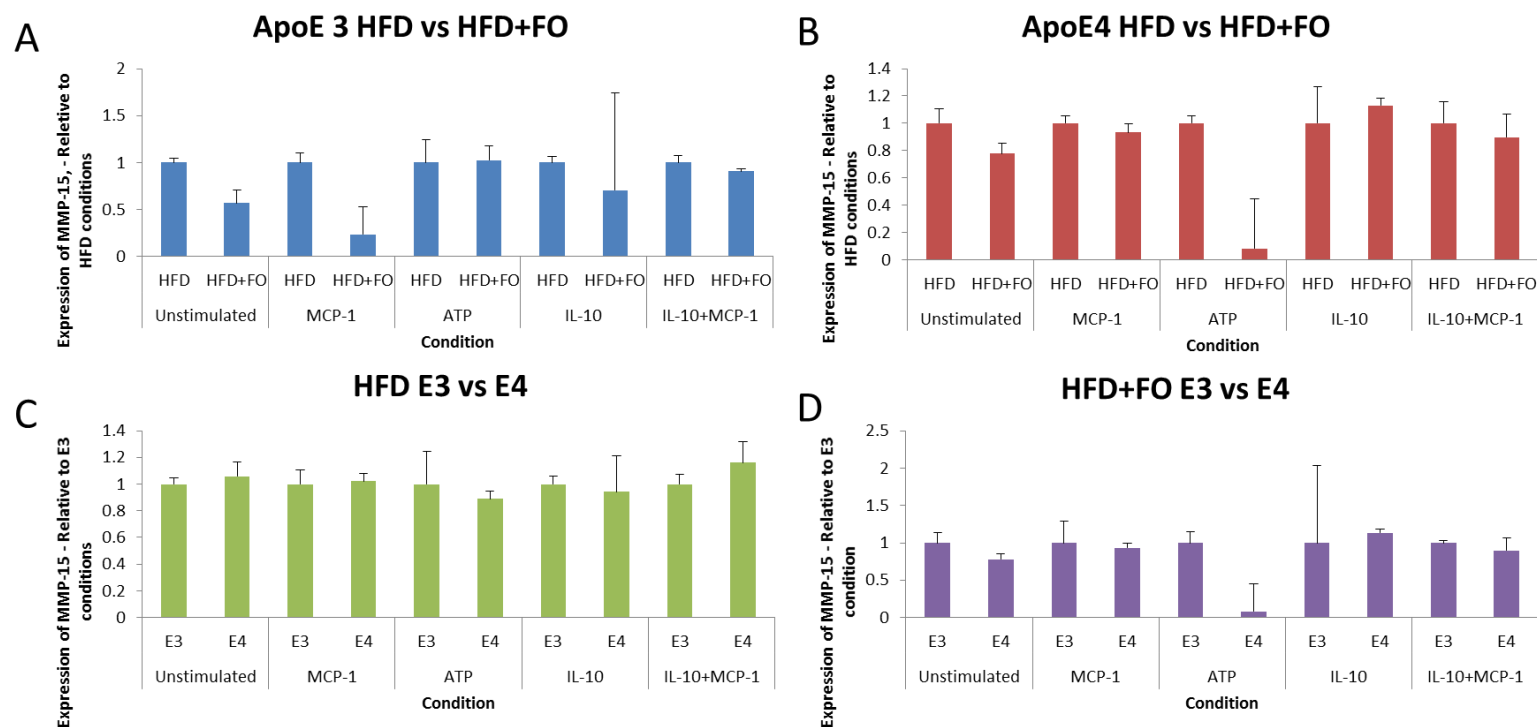


Figure 5.5. Expression of MMP-15 in comparison to either diet or ApoE subtype. (A) shows the comparison between MMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE3 knock in macrophages (B) shows the comparison between MMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE4 knock in macrophages. (C) shows the comparison between MMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet, whereas (D) shows the comparison between MMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet supplemented with fish oils. MMP-15 expression was measured using Taqman on RNA derived using a cells to C_T kit and analysed using the 2^{ΔΔCT} method, results are normalised to 18s and displayed as a fold change relative to the high fat diet result in A and B, or to the E3 result in C and D, these graphs represent the result of one experiment. n=4 unless otherwise stated, bars shown are mean ± 1 S.E.M

In figure 5.5.A it is observed that in control conditions, the HFD + FO fed mice lead to macrophages with a 2-fold reduction in MMP-15 expression than in those cells that were derived from mice on the HFD diet, a similar story is seen in MCP-1 stimulated cells, with the HFD + FO cells expressing less than a fifth MMP-15 than seen in the HFD cells. In ATP stimulated cells however, there appears to be no difference. In IL-10 and IL-10 stimulated cells, MMP-15 is only slightly decreased in the HFD+FO condition (0.8 fold and 0.95 fold respectively).

In Figure 5.5.B, we see the effect of the diet in ApoE4 cells, here it appears that there is no change when cells are stimulated with MCP-1. However, when stimulated with ATP, MMP-15 expression is decreased by 10 times in HFD+FO cells, this decrease was not found to be significant, a trend change was seen. There are negligible changes to MMP-15 expression in both IL-10 and IL-10 + MCP-1 conditions.

Next, the effect of ApoE subtypes was explored. In figure 5.5.C, the difference in HFD cells is explored; however, there are minimal changes dependent on the ApoE subtype.

Figure 5.5.D shows the difference in HFD+FO cells between the ApoE3 variant and ApoE4 variant cells. There is a similar pattern to that of Figure 5.5.B, in which there is a small decrease of MMP-15 expression in the control conditions between E4 cells and E3 cells, and almost no difference in the MCP-1 stimulated cells. However, in ATP there is a large decrease of MMP-15 in E4 cells when compared to E3 cells. There are small insignificant changes in the MMP-15 expression levels between E3 and E4 cells in the IL-10 and IL-10 + MCP-1 conditions.

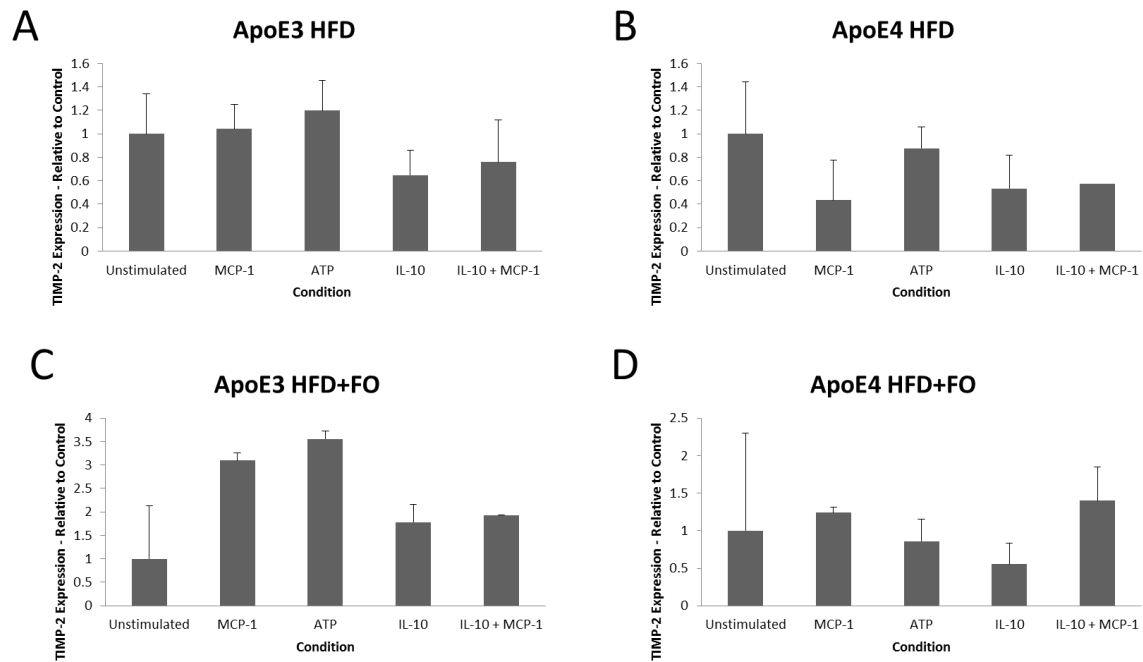


Figure 5.6. Expression of TIMP-2 in primary murine macrophages. Graphs show expression of TIMP-2 in either (A) Primary macrophages derived from mice with a humanised ApoE3 knock-in fed on a high fat diet (B) Primary macrophages derived from mice with a humanised ApoE4 knock-in fed on a high fat diet (C) Primary macrophages derived from mice with a humanised ApoE3 knock-in fed on a high fat diet supplemented with EPA and DHA, or (D) Primary macrophages derived from mice with a humanised ApoE4 knock-in fed on a high fat diet supplemented with EPA and DHA. TIMP-2 expression was measured using Taqman on RNA derived using a cells to C_T kit and analysed using the $2^{-\Delta\Delta CT}$ method, results are normalised to the value for 18s and displayed as a fold change relative to the control condition, these graphs represent the result of one experiment. n=4 unless otherwise stated. Bars shown are mean \pm 1 S.E.M * = p<0.05.

As seen in figure 5.6.A, in ApoE3 HFD cells, there is no change in the expression of TIMP-2 in MCP-1 and ATP conditions, when compared to the control condition. However, in both IL-10 and IL-10 + MCP-1 conditions, there appears to be a decrease in the TIMP-2 expression level, to 0.6 times that of the control level in IL-10 stimulated conditions, and 0.7 times that of the control levels in IL-10 + MCP-1 stimulated cells.

Figure 5.6.B shows TIMP-2 expression in ApoE3 HFD+FO cells, here it is observed that MCP-1 stimulation leads to a decrease in the amount of TIMP-2 expressed, down to 0.4 times that seen in the control value. There is a very minor decrease in the level of TIMP-2 after stimulation with ATP. In both IL-10 and IL-10 + MCP-1, compared to the control conditions, there is a decrease in TIMP-2 expression, to half that of the control conditions.

In figure 5.6.C, we explore the effect of stimulation of ApoE4 HFD cells, in MCP-1 and ATP stimulated cells, it is possible to see an increase in the level of TIMP-2 expression up to 3 fold, and 3.5 fold the control levels respectively. In IL-10 stimulated cells, there is also a rise in the level of TIMP-2 expression, up to 1.8 fold, and there is also a rise in TIMP-2 expression seen in IL-10 + MCP-1 stimulated cells, up to 2 fold the expression of TIMP-2 in the control cells.

Figure 5.6.D investigates the TIMP-2 expression in ApoE4 cells derived from mice fed on a HFD+FO diet. After 8 hours of MCP-1 stimulation, there is a slight increase in the expression of TIMP-2, although in ATP stimulated cells there appears to be very little difference. Stimulation of these cells with IL-10 leads to a decrease down to around half the amount of TIMP-2, when compared to control conditions. IL-10 + MCP-1 conditions were shown to raise TIMP-2 expression, up to 1.5 times the control level.

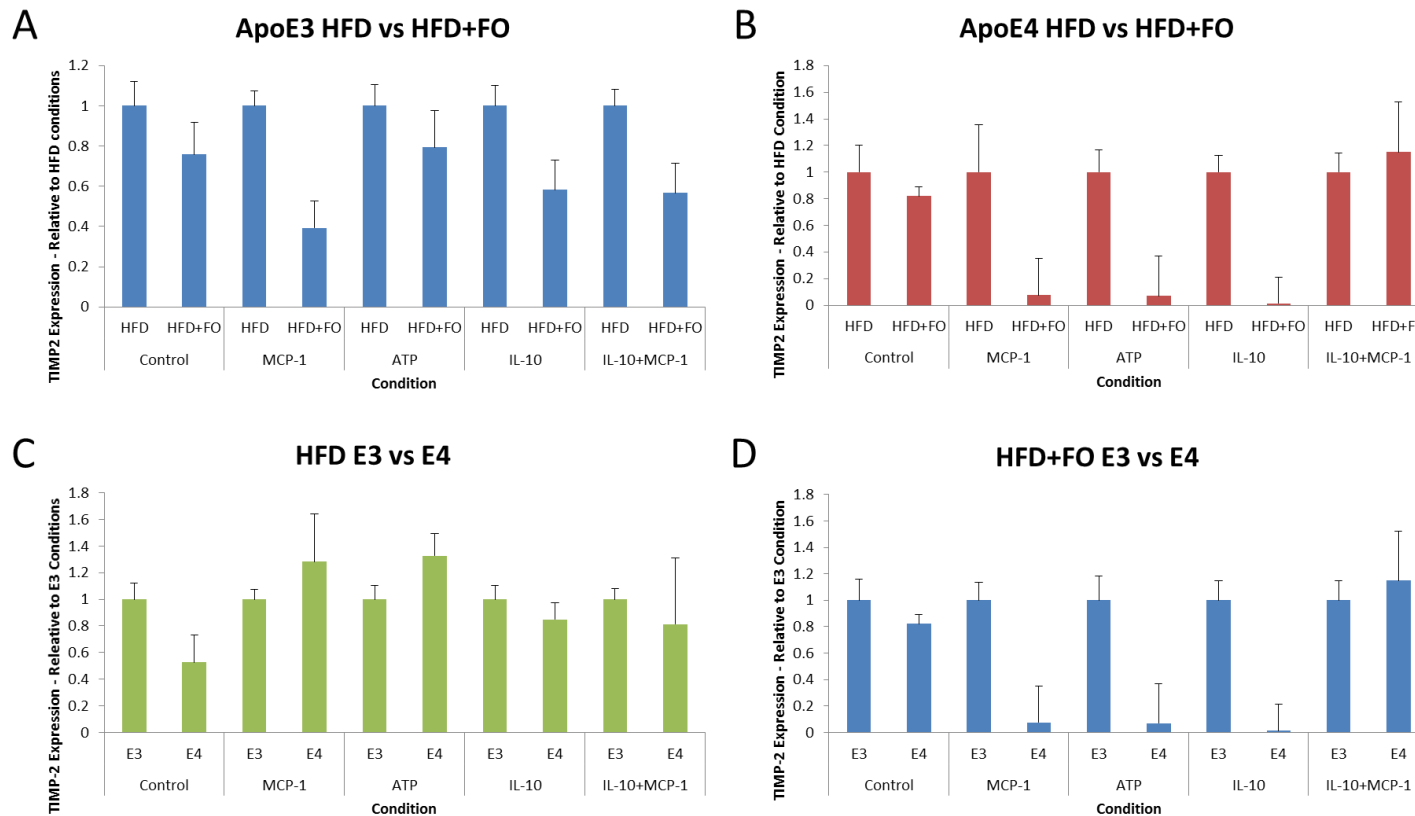


Figure 5.7. Expression of TIMP-2 in comparison to either diet or ApoE subtype. (A) shows the comparison between TIMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE3 knock in macrophages (B) shows the comparison between TIMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE4 knock in macrophages. (C) shows the comparison between TIMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet, whereas (D) shows the comparison between TIMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet supplemented with fish oils. TIMP-2 expression was measured using Taqman on RNA derived using a cells to C_T© kit and analysed using the 2^{ΔΔCT} method, results are normalised to 18s and displayed as a fold change relative to the high fat diet result in A and B, or to the E3 result in C and D, these graphs represent the result of one experiment. n=4 unless otherwise stated, bars shown are mean ± 1 S.E.M

Figure 5.7.A displays the comparison of TIMP-2 between ApoE3 mice fed on either a HFD or a HFD+FO diet. Here it is possible to see a 0.7-fold decrease in the expression of TIMP-2 in the control condition, which falls to 0.4-fold upon MCP-1 stimulation – although this was found to be not significant. The fish oil supplemented diet leads to a slight reduction of TIMP-2 expression in other stimulants.

Figure 5.7.B shows the difference in diet in ApoE4 mice. In these cells, large decreases in TIMP-2 expression are shown after stimulation with MCP-1, ATP and IL-10, however, these were not significant.

The difference between macrophages obtained from ApoE3 and ApoE4 mice fed on a HFD are little, although in the control condition, it can be observed that ApoE4 mice express 0.5-fold TIMP-2 levels than ApoE3 mice (Figure 5.7.C).

Again, Figure 5.7.D shows the difference between macrophages from ApoE3 and ApoE4 fed on a HFD+FO diet, which shows a similar pattern to 5.7.B.

However, when the raw C_T values from this experiment are displayed as a $2^{\Delta C_T}$ expression graph, it is possible to see significant differences in the levels of TIMP-2 expressed between HFD and HFD+FO fed mice (figure 5.8).

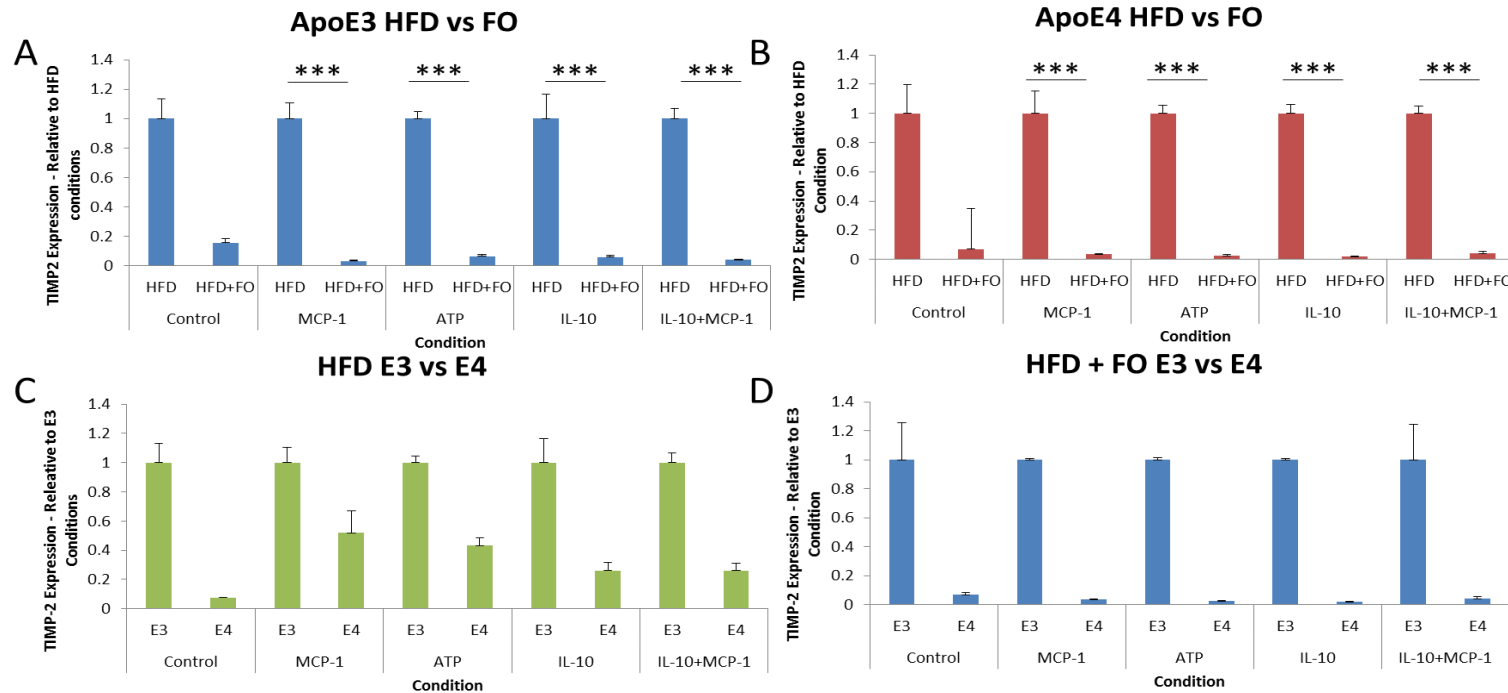


Figure 5.8. Expression of TIMP-2 in comparison to either diet or ApoE subtype. (A) shows the comparison between TIMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE3 knock in macrophages (B) shows the comparison between TIMP expression in both High Fat Diet or in High Fat diet supplemented with Fish Oils (EPA and DHA) in ApoE4 knock in macrophages. (C) shows the comparison between TIMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet, whereas (D) shows the comparison between TIMP expression in both ApoE3 knock-in and ApoE4 knock-in macrophages from mice fed on a high fat diet supplemented with fish oils. TIMP-2 expression was measured using Taqman on RNA derived using a cells to C_T kit and results are expressed as the raw 2^{ΔΔC_T} and displayed as a fold change relative to the high fat diet result in A and B, or to the E3 result in C and D, these graphs represent the result of one experiment. n=4 unless otherwise stated, bars shown are mean ± 1 S.E.M.

In unstimulated cells derived from HFD+FO fed mice, there was an apparent 5-fold decrease in the expression of TIMP-2, although this did not reach significance. With all stimuli there was a 10-fold decrease in TIMP-2 expression in HFD+FO derived cells, when compared to HFD derived cells (Figure 5.8.A).

Similarly, in ApoE4 cells, with all stimulated conditions, there was a 20-fold significant reduction in the levels of TIMP-2 in HFD+FO cells (Figure 5.8.B). TIMP-2 expression is apparently lower in ApoE4 cells than in ApoE3 cells (Figure 5.8.C), although no conditions reveals significance.

The fish oil diet resulted in dramatic reduction in TIMP-2 expression in ApoE4 cells under all conditions compared to ApoE3 cells, although there is no significance in these reductions (Figure 5.8.D).

5.4. Discussion

5.4.1. The Effect of Stimulation on migration and MMP expression of Primary Macrophages.

In this chapter, the characteristics of primary macrophages derived from mice with a humanised ApoE3 knock-in, or a humanised ApoE4 knock-in gene were explored. In wild-type mice, there is only 1 allele for ApoE, which is not identical to any human ApoE gene – but is thought to act similarly to ApoE4, therefore this could be responsible for some differences seen.

5.4.1.1. Cell Migration: Effects of Exogenous Stimuli *in vitro*

No significant differences in random migration speed were observed between stimulants in each set of macrophages studied. In essence, this suggests that each of these stimulants have no effect on the primary macrophages. However, it should be noted that MCP-1 is widely known to stimulate macrophage chemotaxis, and ATP is also widely known as a stimulus for macrophages (as seen in Chapters 3 and 4) (Hall et al., 2009, Kawamura et al., 2012, Li et al., 2014b, Wang et al., 2014, Vogel et al., 2014). IL-10 is an anti-inflammatory cytokine, and an effect would be expected based on previous literature (Kleemann et al., 2008).

Here, it is suggested that in this particular migration experiment, with these particular conditions, that there is no change in the overall migration speed between stimulant effects, although this does not suggest no change in migration overall. Although, it is noted that perhaps in a chemotaxis assay, one would see an effect in which MCP-1 and ATP are seen to be chemoattractants, and thus affect the directionality of the migration. In the appendix, data is presented to support this hypothesis. Chemotaxis assays were performed on ApoE3 HFD derived macrophages, where it was seen that MCP-1 and ATP induced chemotaxis, but with the addition of IL-10 to MCP-1, preliminary data shows that the chemotactic ability of MCP-1 was completely and significantly abolished (See Appendix). Thus, suggesting that the addition of the anti-inflammatory cytokine can influence the migration of macrophages towards chemoattractants (Takayama et al., 2001).

5.4.1.2. The Effect of Exogenous Stimuli on MMP Expression

Despite there being no changes in migration with the stimulants, there are some effects when MMP expression is observed, although due to time constraints, these are

preliminary data arising from experiments with cells from one mouse from each genotype and diet condition only.

Interleukin-10 is a well-studied anti-inflammatory cytokine, and is usually immunosuppressive and can inhibit the production of pro-inflammatory cytokines such as TNF α , IL-1 and IL-6 and thus terminating the immune response (Lippitz, 2013). One would hypothesize that that IL-10 would limit the invasive/migratory capabilities of the primary macrophages studied – although this effect was not observed, the down-regulation of both MMP-13 and MMP-15 (collagenase-3 and MT2-MMP respectively) in ApoE3 HFD macrophages by IL-10 could be related to modulation of a migration phenotype in 3D or chemotaxis (see appendix). This regulation could also suggest that IL-10 could, in the context of atherosclerosis, work to make a plaque more stable – as collagen is the most abundant ECM component in the plaque.

Previously, Interleukin-10 has been found to be able to modulate MMP-9 and TIMP-1 expression in human macrophages and in peripheral blood mononuclear cells through the upregulation of TIMP-1, acting through adiponectin (Nold et al., 2003, Kumada et al., 2004).

It should be noted that in these experiments, ATP did not induce MMP-13 expression as previously seen in Raw 264.7 cell line (Chapter 4), but in this chapter, macrophages are derived directly from mice.

5.4.2. The Effect of diet on macrophage migration and MMP expression.

In this chapter, the effect of fish oils in a diet was explored in primary macrophages, and the data gathered has presented an argument in a favour of the hypothesis that diet can indeed affect the characteristics and behaviour of primary macrophages *in vitro*.

5.4.2.1. Macrophage Migration and Diet

The fish oil supplemented diet perhaps surprisingly resulted in primary macrophages with an increased migration speed in both ApoE3 and ApoE4 macrophages, irrespective of the exogenous stimuli. This is a novel finding, and although there are reports which indicate

that PUFA administration can affect macrophage migration and even tumour cell migration (Li et al., 2014a), there is no previous data available using an *in vitro* model.

One would hypothesize that the increased migration of macrophages derived from mice fed with a HFD+FO have an increased speed due to increased surveillance or circulation. In the context of atherosclerosis – it is suggested that perhaps macrophages do not invade into the intima and continue to circulate, or move away from the atherosclerotic lesion. To our knowledge, the effect of a HFD on macrophage migration compared to a normal diet is unreported in the literature, therefore, it is unknown if HFD results in a change in migration compared to macrophages derived from mice on a normal diet, and in future experiments, a control should be added to test this effect.

5.4.2.2. MMP Expression and Diet

There is also evidence that the supplementation of a high fat diet with fish oil has an effect on the MMP production of macrophages. For example, in ApoE4 cells, there is a trend towards a decrease in the expression in MMP-13 in MCP-1, ATP and IL-10 treated cells, in those cells derived from mice fed on a fish oil supplemented diet. MMP-13 is collagenase-2, and an increased expression would lead to plaque instability as more collagen would be lysed. Therefore the same is true for the opposite, and the decrease in MMP-13 could be beneficial, and therefore infer more stability into the plaque. However, this decrease in MMP-13 expression is only seen in ApoE4 expressing macrophages, and not ApoE3 expressing macrophages - indicating that ApoE4 macrophages respond better to the FO diet.

The most surprising result, however is the reduction of TIMP-2 expression by the fish oil diet, we observe that in both ApoE3 and ApoE4 cells, with all exogenous stimuli, there is a significant decrease in the level of TIMP-2 expressed (with TIMP-2 expression being at least tenfold less than in HFD conditions), and a trend decrease in the control conditions, when the raw C_T values were analysed. This offers a potential mechanism behind why the HFD+FO macrophages migrate faster than the cells derived from HFD fed mice. As TIMP-2 can inhibit all MMPs, a reduction in the level of TIMPs would mean more MMPs can be

active and thus contribute to the remodelling of the extracellular matrix, allowing macrophages to invade and migrate.

The suggestion that n3 PUFAs can have long lasting effects upon the body was implied to as early as the 1980s.(Mahley, 1988, Calder and Kew, 2002) and many dietary intervention studies have further implicated PUFAs in beneficial roles.

Previously, it has been found that n-3 PUFAs can be immunosuppressive, as they are able to decrease superoxide production, and leads to the replacement of arachidonic acid with EPA – meaning that instead of the well-known inflammatory molecule – prostaglandin E₂, the less bioactive prostaglandin E₃ is synthesized, thus leading to a decrease in inflammation (Thies et al., 2001, Grimble, 2007, Ohara et al., 1995, Warnholtz et al., 1999). Thies and colleagues found that a fish oil supplemented diet induced a decrease of expression of adhesion molecules such as sVCAM-1 and sE-selectin, thus suggesting that n-3 PUFAs act to decrease endothelial activation (Thies et al., 2001). It is important to note that the expression of adhesion molecules in endothelial cells is paramount to the development of atherosclerosis (as normal endothelium does not generally support leukocyte binding), in particular, the formation of the initial fatty streak, as the expression of VCAM-1, ICAM-1 and other adhesion molecules by endothelial cells is required to initiate leukocyte rolling and the migration of monocytes into the vessel wall. (Libby et al., 2002, Laxton et al., 2009).

Here, data is presented which confirms that diet can indeed have an effect on individual cellular functions, and that a fish oil supplemented diet alters the migration speed of primary macrophages, and can possibly influence the regulation of MMPs and TIMPs. The mechanism behind this remains unclear, but one can speculate that diet can have epigenetic effects, thus altering the behaviour of primary macrophages in response to certain stimuli. For example, epigenetic changes such as histone deacetylation (HDAC), DNA methylation and miRNA expression can lead to shifts in MMP expression. Culley *et al*, 2013 showed that by inhibiting HDAC, then there was a repression of cytokine induced MMP expression in cartilage (Culley et al., 2013). In addition miRNA-29b has been shown to be upregulated by OxLDL, thus leading to MMP-2 and MMP-9 upregulation in

atherosclerosis (Chen et al., 2011), and in the context of cancer, a number of miRNAs have been shown to regulate cell migration and proliferation (Abba et al., 2014).

5.4.3. The Effect of ApoE subtype on macrophage migration and MMP expression.

The final objective of this chapter was to explore the differences that would arise from the different ApoE subtypes. In WT mice, ApoE is encoded by just a single variant, which is more homogenous to the human ApoE4 gene. However, in these mice, a humanised variant of ApoE3 or ApoE4 has been knocked in, in place of the natural, WT variant of the gene.

The first difference in ApoE subtype is seen in Figure 5.1, although the difference is not obvious, in HFD+FO derived macrophages, there is a slight but significant increase in average migration speed in MCP-1 conditions in ApoE4 cells.

In unstimulated cells there appear to be no major differences in MMP expression between the ApoE variants, although these changes may be subtle and overshadowed by the large effects of the differing diets. Regardless of this, in figure 5.8.C, we can observe that there is a trend towards a decrease in the levels of TIMP-2 in HFD derived E4 cells when compared to E3 cells. This decrease of TIMP-2 in ApoE4 cells could be linked to plaque instability, and thus be partially responsible for the detrimental prognosis of the ApoE4 allele in atherosclerosis.

Previous studies generally observe the difference between ApoE3 and ApoE4 in the context of Alzheimer's disease, whereas studies focussing on atherosclerosis usually focus upon ApoE knockout models. Previous studies into the differences between the ApoE3/3 genotype, and the ApoE4/4 genotype have shown that ApoE4 expressing macrophages and microglia have a stronger innate immune response including an increased NO production in response to IFN γ and LPS, along with an increased proinflammatory cytokine production (Colton et al., 2004, Vitek et al., 2009)

It has been found previously that the possession of the ApoE4 allele, either hetero- or homozygously is associated with an increased activation of microglia, and a pro-inflammatory state *in vitro*.

17 β -estradiol is an anti-inflammatory agent capable of modulating the immune response of macrophages, and it is known that treatment of either macrophages or microglia with 17 β -estradiol results in the reduction of pro-inflammatory factors. Brown et al, 2008 demonstrated that ApoE4 microglia and peritoneal macrophages responded differently to 17 β -estradiol than ApoE3 microglia and macrophages – leading to an increase in the amount of nitrite, TNF α and IL-6 produced in ApoE4 microglia, and a reduced response to 17 β -estradiol (Brown et al., 2008).

Brown suggests that this decreased response to 17 β estradiol is related to the differing levels of oestrogen receptor β levels, and that this may account partly for the pro-inflammatory phenotype of ApoE4 microglia and macrophages. This suggests that the ApoE subtype favours a more pro-inflammatory response and is less responsive to anti-inflammatory agents than the ApoE subtype (Brown et al., 2008). This may explain why ApoE3 HFD macrophages show a decreased production of MMP-13 and MMP-15 in response to treatment with IL-10, whereas ApoE4 macrophages do not.

To conclude, the evidence that presented in Chapter 5 to some extent supports the idea that the ApoE4 results in altered macrophage responses when compared to cells derived from mice with the ApoE3 genotype. No major differences in random macrophage migration speed between those macrophages from ApoE3 mice and those from ApoE4 mice, but unexpectedly in both genotypes the FO diet markedly enhance migration.

There is some evidence, however, that does support the case that ApoE4 may affect the modulation of anti-inflammatory agents – in ApoE3 cells derived from mice in fed on a high fat diet, IL-10 causes a reduction of MMP-13 and MMP-15 (supporting less migration which would be considered anti-inflammatory), but the ApoE4 counterparts fail to deliver this response to the anti-inflammatory agent. The situation is complex however since in mice fed with the FO diet, ApoE4 macrophages were more sensitive to these stimuli than ApoE3 cells.

Overall key findings in this Chapter relate to migration of primary macrophages and reveal that unexpectedly a fish oil diet leads to increased speed of migration in vitro.

Chapter 6: General Discussion

ATP is widely recognised now as an extracellular signalling molecule, capable of altering the behaviour of cells, and inducing migration of macrophages and microglia. In this thesis, the role of ATP and the downstream effects were explored, including the effects of BDNF, along with ATP induced MMP and TIMP expression.

6.1. BDNF abrogates ATP-induced invasion.

In Chapter 3, after first confirming that ATP did indeed induce invasion in the model used, the effect of adding BDNF to the inverted invasion assay was explored, Trang *et al*, (2009), demonstrated that BDNF was released from microglia after stimulation of the P2X4 receptor with ATP via a p38-MAPK pathway (Trang *et al.*, 2009). In a novel finding BDNF alone had no significant effects on invasion when compared to the control level, however, when added in combination with ATP, BDNF was able to abrogate ATP-induced invasion significantly in both BMMs and Raw 264.7 cells. Here, it is speculated that this may be a feedback mechanism to prevent further recruitment of macrophages and microglia to the site of injury.

BDNF is expressed by macrophages along with its receptor, TrkB (Artico *et al.*, 2008). BDNF is known to be involved in the development of neuropathic pain and has shown to be a neuromodulator, able alter neurotransmitter release (including glutamate and aspartate) and thus modulate synaptic efficiency. (Thompson *et al.*, 1999, Pezet, 2002). BDNF in spinal cord neurons is found dense core vesicles, which suggests that BDNF is likely to be released along with the activators of nociceptors. BDNF is also found to be increased in peripheral nerve injury and sciatic axotomy (Thompson *et al.*, 1999).

BDNF has also been implicated in the development of central sensitization. Nociceptive pain is a transient signal which requires a high threshold to activate. Inflammatory and neuropathic pain, however, both have persisting symptoms, and central sensitization is a key step in the progression of neuropathic pain (Ji *et al.*, 2003). Central sensitization is the consequence of the signalling of several factors, including BDNF, Substance P, and ephrin-B. Both neuronal and non-neuronal BDNF is thought to play a role in central sensitization – BDNF-TrkB signalling enhances NMDAR-mediated C-fibre evoked responses and activates ERK, PKC and PKA signalling pathways (Kerr *et al.*, 1999, Latremoliere and Woolf, 2009, Boudes and Menigoz, 2009). Coull *et al*, 2005 suggests that BDNF is responsible for

causing a depolarizing shift in lamina I neurons, leading to ectopic nerve activity and the onset of neuropathic pain (Coull et al., 2005).

Nonetheless, many articles have provided evidence that BDNF may in fact be neuroprotective. In 2002, Eaton *et al*, demonstrated that the overexpression of BDNF (by adenovirus) could ameliorate chronic pain behaviour in CCI of the sciatic nerve (Eaton et al., 2002). Furthermore, the delivery of neurotrophins into the site of injury in several models in adult rats has been shown to rescue injured neurons, promote regeneration and restore function in injured nerves. After nerve injury, injured neurons will atrophy and die in the process of retrograde degeneration. However, it has been found that the transplantation of genetically modified fibroblasts with the ability to secrete BDNF and NT-3 promote the rescuing of injured neurons and axon growth – restoring partial function after SCI and the reversal of chronic pain conditions (Shumsky et al., 2003, Tobias et al., 2003).

More evidence on the neuroprotective effects of BDNF comes from several studies which cite BDNF as being neuroprotective in models of hypoxia-induced brain damage, to such an extent that Zhao *et al* (2014) suggests that BDNF should be used as a treatment to promote neurological recovery after hypoxic ischemia (Zhao et al., 2014, Chen et al., 2013).

Additionally, BDNF has been shown to be essential in neural stem cell survival. Imipramine is a tricyclic antidepressant that has been found to increase the neuronal survival rate. Peng *et al* observed that after treatment with imipramine, there was an increased survival rate of neural stem cells connected with an increase in the mRNA and protein levels of BDNF. It was also shown that imipramine was able to prevent LPS-induced apoptosis via activation of the MAPK pathway, and that siRNA inhibition of BDNF lead to the attenuation of neuroprotective effects seen - suggesting that BDNF is a mediator in the neuroprotective effects of imipramine (Peng et al., 2008).

In summary, BDNF has been shown to be able to regulate neuronal cell survival, synaptic plasticity and cell differentiation. Although BDNF has controversial roles in the onset of chronic pain, numerous papers have outlined its importance in neuronal cell survival. The data presented in this thesis suggests that BDNF can act to reduce macrophage recruitment to the site of injury. Here, we speculate that this may be a neuroprotective effect of BDNF in order to reduce the further infiltration of macrophages and microglia to the site of injury – and to counter the role of pro-inflammatory cytokines which are present.

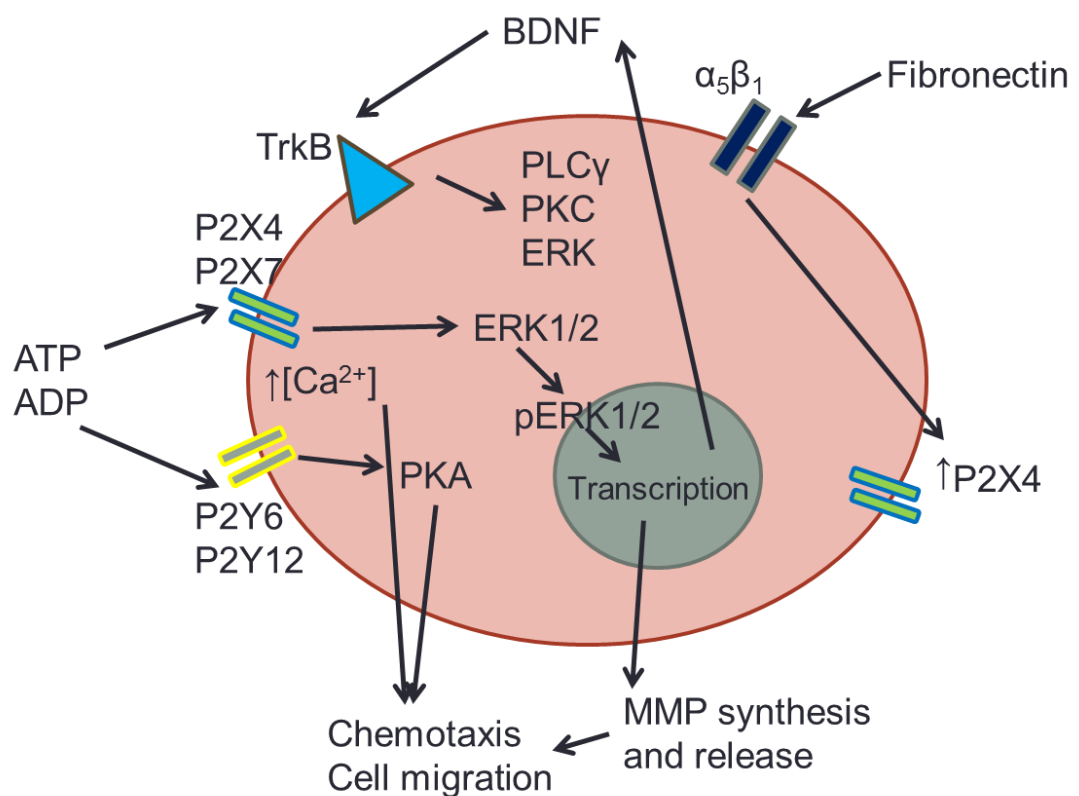


Figure 6.1. What happens in macrophages after nerve injury? This figure displays a graphical representation of the effects of ATP stimulation upon macrophages in the context of nerve injury.

In Figure 6.1 the macrophage response after stimulation of ATP is summarised. ATP is rapidly broken down in the extracellular milieu by ATPases such as CD46 to ADP. ATP activates P2X4 and P2X7, whereas ADP activates P2Y12 and can activate P2Y6. Meanwhile, the activation of surrounding astrocytes produces fibronectin, which cause an increase in the level of P2X4 receptors, through as yet unknown mechanisms (Tsuda et al., 2005). In turn, the activation of purinergic receptors leads an increase in the intracellular calcium levels, and activation of the MAPK pathway through ERK1/2 and p38 and PKA, which act

to promote chemotaxis and cell migration. MAPK activation also leads to the activation of transcription factors and the synthesis and release of MMPs. MAPK activation has also been shown to lead to the release of BDNF, which acts via TrkB to activate a variety of pathways including PLC γ , MAPK, PI3K and PKC – leading to wider effects such as those explored previously.

6.2. Role of Sigma-1 receptor in macrophage migration.

In an attempt to elucidate the mechanisms behind the BDNF abrogation of ATP-induced invasion in both Raw 264.7 cells and BMMs, the effects of agonism, antagonism and eventually siRNA knockdown of Sigma-1 receptor were explored.

Results shown in Chapter 3 of this thesis demonstrates that the agonism of Sigma-1 receptor leads in a decrease in macrophage migration in all conditions, whereas the antagonism and knockdown of sigma-1 receptor results in an increase in migration, and a rescue of the BDNF attenuation of invasion. The role of Sigma-1 receptor has not apparently been previously explored in primary macrophages and Raw 264.7 cells.

Previously, it has been shown that the agonism of sigma-1 receptor with DTG led to the attenuation of various cellular functions in primary microglia, including the migration of microglia towards ATP and MCP-1 (Hall et al., 2009). In addition, the sigma-1 receptor agonist 4-IBP was shown to reduce migration in human cancer cells – including the prostate cancer cell like PC3, the C32 melanoma cell line and U373-MG – a glioblastoma cell line in a scratch wound assay (Megalizzi et al., 2007). A non-selective sigma agonist, afobazole, is an anxiolytic drug currently in use in Russia, was explored by Cuevas *et al* on microglial migration. In a microchemotaxis chamber assay, afobazole was found to block the migration of microglia towards ATP and UTP. In addition, afobazole was found to block the Ca²⁺ increase associated with ATP stimulation in microglia, suggesting that sigma-1 receptors can modulate purinergic receptor function, it was also found that afobazole and thus sigma receptor agonism could reduce microglial cell death after ischemic injury (Cuevas et al., 2011).

Sigma-1 receptor has many links to pain, and has previously been found to be involved in the analgesic effects which are mediated by μ -, δ -, and κ - opioid receptors. The antagonism

of sigma-1 with haloperidol results in the potentiation of opioid induced analgesia (Mei and Pasternak, 2002, Maurice and Su, 2009). Sigma-1 receptor has also been shown to act as a chaperone in the production and maturation of BDNF.

The involvement of Sigma-1 receptor in neuropathic pain has been briefly explored previously in this thesis. However, Kibaly *et al*, 2008 demonstrated that the Sigma-1 agonist, the neurosteroid DHEA, induced a pro-nociceptive effect in rats with chronic pain, and thus implicated cytochrome P450c17 (which is crucial in synthesizing DHEA) in neuropathic pain (Kibaly et al., 2008). Yoon *et al* (2009) also implicated DHEA and DHEAS as pro-nociceptive, and demonstrated that intrathecal injection of DHEAS induced mechanical allodynia in mice (Yoon et al., 2009). This pro-nociceptive effect was blocked with BD1047, a sigma-1 receptor antagonist. However, recently, Francardo and colleagues, found that the agonism of Sigma-1 receptor with PRE-084 led to neurorestoration in the context of Parkinson's disease (Francardo et al., 2014). It was also found that in the context of amyotrophic lateral sclerosis, sigma-1 antagonism by PRE-084 was neuroprotective (Peviani et al., 2014).

Yao, in 2010 and 2011, has implicated Sigma-1 receptor in increase monocyte adhesion and migration into the CNS in response to cocaine abuse in HIV. HIV-1 associated neurodegenerative disorders are a common complication of HIV, and are often exacerbated in response to the abuse of illicit drugs such as cocaine, in these conditions, monocytes can migrate through the blood brain barrier and cause disruption of the BBB, and neuroinflammation, along with activation of glial cells. Yao demonstrates that in microglia, cocaine can induce the translocation of Sigma-1 receptor from the mitochondrion associated membrane to lipid rafts, thus inducing MCP-1 production via Src, MAPK, PI3K and NFκB signalling pathways, and in turn inducing monocyte migration into the CNS. Thus, this demonstrates that sigma-1 receptors could be responsible for the recruitment of further macrophages and monocytes towards the site of injury in chronic pain conditions (Yao et al., 2010, Yao et al., 2011).

Although Sigma-1 receptors were discovered over 30 years ago, the mechanisms behind their actions have proven difficult to find due to the fact that the sequence of Sigma-1 is unlike that of any other mammalian proteins (Maurice and Su, 2009). However, the most

explored molecular mechanism behind the action of Sigma-1 receptor is the interaction with ion channels. It has been found that Sigma-1 receptor antagonist BD-1047 was found to cause an increase in $[Ca^{2+}]$, and activate PLC γ along with elevating IP3 levels. It has also been found that the role of sigma receptors in the regulation of calcium stores concerns Ca^{2+} channels on the plasma membrane, but not intracellular Ca^{2+} stores, and ligands of sigma receptor appear to block all subtypes of Ca^{2+} ion channel (Maurice and Su, 2009).

This block of all calcium channels may go some way to explain the data obtained when cells were exposed to DTG (a sigma agonist), and IPAG, (a sigma receptor antagonist). Data in this thesis shows that when exposed to DTG, macrophage invasion is suppressed and Hall et al. (2009) have shown that calcium channels are then blocked in microglia. Since calcium influx is required for macrophage cell migration, the DTG effect observed is likely mediated through eventual blockade of Calcium channels. However, when cells are treated with IPAG, the base level of invasion in all conditions was increased – for example, in the control conditions and BDNF alone conditions, where previously no invasion was observed. Although Hall et al. did not explore effects of antagonising Sigma 1 receptors it is interesting to note that DTG was able to suppress migration in response to both ATP and MCP-1, which suggests a broad effect, likely due to blockade of calcium channels, as the authors surmise (Hall et al., 2009).

One could speculate that the treatment of cells with IPAG opens calcium channels, leading to an influx of calcium and a subsequent increase in macrophage invasion. RNA silencing of Sigma 1 receptor expression also led to enhanced invasion irrespective of the cell stimulus. Thus antagonism of Sigma 1 receptors appears to release macrophages from a stationary phenotype. Another possible explanation may lie in Sigma 1 receptor interaction with cholesterol in lipid rafts. Gene silencing of Sigma 1 receptor has been shown to block mammary tumour cell adhesion to several different ECM components including laminin (a major component of Matrigel used in invasion assays in this thesis) and fibronectin (with which Matrigel was supplemented in invasion assays) as well as type I collagen (Palmer et al., 2007). The mechanism for reduced adhesion was shown to be due to displacement of $\beta 1$ integrins from lipid rafts. It is interesting to note that although reduced cell adhesion can be correlated with enhanced migration, although some adhesion to the ECM is necessary for traction.

In conclusion, the Sigma-1 receptor has been shown to be involved in macrophage invasion to towards ATP as well as in a general suppression of invasion but the precise mechanisms involved remain to be elucidated.

6.3. ATP can induce MMP and TIMP expression.

Seeking to explore whether pain-related stimuli modulate MMP expression a survey of MMP and TIMP expression in Raw 264.7 macrophages was performed. In the preliminary data obtained. MMP induction after stimulation with ATP was most highly upregulated at earlier time points (4 and 8 hours) – during which MMP-8, MMP-10, MMP-12 and MMP-13, along with TIMP-2 were found to be upregulated after stimulation with 10 μ M and 1mM ATP. To my knowledge, this is the first time that direct stimulation of macrophages with ATP has been demonstrated to induce MMP expression.

Although ATP has not been investigated in the induction of MMPs from macrophages previously, ATP has been shown to be able to induce MMP and TIMP expression in other cell types. For example, in primary microglia and BV-2 microglial cells, it was found that ATP lead to an increase in the secretion and activation of MMP-9, through PI3K activation (Choi et al., 2010). Chang *et al*, demonstrated in human endometrial stromal cells, that 10 μ M ATP lead to the induction of MMP-2, MMP-3, MMP-10 and MMP-24 expression after 24hrs stimulation (Chang et al., 2007). ATP was also found to induce MMP-9 expression in peripheral blood mononuclear cells via activation of P2X7 receptor, it was also found that TIMP-1 expression was decreased – although it has also been shown that TIMP-1 can be degraded by cathepsin B after P2X7 activation (Gu and Wiley, 2006, Murphy and Lynch, 2012). It should be noted that the effects of ATP on MMP expression may be cell or context dependent. Thus in human gingival fibroblasts ATP suppresses IL-1-induced MMP-1 expression (Nemoto et al., 2013).

MMPs have been implicated in Neuropathic Pain conditions previously – in 2008, Kawasaki *et al* implicated both MMP-2 and MMP-9 expression in the early and late phase developments of neuropathic pain, suggesting that MMP-9 was involved in the early phase response of nerve injury, whereas MMP-2 was expressed in a late phase response. It was also found that the intrathecal administration of MMPs -2 and -9 was capable of producing chronic pain behaviours, and that these MMPs are at least, in part, responsible for chronic

pain conditions by activating pro-inflammatory cytokine IL-1 β by cleavage of its precursor (Schönbeck et al., 1998, Kawasaki et al., 2008). MMP-9 has further been implicated in chronic pain conditions, due to the upregulation after SCI, along with MCP-1 and TIMP-1 (Platt et al., 2003, Yong and Guoping, 2008, Sandhir et al., 2011), in particular, the emergence of a MMP-9/TIMP-1 activation axis has suggested that MMP-9 and TIMP-1 co-localised within myelinating Schwann cells. Kim *et al*, demonstrated that MMP-9 knockout lead to an induction of immature myelinating Schwann cells into the injured nerve, suggesting that MMP-9 is required for the differentiation of Schwann cells and important in the regeneration of injured nerves (Kim et al., 2012). MMP-9 upregulation may also play a role in the release of BDNF from injured neurons, microglia and macrophages, as MMP-9 has been implicated in the processing of proBDNF to mature BDNF (Yamamori et al., 2013).

Similarly MMP-3 and TIMP-3 have been shown to have important roles in nerve injury by ischemia. Walker and Rosenberg investigated MMP-3 and TIMP-3 due to their respective roles in the increase of inflammatory conditions and the promotion of apoptosis. It was found that knockout of TIMP-3 levels lead to a correlated reduction in microglial activity and neuronal cell death, and the knockout of MMP-3 lead to a reduction of microglial cells, and neuronal cell death. It was observed that the pharmacological inhibition of MMPs, along with the knockout of TIMP-3 lead to an additive addition and significantly less neuronal cell death in ischemic conditions (Walker and Rosenberg, 2009).

As explored previously, MMPs have been shown to play a role in the remodelling of the extracellular matrix components. In the context of peripheral nerve injury, ECM components have been shown to play a role in creating a nerve regeneration positive environment – for example, it has been shown that laminin is essential for the sustained ability of axonal regeneration. (Gonzalez-Perez et al., 2013). It is thought that using collagen and laminin matrices is beneficial for the regeneration of axons and the restoration of function, and the presence of a collagen matrix is highly beneficial, and that fibronectin matrix presence is capable of improving neurite outgrowth and migration of Schwann cells – it is perhaps unsurprising that after nerve injury astrocytes produce fibronectin (Tsuda et al., 2008, Gonzalez-Perez et al., 2013). However, Koopmans *et al*, argue that after peripheral nerve injury, collagen production is overdriven and thus hinders

the growth of axons (Koopmans et al., 2009). ATP induced MMP expression then could play either a beneficial or detrimental role to nerve regeneration.

As MMP-8 and MMP-13 are Collagenase -2 and -3, respectively, they are able to hydrolyse a number of collagens and other substrates – for example MMP-13 is also capable of cleaving Fibronectin and Laminin. ATP was found (in preliminary data shown in this thesis) to upregulate these MMPs, along with MMP-10 and MMP-12 – which can also degrade type IV collagen and fibronectin as well as chemokines (Dean et al., 2008). *In vivo*, this may be linked to the degradation of excess collagen and thus promote nerve regeneration, or degrade collagen essential to nerve regeneration, thus giving a detrimental effect. The upregulation of TIMP-2 observed could then be a feedback mechanism to inhibit MMP degradation of essential collagen required for nerve regeneration. *In vitro*, in this thesis, ATP was shown to induce invasion in Raw 264.7 cells through a Matrigel™/Fibronectin matrix, as Matrigel™ contains type IV collagen and laminin, one could speculate that the upregulation of these MMPs could offer an explanation for the increased invasion observed, although more data would be required.

6.3.1. BDNF may attenuate MMP expression.

This thesis presents data to demonstrate that when treated with a combination of ATP and BDNF, after 8 hours, there is a trend towards a decrease in the expression of MMP-8, MMP-12, MMP-13 and TIMP-2 observed. To our knowledge, BDNF has only previously been shown to induce levels of MMP-9 (Kuzniewska et al., 2013). Although these decreases in MMP expression were not shown to be significant, it could suggest that BDNF plays a role in reducing MMP degradation of essential extracellular matrix components, or that BDNF acts to reduce the cleavage (and subsequent activation) of pro-inflammatory cytokines by these MMPs – and thus has a neuroprotective effect. However, the cleavage of ECM components may be essential for nerve regeneration (Koopmans et al., 2009), and BDNF could, in contrast, support neurodegeneration.

6.4. A Fish Oil supplemented diet leads to increased macrophage migration and a reduction in TIMP-2 expression.

Data in this thesis demonstrates that when macrophages are derived from mice fed on fish oil supplemented high fat diet, perhaps unexpectedly; these cells migrate significantly faster than macrophages derived from mice fed on a high fat diet. Also, the data shown in

chapter 5 demonstrates that macrophages from HFD+FO fed mice also show a significant reduction in the expression TIMP-2 in conditions when stimulated with ATP, IL-10, MCP-1 and a combination of IL-10 and MCP-1. The difference in ApoE subtype was found to have little effect on the migration and MMP expression of macrophages.

Previously, the supplementation of fish oils has been shown to affect monocyte and macrophage infiltration into the atherosclerotic plaque. For example, Kanai *et al* demonstrated that the supplementation of diet with EPA was able to reduce arterial medial calcification, along with macrophage infiltration into the plaque in rats possibly due to a reduction in the expression of MCP-1 and MMP-2 and MMP-9 – however, an increased migration of macrophages was seen around the calcified area (Kanai et al., 2011). It has also been demonstrated that EPA and DHA supplementation led to decreased macrophage infiltration into gastric cancer tumours, which is due to the blocking of MMP-10 expression via a down-regulation of ERK activation (Wu et al., 2012). Luu *et al* (2007) also observed an EPA induced reduction of monocyte infiltration in atherosclerosis, but suggests that although an EPA supplemented diet leads to the modulation of the ability of monocytes to induce an inflammatory response, the reduction in infiltration of macrophages into the intima may be due to differences seen on the surface of endothelial cells. However, in 1998, Sanderson and Calder found that lymphocytes from rats derived from fish oil supplemented diet fed rats were had a decreased level of CD2, CD11a, CD18 and CD44, which reduced adhesion to TNF α stimulated endothelial cells (Sanderson and Calder, 1998, Luu et al., 2007), hinting that reduced infiltration and thus migration of macrophages is reduced as they lack the necessary adhesion molecules.

In addition to the examples mentioned above, the supplementation of diet with fish oils has been shown to alter MMP expression on numerous occasions and in different conditions. Velten *et al*, demonstrated that in a new-born mouse model of peritoneal inflammation DHA supplementation lead to a decrease in pulmonary fibrosis, perhaps a result of a decrease in the expression of MMP-2, MMP-3 and MMP-8 in macrophages and monocytes (Velten et al., 2014). It was also found that in amnion explants, EPA and DHA lead to a decrease in the level of MMP-9 expression, possibly through a mechanism involving PPAR γ and thus decreasing inflammation (Frew et al., 2013). N-3 PUFA supplemented diet also lead to the decrease in inflammation of adipose tissue in a model

of chronic kidney disease in humans, as a decrease in MMP-9 was found, again suggesting an anti-inflammatory role for dietary fish oil supplementation (Guebre-Egziabher et al., 2013).

In atherosclerosis in humans, fish oil dietary supplements were given to patients awaiting carotid endarterectomy, allowing the contents of the atherosclerotic plaque to be examined. It was found that EPA supplementation led to a decrease in foam cell content of the atherosclerotic plaque, along with a decrease in MMP-7, MMP-9 and MMP-12. Although this led to no significant change in overall plaque stability, it was found that supplementation with EPA led to more stable plaques (Cawood et al., 2010). Derosa *et al*, also found that fish oil supplements lead to a decrease in MMP-2 and MMP-9 levels in circulating blood plasma (Derosa et al., 2012).

Johnson and colleagues (2006) reported that the overexpression of TIMP-2 by adenovirus in ApoE knockout mice with atherosclerosis resulted in the reduction of atherosclerotic lesion size, along with the reduction of many markers of plaque instability – such as the presence of buried fibrous caps and the presence of macrophages in the intima. *In vitro*, overexpression of TIMP-2 was found to inhibit macrophage migration and apoptosis (Johnson et al., 2006).

In this thesis, data presented shows that macrophages derived from HFD+FO fed mice migrate faster, as the majority of evidence suggests that the supplementation of diet with fish oils is anti-inflammatory, one would be inclined to suggest that the increased migration speed is beneficial. This may be due to the formation of a macrophage phenotype that circulates and surveys the environment, but does not migrate into the intima – for example Kanai *et al* reported that FO supplementation led to an increased number of macrophages surrounding the calcified area, but less macrophages infiltrating the calcified area (Kanai et al., 2011).

The observation that mice fed with fish oil supplements produce macrophages with significantly reduced TIMP-2 levels which could be associated with enhanced macrophage migration (although this was not explored directly). In an *in vivo* atherosclerosis context this could support the notion that fish oil could lead to a reduction in TIMP2 expression would likely be associated with plaque instability but further investigation would be

required to confirm or refute this possibility. In conclusion, one would suggest that the increased macrophage migration speed and reduction of TIMP-2 expression in HFD+FO fed mice derived macrophages leads to the speculation that this is indicative of a pro- rather than an anti-inflammatory phenotype.

6.5. Future Work.

6.5.1. BDNF abrogates ATP-induced invasion.

To further explore the effect of BDNF on ATP-induced invasion, in future studies, it is crucial to study the signalling mechanism behind both ATP stimulation – from which purinergic receptor conveys migration to the effect of individual signalling pathways, including PLC γ , MAPK and PI3K – to determine how ATP induces migration in macrophages. It is paramount to explore the activity of signalling pathways, and how the stimulation of each subtype of purinergic receptor influences the activity of these signalling pathways. Likewise, to investigate BDNF stimulation in macrophages in detail and exploring the effect of BDNF signalling not only through TrkB, but also through p75 is of importance for similar reasons – if one is to explore the BDNF abrogation of ATP invasion, it is imperative to explore the crosstalk in these mechanisms and identify the pathways which lead to the BDNF abrogation of ATP-induced invasion. If one were to have the chance to repeat the experience of researching and writing this thesis, it would be a priority to explore signalling mechanisms behind the observed effects of ATP and BDNF.

6.5.2. Sigma receptor studies.

It has been suggested that Sigma-1 receptor can translocate from the mitochondrion-associated-membrane to the cell surface, where it can interfere with calcium, sodium and potassium channels, and other cell surface receptors including displacement of integrins from lipid rafts to modulate the effects of signalling, and thus alter the behaviour of the cell. Future work should aim to explore the mechanism behind this action, and also the mechanism behind which Sigma-1 is able to modulate the signalling of many different receptors. In the context of this thesis, it would be of interest to examine if Sigma-1 can alter the activation of TrkB signalling and that of purinergic signalling by FRET analysis and immunoprecipitation methods. It would also be important to examine the action by which Sigma-1 receptor controls macrophage migration by exploring the effect of the activation

of sigma-1 receptor on β 1 integrin displacement, the activation of various signalling pathways and calcium signalling.

6.5.3. ATP-induced MMP expression.

To understand potential links between ATP regulation of MMPs and migration of Raw 264.7 cells, an approach based on previous studies in invading tumour cells could be exploited. Zhang *et al* observed that ATP up-regulated activation of Rac1 and Cdc42 as well as migration in three different prostate cancer cell lines – in connection with the up-regulation of MMP-3 and MMP-13 (Zhang et al., 2010). To further explore the role of the up-regulation of MMPs induced by ATP stimulation, it would be important to examine the functional role of MMP expression in macrophages. This could be achieved by measuring macrophage migration on different substrates and by zymography (for MMPs 2 and 9). The importance of these MMPs in migration could be explored using hydroxamate inhibitors and TIMPs and eventually siRNA knockdown experiments to determine if ATP induced macrophage migration is MMP-dependent.

6.5.4. Dietary Fish Oils in Atherosclerosis.

To further explore the effect of the dietary supplementation with fish oils on atherosclerosis, it would be important to first explore the effects of chemotaxis towards MCP-1 on macrophages derived from fish oil fed mice, and it would be interesting to explore the effects of anti-inflammatory cytokines on this chemotaxis. It would be important to explore the inflammatory response of macrophages derived from these mice – and the production of pro- and anti-inflammatory cytokines and nitric oxide – as these are measures of how macrophages can influence the surrounding environment and the stability of the plaque, as well as a way of assessing if the response of macrophages to dietary fish oil supplementation is pro- or anti-inflammatory. Furthermore, it would be crucial to explore plaque stability in response to a fish oil supplemented diet, by investigating the composition of the plaques induced by the high fat diet. It would also be of interest to explore chemokine production by macrophages, and also to examine the surrounding environment of the atherosclerotic plaque – for example, the chemokine production from the endothelial cells within the intima, to see if the action of fish oil supplemented diet is more generalised. To determine if fish oil supplemented diet is enough to prevent or promote the development of atherosclerosis would be of interest,

and also to determine factors that would promote the regression of the atherosclerotic plaque.

6.6. Overall Conclusions

Macrophage migration has been shown to play an important role in many normal biological functions, and also in pathologies. In this thesis, it is reported that ATP is capable of inducing macrophage migration, and BDNF can lead to an abrogation of ATP-induced invasion, although the mechanisms behind this effect have yet to be determined. Data is also presented which suggests that sigma-1 receptor is a regulator of macrophage migration, although the actions which allow sigma-1 receptor to do this are yet to be elucidated, previous studies suggest that the blockade of calcium channels and the reduction of adhesion due to β 1 integrin displacement play a role.

This thesis also presents data to show that ATP can induce the expression of MMPs and TIMPs, which may play a role in macrophage migration, although further investigation is required to determine if this conclusion is correct in this model.

Fish oil supplementation of a high fat diet and its effect on macrophage migration and MMP expression was also explored. It was found that a HFD+FO diet led to significant changes in migration speed and TIMP-2 expression. This data hints that the supplementation of diet with EPA and DHA could be pro-inflammatory, rather than anti-inflammatory, however. Further investigation is of importance. Figure 6.2 summarises these findings.

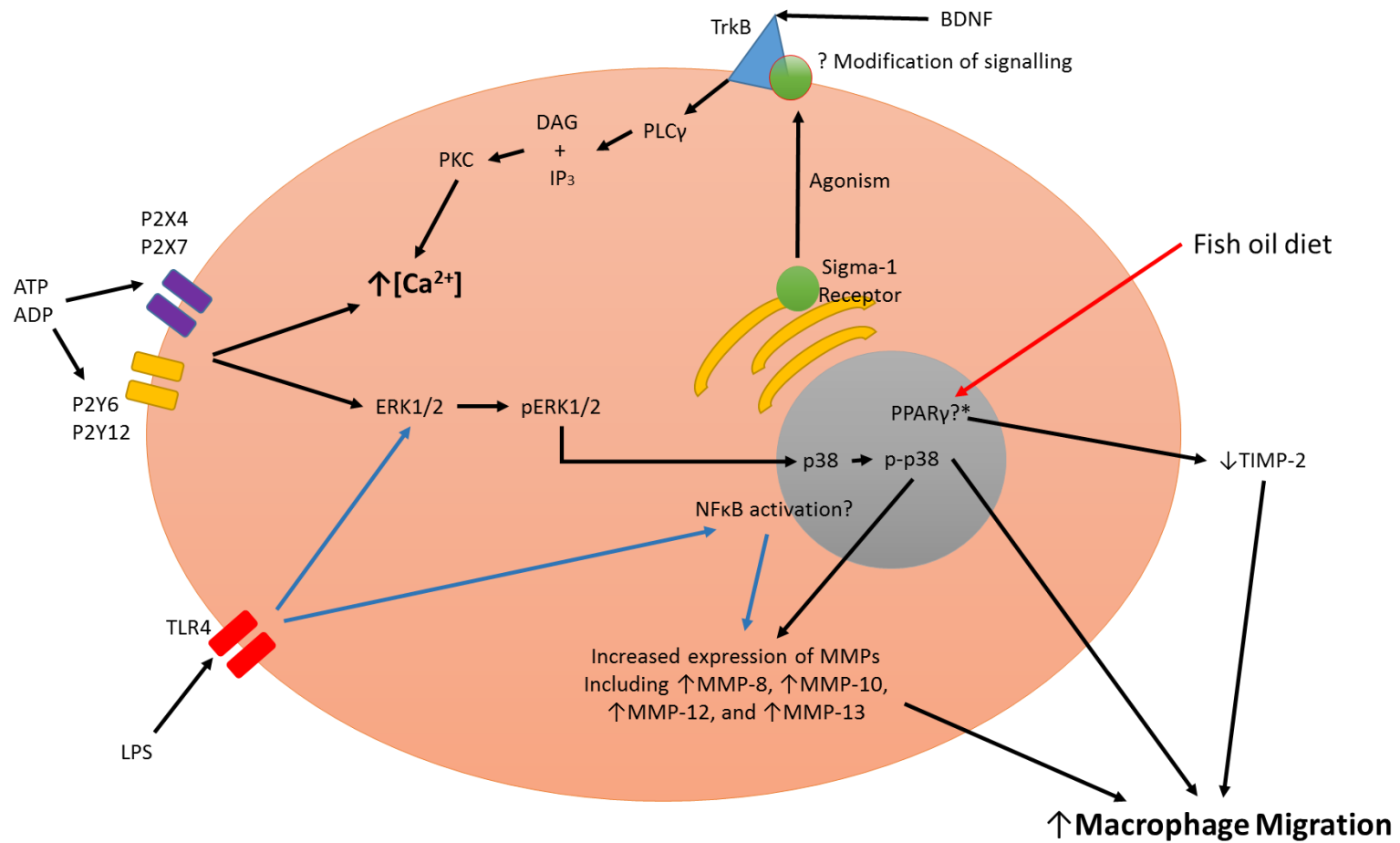


Figure 6.2. A diagram to summarise the speculations and findings of this thesis. This figure demonstrates a graphical representation of a macrophage, and the factors explored in this thesis which lead to increased macrophage migration. Red arrow fish oil diet leads to activation of PPAR γ , but this has not been shown in our particular study. The blue arrows are indicative of a late-phase response as LPS leads to a later phase MMP response of Raw 264.7 than ATP in chapter 4, possibly mediated through ERK1/2 or Nf κ B.

7. Bibliography

- ABBA, M., PATIL, N. & ALLGAYER, H. 2014. MicroRNAs in the Regulation of MMPs and Metastasis. *Cancers (Basel)*, 6, 625-45.
- ABBADIE, C. 2005. Chemokines, chemokine receptors and pain. *Trends in Immunology*, 26, 529-534.
- ABBRACCHIO, M. P. & CERUTI, S. 2007. P1 receptors and cytokine secretion. *Purinergic Signal*, 3, 13-25.
- ABDI, S., LEE, D. H. & CHUNG, J. M. 1998. The Anti-Allodynic Effects of Amitriptyline, Gabapentin, and Lidocaine in a Rat Model of Neuropathic Pain. *Anesthesia & Analgesia*, 87, 1360-1366
10.1213/00000539-199812000-00027.
- ABDULKHALEK, S., GUO, M., AMITH, S. R., JAYANTH, P. & SZEWCZUK, M. R. 2012. G-protein coupled receptor agonists mediate Neu1 sialidase and matrix metalloproteinase-9 cross-talk to induce transactivation of TOLL-like receptors and cellular signaling. *Cell Signal*, 24, 2035-42.
- ADEREM, A. & UNDERHILL, D. M. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*, 17, 593-623.
- ALMEIDA, R. D., MANADAS, B. J., MELO, C. V., GOMES, J. R., MENDES, C. S., GRAOS, M. M., CARVALHO, R. F., CARVALHO, A. P. & DUARTE, C. B. 2005. Neuroprotection by BDNF against glutamate-induced apoptotic cell death is mediated by ERK and PI3-kinase pathways. *Cell Death Differ*, 12, 1329-43.
- ARTICO, M., BRONZETTI, E., FELICI, L. M., ALICINO, V., IONTA, B., BRONZETTI, B., MAGLIULO, G., GRANDE, C., ZAMAI, L., PASQUANTONIO, G. & DE VINCENZI, M. 2008. Neurotrophins and their receptors in human lingual tonsil: an immunohistochemical analysis. *Oncol Rep*, 20, 1201-6.
- ATTAL, N., CRUCCU, G., BARON, R., HAANPÄÄ, M., HANSSON, P., JENSEN, T. S. & NURMIKKO, T. 2010. EFNS guidelines on the pharmacological treatment of neuropathic pain: 2010 revision. *European Journal of Neurology*, 17, 1113-e88.
- AZOULAY, D., URSHANSKY, N. & KARNI, A. 2008. Low and dysregulated BDNF secretion from immune cells of MS patients is related to reduced neuroprotection. *J Neuroimmunol*, 195, 186-93.
- BÄCK, M., KETELHUTH, D. F. J. & AGEWALL, S. 2010. Matrix Metalloproteinases in Atherothrombosis. *Progress in Cardiovascular Diseases*, 52, 410-428.
- BAKER, N., SHARPE, P., CULLEY, K., OTERO, M., BEVAN, D., NEWHAM, P., BARKER, W., CLEMENTS, K. M., LANGHAM, C. J., GOLDRING, M. B. & GAVRILOVIC, J. 2012. Dual regulation of metalloproteinase expression in chondrocytes by Wnt-1-inducible signaling pathway protein 3/CCN6. *Arthritis Rheum*, 64, 2289-99.
- BALÁZS, B., DANKÓ, T., KOVÁCS, G., KÖLES, L., HEDIGER, M. A., ZSEMBERY, Z. 2013. Investigation of the inhibitory effects of the benzodiazepine derivative, 5-BDBD on P2X4 purinergic receptors by two complementary methods. *Cell Physiol Biochem*, 32, 11-24.
- BAR, I., GUNS, P. J., METALLO, J., CAMMARATA, D., WILKIN, F., BOEYNAMS, J. M., BULT, H. & ROBAYE, B. 2008. Knockout mice reveal a role for P2Y6 receptor in macrophages, endothelial cells, and vascular smooth muscle cells. *Mol Pharmacol*, 74, 777-84.

- BARGUT, T. C., FRANTZ, E. D., MANDARIM-DE-LACERDA, C. A. & AGUILA, M. B. 2014. Effects of a diet rich in n-3 polyunsaturated fatty acids on hepatic lipogenesis and beta-oxidation in mice. *Lipids*, 49, 431-44.
- BARON, R., BINDER, A. & WASNER, G. 2010. Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. *Lancet Neural*, 9, 807-19.
- BARRAGAN-IGLESIAS, P., PINEDA-FARIAS, J. B., CERVANTES-DURAN, C., BRAVO-HERNANDEZ, M., ROCHA-GONZALEZ, H. I., MURBARTIAN, J. & GRANADOS-SOTO, V. 2014. Role of spinal P2Y6 and P2Y11 receptors in neuropathic pain in rats: possible involvement of glial cells. *Mol Pain*, 10, 29.
- BAUTISTA, D. M., JORDT, S. E., NIKAI, T., TSURUDA, P. R., READ, A. J., POBLETE, J., YAMOAH, E. N., BASBAUM, A. I. & JULIUS, D. 2006. TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. *Cell*, 124, 1269-82.
- BEGGS, S. & SALTER, M. W. 2010. Microglia-neuronal signalling in neuropathic pain hypersensitivity 2.0. *Curr Opin Neurobiol*, 20, 474-80.
- BEGGS, S., TRANG, T. & SALTER, M. W. 2012. P2X4R+ microglia drive neuropathic pain. *Nat Neurosci*, 15, 1068-73.
- BELFRAGE, M., SOLLEVI, A., SEGERDAHL, M., SJOLUND, K.-F. & HANSSON, P. 1995. Systemic Adenosine Infusion Alleviates Spontaneous and Stimulus Evoked Pain in Patients with Peripheral Neuropathic Pain. *Anesthesia & Analgesia*, 81, 713-717.
- BEN YEBDRI, F., KUKULSKI, F., TREMBLAY, A. & SEVIGNY, J. 2009. Concomitant activation of P2Y(2) and P2Y(6) receptors on monocytes is required for TLR1/2-induced neutrophil migration by regulating IL-8 secretion. *Eur J Immunol*, 39, 2885-94.
- BENDECK, M. P., IRVIN, C. & REIDY, M. A. 1996. Inhibition of Matrix Metalloproteinase Activity Inhibits Smooth Muscle Cell Migration but Not Neointimal Thickening After Arterial Injury. *Circulation Research*, 78, 38-43.
- BENDECK, M. P., ZEMPO, N., CLOWES, A. W., GALARDY, R. E. & REIDY, M. A. 1994. Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. *Circulation Research*, 75, 539-45.
- BERNARDO, M. M. & FRIDMAN, R. 2003. TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type 1)-MMP. *Biochem J*, 374, 739-45.
- BERNIER, L. P., ASE, A. R., BOUE-GRABOT, E. & SEQUELA, P. 2012. P2X4 receptor channels form large noncytolytic pores in resting and activated microglia. *Glia*, 60, 728-37.
- BESSIS, A., BECHADE, C., BERNARD, D. & ROUMIER, A. 2007. Microglial control of neuronal death and synaptic properties. *Glia*, 55, 233-8.
- BIGGS, J. E., LU, V. B., STEBBING, M. J., BALASUBRAMANYAN, S. & SMITH, P. A. 2010. Is BDNF sufficient for information transfer between microglia and dorsal horn neurons during the onset of central sensitization? *Mol Pain*, 6, 44.

- BIRKEDAL-HANSEN, H., MOORE, W. G., BODDEN, M. K., WINDSOR, L. J., BIRKEDAL-HANSEN, B., DECARLO, A. & ENGLER, J. A. 1993. Matrix metalloproteinases: a review. *Crit Rev Oral Biol Med*, 4, 197-250.
- BOUDES, M. & MENIGOZ, A. 2009. Non-neuronal BDNF, a key player in development of central sensitization and neuropathic pain. *The Journal of Physiology*, 587, 2111-2112.
- BOURBOULIA, D. & STETLER-STEVENSON, W. G. 2010. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. *Semin Cancer Biol*, 20, 161-8.
- BREW, K. & NAGASE, H. 2010. The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. *Biochim Biophys Acta*, 1803, 55-71.
- BRIMSON, J. M., BROWN, C. A. & SAFRANY, S. T. 2011. Antagonists show GTP-sensitive high-affinity binding to the sigma-1 receptor. *J Pharmacol.*, 164(2b), 772-780.
- BROWN, C. M., CHOI, E., XU, Q., VITEK, M. P. & COLTON, C. A. 2008. The APOE4 genotype alters the response of microglia and macrophages to 17beta-estradiol. *Neurobiol Aging*, 29, 1783-94.
- BURNSTOCK, G. 1972. Purinergic Nerves. *Pharmacol. Rev.*, 24, 509-81.
- BURNSTOCK, G. 1976. Do some nerve cells release more than one transmitter? *Neuroscience*, 1, 239-248.
- BURNSTOCK, G. 2007. Purine and pyrimidine receptors. *Cell Mol Life Sci*, 64, 1471-83.
- BURNSTOCK, G., KRUGEL, U., ABBRACCHIO, M. P. & ILLES, P. 2011. Purinergic signalling: from normal behaviour to pathological brain function. *Prog Neurobiol*, 95, 229-74.
- BUSS, A., PECH, K., KAKULAS, B. A., MARTIN, D., SCHOENEN, J., NOTH, J. & BROOK, G. A. 2007. Matrix metalloproteinases and their inhibitors in human traumatic spinal cord injury. *BMC Neurol*, 7, 17.
- CALDER, P. C. & KEW, S. 2002. The immune system: a target for functional foods? *Br J Nutr*, 88 Suppl 2, S165-77.
- CALDER, P. C. 2001. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids*, 36, 1007-24.
- CALDER, P. C. 2006. n-3 polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am J Clin Nutr*, 83, 1505S-1519S.
- CASH, J. G., KUHEL, D. G., BASFORD, J. E., JAESCHKE, A., CHATTERJEE, T. K., WEINTRAUB, N. L. & HUI, D. Y. 2012. Apolipoprotein E4 impairs macrophage efferocytosis and potentiates apoptosis by accelerating endoplasmic reticulum stress. *J Biol Chem*, 287, 27876-84.
- CAWOOD, A. L., DING, R., NAPPER, F. L., YOUNG, R. H., WILLIAMS, J. A., WARD, M. J., GUDMUNDSEN, O., VIGE, R., PAYNE, S. P., YE, S., SHEARMAN, C. P., GALLAGHER, P. J., GRIMBLE, R. F. & CALDER, P. C. 2010. Eicosapentaenoic acid (EPA) from highly concentrated n-3 fatty acid ethyl esters is incorporated into advanced atherosclerotic plaques and higher plaque EPA is associated with decreased plaque inflammation and increased stability. *Atherosclerosis*, 212, 252-9.

- CHANG, S. J., WANG, T. Y., LEE, Y. H. & TAI, C. J. 2007. Extracellular ATP activates nuclear translocation of ERK1/2 leading to the induction of matrix metalloproteinases expression in human endometrial stromal cells. *J Endocrinol*, 193, 393-404.
- CHAPLAN, S. R., MALMBERG, A. B. & YAKSH, T. L. 1997. Efficacy of spinal NMDA receptor antagonism in formalin hyperalgesia and nerve injury evoked allodynia in the rat. *J Pharmacol Exp Ther*, 280, 829-38.
- CHATTOPADHYAY, S., MYERS, R. R., JANES, J. & SHUBAYEV, V. 2007. Cytokine regulation of MMP-9 in peripheral glia: implications for pathological processes and pain in injured nerve. *Brain Behav Immun*, 21, 561-8.
- CHEKENI, F. B. & RAVICHANDRAN, K. S. 2011. The role of nucleotides in apoptotic cell clearance: implications for disease pathogenesis. *J Mol Med (Berl)*, 89, 13-22.
- CHEN, A., XIONG, L. J., TONG, Y. & MAO, M. 2013. The neuroprotective roles of BDNF in hypoxic ischemic brain injury. *Biomed Rep*, 1, 167-176.
- CHEN, K. C., WANG, Y. S., HU, C. Y., CHANG, W. C., LIAO, Y. C., DAI, C. Y. & JUO, S. H. 2011. OxLDL up-regulates microRNA-29b, leading to epigenetic modifications of MMP-2/MMP-9 genes: a novel mechanism for cardiovascular diseases. *FASEB J*, 25, 1718-28.
- CHEN, Y., CORRIDEN, R., INOUE, Y., YIP, L., HASHIGUCHI, N., ZINKERNAGEL, A., NIZET, V., INSEL, P. A. & JUNGER, W. G. 2006. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. *Science*, 314, 1792-5.
- CHOI, M., CHO, K., SHIN, S., KO, H., KWON, K., SHIN, C. & KO, K. 2010. ATP induced microglial cell migration through non-transcriptional activation of matrix metalloproteinase-9. *Archives of Pharmacal Research*, 33, 257-265.
- CIRILLO, G., CAVALIERE, C., BIANCO, M. R., DE SIMONE, A., COLANGELO, A. M., SELLITTI, S., ALBERGHINA, L. & PAPA, M. 2010. Intrathecal NGF administration reduces reactive astrocytosis and changes neurotrophin receptors expression pattern in a rat model of neuropathic pain. *Cell Mol Neurobiol*, 30, 51-62.
- CLARK, A. K., WODARSKI, R., GUIDA, F., SASSO, O. & MALCANGIO, M. 2010. Cathepsin S release from primary cultured microglia is regulated by the P2X7 receptor. *Glia*, 58, 1710-26.
- CODERRE, T. J. & MELZACK, R. 1992. The Contribution of Excitatory Amino Acids to Central Sensitization and Persistent Nociception after Formalin-Injury Tissue Injury. *The Journal of Neuroscience*, 12, 3665-3670.
- COLAK, T., CINE, N., BAMAC, B., KURTAS, O., OZBEK, A., BICER, U., SUNNETCI, D. & SAVLI, H. 2012. Microarray-based gene expression analysis of an animal model for closed head injury. *Injury*, 43, 1264-70.
- COLLEONI, M. & SACERDOTE, P. 2010. Murine models of human neuropathic pain. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1802, 924-933.

COLTON, C. A., NEEDHAM, L. K., BROWN, C., COOK, D., RASHEED, K., BURKE, J. R., STRITTMATTER, W. J., SCHMECHEL, D. E. & VITEK, M. P. 2004. APOE genotype-specific differences in human and mouse macrophage nitric oxide production. *Journal of Neuroimmunology*, 147, 62-67.

COMBADIÈRE, C., POTTEAUX, S., RODERO, M., SIMON, T., PEZARD, A., ESPOSITO, B., MERVAL, R., PROUDFOOT, A., TEDGUI, A. & MALLAT, Z. 2008. Combined Inhibition of CCL2, CX3CR1, and CCR5 Abrogates Ly6Chi and Ly6Clo Monocytosis and Almost Abolishes Atherosclerosis in Hypercholesterolemic Mice. *Circulation*, 117, 1649-1657.

CORRIDEN, R. & INSEL, P. A. 2012. New insights regarding the regulation of chemotaxis by nucleotides, adenosine, and their receptors. *Purinergic Signal*, 8, 587-98.

CORRY, D. B., RISHI, K., KANELIS, J., KISS, A., SONG LZ, L. Z., XU, J., FENG, L., WERB, Z. & KHERADMAND, F. 2002. Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. *Nat Immunol*, 3, 347-53.

COULL, J. A., BEGGS, S., BOUDREAU, D., BOIVIN, D., TSUDA, M., INOUE, K., GRAVEL, C., SALTER, M. W. & DE KONINCK, Y. 2005. BDNF from microglia causes the shift in neuronal anion gradient underlying neuropathic pain. *Nature*, 438, 1017-1021.

COULL, J. A., BOURDREAU, D., BARCHAND, K., PRESCOTT, S. A., SILK, A., DE KONINCK, P. & DE KONINCK, Y. 2003. Trans-synaptic shift in anion gradient in spinal lamina I neurons as a mechanism of neuropathic pain. *Nature*, 21, 938-942.

COX, J. H., DEAN, R. A., ROBERTS, C. R. & OVERALL, C. M. 2008. Matrix metalloproteinase processing of CXCL11/I-TAC results in loss of chemoattractant activity and altered glycosaminoglycan binding. *J Biol Chem*, 283, 19389-99.

CUDABACK, E., LI, X., MONTINE, K. S., MONTINE, T. J. & KEENE, C. D. 2011. Apolipoprotein E isoform-dependent microglia migration. *FASEB J*, 25, 2082-91.

CUEVAS, J., RODRIGUEZ, A., BEHENSKY, A. & KATNIK, C. 2011. Afobazole modulates microglial function via activation of both sigma-1 and sigma-2 receptors. *J Pharmacol Exp Ther*, 339, 161-72.

CULLEY, K. L., HUI, W., BARTER, M. J., DAVIDSON, R. K., SWINGLER, T. E., DESTUMENT, A. P. M., SCOTT, J. L., DONELL, S. T., FENWICK, S., ROWAN, A. D., YOUNG, D. A. & CLARK, I. M. 2013. Class I Histone Deacetylase Inhibition Modulates Metalloproteinase Expression and Blocks Cytokine-Induced Cartilage Degradation. *Arthritis & Rheumatism*, 65, 1822-1830.

CURTISS, L. K. 2000. ApoE in Atherosclerosis : A Protein With Multiple Hats. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 20, 1852-1853.

DE LA PUENTE, B., NADAL, X., PORTILLO-SALIDO, E., SANCHEZ-ARROYOS, R., OVALLE, S., PALACIOS, G., MURO, A., ROMERO, L., ENTRENA, J. M., BAEYENS, J. M., LOPEZ-GARCIA, J. A., MALDONADO, R., ZAMANILLO, D. & VELA, J. M. 2009. Sigma-1 receptors regulate activity-induced spinal sensitization and neuropathic pain after peripheral nerve injury. *Pain*, 145, 294-303.

DE RIVERO VACCARI, J. P., BASTIEN, D., YURCISIN, G., PINEAU, I., DIETRICH, W. D., DE KONINCK, Y., KEANE, R. W. & LACROIX, S. 2012. P2X4 receptors influence inflammasome activation after spinal cord injury. *J Neurosci*, 32, 3058-66.

- DEAN, R. A., COX, J. H., BELLAC, C. L., DOUCET, A., STARR, A. E., OVERALL, C. M. 2008. Macrophage-specific metalloelastase (MMP-12) truncates and inactivates ELR+ CXC chemokines and generates CCL2, -7, -8, and -13 antagonists: potential role of the macrophage in terminating polymorphonuclear leukocyte influx. *Blood*, 112, 3455-64.
- DEROSA, G., CICERO, A. F., FOGARI, E., D'ANGELO, A., BONAVENTURA, A., ROMANO, D. & MAFFIOLI, P. 2012. Effects of n-3 PUFAs on postprandial variation of metalloproteinases, and inflammatory and insulin resistance parameters in dyslipidemic patients: evaluation with euglycemic clamp and oral fat load. *J Clin Lipidol*, 6, 553-64.
- DINGLEDINE, R., BORGES, K., BOWIE, D. & TRAYNELIS, S. F. 1999. The glutamate receptor ion channels. *Pharmacol Rev*, 51, 7-61.
- DOLLERY, C. M., MCEWAN, J. R. & HENNEY, A. M. 1995. Matrix Metalloproteinases and Cardiovascular Disease. *Circulation Research*, 77, 863-868.
- EATON, M. J., BLITS, B., RUITENBERG, M. J., VERHAAGEN, J. & OUDEGA, M. 2002. Amelioration of chronic neuropathic pain after partial nerve injury by adeno-associated viral (AAV) vector-mediated over-expression of BDNF in the rat spinal cord. *Gene Ther*, 9, 1387-95.
- ELLIOTT, M. R., CHEKENI, F. B., TRAMPONT, P. C., LAZAROWSKI, E. R., KADL, A., WALK, S. F., PARK, D., WOODSON, R. I., OSTANKOVICH, M., SHARMA, P., LYSIAK, J. J., HARDEN, T. K., LEITINGER, N. & RAVICHANDRAN, K. S. 2009. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature*, 461, 282-6.
- ELOSUA, R., ORDOVAS, J. M., CUPPLES, L. A., FOX, C. S., POLAK, J. F., WOLF, P. A., D'AGOSTINO, R. A., SR. & O'DONNELL, C. J. 2004. Association of APOE genotype with carotid atherosclerosis in men and women: the Framingham Heart Study. *J Lipid Res*, 45, 1868-75.
- EMING, S. A., KRIEG, T. & DAVIDSON, J. M. 2007. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol*, 127, 514-25.
- ENDRES-BECKER, J., HEPPENSTALL, P. A., MOUSA, S. A., LABUZ, D., OKSCHE, A., SCHAFER, M., STEIN, C. & ZOLLNER, C. 2007. Mu-opioid receptor activation modulates transient receptor potential vanilloid 1 (TRPV1) currents in sensory neurons in a model of inflammatory pain. *Mol Pharmacol*, 71, 12-8.
- FALK, E. 2006. Pathogenesis of Atherosclerosis. *Journal of the American College of Cardiology*, 47, C7-C12.
- FINNERUP, N. B., SINDRUP, S. H. & JENSEN, T. S. 2010. The evidence for pharmacological treatment of neuropathic pain. *PAIN*, 150, 573-581.
- FISHER, K. E., SACHARIDOU, A., STRATMAN, A. N., MAYO, A. M., FISHER, S. B., MAHAN, R. D., DAVIS, M. J. & DAVIS, G. E. 2009. MT1-MMP- and Cdc42-dependent signaling co-regulate cell invasion and tunnel formation in 3D collagen matrices. *J Cell Sci*, 122, 4558-69.
- FISHMAN, D. A., LIU, Y., ELLERBROEK, S. M. & STACK, M. S. 2001. Lysophosphatidic Acid Promotes Matrix Metalloproteinase (MMP) Activation and MMP-dependent Invasion in Ovarian Cancer Cells. *Cancer Research*, 61, 3194-3199.

- FRANCARDO, V., BEZ, F., WIELOCH, T., NISSBRANDT, H., RUSCHER, K. & CENCI, M. A. 2014. Pharmacological stimulation of sigma-1 receptors has neurorestorative effects in experimental parkinsonism. *Brain*.
- FREW, L., SUGIARTO, N. U., RAJAGOPAL, S. P., HE, J., LEASK, R., NORMAN, J. E., RILEY, S. C. & STOCK, S. J. 2013. The effect of omega-3 polyunsaturated fatty acids on the inflammatory response of the amnion. *Prostaglandins Leukot Essent Fatty Acids*, 89, 221-5.
- FUJIMOTO, M., HAYASHI, T., URFER, R., MITA, S. & SU, T. P. 2012. Sigma-1 receptor chaperones regulate the secretion of brain-derived neurotrophic factor. *Synapse*, 66, 630-9.
- GALIS, Z. S. & KHATRI, J. J. 2002. Matrix Metalloproteinases in Vascular Remodeling and Atherogenesis: The Good, the Bad, and the Ugly. *Circulation Research*, 90, 251-262.
- GALIS, Z. S., SUKHOVA, G. K., LARK, M. W. & LIBBY, P. 1994. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest*, 94, 2493-503.
- GILL, S. E. & PARKS, W. C. 2008. Metalloproteinases and their inhibitors: regulators of wound healing. *Int J Biochem Cell Biol*, 40, 1334-47.
- GONG, Y., HART, E., SHCHURIN, A. & HOOVER-PLOW, J. 2008. Inflammatory macrophage migration requires MMP-9 activation by plasminogen in mice. *The Journal of Clinical Investigation*, 118, 3012-3024.
- GONZALEZ-PEREZ, F., UDINA, E. & NAVARRO, X. 2013. Extracellular matrix components in peripheral nerve regeneration. *Int Rev Neurobiol*, 108, 257-75.
- GORDON, S. 2007. The macrophage: past, present and future. *Eur J Immunol*, 37 Suppl 1, S9-17.
- GREENOW, K., PEARCE, N. J. & RAMJI, D. P. 2005. The key role of apolipoprotein E in atherosclerosis. *J Mol Med (Berl)*, 83, 329-42.
- GRIMBLE, R. F. 2007. Nutritional modulation of immune function. *Proceedings of the Nutrition Society*, 60, 389-397.
- GROBLEWSKA, M., SIEWKO, M., MROCZKO, B. & SZMITKOWSKI, M. 2012. The role of matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) in the development of esophageal cancer. *Folia Histochem Cytobiol*, 50, 12-9.
- GU, B. J. & WILEY, J. S. 2006. Rapid ATP-induced release of matrix metalloproteinase 9 is mediated by the P2X7 receptor. *Blood*, 107, 4946-53.
- GUEBRE-EGZIABHER, F., DEBARD, C., DRAI, J., DENIS, L., PESENTI, S., BIENVENU, J., VIDAL, H., LAVILLE, M. & FOUQUE, D. 2013. Differential dose effect of fish oil on inflammation and adipose tissue gene expression in chronic kidney disease patients. *Nutrition*, 29, 730-6.
- GUIDETTI, G. F., LOVA, P., BERNARDI, B., CAMPUS, F., BALDANZI, G., GRAZIANI, A., BALDUINI, C. & TORTI, M. 2008. The Gi-coupled P2Y12 receptor regulates diacylglycerol-mediated signaling in human platelets. *J Biol Chem*, 283, 28795-805.

- GUIEU, R., PERAGUT, J. C., HASSANI, H., SAMPIERI, F., BECHIS, G., GOLLA, R. & ROCHAT, H. 1996. Adenosine and neuropathic pain. *Pain*, 68, 271-274..
- GUNS, P. J., HENDRICKX, J., VAN ASSCHE, T., FRANSEN, P. & BULT, H. 2010. P2Y receptors and atherosclerosis in apolipoprotein E-deficient mice. *Br J Pharmacol*, 159, 326-36.
- HAINS, B. C., SAAB, C. Y., KLEIN, J. P., CRANER, M. J. & WAXMAN, S. G. 2004. Altered sodium channel expression in second-order spinal sensory neurons contributes to pain after peripheral nerve injury. *J Neurosci*, 24, 4832-9.
- HALL, A. A., HERRERA, Y., AJMO, C. T., JR., CUEVAS, J. & PENNYPACKER, K. R. 2009. Sigma receptors suppress multiple aspects of microglial activation. *Glia*, 57, 744-54.
- HANOUNE, J. & DEFER, N. 2001. Regulation and role of adenylyl cyclase isoforms. *Annu Rev Pharmacol Toxicol*, 41, 145-74.
- HANSSON, G. K. 2005. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*, 352, 1685-95.
- HAQ, I., LOWREY, G. E., KALSHEKER, N. & JOHNSON, S. R. 2011. Matrix metalloproteinase-12 (MMP-12) SNP affects MMP activity, lung macrophage infiltration and protects against emphysema in COPD. *Thorax*, 66, 970-6.
- HASHIMOTO, K. 2013. Sigma-1 receptor chaperone and brain-derived neurotrophic factor: emerging links between cardiovascular disease and depression. *Prog Neurobiol*, 100, 15-29.
- HASKO, G. & CRONSTEIN, B. 2013. Regulation of inflammation by adenosine. *Front Immunol*, 4, 85.
- HASKÓ, G., PACHER, P., SYLVESTER VIZI, E. & ILLES, P. 2005. Adenosine receptor signaling in the brain immune system. *Trends in Pharmacological Sciences*, 26, 511-516.
- HAUSER, P. S., NARAYANASWAMI, V. & RYAN, R. O. 2011. Apolipoprotein E: from lipid transport to neurobiology. *Prog Lipid Res*, 50, 62-74.
- HAYNES, S. E., HOLLOPETER, G., YANG, G., KURPIUS, D., DAILEY, M. E., GAN, W. B. & JULIUS, D. 2006. The P2Y₁₂ receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci*, 9, 1512-9.
- HE, W. J., CUI, J., DU, L., ZHAO, Y. D., BURNSTOCK, G., ZHOU, H. D. & RUAN, H. Z. 2012. Spinal P2X₇ receptor mediates microglia activation-induced neuropathic pain in the sciatic nerve injury rat model. *Behav Brain Res*, 226, 163-70.
- HENNIGAN, R. F., HAWKER, K. L. & OZANNE, B. W. 1994. Fos-transformation activates genes associated with invasion. *Oncogene*, 9, 3591-3600.
- HERMANSSON, C., LUNDQVIST, A., MAGNUSSON, L. U., ULLSTROM, C., BERGSTROM, G. & HULTEN, L. M. 2014. Macrophage CD14 expression in human carotid plaques is associated with complicated lesions, correlates with thrombosis, and is reduced by angiotensin receptor blocker treatment. *Int Immunopharmacol*, 22, 318-323.

- HERNANDEZ-BARRANTES, S., BERNARDO, M., TOTH, M. & FRIDMAN, R. 2002. Regulation of membrane type-matrix metalloproteinases. *Semin Cancer Biol*, 12, 131-8.
- HETMAN, M., KANNING, K., CAVANAUGH, J. E. & XIA, Z. 1999. Neuroprotection by Brain-derived Neurotrophic Factor Is Mediated by Extracellular Signal-regulated Kinase and Phosphatidylinositol 3-Kinase. *The Journal of Biological Chemistry*, 274, 22569-22580.
- HONG, J., QIAN, T., LE, Q., SUN, X., WU, J., CHEN, J., YU, X. & XU, J. 2012. NGF promotes cell cycle progression by regulating D-type cyclins via PI3K/Akt and MAPK/Erk activation in human corneal epithelial cells. *Mol Vis*, 18, 758-64.
- HONORÉ, P. H., BASNET, A., ELJAJA, L., KRISTENSEN, P., ANDERSEN, L. M., NEUSTRUP, S., MØLLGAARD, P. & BJERRUM, O. J. 2010. Neuropathic pain models in the development of analgesic drugs. *Scandinavian Journal of Pain*, 2, 172-177.
- HOPPS, E. & CAIMI, G. 2012. Matrix metalloproteinases in metabolic syndrome. *Eur J Intern Med*, 23, 99-104.
- HORVATH, R. J. & DELEO, J. A. 2009. Morphine enhances microglial migration through modulation of P2X4 receptor signaling. *J Neurosci*, 29, 998-1005.
- HORWITZ, A. R. & PARSONS, J. T. 1999. Cell migration--movin' on. *Science*, 286, 1102-3.
- IDZKO, M., FERRARI, D., RIEGEL, A. K. & ELTZSCHIG, H. K. 2014. Extracellular nucleotide and nucleoside signaling in vascular and blood disease. *Blood*.
- IGLESIAS, P. A. & DEVREOTES, P. N. 2008. Navigating through models of chemotaxis. *Curr Opin Cell Biol*, 20, 35-40.
- INOUE, K. & TSUDA, M. 2009. Microglia and neuropathic pain. *Glia*, 57, 1469-79.
- INOUE, K. & TSUDA, M. 2012. Purinergic systems, neuropathic pain and the role of microglia. *Exp Neurol*, 234, 293-301.
- IRINO, Y., NAKAMURA, Y., INOUE, K., KOHSAKA, S. & OHSAWA, K. 2008. Akt activation is involved in P2Y12 receptor-mediated chemotaxis of microglia. *J Neurosci Res*, 86, 1511-9.
- ISFORT, K., EBERT, F., BORNHORST, J., SARGIN, S., KARDAKARIS, R., PASPARAKIS, M., BAHLER, M., SCHWERDTLE, T., SCHWAB, A. & HANLEY, P. J. 2011. Real-time imaging reveals that P2Y2 and P2Y12 receptor agonists are not chemoattractants and macrophage chemotaxis to complement C5a is phosphatidylinositol 3-kinase (PI3K)- and p38 mitogen-activated protein kinase (MAPK)-independent. *J Biol Chem*, 286, 44776-87.
- ITO, D., IMAI, Y., OHSAWA, K., NAKAJIMA, K., FUKUUCHI, Y. & KOHSAKA, S. 1998. Microglia-specific localisation of a novel calcium binding protein, Iba1. *Molecular Brain Research*, 57, 1-9.
- ITOH, Y. 2006. MT1-MMP: A key regulator of cell migration in tissue. *IUBMB Life*, 58, 589-596.
- ITZHAK, Y. 1989. Multiple affinity binding states of the sigma receptor: effect of GTP-binding protein-modifying agents. *Mol Pharmacol*, 36, 512-7.

- JAGGI, A. S., JAIN, V. & SINGH, N. 2011. Animal models of neuropathic pain. *Fundamental & Clinical Pharmacology*, 25, 1-28.
- JASMIN, L., VIT, J. P., BHARGAVA, A. & OHARA, P. T. 2010. Can satellite glial cells be therapeutic targets for pain control? *Neuron Glia Biol*, 6, 63-71.
- JEFFERIES, K. 2010. Treatment of Neuropathic Pain. *Semin Neurol*, 30, 425-432.
- JI, R. R., KOHNO, T., MOORE, K. A. & WOOLF, C. J. 2003. Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci*, 26, 696-705.
- JIN, S. X., ZHUANG, Z. Y., WOOLF, C. J. & JI, R. R. 2003. p38 Mitogen-Activated Protein Kinase Is Activated after a Spinal Nerve Ligation in Spinal Cord Microglia and Dorsal Root Ganglion Neurons and Contributes to the Generation of Neuropathic Pain. *The Journal of Neuroscience*, 23, 4017-4022.
- JOHNSON, J. L. & NEWBY, A. C. 2009. Macrophage heterogeneity in atherosclerotic plaques. *Curr Opin Lipidol*, 20, 370-8.
- JOHNSON, J. L., BAKER, A. H., OKA, K., CHAN, L., NEWBY, A. C., JACKSON, C. L. & GEORGE, S. J. 2006. Suppression of atherosclerotic plaque progression and instability by tissue inhibitor of metalloproteinase-2: involvement of macrophage migration and apoptosis. *Circulation*, 113, 2435-44.
- JOHNSON, J. L., DEVEL, L., CZARNY, B., GEORGE, S. J., JACKSON, C. L., ROGAKOS, V., BEAU, F., YIOTAKIS, A., NEWBY, A. C. & DIVE, V. 2011. A Selective Matrix Metalloproteinase-12 Inhibitor Retards Atherosclerotic Plaque Development in Apolipoprotein E-Knockout Mice. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31, 528-535.
- JULIUS, D. & BASBAUM, A. I. 2001. Molecular mechanisms of nociception. *Nature*, 413, 203-10.
- JUNG, H., BHANGOO, S., BANISADR, G., FREITAG, C., REN, D., WHITE, F. A. & MILLER, R. J. 2009. Visualization of chemokine receptor activation in transgenic mice reveals peripheral activation of CCR2 receptors in states of neuropathic pain. *J Neurosci*, 29, 8051-62.
- KANAI, S., UTO, K., HONDA, K., HAGIWARA, N. & ODA, H. 2011. Eicosapentaenoic acid reduces warfarin-induced arterial calcification in rats. *Atherosclerosis*, 215, 43-51.
- KANDEL, E. S., JH. JESSEL, TM. 2000. *Principles of Neural Science*, 4th Edition. McGraw-Hill, New York.
- KAWAMURA, H., KAWAMURA, T., KANDA, Y., KOBAYASHI, T. & ABO, T. 2012. Extracellular ATP-stimulated macrophages produce macrophage inflammatory protein-2 which is important for neutrophil migration. *Immunology*, 136, 448-58.
- KAWASAKI, Y., XU, Z. Z., WANG, X., PARK, J. Y., ZHUANG, Z. Y., TAN, P. H., GAO, Y. J., ROY, K., CORFAS, G., LO, E. H. & JI, R. R. 2008. Distinct roles of matrix metalloproteases in the early- and late-phase development of neuropathic pain. *Nat Med*, 14, 331-6.
- KERR, B. J., BRADBURY, E. J., BENNETT, D. L. H., TRIVEDI, P. M., DASSAN, P., FRENCH, J., SHELTON, D. B., MCMAHON, S. B. & THOMPSON, S. W. N. 1999. Brain-Derived Neurotrophic Factor

Modulates Nociceptive Sensory Inputs and NMDA-Evoked Responses in the Rat Spinal Cord. *The Journal of Neuroscience*, 19, 5138-5148.

KESSENBROCK, K., PLAKS, V. & WERB, Z. 2010. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*, 141, 52-67.

KETELHUTH, D. J. & BÄCK, M. 2011. The Role of Matrix Metalloproteinases in Atherothrombosis. *Current Atherosclerosis Reports*, 13, 162-169.

KHOKHA, R., MURTHY, A. & WEISS, A. 2013. Metalloproteinases and their natural inhibitors in inflammation and immunity. *Nat Rev Immunol*, 13, 649-65.

KIBALY, C., MEYER, L., PATTE-MENSAH, C. & MENSAH-NYAGAN, A. G. 2008. Biochemical and functional evidence for the control of pain mechanisms by dehydroepiandrosterone endogenously synthesized in the spinal cord. *The FASEB Journal*, 22, 93-104.

KIM, H. J., AJIT, D., PETERSON, T. S., WANG, Y., CAMDEN, J. M., GIBSON WOOD, W., SUN, G. Y., ERB, L., PETRIS, M. & WEISMAN, G. A. 2012. Nucleotides released from Abeta(1-)(4)(2)-treated microglial cells increase cell migration and Abeta(1-)(4)(2) uptake through P2Y(2) receptor activation. *J Neurochem*, 121, 228-38.

KIM, K. & CHUNG, J. 1997. Comparison of three rodent neuropathic pain models. *Experimental Brain Research*, 113, 200-206.

KIM, M., JANG, H. R., KIM, J. H., NOH, S. M., SONG, K. S., CHO, J. S., JEONG, H. Y., NORMAN, J. C., CASWELL, P. T., KANG, G. H., KIM, S. Y., YOO, H. S. & KIM, Y. S. 2008. Epigenetic inactivation of protein kinase D1 in gastric cancer and its role in gastric cancer cell migration and invasion. *Carcinogenesis*, 29, 629-37.

KIM, Y., REMACLE, A. G., CHERNOV, A. V., LIU, H., SHUBAYEV, I., LAI, C., DOLKAS, J., SHIRYAEV, S. A., GOLUBKOV, V. S., MIZISIN, A. P., STRONGIN, A. Y. & SHUBAYEV, V. I. 2012. The MMP-9/TIMP-1 axis controls the status of differentiation and function of myelin-forming Schwann cells in nerve regeneration. *PLoS One*, 7, e33664.

KLEEMANN, R., ZADELAAR, S. & KOOISTRA, T. 2008. Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovasc Res*, 79, 360-76.

KNOUFF, C., HINSDALE, M. E., MEZDOUR, H., ALTENBURG, M. K., WATANABE, M., QUARFORDT, S. H., SULLIVAN, P. M. & MAEDA, N. 1999. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *J Clin Invest*, 103, 1579-86.

KOFLER, B. M., MILES, E. A., CURTIS, P., ARMAH, C. K., TRICON, S., GREW, J., NAPPER, F. L., FARRELL, L., LIETZ, G., PACKARD, C. J., CASLAKE, M. J., MATHERS, J. C., WILLIAMS, C. M., CALDER, P. C. & MINIHANE, A. M. 2012. Apolipoprotein E genotype and the cardiovascular disease risk phenotype: impact of sex and adiposity (the FINGEN study). *Atherosclerosis*, 221, 467-70.

KOIZUMI, S., OHSAWA, K., INOUE, K. & KOHSAKA, S. 2013. Purinergic receptors in microglia: Functional modal shifts of microglia mediated by P2 and P1 receptors. *Glia*, 61, 47-54.

- KOIZUMI, S., SHIGEMOTO-MOGAMI, Y., NASU-TADA, K., SHINOZAKI, Y., OHSAWA, K., TSUDA, M., JOSHI, B. V., JACOBSON, K. A., KOHSAKA, S. & INOUE, K. 2007. UDP acting at P2Y6 receptors is a mediator of microglial phagocytosis. *Nature*, 446, 1091-5.
- KONNECKE, H. & BECHMANN, I. 2013. The role of microglia and matrix metalloproteinases involvement in neuroinflammation and gliomas. *Clin Dev Immunol*, 2013, 914104.
- KONSTANTINOV, I. E., MEJEVOI, N. & ANICHKOV, N. M. 2006. Nikolai N. Anichkov and his theory of atherosclerosis. *Tex Heart Inst J*, 33, 417-23.
- KOOPMANS, G., HASSE, B. & SINIS, N. 2009. Chapter 19 The Role of Collagen in Peripheral Nerve Repair. In: STEFANO, G., PIERLUIGI, T. & BRUNO, B. (eds.) *International Review of Neurobiology*. Academic Press.
- KOUPENOVA, M., JOHNSTON-COX, H. & RAVID, K. 2012. Regulation of atherosclerosis and associated risk factors by adenosine and adenosine receptors. *Curr Atheroscler Rep*, 14, 460-8.
- KRAUSE, K. H. 2000. Professional phagocytes: predators and prey of microorganisms. *Schweiz Med Wochenschr*, 29, 97-100.
- KRONLAGE, M., SONG, J., SOROKIN, L., ISFORT, K., SCHWERDTLE, T., LEIPZIGER, J., ROBAYE, B., CONLEY, P. B., KIM, H. C., SARGIN, S., SCHON, P., SCHWAB, A. & HANLEY, P. J. 2010. Autocrine purinergic receptor signaling is essential for macrophage chemotaxis. *Sci Signal*, 3, ra55.
- KUMADA, M., KIHARA, S., OUCHI, N., KOBAYASHI, H., OKAMOTO, Y., OHASHI, K., MAEDA, K., NAGARETANI, H., KISHIDA, K., MAEDA, N., NAGASAWA, A., FUNAHASHI, T. & MATSUZAWA, Y. 2004. Adiponectin specifically increased tissue inhibitor of metalloproteinase-1 through interleukin-10 expression in human macrophages. *Circulation*, 109, 2046-9.
- KURPIUS, D., NOLLEY, E. P. & DAILEY, M. E. 2007. Purines induce directed migration and rapid homing of microglia to injured pyramidal neurons in developing hippocampus. *Glia*, 55, 873-84.
- KUZNIEWSKA, B., REJMAK, E., MALIK, A. R., JAWORSKI, J., KACZMAREK, L. & KALITA, K. 2013. Brain-derived neurotrophic factor induces matrix metalloproteinase 9 expression in neurons via the serum response factor/c-Fos pathway. *Mol Cell Biol*, 33, 2149-62.
- LA ROCCA, R. V., DANESI, R., COOPER, M. R., JAMIS-DOW, C. A., EWING, M. W., LINEHAM, W. M. & MYERS, C. E. 1991. Effect of Suramin on human prostate cancer cells in vitro. *Journal of Urology*, 145, 393-8.
- LAKHAN, S. E. & AVRAMUT, M. 2012. Matrix metalloproteinases in neuropathic pain and migraine: friends, enemies, and therapeutic targets. *Pain Res Treat*, 2012, 952906.
- LAMBERT, C., ASE, A. R., SEQUELA, P. & ANTEL, J. P. 2010. Distinct migratory and cytokine responses of human microglia and macrophages to ATP. *Brain Behav Immun*, 24, 1241-8.
- LANCELOT, E., AMIRBEKIAN, V., BRIGGER, I., RAYNAUD, J.-S., BALLEST, S., DAVID, C., ROUSSEAU, O., LE GRENEUR, S., PORT, M., LIJNEN, H. R., BRUNEVAL, P., MICHEL, J.-B., OUIMET, T., ROQUES, B., AMIRBEKIAN, S., HYAFIL, F., VUCIC, E., AGUINALDO, J. G. S., COROT, C. & FAYAD, Z. A. 2008. Evaluation of Matrix Metalloproteinases in Atherosclerosis Using a Novel Noninvasive Imaging Approach. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 28, 425-432.

- LATREMOLIERE, A. & WOOLF, C. J. 2009. Central sensitization: a generator of pain hypersensitivity by central neural plasticity. *J Pain*, 10, 895-926.
- LAXTON, R. C., HU, Y., DUCHENE, J., ZHANG, F., ZHANG, Z., LEUNG, K. Y., XIAO, Q., SCOTLAND, R. S., HODGKINSON, C. P., SMITH, K., WILLEIT, J., LOPEZ-OTIN, C., SIMPSON, I. A., KIECHL, S., AHLUWALIA, A., XU, Q. & YE, S. 2009. A role of matrix metalloproteinase-8 in atherosclerosis. *Circ Res*, 105, 921-9.
- LEE, Y. L., CH. OH, U. 2005. Painful Channels in Sensory Neurons. *Molecules and Cells*, 20, 315-324.
- LEVANT, B. & NEMEROFF, C. B. 1990. Sigma receptor "antagonist" BMY 14802 increases neurotensin concentrations in the rat nucleus accumbens and caudate. *J Pharmacol Exp Ther*, 254, 330-5.
- LEVERRIER, Y. & RIDLEY, A. J. 2001. Requirement for Rho GTPases and PI 3-kinases during apoptotic cell phagocytosis by macrophages. *Curr Biol*, 11, 195-9.
- LEWIN, G. R., RUEFF, A. & MENDELL, L. M. 1994. Peripheral and central mechanisms of NGF-induced hyperalgesia. *Eur J Neurosci*, 6, 1903-12.
- LI, C. H., CHENG, Y. W., LIAO, P. L., YANG, Y. T. & KANG, J. J. 2010. Chloramphenicol causes mitochondrial stress, decreases ATP biosynthesis, induces matrix metalloproteinase-13 expression, and solid-tumor cell invasion. *Toxicol Sci*, 116, 140-50.
- LI, N. N., ZHOU, Y., QIN, X. P., CHEN, Y., HE, D., FENG, J. Y. & WU, X. T. 2014a. Does intravenous fish oil benefit patients post-surgery? A meta-analysis of randomised controlled trials. *Clin Nutr*, 33, 226-39.
- LI, X., ZHANG, Y., XIA, M., GULBINS, E., BOINI, K. M. & LI, P. L. 2014b. Activation of Nlrp3 inflammasomes enhances macrophage lipid-deposition and migration: implication of a novel role of inflammasome in atherogenesis. *PLoS One*, 9, e87552.
- LIBBY, P., RIDKER, P. M. & MASERI, A. 2002. Inflammation and Atherosclerosis. *Circulation*, 105, 1135-1143.
- LIPPITZ, B. E. 2013. Cytokine patterns in patients with cancer: a systematic review. *Lancet Oncol*, 14, e218-28.
- LIVERANI, E., KILPATRICK, L. E., TSYGANKOV, A. Y. & KUNAPULI, S. P. 2014. The role of P2Y₁(2) receptor and activated platelets during inflammation. *Curr Drug Targets*, 15, 720-8.
- LOBOV, I. B., RAO, S., CARROLL, T. J., VALLANCE, J. E., ITO, M., ONDR, J. K., KURUP, S., GLASS, D. A., PATEL, M. S., SHU, W., MORRISEY, E. E., MCMAHON, A. P., KARSENTY, G. & LANG, R. A. 2005. WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. *Nature*, 437, 417-421.
- LODISH, H., BERK, A., ZIPURSKY, S. L. 2000. *Molecular Cell Biology*, 4th Edition. W. H. Freeman.
- LORAM, L. C., HARRISON, J. A., SLOANE, E. M., HUTCHINSON, M. R., SHOLAR, P., TAYLOR, F. R., BERKELHAMMER, D., COATS, B. D., POOLE, S., MILLIGAN, E. D., MAIER, S. F., RIEGER, J. &

- WATKINS, L. R. 2009. Enduring reversal of neuropathic pain by a single intrathecal injection of adenosine 2A receptor agonists: a novel therapy for neuropathic pain. *J Neurosci*, 29, 14015-25.
- LU, V. B., BIGGS, J. E., STEBBING, M. J., BALASUBRAMANYAN, S., TODD, K. G., LAI, A. Y., COLMERS, W. F., DAWBARN, D., BALLANYI, K. & SMITH, P. A. 2009. Brain-derived neurotrophic factor drives the changes in excitatory synaptic transmission in the rat superficial dorsal horn that follow sciatic nerve injury. *J Physiol*, 587, 1013-32.
- LUU, N. T., MADDEN, J., CALDER, P. C., GRIMBLE, R. F., SHEARMAN, C. P., CHAN, T., DASTUR, N., HOWELL, W. M., RAINGER, G. E. & NASH, G. B. 2007. Dietary supplementation with fish oil modifies the ability of human monocytes to induce an inflammatory response. *J Nutr*, 137, 2769-74.
- MA, J., HARNETT, K. M., BEHAR, J., BIANCANI, P. & CAO, W. 2010. Signaling in TRPV1-induced platelet activating factor (PAF) in human esophageal epithelial cells.
- MAHLEY, R. W. & RALL, S. C., JR. 2000. Apolipoprotein E: far more than a lipid transport protein. *Annu Rev Genomics Hum Genet*, 1, 507-37.
- MAHLEY, R. W. 1988. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*, 240, 622-30.
- MAHLEY, R. W., WEISGRABER, K. H. & HUANG, Y. 2009. Apolipoprotein E: structure determines function, from atherosclerosis to Alzheimer's disease to AIDS. *J Lipid Res*, 50 Suppl, S183-8.
- MALMBERG, A. B. & BASBAUM, A. I. 1998. Partial sciatic nerve injury in the mouse as a model of neuropathic pain: behavioral and neuroanatomical correlates. *Pain*, 76, 215-222.
- MAMEDOVA, L. K., JOSHI, B. V., GAO, Z. G., VON KUGELGEN, I. & JACOBSON, K. A. 2004. Diisothiocyanate derivatives as potent, insurmountable antagonists of P2Y6 nucleotide receptors. *Biochem Pharmacol*, 67, 1763-70.
- MARAI, I. & MASSALHA, S. 2014. Effect of omega-3 polyunsaturated fatty acids and vitamin D on cardiovascular diseases. *Isr Med Assoc J*, 16, 117-21.
- MARTIN, W. R., EADES, C. G., THOMPSON, J. A., HUPPLER, R. E. & GILBERT, P. E. 1976. The effects of morphine- and nalorphine- like drugs in the nondependent and morphine-dependent chronic spinal
- MAURICE, T. & LOCKHART, B. P. 1997. Neuroprotective and anti-amnesic potentials of sigma (sigma) receptor ligands. *Prog Neuropsychopharmacol Biol Psychiatry*, 21, 69-102.
- MAURICE, T. & SU, T. P. 2009. The pharmacology of sigma-1 receptors. *Pharmacol Ther*, 124, 195-206.
- MCCAWLEY, L. J. & MATRISIAN, L. M. 2001. Matrix metalloproteinases: they're not just for matrix anymore! *Current Opinion in Cell Biology*, 13, 534-540.
- MEGALIZZI, V., MATHIEU, V., MIJATOVIC, T., GAILLY, P., DEBEIR, O., DE NEVE, N., VAN DAMME, M., BONTEMPI, G., HAIBE-KAINS, B., DECAESTECKER, C., KONDO, Y., KISS, R. & LEFRANC, F. 2007. 4-IBP, a sigma1 receptor agonist, decreases the migration of human cancer cells, including

- glioblastoma cells, in vitro and sensitizes them in vitro and in vivo to cytotoxic insults of proapoptotic and proautophagic drugs. *Neoplasia*, 9, 358-69.
- MEI, J. & PASTERNAK, G. W. 2002. Sigma1 receptor modulation of opioid analgesia in the mouse. *J Pharmacol Exp Ther*, 300, 1070-4.
- MICÓ, J. A., ARDID, D., BERROCOSO, E. & ESCHALIER, A. 2006. Antidepressants and pain. *Trends in Pharmacological Sciences*, 27, 348-354.
- MILLER, R. J., ROSTENE, W., APARTIS, E., BANISADR, G., BIBER, K., MILLIGAN, E. D., WHITE, F. A. & ZHANG, J. 2008. Chemokine action in the nervous system. *J Neurosci*, 28, 11792-5.
- MILLS, C. D. 2012. M1 and M2 Macrophages: Oracles of Health and Disease. *Crit Rev Immunol*, 32, 463-88.
- MIYAZAWA, Y., UEKITA, T., ITO, Y., SEIKI, M., YAMAGUCHI, H. & SAKAI, R. 2013. CDCP1 regulates the function of MT1-MMP and invadopodia-mediated invasion of cancer cells. *Mol Cancer Res*, 11, 628-37.
- MIZOGUCHI, Y., KATO, T. A., SEKI, Y., OHGIDANI, M., SAGATA, N., HORIKAWA, H., YAMAUCHI, Y., SATO-KASAI, M., HAYAKAWA, K., INOUE, R., KANBA, S. & MONJI, A. 2014. Brain-derived Neurotrophic Factor (BDNF) Induces Sustained Intracellular Ca²⁺ Elevation through the Up-regulation of Surface Transient Receptor Potential 3 (TRPC3) Channels in Rodent Microglia. *J Biol Chem*, 289, 18549-55.
- MONK, J. M., HOU, T. Y., TURK, H. F., MCMURRAY, D. N. & CHAPKIN, R. S. 2013. n3 PUFAs reduce mouse CD4+ T-cell ex vivo polarization into Th17 cells. *J Nutr*, 143, 1501-8.
- MOON, J. Y., ROH, D. H., YOON, S. Y., KANG, S. Y., CHOI, S. R., KWON, S. G., CHOI, H. S., HAN, H. J., BEITZ, A. J. & LEE, J. H. 2013. Sigma-1 receptor-mediated increase in spinal p38 MAPK phosphorylation leads to the induction of mechanical allodynia in mice and neuropathic rats. *Exp Neurol*, 247, 383-91.
- MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*, 8, 958-69.
- MURPHY, G. & GAVRILOVIC, J. 1999. Proteolysis and cell migration: creating a path? *Curr Opin Cell Biol*, 11, 614-21.
- MURPHY, G. & NAGASE, H. 2008. Progress in matrix metalloproteinase research. *Mol Aspects Med*, 29, 290-308.
- MURPHY, G. 2011. Tissue inhibitors of metalloproteinases. *Genome Biol*, 12, 233.
- MURPHY, N. & LYNCH, M. A. 2012. Activation of the P2X7 receptor induces migration of glial cells by inducing cathepsin B degradation of tissue inhibitor of metalloproteinase 1. *Journal of Neurochemistry*, 123, 761-770.
- MURRAY, M. Y., BIRKLAND, T. P., HOWE, J. D., ROWAN, A. D., FIDOCK, M., PARKS, W. C. & GAVRILOVIC, J. 2013. Macrophage migration and invasion is regulated by MMP10 expression. *PLoS One*, 8, e63555.

- MURRAY, M. Y., BIRKLAND, T. P., HOWE, J. D., ROWAN, A. D., FIDOCK, M., PARKS, W. C. & GAVRILOVIC, J. 2013. Macrophage migration and invasion is regulated by MMP10 expression. *PLoS One*, 8, e63555.
- MURRAY, M. Y., BIRKLAND, T. P., HOWE, J. D., ROWAN, A. D., FIDOCK, M., PARKS, W. C. & GAVRILOVIC, J. 2013. Macrophage migration and invasion is regulated by MMP10 expression. *PLoS One*, 8, e63555.
- NABESHIMA, K., INOUE, T., SHIMAO, Y. & SAMESHIMA, T. 2002. Matrix metalloproteinases in tumor invasion: role for cell migration. *Pathol Int*, 52, 255-64.
- NAGAHARA, A. H., MERRILL, D. A., COPPOLA, G., TSUKADA, S., SCHROEDER, B. E., SHAKED, G. M., WANG, L., BLESCH, A., KIM, A., CONNER, J. M., ROCKENSTEIN, E., CHAO, M. V., KOO, E. H., GESCHWIND, D., MASLIAH, E., CHIBA, A. A. & TUSZYNSKI, M. H. 2009. Neuroprotective effects of brain-derived neurotrophic factor in rodent and primate models of Alzheimer's disease. *Nat Med*, 15, 331-7.
- NAGASE, H., VISSE, R. & MURPHY, G. 2006. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc Res*, 69, 562-73.
- NASU-TADA, K., KOIZUMI, S. & INOUE, K. 2005. Involvement of beta1 integrin in microglial chemotaxis and proliferation on fibronectin: different regulations by ADP through PKA. *Glia*, 52, 98-107.
- NASU-TADA, K., KOIZUMI, S., TSUDA, M., KUNIFUSA, E. & INOUE, K. 2006. Possible involvement of increase in spinal fibronectin following peripheral nerve injury in upregulation of microglial P2X4, a key molecule for mechanical allodynia. *Glia*, 53, 769-75.
- NAVARATNA, D., FAN, X., LEUNG, W., LOK, J., GUO, S., XING, C., WANG, X. & LO, E. H. 2013. Cerebrovascular degradation of TRKB by MMP9 in the diabetic brain. *J Clin Invest*, 123, 3373-7.
- NEMOTO, E., GOTOH, K., TSUCHIYA, M., SAKISAKA, Y. & SHIMAUCHI, H. 2013. Extracellular ATP inhibits IL-1-induced MMP-1 expression through the action of CD39/nucleotidase triphosphate dephosphorylase-1 on human gingival fibroblasts. *Int Immunopharmacol*, 17, 513-8.
- NEWBY, A. C. 2005. Dual Role of Matrix Metalloproteinases (Matrixins) in Intimal Thickening and Atherosclerotic Plaque Rupture.
- NEWBY, A. C. 2006. Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. *Cardiovascular Research*, 69, 614-624.
- NIETO, F. R., CENDAN, C. M., SANCHEZ-FERNANDEZ, C., COBOS, E. J., ENTRENA, J. M., TEJADA, M. A., ZAMANILLO, D., VELA, J. M. & BAEYENS, J. M. 2012. Role of sigma-1 receptors in paclitaxel-induced neuropathic pain in mice. *J Pain*, 13, 1107-21.
- NITZAN-LUQUES, A., DEVOR, M. & TAL, M. 2011. Genotype-selective phenotypic switch in primary afferent neurons contributes to neuropathic pain. *PAIN*, 152, 2413-2426.
- NOLD, M., GOEDE, A., EBERHARDT, W., PFEILSCHIFTER, J. & MUHL, H. 2003. IL-18 initiates release of matrix metalloproteinase-9 from peripheral blood mononuclear cells without affecting tissue

inhibitor of matrix metalloproteinases-1: suppression by TNF alpha blockage and modulation by IL-10. *Naunyn Schmiedebergs Arch Pharmacol*, 367, 68-75.

NUTTALL, R. K., SILVA, C., HADER, W., BAR-OR, A., PATEL, K. D., EDWARDS, D. R. & YONG, V. W. 2007. Metalloproteinases are enriched in microglia compared with leukocytes and they regulate cytokine levels in activated microglia. *Glia*, 55, 516-26.

OH, S. B., TRAN, P. B., GILLARD, S. E., HURLEY, R. W., HAMMOND, D. L. & MILLER, R. J. 2001. Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. *J Neurosci*, 21, 5027-35.

OHARA, Y., PETERSON, T. E., SAYEGH, H. S., SUBRAMANIAN, R. R., WILCOX, J. N. & HARRISON, D. G. 1995. Dietary correction of hypercholesterolemia in the rabbit normalizes endothelial superoxide anion production. *Circulation*, 92, 898-903.

OHSAWA, K., IRINO, Y., SANAGI, T., NAKAMURA, Y., SUZUKI, E., INOUE, K. & KOHSAKA, S. 2010. P2Y12 receptor-mediated integrin-beta1 activation regulates microglial process extension induced by ATP. *Glia*, 58, 790-801.

OKUBO, M., YAMANAKA, H., KOBAYASHI, K., KANDA, H., DAI, Y. & NOGUCHI, K. 2012. Up-regulation of platelet-activating factor synthases and its receptor in spinal cord contribute to development of neuropathic pain following peripheral nerve injury. *Mol Pain*, 8, 8.

ORTEGA, F., PEREZ-SEN, R., MORENTE, V., DELICADO, E. G. & MIRAS-PORTUGAL, M. T. 2010. P2X7, NMDA and BDNF receptors converge on GSK3 phosphorylation and cooperate to promote survival in cerebellar granule neurons. *Cell Mol Life Sci*, 67, 1723-33.

PALMER, C. P., MAHEN, R., SCHNELL, E., DJAMGOZ, M. B. & AYDAR, E. 2007. Sigma-1 receptors bind cholesterol and remodel lipid rafts in breast cancer cell lines. *Cancer Res*, 67, 11166-75.

PARIKH, P., MCDANIEL, M. C., ASHEN, M. D., MILLER, J. I., SORRENTINO, M., CHAN, V., BLUMENTHAL, R. S. & SPERLING, L. S. 2005. Diets and Cardiovascular Disease An Evidence-Based Assessment. *Journal of the American College of Cardiology*, 45, 1379-1387.

PARKS, W. C., WILSON, C. L. & LOPEZ-BOADO, Y. S. 2004. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol*, 4, 617-29.

PASERO, C. 2004. Pathophysiology of neuropathic pain. *Pain Manag Nurs*, 5, 3-8.

PENG, C.-H., CHIOU, S.-H., CHEN, S.-J., CHOU, Y.-C., KU, H.-H., CHENG, C.-K., YEN, C.-J., TSAI, T.-H., CHANG, Y.-L. & KAO, C.-L. 2008. Neuroprotection by Imipramine against lipopolysaccharide-induced apoptosis in hippocampus-derived neural stem cells mediated by activation of BDNF and the MAPK pathway. *European Neuropsychopharmacology*, 18, 128-140.

PEREZ-SAYANS GARCIA, M., SUAREZ-PENARANDA, J. M., GAYOSO-DIZ, P., BARROS-ANGUEIRA, F., GANDARA-REY, J. M. & GARCIA-GARCIA, A. 2012. Tissue inhibitor of metalloproteinases in oral squamous cell carcinomas - a therapeutic target? *Cancer Lett*, 323, 11-9.

PETH, G. & REEH, P. W. 2012. Sensory and Signaling Mechanisms of Bradykinin, Eicosanoids, Platelet-Activating Factor, and Nitric Oxide in Peripheral Nociceptors.

- PEVIANI, M., SALVANESCHI, E., BONTEMPI, L., PETESE, A., MANZO, A., ROSSI, D., SALMONA, M., COLLINA, S., BIGINI, P. & CURTI, D. 2014. Neuroprotective effects of the Sigma-1 receptor (S1R) agonist PRE-084, in a mouse model of motor neuron disease not linked to SOD1 mutation. *Neurobiol Dis*, 62, 218-32.
- PEZET, S. 2002. Noxious Stimulation Induces Trk Receptor and Downstream ERK Phosphorylation in Spinal Dorsal Horn. *Molecular and Cellular Neuroscience*, 21, 684-695.
- QUILLARD, T., ARAUJO, H. A., FRANCK, G., TESMENITSKY, Y. & LIBBY, P. 2014. Matrix Metalloproteinase-13 Predominates Over Matrix Metalloproteinase-8 as the Functional Interstitial Collagenase in Mouse Atheromata. *Arterioscler Thromb Vasc Biol*.
- QUIRION, R., BOWEN, W. D., ITZHAK, Y., JUNIEN, J. L., MUSACCHIO, J. M., ROTHMAN, R. B., SU, T. P., TAM, S. W. & TAYLOR, D. P. 1992. A proposal for the classification of sigma binding sites. *Trends Pharmacol Sci*, 13, 85-6.
- RALEVIC, V. & BURNSTOCK, G. 1998. Receptors for purines and pyrimidines. *Pharmacol Rev*, 50, 413-92.
- RAMESH, G., MACLEAN, A. G. & PHILIPP, M. T. 2013. Cytokines and chemokines at the crossroads of neuroinflammation, neurodegeneration, and neuropathic pain. *Mediators Inflamm*, 2013, 480739.
- RANDOLPH, G. J. 2014. Mechanisms that regulate macrophage burden in atherosclerosis. *Circ Res*, 114, 1757-71.
- RIDLEY, A. J., SCHWARTZ, M. A., BURRIDGE, K., FIRTEL, R. A., GINSBERG, M. H., BORISY, G., PARSONS, J. T. & HORWITZ, A. R. 2003. Cell Migration: Integrating Signals from Front to Back. *Science*, 302, 1704-1709.
- RISTOIU, V. 2013. Contribution of macrophages to peripheral neuropathic pain pathogenesis. *Life Sci*, 93, 870-81.
- ROBINSON, S.C., S. K. A., BALKWILL, F. R. 2002. Chemokinin stimulation of monocyte matrix metalloproteinase-9 requires endogenous TNF-alpha. *Eur J Immunol*, 32, 404-412.
- ROH, D.-H., CHOI, S.-R., YOON, S.-Y., KANG, S.-Y., MOON, J.-Y., KWON, S.-G., HAN, H.-J., BEITZ, A. J. & LEE, J.-H. 2011. Spinal neuronal NOS activation mediates sigma-1 receptor-induced mechanical and thermal hypersensitivity in mice: involvement of PKC-dependent GluN1 phosphorylation. *British Journal of Pharmacology*, 163, 1707-1720.
- ROMERO, L., ZAMANILLO, D., NADAL, X., SANCHEZ-ARROYOS, R., RIVERA-ARCONADA, I., DORDAL, A., MONTERO, A., MURO, A., BURRA, A., SEGALES, C., LALOYA, M., HERNANDEZ, E., PORTILLO-SALIDO, E., ESCRICHE, M., CODONY, X., ENCINA, G., BURGUENO, J., MERLOS, M., BAEYENS, J. M., GIRALDO, J., LOPEZ-GARCIA, J. A., MALDONADO, R., PLATA-SALAMAN, C. R. & VELA, J. M. 2012. Pharmacological properties of S1RA, a new sigma-1 receptor antagonist that inhibits neuropathic pain and activity-induced spinal sensitization. *Br J Pharmacol*, 166, 2289-306.
- ROSS, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature*, 362, 801-809.

- ROSS, R. 1999. Atherosclerosis — An Inflammatory Disease. *New England Journal of Medicine*, 340, 115-126.
- ROSS, R., GLOMSET, J. & HARKER, L. 1977. Response to injury and atherogenesis. *Am J Pathol*, 86, 675-84.
- RUSCHER, K., SHAMLOO, M., RICKHAG, M., LADUNGA, I., SORIANO, L., GISSELISSON, L., TORESSON, H., RUSLIM-LITRUS, L., OKSENBERG, D., URFER, R., JOHANSSON, B. B., NIKOLICH, K. & WIELOCH, T. 2011. The sigma-1 receptor enhances brain plasticity and functional recovery after experimental stroke. *Brain*, 134, 732-46.
- SALVAMANI, S., GUNASEKARAN, B., SHAHARUDDIN, N. A., AHMAD, S. A. & SHUKOR, M. Y. 2014. Antiatherosclerotic effects of plant flavonoids. *Biomed Res Int*, 2014, 480258.
- SANDERSON, P. & CALDER, P. C. 1998. Dietary fish oil diminishes lymphocyte adhesion to macrophage and endothelial cell monolayers. *Immunology*, 94, 79-87.
- SANDHIR, R., GREGORY, E., HE, Y. Y. & BERMAN, N. E. 2011. Upregulation of inflammatory mediators in a model of chronic pain after spinal cord injury. *Neurochem Res*, 36, 856-62.
- SANDKUHLER, J. 2009. Models and mechanisms of hyperalgesia and allodynia. *Physiol Rev*, 89, 707-58.
- SCADDING, J. W. 2004. Treatment of neuropathic pain: historical aspects. *Pain Med*, 5 Suppl 1, S3-8.
- SCHÄFERS, M., SCHOBER, O. & HERMANN, S. 2010. Matrix-Metalloproteinases as Imaging Targets for Inflammatory Activity in Atherosclerotic Plaques. *Journal of Nuclear Medicine*, 51, 663-666.
- SCHOLTES, V. P. W., JOHNSON, J. L., JENKINS, N., SALA-NEWBY, G. B., DE VRIES, J.-P. P. M., BORST, G. J. D., DE KLEIJN, D. P. V., MOLL, F. L., PASTERKAMP, G. & NEWBY, A. C. 2012. Carotid Atherosclerotic Plaque Matrix Metalloproteinase-12–Positive Macrophage Subpopulation Predicts Adverse Outcome After Endarterectomy. *Journal of the American Heart Association*, 1.
- SCHOLZ, J. & WOOLF, C. J. 2007. The neuropathic pain triad: neurons, immune cells and glia. *Nat Neurosci*, 10, 1361-1368.
- SCHÖNBECK, U., MACH, F. & LIBBY, P. 1998. Generation of Biologically Active IL-1 β by Matrix Metalloproteinases: A Novel Caspase-1-Independent Pathway of IL-1 β Processing. *The Journal of Immunology*, 161, 3340-3346.
- SELTZER, Z. E., DUBNER, R. & SHIR, Y. 1990. A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain*, 43, 205-218.
- SEMEL, D., MURPHY, T. K., ZLATEVA, G., CHEUNG, R. & EMIR, B. 2010. Evaluation of the safety and efficacy of pregabalin in older patients with neuropathic pain: results from a pooled analysis of 11 clinical studies. *BMC Fam Pract*, 11, 85.
- SHIN, S. M., CHO, K. S., CHOI, M. S., LEE, S. H., HAN, S. H., KANG, Y. S., KIM, H. J., CHEONG, J. H., SHIN, C. Y., KO, K. H. 2010. Urokinase-type plasminogen activator induces BV-2 microglial cell migration through activation of matrix metalloproteinase-9. *Neurochem Res*, 35, 976-85.

- SHORE, V. G. & SHORE, B. 1973. Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry*, 12, 502-507.
- SHUMSKY, J. S., TOBIAS, C. A., TUMOLO, M., LONG, W. D., GISZTER, S. F. & MURRAY, M. 2003. Delayed transplantation of fibroblasts genetically modified to secrete BDNF and NT-3 into a spinal cord injury site is associated with limited recovery of function. *Exp Neurol*, 184, 114-30.
- SKAPER, S. D. 2012. The neurotrophin family of neurotrophic factors: an overview. *Methods Mol Biol*, 846, 1-12.
- SU, T. P., HAYASHI, T., MAURICE, T., BUCH, S. & RUOHO, A. E. 2010. The sigma-1 receptor chaperone as an inter-organelle signalling modulator. *Trends Pharmacol Sci*, 31, 557-566.
- SU, T. P., LONDON, E. D. & JAFFE, J. H. 1988a. Steroid Binding at Sigma Receptors Suggests a Link Between Endocrine, Nervous, and Immune Systems. *Science*, 240, 219-221.
- SU, T. P., SCHELL, S. E., FORD-RICE, F. Y. & LONDON, E. D. 1988b. Correlation of inhibitory potencies of putative antagonists for sigma receptors in brain and spleen. *Eur J Pharmacol*, 148, 467-70.
- SULLIVAN, P. M., MEZDOUR, H., ARATANI, Y., KNOUFF, C., NAJIB, J., REDDICK, R. L., QUARFORDT, S. H. & MAEDA, N. 1997. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. *J Biol Chem*, 272, 17972-80.
- SUN, J. H., YANG, B., DONNELLY, D. F., MA, C. & LAMOTTE, R. H. 2006. MCP-1 enhances excitability of nociceptive neurons in chronically compressed dorsal root ganglia. *J Neurophysiol*, 96, 2189-99.
- SUTER, M. R., SIEGENTHALER, A., DECOSTERD, I. & JI, R. R. 2011. Perioperative Nerve Blockade: Clues from the Bench. *Anesthesiology Research and Practice*, 2011, 12.
- TAKAYAMA, T., MORELLI, A. E., ONAI, N., HIRAO, M., MATSUSHIMA, K., TAHARA, H. & THOMSON, A. W. 2001. Mammalian and Viral IL-10 Enhance C-C Chemokine Receptor 5 but Down-Regulate C-C Chemokine Receptor 7 Expression by Myeloid Dendritic Cells: Impact on Chemotactic Responses and In Vivo Homing Ability. *The Journal of Immunology*, 166, 7136-7143.
- TEDGUI, A. & MALLAT, Z. 2006. Cytokines in atherosclerosis: pathogenic and regulatory pathways. *Physiol Rev*, 86, 515-81.
- TEIXEIRA, J. M., OLIVEIRA, M. C. G., PARADA, C. A. & TAMBELI, C. H. 2010. Peripheral mechanisms underlying the essential role of P2X7 receptors in the development of inflammatory hyperalgesia. *European Journal of Pharmacology*, 644, 55-60.
- TESTER, A. M., COX, J. H., CONNOR, A. R., STARR, A. E., DEAN, R. A., PUENTE, X. S., LÓPEZ-OTÍN, C. & OVERALL, C. M. 2007. LPS Responsiveness and Neutrophil Chemotaxis In Vivo Require PMN MMP-8 Activity. *PLoS ONE*, 2, e312.
- THIBERT, K. A., RAYMOND, G. V., NASCENE, D. R., MILLER, W. P., TOLAR, J., ORCHARD, P. J. & LUND, T. C. 2012. Cerebrospinal fluid matrix metalloproteinases are elevated in cerebral

adrenoleukodystrophy and correlate with MRI severity and neurologic dysfunction. *PLoS One*, 7, e50430.

THIES, F., MILES, E. A., NEBE-VON-CARON, G., POWELL, J. R., HURST, T. L., NEWSHOLME, E. A. & CALDER, P. C. 2001. Influence of dietary supplementation with long-chain n-3 or n-6 polyunsaturated fatty acids on blood inflammatory cell populations and functions and on plasma soluble adhesion molecules in healthy adults. *Lipids*, 36, 1183-93.

THIRKETTLE, S., DECOCK, J., ARNOLD, H., PENNINGTON, C. J., JAWORSKI, D. M. & EDWARDS, D. R. 2013. Matrix metalloproteinase 8 (collagenase 2) induces the expression of interleukins 6 and 8 in breast cancer cells. *J Biol Chem*, 288, 16282-94.

THOMPSON, S. W. N., BENNETT, D. L. H., KERR, B. J., BRADBURY, E. J. & MCMAHON, S. B. 1999. Brain-derived neurotrophic factor is an endogenous modulator of nociceptive responses in the spinal cord. *Proceedings of the National Academy of Sciences*, 96, 7714-7718.

TIWARI, R. L., SINGH, V. & BARTHWAL, M. K. 2008. Macrophages: an elusive yet emerging

TOBIAS, C. A., SHUMSKY, J. S., SHIBATA, M., TUSZYNSKI, M. H., FISCHER, I., TESSLER, A. & MURRAY, M. 2003. Delayed grafting of BDNF and NT-3 producing fibroblasts into the injured spinal cord stimulates sprouting, partially rescues axotomized red nucleus neurons from loss and atrophy, and provides limited regeneration. *Exp Neurol*, 184, 97-113.

TODA, S., SAKAI, A., IKEDA, Y., SAKAMOTO, A. & SUZUKI, H. 2011. A local anesthetic, ropivacaine, suppresses activated microglia via a nerve growth factor-dependent mechanism and astrocytes via a nerve growth factor-independent mechanism in neuropathic pain. *Mol Pain*, 7, 2.

TOPP, K. S. & BOYD, B. S. 2006. Structure and biomechanics of peripheral nerves: nerve responses to physical stresses and implications for physical therapist practice. *Phys Ther*, 86, 92-109.

TOZAKI-SAITOH, H., TSUDA, M., MIYATA, H., UEDA, K., KOHSAKA, S. & INOUE, K. 2008. P2Y12 receptors in spinal microglia are required for neuropathic pain after peripheral nerve injury. *J Neurosci*, 28, 4949-56.

TRANG, T., BEGGS, S. & SALTER, M. W. 2012. ATP receptors gate microglia signaling in neuropathic pain. *Exp Neurol*, 234, 354-61.

TRANG, T., BEGGS, S., WAN, X. & SALTER, M. W. 2009. P2X4-receptor-mediated synthesis and release of brain-derived neurotrophic factor in microglia is dependent on calcium and p38-mitogen-activated protein kinase activation. *J Neurosci*, 29, 3518-28.

TSUDA, M., INOUE, K. & SALTER, M. W. 2005. Neuropathic pain and spinal microglia: a big problem from molecules in 'small' glia. *Trends in Neurosciences*, 28, 101-107.

TSUDA, M., SHIGEMOTO-MOGAMI, Y., KOIZUMI, S., MIZOKOSHI, A., KOHSAKA, S., SALTER, M. W. & INOUE, K. 2003. P2X4 receptors induced in spinal microglia gate tactile allodynia after nerve injury. *Nature*, 424, 778-83.

TSUDA, M., TOYOMITSU, E., KOMATSU, T., MASUDA, T., KUNIFUSA, E., NASU-TADA, K., KOIZUMI, S., YAMAMOTO, K., ANDO, J. & INOUE, K. 2008. Fibronectin/integrin system is involved in P2X(4) receptor upregulation in the spinal cord and neuropathic pain after nerve injury. *Glia*, 56, 579-85.

- TSUDA, M., TOZAKI-SAITOH, H. & INOUE, K. 2010. Pain and purinergic signaling. *Brain Res Rev*, 63,
- TSUDA, M., TOZAKI-SAITOH, H. & INOUE, K. 2012. Purinergic system, microglia and neuropathic pain. *Curr Opin Pharmacol*, 12, 74-9.
- TSUTSUI, S., SCHNERMANN, J., NOORBAKHS, F., HENRY, S., YONG, V. W., WINSTON, B. W., WARREN, K. & POWER, C. 2004. A1 adenosine receptor upregulation and activation attenuates neuroinflammation and demyelination in a model of multiple sclerosis. *J Neurosci*, 24, 1521-9.
- UEDA, H. & RASHID, M. H. 2003. Molecular mechanism of neuropathic pain. *Drug News Perspect*, 16, 605-13.
- UGALDE, A., ORDÓÑEZ, G., QUIRÓS, P., PUENTE, X. & LÓPEZ-OTÍN, C. 2010. Metalloproteases and the Degradosome. In: CLARK, I. M. (ed.) *Matrix Metalloproteinase Protocols*. Humana Press.
- URATSUJI, H., TADA, Y., KAWASHIMA, T., KAMATA, M., HAU, C. S., ASANO, Y., SUGAYA, M., KADONO, T., ASAHINA, A., SATO, S. & TAMAKI, K. 2012. P2Y6 receptor signaling pathway mediates inflammatory responses induced by monosodium urate crystals. *J Immunol*, 188, 436-44.
- VAN DEN ELZEN, P., GARG, S., LEON, L., BRIGL, M., LEADBETTER, E. A., GUMPERZ, J. E., DASCHER, C. C., CHENG, T. Y., SACKS, F. M., ILLARIONOV, P. A., BESRA, G. S., KENT, S. C., MOODY, D. B. & BRENNER, M. B. 2005. Apolipoprotein-mediated pathways of lipid antigen presentation. *Nature*, 437, 906-10.
- VAN DER WAL, A. C. & BECKER, A. E. 1999. Atherosclerotic plaque rupture – pathologic basis of plaque stability and instability. *Cardiovascular Research*, 41, 334-344.
- VANDENBROUCKE, R. E., DEJONCKHEERE, E., VAN LINT, P., DEMEESTERE, D., VAN WONTERGHEM, E., VANLAERE, I., PUIMEGE, L., VAN HAUWERMEIREN, F., DE RYCKE, R., MC GUIRE, C., CAMPESTRE, C., LOPEZ-OTIN, C., MATTHYS, P., LECLERCQ, G. & LIBERT, C. 2012. Matrix metalloprotease 8-dependent extracellular matrix cleavage at the blood-CSF barrier contributes to lethality during systemic inflammatory diseases. *J Neurosci*, 32, 9805-16.
- VELTEN, M., BRITT, R. D., JR., HEYOB, K. M., TIPPLE, T. E. & ROGERS, L. K. 2014. Maternal dietary docosahexaenoic acid supplementation attenuates fetal growth restriction and enhances pulmonary function in a newborn mouse model of perinatal inflammation. *J Nutr*, 144, 258-66.
- VEREYKEN, E. J., HEIJNEN, P. D., BARON, W., DE VRIES, E. H., DIJKSTRA, C. D. & TEUNISSEN, C. E. 2011. Classically and alternatively activated bone marrow derived macrophages differ in cytoskeletal functions and migration towards specific CNS cell types. *J Neuroinflammation*, 8, 58.
- VERKHRATSKY, A. & BURNSTOCK, G. 2014. Biology of purinergic signalling: Its ancient evolutionary roots, its omnipresence and its multiple functional significance. *BioEssays*, 36, 697-705.
- VEROLLET, C., CHARRIERE, G. M., LABROUSSE, A., COUGOULE, C., LE CABEC, V. & MARIDONNEAU-PARINI, I. 2011. Extracellular proteolysis in macrophage migration: losing grip for a breakthrough. *Eur J Immunol*, 41, 2805-13.
- VICENTE-MANZANARES, M. & HORWITZ, A. R. 2011. Cell migration: an overview. *Methods Mol Biol*, 769, 1-24.

- VITEK, M. P., BROWN, C. M. & COLTON, C. A. 2009. APOE genotype-specific differences in the innate immune response. *Neurobiol Aging*, 30, 1350-60.
- VOGEL, D. Y., HEIJNEN, P. D., BREUR, M., DE VRIES, H. E., TOOL, A. T., AMOR, S. & DIJKSTRA, C. D. 2014. Macrophages migrate in an activation-dependent manner to chemokines involved in neuroinflammation. *J Neuroinflammation*, 11, 23.
- VON SCHACKY, C., FISCHER, S. & WEBER, P. C. 1985. Long-term effects of dietary marine omega-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. *J Clin Invest*, 76, 1626-31.
- WALDMAN, S. D., USPRECH, J., FLYNN, L. E. & KHAN, A. A. 2010. Harnessing the purinergic receptor pathway to develop functional engineered cartilage constructs. *Osteoarthritis Cartilage*, 18, 864-72.
- WALDO, S. W., LI, Y., BUONO, C., ZHAO, B., BILLINGS, E. M., CHANG, J. & KRUTH, H. S. 2008. Heterogeneity of human macrophages in culture and in atherosclerotic plaques. *Am J Pathol*, 172, 1112-26.
- WALKER, E. J. & ROSENBERG, G. A. 2009. TIMP-3 and MMP-3 contribute to delayed inflammation and hippocampal neuronal death following global ischemia. *Experimental Neurology*, 216, 122-131.
- WALLENTIN, L. 2009. P2Y₁₂ inhibitors: differences in properties and mechanisms of action and potential consequences for clinical use. *Eur Heart J*, 30, 1964-77.
- WANG, X. X., TAN, M. S., YU, J. T. & TAN, L. 2014. Matrix Metalloproteinases and Their Multiple Roles in Alzheimer's Disease. *Biomed Res Int*, 2014, 908636.
- WANG, Z., LEE, Y., EUN, J. S. & BAE, E. J. 2014. Inhibition of adipocyte inflammation and macrophage chemotaxis by butein. *Eur J Pharmacol*, 738C, 40-48.
- WARNHOLTZ, A., NICKENIG, G., SCHULZ, E., MACHARZINA, R., BRASEN, J. H., SKATCHKOV, M., HEITZER, T., STASCH, J. P., GRIENDLING, K. K., HARRISON, D. G., BOHM, M., MEINERTZ, T. & MUNZEL, T. 1999. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation*, 99, 2027-33.
- WATANABE, N. & IKEDA, U. 2004. Matrix metalloproteinases and atherosclerosis. *Current Atherosclerosis Reports*, 6, 112-120.
- WHITE, F. A., BHANGOO, S. K. & MILLER, R. J. 2005. Chemokines: integrators of pain and inflammation. *Nat Rev Drug Discov*, 4, 834-44.
- WHITE, F. A., JUNG, H. & MILLER, R. J. 2007. Chemokines and the pathophysiology of neuropathic pain. *Proc Natl Acad Sci U S A*, 104, 20151-8.
- WILD, K. D., BIAN, D., ZHU, D., DAVIS, J., BANNON, A. W., ZHANG, T. J. & LOUIS, J. C. 2007. Antibodies to nerve growth factor reverse established tactile allodynia in rodent models of neuropathic pain without tolerance. *J Pharmacol Exp Ther*, 322, 282-7.

WODARSKI, R., CLARK, A. K., GRIST, J., MARCHAND, F. & MALCANGIO, M. 2009. Gabapentin reverses microglial activation in the spinal cord of streptozotocin-induced diabetic rats. *Eur J Pain*, 13, 807-11.

WOLF, K., TE LINDERT, M., KRAUSE, M., ALEXANDER, S., TE RIET, J., WILLIS, A. L., HOFFMAN, R. M., FIGDOR, C. G., WEISS, S. J. & FRIEDL, P. 2013. Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *J Cell Biol*, 201, 1069-84.

WOOLF, C. J. & MANNION, R. J. 1999. Neuropathic pain: aetiology, symptoms, mechanisms, and management. *The Lancet*, 353, 1959-1964.

WOOLF, C. J. 1996. Phenotypic modification of primary sensory neurons: the role of nerve growth factor in the production of persistent pain. *Philos Trans R Soc Lond B Biol Sci*, 351, 441-8.

WU, M.-H., TSAI, Y.-T., HUA, K.-T., CHANG, K.-C., KUO, M.-L. & LIN, M.-T. 2012. Eicosapentaenoic acid and docosahexaenoic acid inhibit macrophage-induced gastric cancer cell migration by attenuating the expression of matrix metalloproteinase 10. *The Journal of nutritional biochemistry*, 23, 1434-1439.

YAGASAKI, Y., NUMAKAWA, T., KUMAMARU, E., HAYASHI, T., SU, T. P. & KUNUGI, H. 2006. Chronic antidepressants potentiate via sigma-1 receptors the brain-derived neurotrophic factor-induced signaling for glutamate release. *J Biol Chem*, 281, 12941-9.

YAMAMORI, H., HASHIMOTO, R., ISHIMA, T., KISHI, F., YASUDA, Y., OHI, K., FUJIMOTO, M., UMEDA-YANO, S., ITO, A., HASHIMOTO, K. & TAKEDA, M. 2013. Plasma levels of mature brain-derived neurotrophic factor (BDNF) and matrix metalloproteinase-9 (MMP-9) in treatment-resistant schizophrenia treated with clozapine. *Neurosci Lett*, 556, 37-41.

YAMAOKA, G., HORIUCHI, H., MORINO, T., MIURA, H. & OGATA, T. 2013. Different analgesic effects of adenosine between postoperative and neuropathic pain. *Journal of Orthopaedic Science*, 18, 130-136.

YAO, H., KIM, K., DUAN, M., HAYASHI, T., GUO, M., MORGELLO, S., PRAT, A., WANG, J., SU, T.-P. & BUCH, S. 2011. Cocaine Hijacks σ 1 Receptor to Initiate Induction of Activated Leukocyte Cell Adhesion Molecule: Implication for Increased Monocyte Adhesion and Migration in the CNS. *The Journal of Neuroscience*, 31, 5942-5955.

YAO, H., YANG, Y., KIM, K. J., BETHEL-BROWN, C., GONG, N., FUNA, K., GENDELMAN, H. E., SU, T. P., WANG, J. Q. & BUCH, S. 2010. Molecular mechanisms involving sigma receptor-mediated induction of MCP-1: implication for increased monocyte transmigration. *Blood*, 115, 4951-62.

YONG, N. & GUOPING, C. 2008. Upregulation of matrix metalloproteinase-9 dependent on hyaluronan synthesis after sciatic nerve injury. *Neurosci Lett*, 444, 259-63.

YOO, J., RODRIGUEZ PEREZ, C. E., NIE, W., SINNETT-SMITH, J. & ROZENGURT, E. 2011. Protein kinase D1 mediates synergistic MMP-3 expression induced by TNF-alpha and bradykinin in human colonic myofibroblasts. *Biochem Biophys Res Commun*, 413, 30-5.

- YOON, S. Y., ROH, D. H., SEO, H. S., KANG, S. Y., HAN, H. J., BEITZ, A. J. & LEE, J. H. 2009. Intrathecal injection of the neurosteroid, DHEAS, produces mechanical allodynia in mice: involvement of spinal sigma-1 and GABA receptors. *Br J Pharmacol*, 157, 666-73.
- YOSHII, A. & CONSTANTINE-PATON, M. 2010. Postsynaptic BDNF-TrkB signaling in synapse maturation, plasticity, and disease. *Dev Neurobiol*, 70, 304-22.
- YU, W., SUN, X., ROBSON, S. C. & HILL, W. G. 2013. Extracellular UDP enhances P2X-mediated bladder smooth muscle contractility via P2Y(6) activation of the phospholipase C/inositol trisphosphate pathway. *FASEB J*, 27, 1895-903.
- ZAMANILLO, D., ROMERO, L., MERLOS, M. & VELA, J. M. 2013. Sigma 1 receptor: A new therapeutic target for pain. *European Journal of Pharmacology*, 716, 78-93.
- ZHANG, X., XU, Y., WANG, J., ZHOU, Q., PU, S., JIANG, W. & DU, D. 2012. The effect of intrathecal administration of glial activation inhibitors on dorsal horn BDNF overexpression and hind paw mechanical allodynia in spinal nerve ligated rats. *J Neural Transm*, 119, 329-36.
- ZHANG, Y., GONG, L. H., ZHANG, H. Q., DU, Q., YOU, J. F., TIAN, X. X. & FANG, W. G. 2010. Extracellular ATP enhances in vitro invasion of prostate cancer cells by activating Rho GTPase and upregulating MMPs expression. *Cancer Lett*, 293, 189-97.
- ZHAO, F., QU, Y., LIU, H., DU, B. & MU, D. 2014. Umbilical cord blood mesenchymal stem cells co-modified by TERT and BDNF: A novel neuroprotective therapy for neonatal hypoxic-ischemic brain damage. *Int J Dev Neurosci*.
- ZHAO, J., YUAN, G., CENDAN, C. M., NASSAR, M. A., LAGERSTROM, M. C., KULLANDER, K., GAVAZZI, I. & WOOD, J. N. 2010. Nociceptor-expressed ephrin-B2 regulates inflammatory and neuropathic pain. *Mol Pain*, 6, 77.
- ZHOU, H. Y., CHEN, S. R., CHEN, H. & PAN, H. L. 2011. Functional plasticity of group II metabotropic glutamate receptors in regulating spinal excitatory and inhibitory synaptic input in neuropathic pain. *J Pharmacol Exp Ther*, 336, 254-64.