Identification of Novel Biomarkers of Neuropathic Pain: A Translational Study from Rat to Human

Bethan C Young

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Supervisor: Dr Patrick C McHugh

The Centre for Biomarker Research at the University of Huddersfield

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Abbreviations

A3GALT2 – Alpha 1,3-Galactosyltransferase 2
ANXA1 – Annexin A1
ATM – Ataxia-telangiectasia mutated
ATP – Adenosine triphosphate
BBB – Blood brain barrier
BDNF – Brain derived neuro
CART – Cocaine and amphetamine regulated transcript prepropeptide
CASP – Caspase
CB - Cannabinoid
CBD - Cannabidiol
C1QTNF3 – C1q and tumour necrosis factor protein 3
CCI – Chronic Constriction Injury
CCK - Cholecystokinin
CCR5 – C-C Chemokine Receptor 5
CD209E – Cluster of Differentiation
CD4 – Cluster of Differentiation 4
CNS – Central Nervous System
CYCS – Cytochrome C
DAMPs – Damage-associated molecular patterns
DEF – Defensin
DH – Dorsal Horn
DMEM – Dubecco's Modified Eagles Medium
DNA - deoxyribonucleic acid
DPN – Diabetic peripheral neuropathy
DRG – Dorsal root ganglion
ECG - Electrocardiograph
EDTA - Ethylenediaminetetraacetic acid
EEG - Electroencephalograph
EIF – Eukaryotic Initiation F actor
ELISA – enzyme-linked immunosorbent assay
ERK – Extracellular signal-regulated kinases
FBS – Foetal Bovine Serum
FPR2 – N-formyl peptide receptor 2
GABA - gamma-aminobutyric acid
GCPS – Generalised Chronic Pain Scale
GPCR – G-protein Coupled Receptor
HAT - Histone acetyltransferases
HDAC - Histone deacetylases
HOX – Homeobox
ICAM – Intercellular Adhesion Molecule
IFM – Inflammatory mediator
IFNγ – Interferon gammma
IL – Interleukin
IMM – Inner mitochondrial membrane
IPA – Ingenuity Pathway Analysis
JNK – c-Jun N-terminal kinases
KCC2 – Potassium/Chloride Transporter 2
LC – Locus Coeruleus

MAPK – Mitogen-activated protein kinase
MCP – Monocyte chemoattractant protein
MS – Multiple Sclerosis
MiRNA - microRNA
mRNA – messenger RNA
MRPL6L – Myosin, light polypeptide 6
mTOR – Mammalian Target of Rapamycin
MYL6L – Mitochondrial ribosomal protein L20
Na_V – Sodium Voltage Gated Ion Channel
Ndufal13 – Reduced Nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase subunit A13
NMDA – N-methyl-D-aspartate receptor
NGF – Nerve growth factor
NP – Neuropathic Pain
NRM – Nucleus raphe magnus
NSAIDs – Non-steroidal anti-inflammatory drugs
NT3 – Neurotrophin 3
OMM – Outer mitochondrial membrane
OMP – Olfactory molecular patterns
OR – Olfactory receptor
PAG – Periaquductal Grey
PAMPs - Pathogen-associated molecular patterns
PCS – Pain Catastrophising Scale
PD – Parkinson's Disease
PECAMS – Platelet-endothelial cell adhesion molecules
PET - Positron emission tomography
PGE – Prostaglandin E
PHN – Post-hepatic Neuralgia
PHQ-9 – Patient Health Questionnaire 9 (Depression)
PKA – Protein Kinase A
PKC – Protein Kinase C
PLAC8 – Placenta-specific 8
P2XR – Purogenic 2 X Receptor
QPCR – Quantitative Polymerase Chain Reaction
REG3B – Regenerating islet-derived protein 3-beta
RNA – Ribonucleic Acid
ROC – Receiver Operating Characteristic
ROMO1 – Reactive Oxygen Species Modulator 1
ROS – Reactive Oxygen Species
RTA – Rat Transcriptome Array
S-LANSS – (Self-assessed) Leeds Assessment of Neuropathic Symptoms and Signs
SDS – Sodium dodecyl sulfate
SGC – Satellite Glial Cells
SH3BGRL3 – SH3 Domain Binding Glutamate Rich Protein Like-3
SNI – Spared Nerve Injury
SNL – Spinal Nerve Ligation
SNRIs - Serotonin–norepinephrine reuptake inhibitors
SSRIs - Selective serotonin reuptake inhibitors
STAT3 - Signal transducer and activator of transcription 3
TAC – Transcriptome Analysis Console (Affymetrix, ThermoFisher Scientific)
TCA – Tricyclic Antidepressant

TENS – Transcutaneous Electrical Nerve Stimulation
TF – Transcription Factor
THC – Tetrahydrocannabinol
TMEM88 – Transmembrane Protein 88
TNFα – Tumour necrosis factor alpha
TRAV3D-3 – T-cell receptor alpha variable
TRK – Tyrosine Receptor Kinase
TRPV1 – Transient receptor potential vanilloid 1
TXN1 – Thioredoxin 1
UCN – Urocortin
VG – Voltage-gated

Abstract

Neuropathic pain is a common chronic condition which remains poorly understood. Twenty percent of patients receiving treatment continue to experience moderate to severe pain, due to limited diagnostic and symptom management programmes. The development of objective diagnostic strategies and more effective medications requires identification of robust biomarkers of neuropathic pain. To this end, several potential biomarkers of chronic neuropathic pain were identified by assessing gene expression profiles of an animal model of neuropathic pain, and differential gene expression in patients to determine the potential translational mechanisms of neuropathic pain in an animal model to a clinical cohort.

Dorsal horn tissue extracted from a Sprague Dawley rat spinal nerve ligation model (35 days post-surgery, n=8) and sham operated controls (n=8) was used for Affymetrix Rat Transcriptome Array 1.0 to identify differentially expressed genes. Genes with significant expression changes (p<0.05, fold change ±1.25) were also measured by qPCR in clinical neuropathic pain blood samples (n=53) and non- neuropathic pain control samples (n=65).

The gene expression analysis revealed a subset of significant differentially regulated genes involved in inflammatory processes and apoptosis. This demonstrated cross-species validation of eight genes by assessing their expression in blood samples from neuropathic pain patients. These include *A3GALT2, CASP1, CASP4, CASP5, CCR5, FPR2, SH3BGRL3, and TMEM88*. Molecules which demonstrate an active role in human neuropathic pain have the potential to be developed into a biological measure for objective diagnostic tests, or as novel drug targets for improved pain management. Such developments could help to relieve the social and economic burden of neuropathic pain by restoring patient health-related quality of life.

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“Of pain you could wish only one thing: that it should stop. Nothing in the world was so bad as physical pain. In the face of pain there are no heroes.”

— 1984, George Orwell

Chapter 1: Introduction

1.1 Introduction to the Literature Review

Neuropathic pain occurs when damaged peripheral nerves send pain signals to the brain in the absence of a painful stimulus. The subjective nature and psychological aspect of the pain experience make it difficult to objectively assess and can lead to incorrect diagnosis and inappropriate treatment recommendations. Current treatment strategies are not efficacious or cost effective. Patients receiving neuropathic pain treatment still experience moderate to severe pain (O'Connor, 2009, Colloca *et al.,* 2017), and adverse side effects of medications can compromise patient adherence (Moulin *et al.*, 2007). The literature review gives a detailed background into pain mechanisms in health, and how neuropathic pain can be established and maintained. The need for clinical biomarkers to improve diagnostic tools and pain management programs for neuropathic pain patients will also be explained.

1.2 Neuropathic Pain and Its Socioeconomic Implications

1.2.1 Prevalence of Neuropathic Pain

Neuropathic pain affects approximately 7.4 million people in the UK, and most patients receiving neuropathic pain treatment still experience moderate to severe pain (O'Connor, 2009). The lack of effective treatment strategies results in a great social and economic cost and patient quality of life remains compromised (Figure 1.1). There is some evidence that certain lifestyle and environmental factors are linked to neuropathic pain, such as the link to high alcohol consumption and alcoholic neuropathy (Julian *et al.*, 2019). Peripheral neuropathy is common among smokers, and smoking is a recognised risk factor for neuropathic pain (Celik *et al.*, 2017). Smoking is associated with a higher risk of peripheral neuropathy in diabetic patients (Clair *et al.*, 2015). However, it has also been suggested that neuropathic pain increases smoking

duration and addiction levels (Celik *et al.,* 2017), which may result from individuals with nicotine addiction seeking the relief that smoking provides to detract from pain.

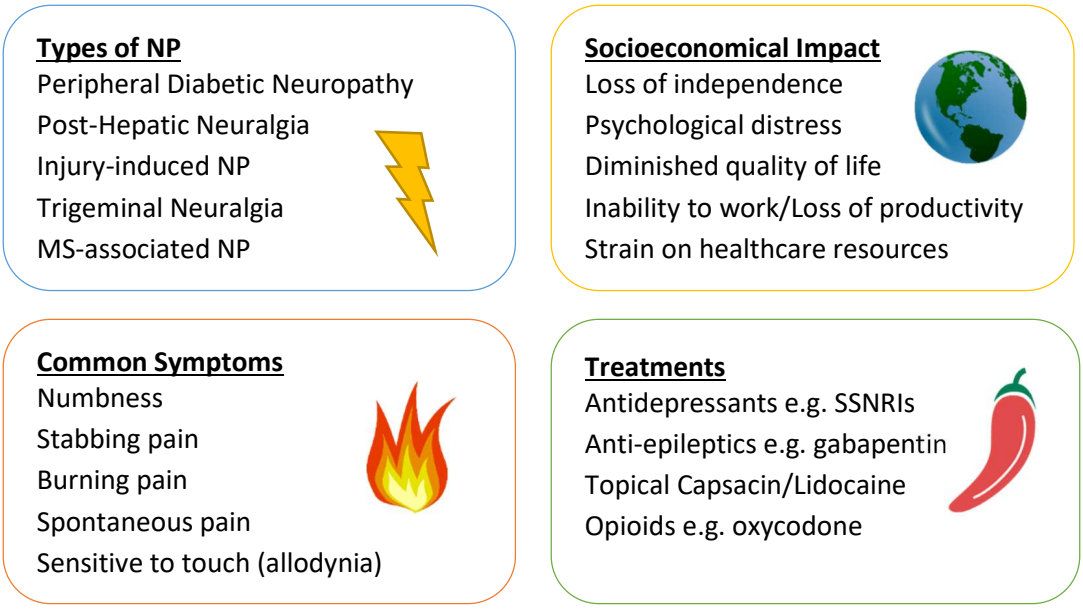


Figure 1.1 Neuropathic pain summary

Reported in Woolf & Mannion, 1999, O'Connor 2009, Dworkin *et al.,* 2007

Plant-based diets may improve some pain measures in diabetic neuropathy (Bunner *et al.*, 2015). Calorie-restricted rats present reduced mechanical and thermal hypersensitivity in the chronic constriction injury model of neuropathic pain, versus control rats with ad-libitum access to food (Liu *et al.*, 2018). A calorie-controlled diet may help attenuate symptoms in neuropathic pain patients, but a randomised clinical trial with a sufficiently large sample size is needed to assess the efficacy and safety in humans before calorie restriction becomes a realistic consideration for pain management. Exercise has also been linked to improvements in neuropathic pain (Dobson *et al.*, 2014) and can delay the onset of neuropathic pain in diabetic rats (Shankarappa *et al.*, 2011). It is possible that exercise could help neuropathic pain patients by providing an outlet for the stress and anxiety associated with chronic pain (Stubbs *et al.*, 2017, Wegner *et al.*, 2014, Stonerock *et al.*, 2015).

1.2.2 Clinical Presentation of Neuropathic Pain

Neuropathic pain results from damage to the nervous system. It clinically manifests as pain in the absence of a painful stimulus (spontaneous pain), painful responses to innocuous stimuli (allodynia), or a heightened pain response (hyperalgesia) (Woolf & Mannion, 1999). Paraesthesia, the presence of a prickling or tingling sensation without an apparent cause, is another common symptom. Symptoms vary in intensity over time and between cases. Allodynia and hyperalgesia are clinical markers of neuropathic pain, and neuropathic pain itself is a symptom associated with several diseases. These include multiple sclerosis and diabetes, and can result from physical trauma to nerve endings such as a crush injury or broken bones (Table 1.1).

Table 1.1: Types of Neuropathic Pain

Type	Description	Characteristics
Peripheral diabetic neuropathy	Damage to the peripheral nerves in the hands and feet due to high blood glycose can cause pain (Rosenberger *et al.*, 2020)	60-70% of diabetic patients (Javed *et al.*, 2015)
Postherpetic neuralgia	Persistent nerve pain caused by shingles (Saguil *et al.*, 2017)	Can develop in anyone who has had shingles Full recovery within 12 months is common, but symptoms can become chronic (Johnson & Rice, 2014)
Injury Induced NP	Damage to peripheral nerves or spinal cord caused by physical traumatic injury, such as crush or severance of a nerve (Shiao & Lee-Kubli 2018) e.g. phantom limb pain	53% of spinal cord injury patients develop neuropathic pain (Burke *et al.,* 2017) 33% of lower limb amputees report chronic pain (Ahmed *et al.,* 2017) but other reports suggest a higher incidence (Limakatso *et al.,* 2019)
Trigeminal neuralgia	Compression of the trigeminal nerve in the face causes sudden and severe pain (Maarbjerg *et al.*, 2017)	Approximately 10 people in 100,000 in the UK develop it each year

		More common in women aged 50-60 (Obermann, 2010)
Multiple sclerosis (MS)	Nerve damage (Duffy *et al.*, 2018)	25% of MS patients (MS Trust)
Carpal Tunnel Syndrome	Compression of the median nerve in the wrist that causes numbness and pain in your hand (Padua *et al.*, 2016)	Common in people with jobs or hobbies which involve bending the wrist or tight gripping (Burton *et al.*, 2014)
Phantom limb pain	Nerves are severed during amputation	80% of amputees (Nikolajsen *et al.*, 1998)

Prevalence information from www.NHS.uk: https://www.nhs.uk/conditions/peripheral-neuropathy/ https://www.nhs.uk/conditions/post-herpetic-neuralgia/ https://www.nhs.uk/conditions/trigeminal-neuralgia/ https://www.nhs.uk/conditions/multiple-sclerosis/symptoms/ https://www.nhs.uk/conditions/carpal-tunnel-syndrome/ https://www.nhs.uk/conditions/peripheral-neuropathy/

1.2.3 Impact of Neuropathic Pain on Quality of Life

Pain is an aversive experience, and chronic pain can produce prolonged negative emotions such as anger, fear, and despondency. Biological stress responses to chronic pain and further contribute to the negative pain experience (Hannibal & Bishop, 2014). This can greatly reduce patient quality of life, and increases their risk of anxiety, insomnia and depression (Campbell *et al.*, 2015; Hammen, 2005; Hammen *et al.*, 2009; Sheng *et al.*, 2017). Further, chronic pain can impair the patient's ability to perform daily tasks, and in severe cases patients may require the help of a carer. Reduced independence can also have a negative effect on self-esteem, and increase depression risk (Biegler, 2008). Depression is a common comorbidity in chronic pain, with over half of American chronic pain patients also with depression (Lerman *et al.*, 2015). Studies in animal models of neuropathic pain report depressive behaviours but these do not appear until after six weeks post-injury (Mitsi *et al.*, 2015, Yalcin *et al.*, 2011) which may indicate the importance of pain chronicity in depression risk.

Chronic stress is a major risk factor for depression (Hammen, 2005). Anti-depressant medications including tricyclics (TCAs) (Moore *et al.*, 2015) are often prescribed to chronic neuropathic pain patients. Though the exact mechanism of action is not known and is thought

to be independent of the mood effects (Bohren *et al.*, 2013, Mitsi *et al.*, 2015). It is possible that the concurrent treatment of depressive symptoms and mood improvement benefits also patients. Treatment of depression has been linked to improved medication adherence in multiple sclerosis (MS) patients (Mohr 1997), a disease cohort which often experience neuropathic pain.

Stress, anxiety, and chronic pain can feed into one another and lead to amplification of the respective states. The stress response involves several neural and hormonal mechanisms that result in acute heightened awareness that is not sustainable over a prolonged length of time. Suffering in spinal cord injury patients with neuropathic pain is associated with increased psychological distress (Gruener *et al.*, 2018) and may contribute to pain catastrophising in which patients experience heightened emotional response to pain resulting from the anticipation of pain (Quartana *et al.*, 2009).

The stress hormone corticosterone acts at glucocorticoid receptors (Murphy *et al.*, 1998) and the glucocorticoid receptor antagonist RU486 prevents stress-induced allodynia in spared nerve injury (SNI) mice (Alexander *et al.*, 2009). Stress hormones including glucocorticoids and norepinephrine mediate the activation and proliferation of DH microglia, and this has implications for stress-induced cognitive impairments and in neuropathic pain (Yuan *et al.*, 2015). Central glucocorticoid receptor activation also increased the expression of spinal N-methyl-D-aspartate receptor (NMDA) receptors after spinal nerve injury (Wang *et al.*, 2005), which contributes to neuropathic pain (Petrenko *et al.*, 2003).

Anxiety is highly prevalent in chronic pain patients, with double the incidence than that seen in the general population (McWilliams *et al.*, 2003). In a study of both SNI and chronic unpredictable stress mouse models, gene expression and pathway analysis reported comparable changes across several brain areas including the nucleus accumbens, the medial prefrontal cortex, and the periaqueductal grey (Descalzi *et al.*, 2017). In a sustained stress

response, increased levels of the potent anti-inflammatory neuroendocrine cortisol, could influence the complex inflammatory mechanisms active in neuropathic pain (Hannibal & Bishop, 2014). It could also contribute to pain catastrophising behaviours given its role in consolidation of fear and avoidance behaviours. Pain catastrophising is recognised as both a source of inaccuracy for pain patient questionnaires, and as a contributing factor of the pain experience and patient discomfort. Catastrophising pain patients have more negative emotions about their pain than patients who do not catastrophise (Newton, 2013). This practice leads to pain anticipation and worsening of the pain experience. This is measurable by the pain catastrophizing scale (PCS), a set thirteen questions answered using a 0-4 scale by the patient themselves, devised by Sullivan, Bishop and Pivik (1995) and has been adapted further for daily use (Darnall *et al.*, 2017). The PCS measures three aspects of catastrophizing: magnification, rumination, and helplessness. PC has been linked to physical disability and insomnia (Glette *et al.*, 2018). By identifying pain catastrophizing in patients and incorporating suitable approaches into treatment regimens (e.g. cognitive behavioural therapy, CBT), this could help improve treatment response and patient quality of life. (Darnall *et al.*, 2014) carried out a pilot study and reported that CBT reduced pain catastrophising in patients. However, CBT follows a highly structured format and takes place over multiple sessions and may not be suitable for all patients.

The relationship between stress, depression, and anxiety with chronic pain are therefore likely to be cyclical in nature and provide a positive feedback mechanism which intensifies both pain and maladaptive psychological states. Therefore, the multi-faceted psychological component of the pain experience represents a critical feature of pain management strategies often overlooked or insufficiently addressed.

Chronic pain is linked to poor sleeping habits and insomnia, which contributes to reduced quality of life in neuropathic pain patients (Cheatle *et al.*, 2016). Current neuropathic pain medications have varying effects on sleep; pregabalin improves sleep quality whilst opioids reduce it (Ferini-

Strambi, 2017). Insomnia has been linked to pain catastrophising (Campbell *et al.*, 2015). Insomnia can be treated with CBT, though access is limited (Cheatle *et al.,* 2016). Anti-convulsant medications such as the gabapentinoids are thought to improve sleep disturbances (Roth *et al.*, 2010), whereas opioid medications can exacerbate them (Trenkwalder *et al.*, 2017). Disturbed sleeping patterns and circadian rhythm can have knock-on adverse health effects, including cluster headache risk (Burish *et al.*, 2019), and depression (Boyce & Barriball, 2010). Cognitive impairment has also been linked to chronic pain, which could impact a patient's ability to participate in certain activities and decrease their quality of life (Campbell *et al.*, 2017; Gorgoraptis *et al.*, 2019; Hill *et al.*, 2017). Cognitive impairment could be a consequence of the stress response caused by chronic pain (Hart *et al.*, 2003).

Pain is difficult to ignore because the pain signal is immediate, urgent, and overrides other activities being performed at the time to provoke a reaction to remove the offending stimulus. Normally, once the stimulus is removed pain stops, and the individual can continue with other activities. In chronic pain, this process is never completed because the pain signal cannot be resolved by stimulus removal. Chronic pain can therefore present a barrier to activities performed in daily life, including tasks that require a higher level of cognition that the patient would otherwise be capable of doing. According to Maslow's Hierarchy of Needs (Figure 1.2) chronic pain patients could become unable to progress through the hierarchy as their physiological needs (i.e. pain relief) are not sufficiently met. This theory of human motivation can demonstrate why the daily lives of chronic pain patients are disrupted in many ways, including the breakdown of relationships and the ability to perform well at work.

Figure 1.2: Maslow's Hierarchy of Needs. Proposed by Abraham Maslow in 1943 (Maslow, 1943), the Hierarchy of Needs describes the theory that an individual's needs must be addressed in the order according to how essential they are to life. These are physiological needs (food and water), safety (shelter and security), social needs (belonging and love), esteem (feeling accomplished), and self-actualisation (described as achieving one's full potential). According to the theory to progress through the hierarchy an individual must be satisfied at the preceding stage. This framework remains relevant and popular in sociology and psychological sciences today (Henwood *et al.*, 2015, Healy 2016, Lussier, 2019).

Chronic pain patients would not satisfy their physiological needs, which prevents subsequent needs to be achieved including social relationships (Beyaz *et al.*, 2016) and self-esteem (Elton *et al.*, 1978). When considering these social consequences in a treatment strategy for chronic pain, it is important to remember that successful management of the pain itself (or causal treatment) will address these problems. For example, successful treatment of pain improves sleep (Mehta *et al.*, 2016). Catastrophising is a consequence of chronic pain (not present in all patients) which intensifies the pain experience, and is therefore worth addressing separately. In the absence of successful chronic pain management, treatment of symptoms such as stress and insomnia may be worthwhile to improve quality of life, though (if treated with medication) increases risk of adverse reactions, and drug interaction in polypharmacy.

1.2.4 Healthcare and the Economy

Chronic pain has a negative impact on a patient's ability to maintain employment (Alleaume *et al.*, 2018) which means that neuropathic pain patients find it more difficult to remain in employment that the general population, reducing independence and quality of life. Patients

unable to work due to poorly managed chronic pain causes to huge losses to the economy. This has knock-on effects on close family members, particularly if relatives take on carer responsibilities for the patient.

Healthcare costs on inefficacious medicines is clearly an ineffective use of funds, and additional resources are spent to manage the adverse side effects of ineffective medications. Polypharmacy resulting from the taking of multiple (>5) medications is also a concern, as patients take additional medications to manage side effects. Polypharmacy in elderly patients, with weakened excretion systems, increases mortality risk (Hajjar *et al.*, 2007). One study suggests that by educating neuropathic pain patients about disease pathology and the mechanism of actions of their medications, their intake of pain medications can be reduced whilst maintaining the pain attenuation they had previously achieved (Shin *et al.*, 2017). However, higher medication intake may also reduce adherence in adults, if it becomes difficult for patients to remember what their medications are taken for (Conn *et al.*, 1991).

1.2.5 Neuropathic Pain as a Comorbidity in Neurodegenerative Diseases

Neuropathic pain is prevalent in neurodegenerative diseases where it can occur as a symptom of the disease or as a comorbidity. In addition to high prevalence of neuropathic pain among diabetes and multiple sclerosis (MS) patients, it is also prevalent in Parkinson's Disease (PD) patients (Blanchet & Brefel-Courbon, 2018). PD is a neurodegenerative disease which primarily impairs the motor system (Sveinbjornsdottir, 2016). PD affects around 1% of people over the age of 65 (Y Yang & Lu, 2009). Symptoms have a slow onset and include tremor, rigidity, bradykinesia, impaired posture and balance, and speech changes (Sveinbjornsdottir, 2016). Symptoms are caused by the death of dopaminergic neurons in the substantia nigra of the midbrain (Michel *et al.*, 2016). Pain is also present in Alzheimer's Disease (AD) but due to the nature of the disease and impaired cognition, accurate self-reported data is difficult to obtain (Frank *et al.*, 2011).

1.3 The Physiology of Pain

1.3.1 Injury and Nociceptive Pain

Nociceptors are pain-specific sensory neurons which innervate the periphery, including the skin and internal organs (Dubin & Patapoutian, 2010). Nociceptors specialise in detecting harmful stimuli by evoking pain to warn of noxious stimuli. They are activated by stimuli likely to cause tissue damage if exposure is prolonged, to prompt stimulus removal to reduce exposure time and subsequent tissue damage (Woolf & Ma, 2007). Extremes of temperature, intense pressure (eg. stretching, pinching, or compression), and noxious chemicals (e.g. extreme pH) activate their respective nociceptors receptors in the nociceptor endings, to send a pain signal to the brain. These are thermoreceptors, chemoreceptors, and mechanoreceptors. Nociceptors have high threshold to prevent pain signals being elicited by touch or non-noxious temperatures (Woolf & Ma, 2007).

During an inflammatory response, inflammatory mediators (IFMs) released locally during acute inflammation sensitise the neighbouring nociceptors to reduce the activation threshold (Lembeck *et al.*, 1976, Juan *et al.*, 1984). This lowered firing threshold in sensitised neurones causes painful touch, to reduce wound interference and promotes healing. In health, this mechanism is localised and time-limited, and sensitivity returns to normal after healing. When this mechanism is prolonged, it can lead to a heightened state of sensitivity and neuropathic pain (Section 1.4).

1.3.2 Peripheral Nociceptors and Pain Transmission

Nociceptor endings contain voltage and ligand gated ion channels, including the heat and mechanically sensitive transient receptor potential (TRP) ion channels, and the voltage-gated (VG) sodium channels (e.g. $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$) (Dubin & Patapoutian, 2010). Opening these channels polarises the neuronal membrane, as positively charged ions move into the neuron and trigger an action potential. The action potential propagates as voltage-gated ion

channels open along the axon (Figure 1.3). Schwann cells are closely associated with neurons and provide the myelin sheath by wrapping around the axon of A fibres (Table 1.1) (Bhatheja & Field, 2006). This insulates the axon and allows the signal to 'jump' between the gaps, the Nodes of Ranvier, in the myelin sheath in a process termed salutary conduction (Huxley & Stampfli, 1949).

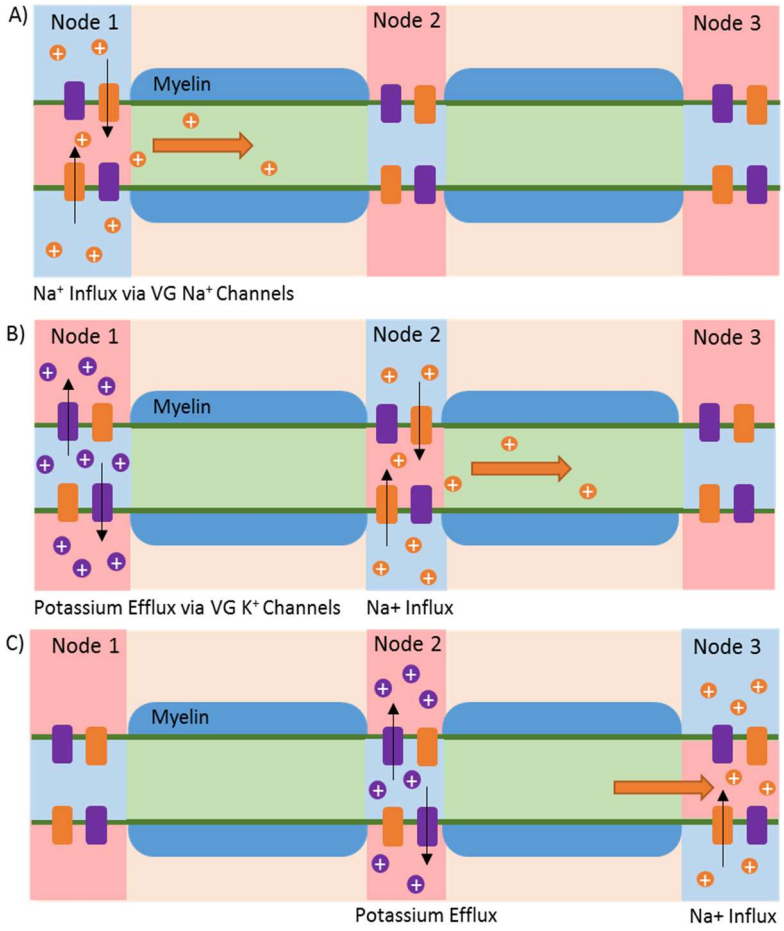


Figure 1.3: Action potential propagation along a myelinated axon. A) The resting potential of a neuron is -75mV. As the membrane potential rises above the threshold of -55mV, the action potential is triggered and Na^+ voltage gated (VG) ion channels are opened. Na^+ ion influx via these channels depolarises the membrane, and the axon becomes positively charged on the inside and negatively charged on the outside. Resting membrane potential is maintained at subsequent nodes until Na^+ diffuse along the axon. **B)** This change in the membrane potential opens the VG K^+ ion channels and K^+ ions then leave the cell at Node 1. **C)** Na^+ ion channels begin to close, and as K^+ continues to leave the neuron the membrane potential falls to below that of the resting potential. The resting membrane potential is restored as extracellular K^+ diffuses away from the neuron. At Node 2, the intracellular Na^+ ions open the VG ion channels and the axon is depolarised here.

Pain signals are relayed through the spinal cord to the brain via two different types of nociceptor neurone, Aδ and C fibres (Table 1.2) (Debanne et al., 2011). These have distinct axonal structures and function. Aδ fibres are myelinated with thin diameters for a fast conduction speed, and relay sharp easy-to-locate distinctive pain (Djouhri & Lawson, 2004). Aδ fibres pain perception usually occurs when the stimulus is present (Garland, 2012).

C fibres have a thinner diameter, but the lack of myelination causes a slower conduction (FitzGibbon & Nestorovski, 2013). C fibre axons are grouped together in Remak bundles with non-myelinating Schwann cells (Murinson & Griffin 2004). Not all C fibres are nociceptive, some respond to pleasant touch (Marshall *et al.* 2009). Nociceptive C fibres are mostly polymodal and respond to both thermal and mechanical stimuli (Basbaum *et al.,* 2009). In the event of acute injury from a noxious stimulus, Aδ fibres are activated first, and nociceptive C fibres are subsequently activated if the stimulus strength increases (Dubin & Patapoutian, 2010), and is often perceived as a slow dull ache and difficult to locate and persists after the stimulus has been removed.

Table 1.2: Physiology of Nerve Fibres

Fibre	Myelination	Diameter	Conduction speed (m/s)	Function
Aα	Myelinated	Thick (15um)	60-80	Efferent motor neurons, initiates skeletal muscle contraction (Vleggeert-Lankamp *et al.,* 2004)
Aβ	Myelinated	Thin (10um)	30-60	Non-noxious touch (Lokan 2009)
Aδ	Myelinated	Thinner (2-5um)	2-30	Thermal and mechanical pain signals (Djouhri & Lawson, 2004)
C	Non-myelination	Thinnest (0.5 – 1.5um)	3-15	Carry chemically induced pain often characterised as a dull ache and poorly localised pain (Staud *et al.* 2007)

1.3.3 The Ascending Pain Pathway

The pain signal travels from the along the peripheral nociceptor (first order neuron) where it terminates in the dorsal horn (DH) in the spinal cord (Figure 1.4) of the central nervous system (CNS). Sensory afferents within cranial nerves of the face, *e.g.* trigeminal nerve, do not terminal at the DH, and terminate in the trigeminal nucleus of the brainstem (Gambeta 2020). The spinal cord encapsulates lateral and anterior spinothalamic tracts, which relay pain/temperature, and

touch/pressure signals respectively to the anterolateral system in the thalamus (Kerr, 1975). Third order neurons then project from the thalamus to the area in the cortex corresponding to injury location and the brain perceives pain (Todd, 2010).

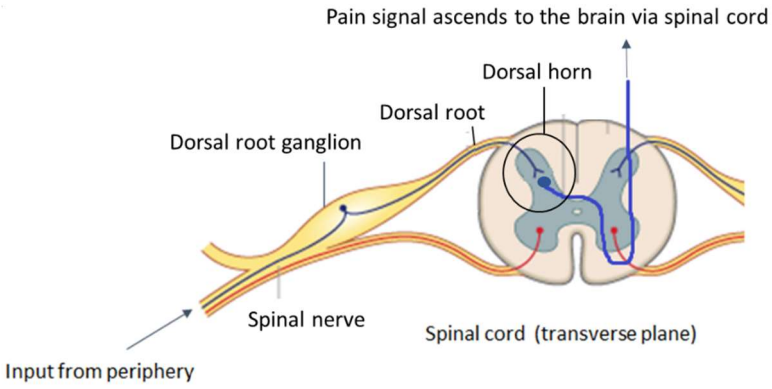
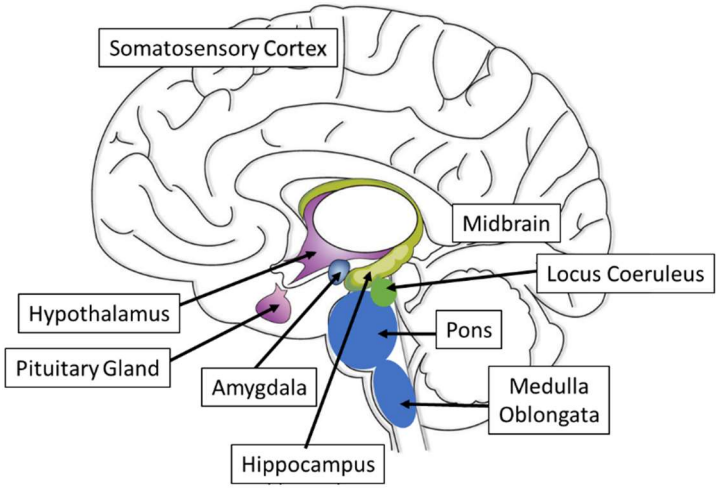


Figure 1.4: The Dorsal Horn in the Spinal Cord. Pain signals transmitted via the peripheral nerve pass through the dorsal root ganglion. The second neuron then relays the signal through the brainstem to the thalamus along the spinothalamic tract. Adapted from (Campbell & Meyer, 2006).

1.3.4 The Descending Pain Pathway and Pain Modulation

The descending pathway modulates the perception of pain by dampening down the ascending pathway (Millan, 2002) and originates in the somatosensory cortex (Dafny *et al.*, 1996). It represents a network of interactions between different areas in the brain (Figure 1.5, 1.6). The brainstem (composed of the midbrain, pons, and the medulla) contains several key areas of the descending pathway, including the periaqueductal grey (PAG) in the midbrain, and the nucleus raphe magnus (NRM) in the medulla (Figure 1.5). Serotonergic neurons originating in the NRM terminate in the DH and modulate the signalling between the first and second order neurons (Dogrul *et al.*, 2009).



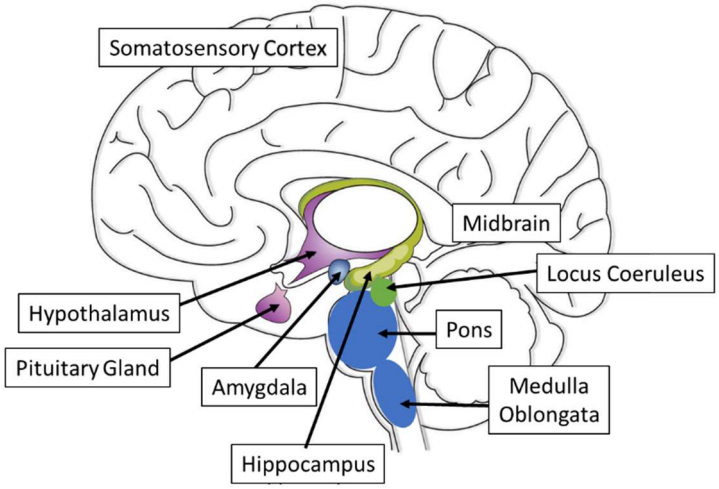


Figure 1.5: Anatomy of the Brainstem. The brainstem is composed of the midbrain, pons, and the medulla) contains several key areas of the descending pathway, including the periaqueductal grey (PAG) in the midbrain, and the nucleus raphe magnus (NRM) in the medulla.

Several neurotransmitters are involved in the descending pathway. Serotonin acts on the first order neuron and blocks substance P release into the synapse, stimulating enkephalin release from interneurons (Fischer *et al.*, 2017; Reiser & Hamprecht, 1989). Enkephalin inhibits the depolarisation of the second order neuron, as well as blocking first order neuron release of substance P (Gothert *et al.*, 1979). In the absence of a pain signal the serotonergic neurons are inhibited by GABA releasing neurons (Figure 1.6) (Huang & Grau, 2018). Noradrenaline is also

synthesised by neurons in the locus coeruleus (LC) in the pons of the brainstem, and acts at the substantia gelatinosa to suppress pain signals (Figure 1.6) (Mehler & Purpura, 2009).

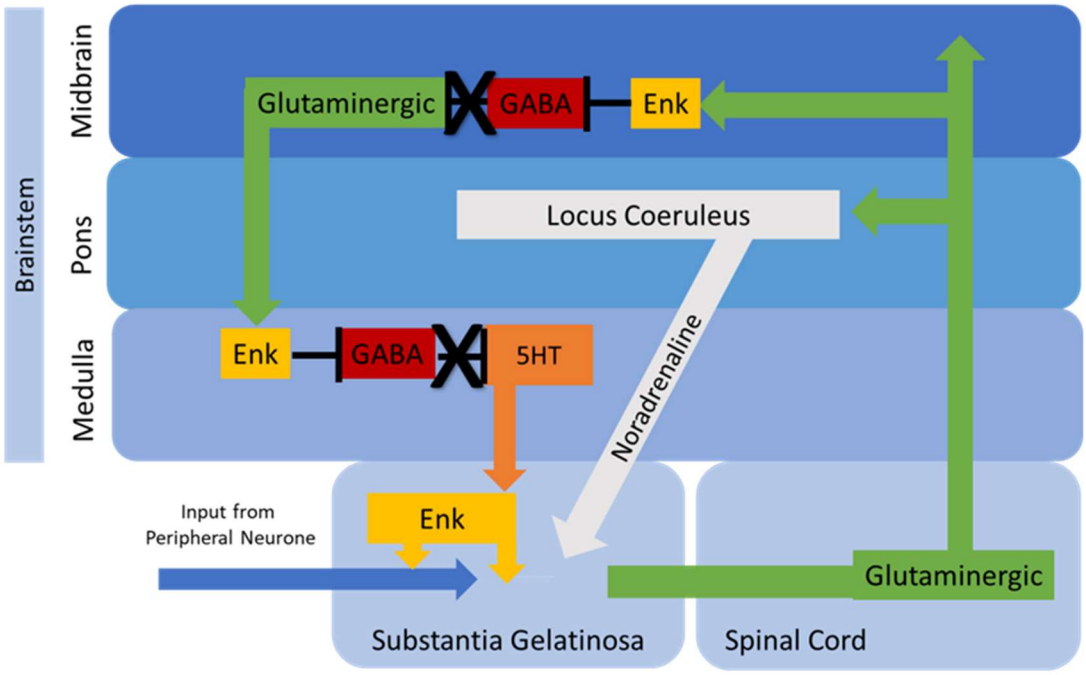


Figure 1.6: Descending Pain Pathways and Dorsal Horn Modulation. The pain signal is modulated by the descending pain pathways, which converge at the synapse between first and second order neurons in the substantia gelatinosa in the dorsal horn (DH). Glutaminergic neurons are stimulated by the initial peripheral neuron stimulation (1). These neurons activate noradrenaline release from locus coeruleus neurons (2), which innervate the DH. Release stimulates enkephalin (Enk) interneurons in the midbrain (3), which inhibits the (inhibitory) GABA signals (4) and allow glutaminergic neurons to activate (5). These activate enkephalin interneurons in the medulla (6), which inhibit GABA neurons and allow 5HT neurons to release serotonin (7). This activates enkephalin interneurons in the DH, which acts on the peripheral and at the synapse (8). Adapted from Michael Bentley [Available at https://www.youtube.com/watch?v=uCFtvmlOZTs].

Interneurons are a key component of pain modulation in the DH, and are classed as excitatory (utilising glutamine), or inhibitory (GABA and/or glycine) neurons (Todd, 2010). Interneurons can modulate the input signal from a peripheral neuron, in a process called “dorsal horn pain processing” as the DH integrates afferent signals from the periphery (English *et al.*, 2011; Koos & Tepper, 1999). The DH substantia gelatinosa contains high levels of opioid peptides and

receptors making it a key site for pain modulation (Kohno *et al.*, 1999). The descending pain modulation pathway plays a key role in the Motivation – Decision Model, which evaluates environmental cues and behavioural outcomes to promote usually pain aversive behaviours in situations where they can promote survival (Salamone *et al.*, 2018). Pain modification can explain how people can withstand painful situations to survive, such as Aron Ralston who amputated his own arm after it became trapped during a canyoneering accident (Botvin, 2003).

1.3.5 Pain and Learning Behaviours

The limbic system of the CNS, including the amygdala and the hippocampus, is involved in memory formation, emotion, motivation, and arousal (Catani *et al.*, 2013). It mediates the emotional responses to pain and the avoidance. The amygdala is a key area in emotional responses, such as fear and anxiety (Ziabreva *et al.*, 2003, Feinstein *et al.*, 2011), and has been implicated in pain processing (Veinante *et al.*, 2013) and pain chronicity (Andreoli *et al.*, 2017). Neurotransmitters involved in the descending pathway of pain modulation also participate in the emotional experience of pain (Bee & Dickenson, 2009).

Pain is perceived as a negative consequence of behaviour (punishment) and learned pain behaviour enables us to avoid potentially painful stimuli based on experiences (Tyrer, 1986). Thus, pain relief is perceived as pleasurable and rewarding. The mesolimbic pathway is involved in reward motivation, and dopaminergic signalling in the nucleus accumbens mediates the reward associated with pain relief (Taylor *et al.*, 2016). Endogenous opioids also mediate a reward response, as hedonic pleasure is driven by opioid release in brain several regions including the nucleus accumbens and amygdala (Le Merrer *et al.*, 2009, Gomtsian *et al.*, 2018). Motivation to seek pleasure is mediated by mesolimbic dopamine signalling (Berridge, 2004). Overlap of pain modulation systems with reward pathways has a profound impact on these patients (Becker *et al.*, 2012), with impaired reward sensitivity and motivation seeking neuropathic pain (Ozaki *et al.*, 2002).

1.4 The Pathophysiology of Neuropathic Pain

1.4.1 Insult and Inflammation

Inflammation occurs after injury as the innate immune system is activated to remove infiltrating pathogens or debris from damaged cells. It begins when local immune cells recognise damage or pathogen associated molecular patterns (DAMPs and PAMPs) via specialised receptors on their surface (Amarante-Mendes *et al.*, 2018). These immune cells include tissue-resident macrophages (which remove debris by phagocytosis), dendritic cells (antigen-presenting cells which can activate T and B cells of the adaptive immune system), and mast cells (a granulocyte containing histamine).

Damaged cells release inflammatory mediators (IFMs), including cytokines interleukin-1 (IL-1), TNFα, vasodilators, and chemokines (Table 1.3). Cytokines facilitate the expression of adhesion molecules by the endothelial cells that line the blood vessel walls at the injury site (Reglero-Real *et al.*, 2016). Mast cell degranulation releases tumour necrosis factor alpha (TNFα) and histamine into the extracellular matrix (Zhao *et al.*, 1996) causing vasodilation and increased blood flow. This increased vascular permeability allows circulating IFM-producing immune cells e.g. neutrophils - phagocytes which remove the cellular debris from the injury site (Rosales *et al.*, 2016) to migrate into the damaged tissue (Aplin *et al.*, 1998).

P- and E-selectins are released from endothelial cells and displayed on the cell surface (Lorenzon *et al.*, 1998). The selectins attract circulating leukocytes (a process called chemoattraction) to the site. Leukocytes roll along the vessel wall as the selectins bind to its surface glycoproteins with low affinity (Langer & Chavakis, 2009). Tight adhesion occurs when integrins on the neutrophil surface shift from a low-affinity to a high-affinity state in response to chemokines released by macrophages. The integrins then bind tightly to Intercellular Adhesion Molecule 1 (ICAM-1) on the endothelium (Yang *et al.*, 2005).

This process of extravasation is a key component in the innate immune response. The leukocyte cytoskeleton extends out, a process called diapedesis, as the cells pass through the gaps in the vessel wall. Platelet-endothelial cell adhesion molecules (PECAMs) on endothelial cells and leukocytes interact and facilitate leukocyte movement through the endothelial layer. Leukocyte penetration of the outer basement membrane layer of the vessel wall is not fully described or understood, but proposed theories include physical force and/or enzymatic digestion (Isfahani & Freund, 2012). Leukocytes continue to migrate towards the injury along an IL-8 chemotactic gradient.

IFMs, including prostaglandins, sensitise neurons to pain signals and temporarily lower the activation threshold to discourage wound contact and prevent further injury to the area and allow healing. Following removal of the insult, inflammation is resolved to limit tissue damage and allow healing of the injured tissue. This is an active process involving several mechanisms which halt chemokine signalling and subsequent tissue infiltration of neutrophils (Sugimoto *et al.*, 2016). Neutrophils apoptose after dealing with the threat in response to anti-inflammation and attenuation signals, and the debris is removed by macrophages (Fox *et al.*, 2010) which then transition into the inflammation resolving phenotype (Ortega-Gomez *et al.*, 2013, Michlewska *et al.*, 2009).

When acute inflammation persists and becomes chronic, the inflammatory mediators themselves cause further tissue damage, and prolonged sensitivity of the peripheral nerves occurs. Nociceptors also release neurotransmitters and neuropeptides which modulate inflammatory immune cells. This crosstalk between the inflammatory and nociceptive systems can develop peripheral sensitisation.

1.4.2 Peripheral Sensitisation

Peripheral sensitisation describes the series of events which lead to the prolonged reduced activation threshold in nociceptors in neuropathic pain resulting in allodynia, hyperalgesia, and

spontaneous pain. This is mediated through gene upregulation of the voltage-gated and ligand-gated ion channels, and increased concentration at the neuronal membrane (Figure 1.7). Active nociceptors release IFMs and neurotransmitters at the peripheral terminal in a process known as neurogenic inflammation (Littlejohn & Guymer, 2018). Substance P is an inflammatory peptide which causes vasodilation and activates mast cells to produce histamine, released by inflammatory cells (eg. macrophages) and sensory nerve terminals (Galli *et al.*, 2005, Basbaum, 1999). It is actively involved in peripheral and central inflammatory responses in animal models (Meert *et al.*, 2003) acting at the neurokinin 1 (NK-) receptor (Gerard *et al.*, 1991). Substance P or NK-1 receptor knockout causes an inhibited cytokine response in mice granuloma (Garza *et al.*, 2008), but also prevents normal pain responses (De Felipe *et al.*, 1998).

Substance P has a range of pro-inflammatory effect on immune cells, including inducing the production of pro-inflammatory cytokines (Rameshwar *et al.*, 1992, Palma & Manzini, 1998) (ie. from monocytes (Lotz *et al.*, 1988), macrophages (Ho *et al.*, 1996), degranulation and oxidative burst in lymphocytes (CJ Guo *et al.*, 2002) and neutrophils (Serra *et al.*, 1988). Independent of NK-1, substance P promotes mast cell activation (degranulation, serotonin and histamine release) in both human and rat tissues (Shanahan *et al.*, 1985, Repke & Bienert, 1987), and in murine mast cells induces TNFα gene expression and release (Ansel *et al.*, 1993). Along with bradykinin, substance P also directly acts on sensory nociceptive terminals and causes sensitisation (Jessell, 1982).

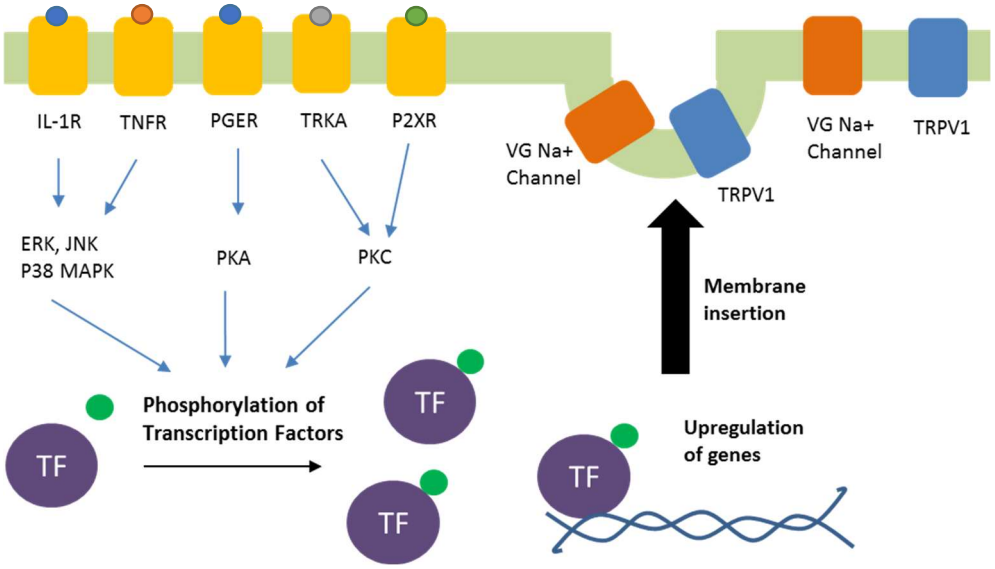


Figure 1.7: Peripheral sensitisation at the sensory nerve terminal. The IFMs activate their receptors on the neuronal membrane, which activates secondary messengers and their associated pathways. Prolonged stimulation of these leads to increased phosphorylation of transcription factors via secondary messenger pathways. In turn expression the voltage gated (VG) sodium channels and transient receptor potential vanilloid 1 (TRPV1) channels are upregulated and expressed at the neuronal surface. With an increased chance of ion influx, the neuron is now more likely to become depolarised and fire an action potential. This is peripheral sensitisation. TF = Transcription Factor. Adapted from (Ellis & Bennett, 2013).

IFMs released by the innate immune cells can interact with receptors on the neuronal cell and alter gene expression via intracellular signalling pathways (Figure 1.7). Increased transcription and membrane insertion of voltage and ligand gated ion channels (Figure 1.7) alters the ion trafficking and decreases the activation threshold. In this state neurons are more susceptible to ectopic axonal firing. For example, TRPV1 is sensitised by bradykinin, ATP (released by lysed ells), and prostaglandins (Huang *et al.*, 2006, Malek *et al.*, 2015), which are released during the inflammatory response. This lowers its activation threshold and increases neuronal sensitivity to thermal stimuli in neuropathic pain. These effects are not limited to damaged neurons, as the shared inflammatory environment causes sensitisation of neighbouring uninjured neurons (Ma *et al.*, 2003).

Some IFMs induce further IFM production and release to maintain the inflammatory state. For example, prostaglandin E2 (PGE2) synthesis is upregulated in response to IL-1β (Binshtok *et al.*, 2008), and PGE2 induces mast cell degranulation (Kalinski, 2012). This causes a positive feedback mechanism and amplification of sensitivity. These effects are not limited to damaged neurons, as the shared inflammatory environment causes sensitisation of neighbouring uninjured neurons (Ma 2003). Lower thresholds and inappropriate neuronal activation cause pain signals to be sent in the absence of a stimulus, and the patient experiences spontaneous pain. When axonal disruption occurs due to lesion, disease, chemotherapy, or inflammatory response to infection, the Schwann cells react by expressing IFMs to recruit macrophages and mast cells to the site (Napoli *et al.*, 2012).

Table 1.3: Role of Inflammatory Mediators in Injury Resolution and Peripheral Sensitisation

Mediator	Sources	Function
IL1	Mast Cells (Galli *et al.*, 2005), Neutrophils (Lindemann *et al.*, 1988), Lymphocytes (Moalem *et al.*, 2004)	Inflammatory cytokine, mediates the acute phase response and tissue repair (Ishida *et al.*, 2006) Stimulates proliferation, maturation, and activation of T helper cells (Lichtman *et al.*, 1988)
TNFα	Activated macrophages (Parameswaran & Patial, 2010), CD4+ lymphocytes, NK cells, neutrophils, eosinophils, mast cells (Bissonnette *et al.*, 1995)	Inflammatory cytokine involved in the acute phase response and immune cell regulation, tissue repair and apoptotic cell death (Kanaji *et al.*, 2011) (Leung & Cahill, 2010) Stimulates further pro-inflammatory cytokine release from other immune cells e.g. stimulates production of IL-1 oxidants and

		prostaglandin E2 in macrophages (Bachwich *et al.*, 1986) Increases excitatory activity in neurons (Ming *et al.*, 2013)
IL6	T cells (Banning *et al.*, 1998), macrophages (Arango Duque & Descoteaux, 2014)	Inflammatory cytokine, involved in the acute phase response (Rose-John 2018) Permeates the blood brain barrier (BBB) contributing to neuroinflammation in the CNS (Banks *et al.*, 1994)
Histamine	Mast cells (TC Moon *et al.*, 2014)	Vasodilation and increased vascular permeability (Panula *et al.*, 2015)
Substance P	Sensory nerve terminals (White, 1997)	Neurotransmitter (Pernow, 1983) Vasodilation via nitric oxide release (Bossaller *et al.*, 1992) and stimulates pro-inflammatory cytokine production (Rameshwar *et al.*, 1992) (Palma & Manzini, 1998) Evidence for a key role in neurogenic inflammation (Donkin *et al.*, 2007) Analgesic (Harris & Peng, 2020)
Bradykinin	Produced by the kinin-kallikrein system in the blood (Marcos-Contreras *et al.*, 2016)	Induces the release of endothelium-derived vasodilators nitric oxide (Palmer *et al.*, 1987) TRPV1 phosphorylation and contributes to heat sensitisation (Cesare & McNaughton, 1996)

Monocyte chemotactic protein 1 (MCP-1)	Neutrophils, mast cells, and macrophages (Deshmane *et al.*, 2009)	Recruitment of more immune cells (e.g. macrophages, neutrophils, helper T cells, microglia) (Deshmane *et al.* 2009) BBB disruption (Echeverry *et al.*, 2011)
Prostaglandin E2 (PGE2) (Eicosanoid)	Arachidonic acid, the precursor for eicosanoids, is released from phospholipid membranes and converted to PGE2 by cyclooxygenase 2 (COX2) enzyme (Heller *et al.*, 1998)	Vasodilation and vascular permeability (Williams, 1982) Increases the production of chemokines, mast cell degranulation (Kalinski, 2012) Blocks T cell receptor signalling (Wiemer *et al.*, 2011)
Calcitonin gene-related peptide (CGRP)	CGRP is located in the CNS, primarily in C and Aδ sensory fibres originating in the trigeminal ganglia (Iyengar *et al.,* 2014)	CGRP is a key mediator in neuropathic inflammation and peripheral sensitisation and is upregulated in both inflammatory and neuropathic pain. (Iyengar *et al.,* 2014) Key mediator of migraine pain (Buzzi *et al.,* 1995), released from activated meningeal nociceptors in the trigeminal ganglia in migraine (Durham 2006)

1.4.3 Phenotypic Switch

The dorsal root ganglion (DRG) is situated between adjacent vertebrae, as the dorsal (afferent sensory) gather and meet the ventral (efferent motor) filaments, at each spinal nerve (Figure 1.4), although the exact location varies depending on the section of the spine (there are 31 pairs of spinal nerves in humans). The structure plays an important part in modulating sensory processing, including in neuropathic pain (Krames, 2015). From a clinical perspective, the DRG is a good target for neuropathic pain interventions as it is more accessible than other areas of the CNS because it lacks the protective blood brain barrier (BBB) and capsular membrane (Sapunar

et al., 2012). Instead, the DRG is surrounded by permeable connective tissue which contains a very high density of blood vessels (Jimenez-Andrade *et al.*, 2008). Unlike the spinal cord, the DRG is not encapsulated by bone. However, this high perfusion is thought to explain why the DRG is resistant to intraneuronal local anaesthetic, as adequate concentration rates cannot be reached (Pfirrmann *et al.*, 2001), though the DRG is already an established steroidal drug target (Manchikanti, 2000).

The DRG contains the cell bodies of the bipolar sensory neuron, which surrounded by satellite glial cells (SGCs). SCGs respond to and control the extracellular microenvironment and supply essential nutrients to maintain nerve cell function (Mizisin & Weerasuriya, 2011). SGCs also physically separate nerve cell bodies from each other and influence neuronal excitability via controlling the extracellular potassium ion concentration (Hanani, 2005). Abnormal spontaneous activity in the DRG neurons contributes to neuropathic pain (North *et al.*, 2018).

Upregulated neuronal expression of MCP-1 by primary sensory neurons in the DRG and spinal cord astrocytes promote the chemotaxis of reactive microglia to the immediate area (Tanaka *et al.*, 2004) (Gao *et al.*, 2009). Normally, the microglia of the CNS monitors and maintains the environment required for physiological neuronal functioning (Aloisi, 2001). These cells become reactive in neuropathic pain (Eggen *et al.*, 2013), exhibiting phagocytic properties (Liu *et al.*, 1998) and expressing P2X7 and P2X4 (Beggs *et al.*, 2012). ATP activation of these receptors induces IL-1β release (Clark *et al.*, 2010). Overactive microglia and the subsequent cytokine production are present in persistent hyperalgesia (Hulsebosch, 2008), after both clinical (AL Davies *et al.*, 2007) and experimental peripheral nerve injury (Scholz *et al.*, 2008). An increased noradrenaline release during the stress response may feed into pain modulation pathways (Pertovaara, 2006).

Neurotrophic factors are upregulated after neuronal injury. Nerve growth factor (NGF) and neurotrophin-3 (NT3) are increasingly expressed by SGCs in the DRG (Sapunar *et al.*, 2012). NGF

is a regulatory protein involved in the growth, maintenance, proliferation, and survival of neurons (Rocco *et al.*, 2018), and NT3 supports the growth and differentiation of neurons and encourages the development of new synapses and neurons. The upregulation of these factors indicates the development of neuronal changes and sprouting, which contributes to neuropathic pain (Ramer *et al.*, 1999, Siniscalco *et al.*, 2011). Local inflammation in the DRG also contributes to neuropathic pain development. Li *et al.* demonstrated that corticosteroid injection near axotomised DRG in SNL rat models reduced mechanical sensitivity, as well as sprouting and SGC activation in the DRG, and activation of microglia in the spinal cord (Li *et al.*, 2011).

1.4.4 Dorsal Horn Modulation in Neuropathic Pain

Peripheral nociceptive signals are received and processed at the DH and relayed to the CNS (Figure 1.4). In peripheral sensitisation, there is increased stimulation at the postsynaptic neuron in the DH. Intracellular Ca^{2+} levels in the postsynaptic neuron are raised via prolonged activation of AMPA glutamate receptors (Latremoliere & Woolf, 2009). This results in extended depolarisation, which removes the Mg^{2+} block from NMDA glutamate receptors (Moriyoshi *et al.*, 1991), allowing these to be activated and contribute to the inward current. Sustained C fibre activity at the presynaptic terminals in the DH and subsequent neurotransmitter release, initiates multiple signalling cascades in postsynaptic neurons. This enhanced activity of the DH neurons feeds into the underlying mechanisms of central sensitisation.

Brain derived neurotrophic factor (BDNF) is released from presynaptic nerves and acts at tropomyosin receptor kinase B (TrkB) receptors on the postsynaptic neuron (Coull *et al.*, 2005). This downregulates potassium/chloride transporter KCC2, which mediates ion efflux (Chamma *et al.*, 2012). Activation of GABA receptors in response to this gradient shift causes further depolarising chloride ion efflux (Spitzer, 2010).

1.4.5 Central Sensitisation

The increased sensory input leads to central sensitisation of the CNS and brainstem, and this is critical to the transition to a chronic pain state. Breakdown of the BBB in the inflamed state (Echeverry *et al.*, 2011) allows infiltration of pro-inflammatory immune cells otherwise prevented in health (Cao & DeLeo, 2008).

Table 1.4: Key Players in Central Sensitisation

Mediator	Origin	Effect
Pro-inflammatory cytokines e.g. IL1β	Sensitised peripheral neurons (Fregnan *et al.*, 2012) Activated microglia (Wang *et al.*, 2015)	Activation of microglia and astrocytes (Zhang & An, 2007)
ATP	Cell damage (Burnstock, 2013)	Activation of G-Protein Coupled Receptors P2Y, and purogenic ligand gated ion channels P2X, on neurons and glial cells, resulting in the activation of macrophages (Kobayashi *et al.*, 2011, North, 2002)
BDNF	Activated microglia (Nakajima *et al.*, 2002)	Functional maturation of astrocytes (Holt *et al.*, 2019) Involved in synaptogenesis (Gonzalez *et al.*, 2019) and synaptic plasticity (Yoshii & Constantine-Paton, 2010)

1.5 Current Diagnostic Methods

1.5.1 Questionnaires

There is no exact correlation between the pain experience and disease pathology, so the use of questionnaires in the diagnosis of chronic pain, including neuropathic pain, has its limitations. However, they are widely used as there are no robust objective biomarkers for chronic pain of any type that can be used for diagnosis. Further, extensive laboratory testing to assess changes to the nervous system are unsuitable for routine use due to the high cost and time-consuming nature (Finnerup *et al.*, 2005), (May & Serpell, 2009). Signs and symptoms have limited

implications for treatment strategy because different underlying mechanisms may produce the same outward symptom. In the absence of biological diagnostic criteria, pain disorders are currently assessed by patient interview (Banerjee *et al.*, 2007). Screening tools such as the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) pain scale are implemented (Bennett, 2001). There are several limitations to this approach. Self-assessment is influenced by the psychological and emotional aspects of pain, which varies within and between cases (Yawn *et al.*, 2009), and 10-20% of neuropathic pain cases may not be identified this way (Cruccu *et al.*, 2010).

Patient answers may describe the pain they are experiencing in that moment, thus may be influenced by their mood or environment, and may not accurately reflect their daily pain experience. Chronic pain patients often exhibit attentional hypervigilance of pain which contributes to the phenomenon of catastrophizing, in which patients anticipate pain and subsequently (He *et al.*, 2014). It is also difficult to assess pain in its raw state without medication. When the patient focuses their attention on the pain, then the pain is perceived as more intense (McCracken, 2007), and the opposite is also true with less intense pain reported when attention is focused elsewhere, or the patient is distracted (Verhoeven *et al.*, 2010).

Inaccurate assessment of the pain can lead to incorrect diagnosis and inappropriate treatment recommendations from clinicians. Improved diagnostic tools and pain management programs of neuropathic pain are needed to help relieve the social and economic burden of neuropathic pain (Dworkin, 2007). For objective diagnoses and effective treatments, comprehensive understanding of the underlying neuropathic pain mechanisms is essential. To this end, gene expression studies in animal neuropathic pain models and neuropathic pain patients have been central, yet distinguishable neuropathic pain biomarkers are yet to be identified. A disease biomarker is defined as “a characteristic that can be measured and evaluated as an indicator of normal biologic processes, pathologic processes or pharmacologic responses to therapeutic

intervention" by the *Biomarkers and surrogate endpoints: preferred definitions and conceptual framework* (Biomarkers Definitions Working Group 2001).

1.5.2 Clinical Parameters

Several clinical parameters have been recognised as potential measurable outcomes in pain diagnosis and monitoring. These include changes to the autonomic nervous system (heart rate variability, blood pressure changes, and electrodermal activity), biopotentials, and neuroimaging. The stress hormone cortisol and serum lipid levels have been investigated as potential biomarkers of chronic pain but are not yet used clinically (Ferrara *et al.*, 2013). Though these parameters have been investigated in the context of general chronic pain, these may be relevant neuropathic cases as they measure physiological aspects of the chronic pain state rather than components of the underlying pathology. These findings are reviewed by Cowen (2015) and summarised in Table 1.5 (Cowen *et al.*, 2015).

Table 1.5: Potential Clinical Parameters of Chronic Pain

Parameter / Technique	Supporting Key Findings	Considerations
Heart rate variability	Correlates with pain scores (Chang *et al.*, 2012) Responds to nociceptive stimulation (Koenig *et al.*, 2014) Correlation with noxious stimuli (Gruenewald *et al.*, 2013)	No correlation with pain intensity (Meeuse *et al.*, 2013) Can be measured via blood pressure or electrocardiography (ECG)
Blood pressure changes	Correlation with noxious stimulation (Rossi *et al.*, 2012)	Non-invasive, standard procedure that can be taken relatively easily at different timepoints to measure response to treatment
Electrodermal activity	Correlates with pain scores in adults (Ledowski *et al.*, 2007) and children (Choo *et al.*, 2010)	Non-invasive procedure

		Results may be influenced by fluctuations in temperature and humidity
Biopotentials as measured by electrocardiography (ECG) or electo-encephalography (EEG)	Predicts intensity of pain perception (G Huang *et al.*, 2013) (ZG Zhang, Hu, *et al.*, 2012)	Unable to predict motor response to noxious stimuli (Takamatsu *et al.*, 2006) Non-invasive procedure but may be time-consuming
Neuroimaging e.g. positron emission tomography (PET)	Correlates with opioid system activation and pain scores (Casey *et al.*, 2000) Correlates with clinical chronic lower back pain. (Loggia *et al.*, 2013)	Non-invasive procedure but relatively time-consuming and cause discomfort, requires specialist equipment
Serum cortisol levels	Increases during noxious stimulation (Greisen *et al.*, 2001)	Measurement of stress hormones could give insight into patient's stress levels
Serum lipid levels	Increased during persistent pain (Krikava *et al.*, 2004)	

Adapted from Cowen 2015

1.6 Treatments for Neuropathic Pain

1.6.1 Currently Used Treatments for Neuropathic Pain

Neuropathic pain is currently managed with drugs available for other disorders (Table 1.6), which have demonstrated varying efficacies in pain management across different types (Finnerup *et al.*, 2016). First-line treatments include anti-depressants, anti-epileptic drugs, and topical lidocaine. Opioid analgesics are generally considered second-line treatments.

Table 1.6: Strengths and Limitations for Current Treatments of Neuropathic Pain

Drug/Drug Class	Mechanism of Action	Strengths	Limitations
Anti-depressants	Tricyclic anti-depressants (TCAs) and selective serotonin and noradrenaline reuptake inhibitors (SSNRIs) increase synaptic levels of these neurotransmitters in the brain by inhibiting pre-synaptic reuptake	Use in neuropathic pain is well established and supported by systematic review evidence (Saarto & Wiffen, 2007) Inexpensive Manages psychological aspects e.g. sleep disturbance, depression, and anxiety (Dworkin *et al.*, 2010) SSNRIs are more selective and exhibit fewer side effects than TCA (Dworkin *et al.*, 2007)	Mechanism in neuropathic pain not fully understood (Kremer *et al.*, 2016) Adverse reactions include cholinergic effects, sedation, blurred vision, orthostatic hypotension, weight gain, heightened suicide risk (Coupland *et al.*, 2018). TCA treatment does not differ from placebo in some neuropathic pain types (Cardenas 2002, Robinson *et al.*, 2004) Trials limited in scope and duration

Anti-convulsants	Gabapentin binds to the calcium channel α2δ subunit, decreasing excitatory neurotransmitter release and reducing membrane expression of the voltage-gated calcium channels (Kukkar *et al.*, 2013) Carbamazepine binds to the inactive voltage-gated sodium channel which prevents re-opening and reduces neuronal excitability (Kawata *et al.*, 2001)	According to Cochrane Review (Wiffen *et al.,* 2017), support for gabapentin use mostly in studies for peripheral diabetic neuropathy Evidence for carbamazepine efficacy in neuropathic pain (Kawata *et al.*, 2001)	Side effects include dizziness, fatigue, drowsiness, ataxia, tremor, increased depression risk, and suicidal behaviours (Patorno *et al.*, 2010) Arguably weak evidence for gabapentin in neuropathic pain (Moore 2014) and adverse side effects occur in 10% of patients who took gabapentin (Wiffen *et al.,* 2017) Trials limited in scope and duration
Lidocaine (Topical)	Lidocaine blocks the sodium channels and prevents the pain signal propagation along the axon (Tikhonov & Zhorov, 2017)	Lidocaine patch (5%) has proven efficacious across many neuropathies, including PHN (Davies & Galer, 2004) Patch method provides a physical barrier provides added protection against painful touch where allodynia occurs (Baron *et al.*, 2016) Milder side effects than other treatments when used topically	Topical applications are only appropriate where neuropathic pain occurs in or closely underneath the skin, not useful in other cases – there is no significant benefit with intravenous lidocaine in chronic peripheral neuropathic pain (Moulin *et al.*, 2019) May cause local erythema and rash in some patients

		Gel application may trigger gate control theory, overriding pain signals with touch and pressure stimulation	
Opioids	Opioids mimic the effects of endogenous opiates in the brain, primarily targeting the μ-receptor (Hill, 1981).	Evidence to support the use of some opioids here including oxycodone and morphine in neuropathic pain (Rosenblum *et al.*, 2008)	Strongly associated with addiction and overdose risk (Ballantyne & LaForge, 2007) and therefore contradicted in patients with history of addiction/substance abuse Inadequate study of long-term use in neuropathic pain (Furlan *et al.*, 2006) but altered structure and activity of opioid receptors in neuropathic pain limits efficacy. For Review see (Smith, 2012). Evidence for causing hypogonadism (Daniell, 2002) and immunosuppression (Vallejo *et al.*, 2004) Tramadol exhibits abuse potential and increases the risk of seizures in susceptible patients (Boostani & Derakhshan, 2012)

Medication regimes are often developed through trial and error, as the combination of drugs with the greatest pain relief and fewest adverse side effects is determined gradually (Dworkin, 2010), delaying symptom management and prolonging patient discomfort. Severe side effects may also require patients to seek additional medical assistance to manage these effects. There is an obvious need for more effective treatment and management strategies in neuropathic pain, with greater safety and tolerability (Dworkin, 2010). This will likely be a multidisciplinary approach, with specific pharmacological and non-pharmacological regimes (eg. physiotherapy or counselling) tailored to each patient according to the underlying pathophysiology of their disease. A curative treatment, rather than symptom management, would help relieve the economic costs and social burden of neuropathic pain, by eliminating the need for long-term medication programs.

1.6.2 Experimental Drugs Not Currently Available for Clinical Treatment of Neuropathic Pain

1.6.2.1 Cannabinoids

The endocannabinoid system in the CNS includes the cannabinoid receptors CB1 and CB2 and their endogenous ligands anandamide and 2-arachidoylglycerol. CB1 and CB2 are G-protein coupled receptors, negatively coupled to adenylate cyclase (Rahn & Hohmann, 2009), and are thought to be involved in several physiological processes including arousal and memory (Broyd *et al.*, 2016) though research into the endocannabinoid system is ongoing. The endocannabinoid system has been implicated in pain pathways (Woodhams *et al.*, 2015), as well as diseases including MS, Huntington's Disease, and PD (Micale *et al.*, 2007). The Cochrane report on "Cannabis products for adults with chronic neuropathic pain" (March 2018), "There is a lack of good evidence that any cannabis-derived product works for any chronic neuropathic pain". Despite this, the pain-relieving effects of cannabis is well-known (Li, Vigil*, et al.*, 2019), though

whether this due to the dissociative effect on the user is not well understood. Medical cannabis is legal in some US states and is prescribed for painful neuropathies such as MS and spinal cord injury (Hall 2016). Tetrahydrocannabinol (THC) is the psychoactive ingredient in cannabis that mediates the effects which promote its recreational use. Cannabidiol (CBD) and hemp (a strain of cannabis low in THC) have recently entered consumer markets. CBD oils marketed as an over-the-counter anxiety remedy and hemp is used as a non-dairy alternative to soya products.

THC acts at the CB1 receptor and increases dopamine release (Oleson & Cheer, 2012), thought to promote recreational use. Cannabinol is a positive allosteric modulator of μ and δ opioid receptors, leading to a stronger analgesic effect (Kathmann *et al.*, 2006). THC and cannabinoid also reportedly potentiate glycine receptors at respective sites (Hejazi *et al.*, 2006, Wells *et al.*, 2015), causing an analgesic effect by increasing inhibitory signals (Sun *et al.*, 2012).

1.6.2.2 Ketamine

Ketamine is a general anaesthetic and Class B illegal drug in the UK, and is taken recreationally for its sedative effects. Ketamine has demonstrated efficacy in chronic pain management (Bell & Kalso, 2018) and has long-lasting and potent anti-depressive effects (Yang *et al.*, 2015). This means that use of ketamine in neuropathic pain may also address the mood component in patients. Aside from the lack of confirmatory evidence, ketamine is known to produce undesirable effects such as abnormal heart rhythms and respiratory depression (Stoker *et al.*, 2019). Ketamine is a non-competitive NMDA receptor antagonist, binding to the receptor in the open activated state and prevents the Ca^{2+} channel closing. This is thought to be the primary mechanism for ketamine efficacy in neuropathic pain, and NMDA receptors in the DH are a key player in central sensitisation (Petrenko *et al.*, 2003).

1.6.2.3 AT-121

The experimental analgesic compound AT-121 gained interest in August 2018 when it was described as a breakthrough non-addictive drug and potential alternative to the likes of morphine, oxycodone, and fentanyl (Ding *et al.*, 2018). AT-121 targets the μ-opioid receptor to induce pain relief (like traditional opioids), and the nociceptin/orphanin FQ receptor (NOP). NOP has been a target of interest in substance abuse management for several years due to its role in the regulation of motivation and reward pathways (Zaveri, 2016). In non-human primates, Ding *et al.* report that AT-121 produces pain relief comparable to morphine, at a much lower dose, without the dangerous side effects including dependence and respiratory depression (Ding et al., 2018).

1.6.2.4 Nocebo Effect and Other Clinical Trial Considerations

The nocebo effect describes the negative non-pharmacological effects which occur after a drug therapy. As the opposite of the placebo effect, the nocebo effect is displayed in the increased frequency of side effects when patients are informed of potential side effects of a given treatment. Nocebo has been reported in 52% of neuropathic pain cases (Papadopoulos & Mitsikostas, 2012), with higher prevalence in other neurological diseases such as Parkinson's (64.7%), epilepsy (60.8%), and MS (74.4%). Given the well-recognised shortcomings of current neuropathic pain treatments, it is not unreasonable to assume that some of the reported adverse reactions to the medications may be due to the nocebo effect. Thus, by reducing the nocebo effect, patient response to current neuropathic pain medications may be improved and associated side effects may be reduced. Nocebo may be reduced by improving patient understanding of how their medication works and the frequency of possible side effects, similar findings were reported by Shin (2017). There may also be a link between patients susceptible to PC and prevalence of the nocebo effect, though there are no studies currently published, this

may represent a potential area for investigation. Jutzeler *et al.* (2018) also reported that placebo effects occur regardless of injury characteristics.

1.6.3 Currently Used Non-Pharmaceutical Pain Management

Transcutaneous Electrical Nerve Stimulation

Transcutaneous electrical nerve stimulation (TENS) is a nonpharmacological intervention used in pain treatment, including chronic pain conditions (Vance *et al.*, 2014). TENS is a small battery-operated device that delivers small electrical impulses via dermal electrodes. TENS machines exploit the Gate Control theory by providing mechanical stimulation to block pain signals. The gate control theory states that non-noxious sensation prevents pain perception by blocking pain signal transmission to the CNS, as proposed by Melzack and Wall in 1965. This theory can explain why rubbing a painful area can ease the pain. Melzack and Wall proposed that pain signals from the periphery, carried by Aδ and C fibres, could be inhibited by interneuron interference stimulated by Aβ fibre activation (Katz & Rosenbloom, 2015).

Tolerance to TENS can develop after repeated application when used at the same frequency and intensity daily. Systematic reviews have suggested that TENS is effective for PDN and is a widely available method of pain relief during labour though evidence for its efficacy is limited (Jones *et al.*, 2012). TENS machines are popular, with one brand called Livia patented in August 2016 and publicly funded through Indiegogo.com. The element of control may appeal to patients, as they can configure the intensity and frequency as they desire.

Acupuncture

Acupuncture is the practice of inserting fine needles at specific points on the body derived from ancient Chinese medicine (Grant *et al.*, 2016). It is thought to stimulate sensory nerves (Haker *et al.*, 2000). It is currently only recommended by NICE for migraine and tension headaches but

has demonstrated some efficacy in neuropathic pain relief (Schroder *et al.*, 2007) and is thought to stimulate the opioid system to inhibit signals in neuropathic pain and facilitate an analgesic effect (Cidral-Filho *et al.*, 2011).

1.7 Animal Models of Neuropathic Pain and Clinical Research

1.7.1 Types of Animal Models of Neuropathic Pain

There is a vast range of established methods used to produce animal models of neuropathic pain (Jaggi *et al.*, 2011), where sensory abnormalities in rodents are induced by various surgical procedures or drugs, and the behavioural characteristics of pain are recorded. The Sprague Dawley rat strain is commonly used due to its calm nature and ease of handling. The Wistar Kyoto strain has a more anxious phenotype (Will *et al.*, 2003), which is useful in pain research because clinical neuropathic pain is associated with depression and anxiety (Sullivan *et al.*, 2004). The most popular surgical methods are the chronic constriction injury (CCI) model (GJ Bennett & Xie, 1988) and the spinal nerve ligation (SNL) model (SH Kim & Chung, 1992) (Figure 1.8).

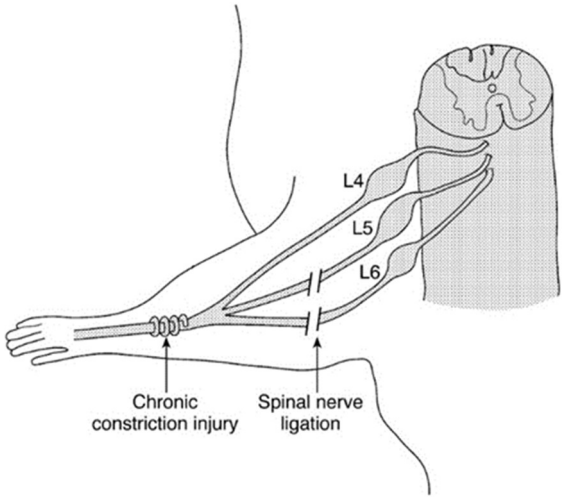


Figure 1.8: Schematic of the Common Models of Neuropathic Pain in Rat. CCI involves four ligatures tied loosely around the spinal nerve of mice or rats, whereas in SNL the L5 and L6 spinal nerves are fully ligated. Image from the British Journal of Anaesthesia (Campbell & Meyer, 2006).

Table 1.7: Strengths and Limitations of Surgical and Drug-Induced Animal Models (Rat and Mouse) of Neuropathic Pain

Model	Strengths	Limitations
Spinal Nerve Ligation Model Surgical model – tight ligation of L5 and/or L6 spinal nerves (Kim & Chung, 1992)	Mimics symptoms of human patients after nerve injury Produces "large and stable magnitude of pain behaviour" (Jaggi *et al.*, 2011) Reproducible (Huang *et al.*, 2016) Well-established and frequently used Surgical models have less variability as damage to each animal is uniform, compared to drug or chemotherapy induced models	Lesser degree of allodynia compared to other models (Jaggi *et al.*, 2011)
Chronic Constriction Injury CCI) Model Surgical model – four loose ligatures around	Reproducible (Huang *et al.,* 2016) Well-established and frequently used Mimics clinical post-traumatic painful neuropathies	Tension of ligations can be a source of variation

sciatic nerve (Bennett & Xie, 1988)	Behavioural signs of spontaneous pain	Thermal hyperalgesia is displayed in CCI model, which is not a symptom of clinical neuropathic pain
Chemotherapy-induced Model anti-cancer agents e.g. paclitaxel (Hama & Takamatsu, 2016)	Paclitaxel-induced neuropathic pain model well established (Liang *et al.*, 2018) Highly relevant to chemotherapy-induced neuropathic pain research (HJ Park, 2014)	Vincristine model failed to produce pre-pulse inhibition in rat models (Borzan *et al.*, 2004) Relevance may be limited for patients whose neuropathy is not chemotherapy-induced
Diabetes-induced Neuropathy Model Steptozotocin-incuded or genetic models (Jolivalt *et al.*, 2016)	Relevant to diabetic neuropathy, a common type of neuropathic pain Mechanical allodynia (Cheng *et al.*, 2014)	Blunted responses to heat (Cheng *et al.,* 2014)

1.7.2 Ethical Issues with Animal Models of Neuropathic Pain

All research involving animals is associated with ethical issues as animals cannot consent and will suffer during and after the procedure. These are addressed with by limiting the number of models used in a study and altering the procedure to keep undue suffering to a minimum if not eliminated. Neuropathic pain research has additional inherent ethical issues as they involve establishing chronic pain to the animal. However, animal models, particularly vertebrate models including rat, are invaluable to neuropathic pain research (Mogil *et al.* 2010) in the absence of alternatives with equal relevance to clinical neuropathic pain.

Alternative invertebrate models such as *Drosophila* or *C. Elegans* could be used, but these are of lesser relevance to human disease, and are considered to have a nociceptive, reflexive response to pain without the emotional component (Hesselson *et al.* 2020). Currently, a suitable *in vitro* model of neuropathic pain does not exist, but potential biomarkers can be investigated in relevant cell lines under appropriate conditions such as inflammation. Clinical research

involving patients with neuropathic pain diagnosis also provides valuable data in a real-life setting, but with disadvantages. The major disadvantage is lifestyle factors such as comorbidities and diet, cannot be controlled, though they may be accounted for if accurately recorded, and pain response is difficult to directly measure and monitor in humans (Ong & Seymour 2004). Animal models provide invaluable insight for gene expression changes in a controlled environment, and a relevant and appropriate alternative to human research, and remain necessary for further developments to neuropathic pain research.

1.7.3 Translational Results from Animal Models to Clinical Neuropathic Pain

The role of the immune system in peripheral and central sensitisation is well established in animal models (Calvo *et al.*, 2012), but to prove applicable to human neuropathic pain management these biomarkers must be present in humans and serve a similar, disease-associated pathophysiological function to that in the animal model. Biomarkers of neuropathic pain are investigated in clinical cohorts by analysing gene and protein expression in samples from patients, including blood and cerebrospinal fluid (Sisignano *et al.*, 2019) (Davies et al., 2007), as the blood brain barrier is compromised in neuropathic pain (Yao et al., 2014).

An example of good example of model to human translation is the melanocortin-1 receptor (MC1R) knockout animal models a as MC1R loss of function variants (rs1805007, rs1805008, and rs1805009) in humans, and demonstrate similar pain responses (Mogil et al., 2003). These MC1R variants are responsible for red hair (Rees et al., 1999) and are associated with reduced sensitivity to noxious stimuli and increased responsiveness to μ-opioid analgesics (Lacroix-Fralish et al., 2009). The melanocortin system, which primarily involved in skin pigmentation, is thought to modify pain via the opioid system (Vrinten et al., 2003).

Biomarkers are used clinically in several diseases. Cerebrospinal fluid and blood biopsies are sometimes used in the diagnosis of Alzheimer's disease, to measure levels of CNS plaque and

tangle components, beta-amyloid 42 and tau (Olsson *et al.* 2016). A well-known genetic variant used in clinical diagnosis and prognosis is the number of CAG (cytosine, adenine, and guanine) trinucleotide repeats in the *HTT* gene for Huntington's disease (Dayalu & Albin 2009). Tumour biopsies from cancer are widely-used patients are taken to diagnose the cancer types and determine the most appropriate treatment course. For example, the monoclonal antibody trastuzumab is only appropriate for herceptin positive tumours and is effective at improving long-term disease-free survival (Cameron *et al.*, 2007). Biomarkers can also be used as surrogate endpoints to monitor disease progression and response to treatment, such as in heart failure (Nadar & Shaikh, 2019).

1.8 Biomarkers of Neuropathic Pain

Several studies have proposed molecules as potential biomarkers of neuropathic pain (Table 1.8). Some biomarkers for pain have been subsequently disproven, as in plasma Cystatin C first described as a plasma marker of renal function by (Shimizu-Tokiwa *et al.*, 2002) and proposed as a pain biomarker by (Mannes *et al.*, 2003) in a small study of women in prolonged labour pain. This was then disputed after a validation study in a larger cohort (Eisenach *et al.*, 2004).

There are several molecules which recur in publications in neuropathic pain research (Table 1.8). TNF-α, IL-1β, and IL-6 levels are often measured as a surrogate marker for neuropathic pain (Hong *et al.*, 2019). Ding performed a series of investigated and found that TNFα induces the upregulation of $Na_v1.6$ via the STAT3 pathway (Ding *et al.*, 2019). In the rat L5 ventral root transection model of neuropathic pain, both $Na_v1.6$ were upregulated STAT3, and TNFα incubation of primary cultured DRG neurons upregulated $Na_v1.6$ expression.

Table 1.8: Recurring Candidates in Neuropathic Pain Research

Candidate	Methods and Evidence
TRPV1 in the brain	CCI mouse model (Arribas-Blazquez *et al.*, 2019) Highly expressed in microglia, and stimulation of microglial TRPV1 enhances neuronal glutamatergic transmission in CCI mice (Marrone *et al.*, 2017) p38 MAPK activation increases TRPV1 membrane expression and contributes to pain hypersensitivity (Ji *et al.*, 2002) Direct phosphorylation by PKA induces neuropathic pain in models (Chen *et al.*, 2011) Block by antagonist AMG-517 promotes axonal regeneration in sciatic nerve injury rat model (Bai *et al.*, 2018)
Voltage Gated Sodium Channels (e.g. $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$)	Neuronal excitation and the generation of action potential is mediated by voltage-gated sodium channels in nociceptors (Jurcakova *et al.*, 2018) Gain of function mutations in NaV channels results in hyperexcitable nociceptors (Garrison *et al.*, 2014) Presence of Nav1.8 and Nav1.9 in painful human lingual nerve neuromas, with correlation of pain sympotoms and Nav1.8 expression (Bird *et al.* 2013) Inhibitors attenuate mechanical allodynia in rat models of neuropathic pain (Suter *et al.*, 2013)
Purogenic receptor P2X7	Important mediator of neuropathic pain following nerve injury (Li *et al.*, 2017) Implicated in PDN and comorbid depressive symptoms, which can be reduced with P2X7 antagonist treatment (Guan *et al.*, 2019) Selective antagonists demonstrate dose-dependent antinociceptive effects in both neuropathic and inflammatory models (Carroll *et al.*, 2009)

Previous work by members of the Centre for Biomarker Research at the University of Huddersfield has identified several potential biomarkers. Buckley *et al.* 2017 identified strong

upregulation of tissue inhibitor of matrix metalloproteinase-1 (*Timp1*) and melanocortin-1 receptor (*Mc1r*) in rat SNL models, with increased TIMP1 levels in plasma samples of chronic neuropathic pain patients (Buckley *et al.*, 2017).

Thus far no potential biomarkers of neuropathic pain have been developed clinically for use in neither diagnosis nor treatment. There are major challenges for the clinical translation of potential biomarkers which have inhibited this process. These include the complex and dynamic nature of neuropathic pain which involves changes in pathophysiology over time (e.g early stages post-injury, wound healing, and pain chronicity), etiological differences, and response to medications (including tolerance e.g. opioid analgesics).

The biomarker evolution pipeline involves:

1. Identification of potential biomarkers (candidates)
2. Determination of the best candidate and best assay
3. Scientific validation of biomarker and assay performance (e.g. sensitivity and reproducibility)
4. Demonstration in clinical population
5. Biomarker becomes accepted as a clinical endpoint by regulators

These are outlined in the review by Borsook and colleagues (Borsook *et al.*, 2011). Once a biomarker candidate is identified, it is essential to fully investigate its function in both health and disease. This allows the changes in its expression or function to be accurately utilised to assess clinical state in a patient. A biomarker may be differentially expressed at different stages of the neuropathic pain disease states, or could be a gene with alleles or mutations associated with increased risk of neuropathic pain development.

If a biomarker is directly involved in the development of neuropathic pain may also have potential for pharmaceutical knockdown in the early stages of the disease, for either the prevention of further development or to slow it down. Such a biomarker would also have to be detectable in earlier stages of neuropathic pain. There is the additional challenge that patients may only seek medical help for pain once it presents outward symptoms or becomes chronic, which may be too late for such pharmaceutical intervention. Given that there is strong evidence that nervous system plasticity is the driving force behind neuropathic pain (Costigan *et al.*, 2009), it is possible that neuropathic pain may be reversed. This is an active area of research (Moutal *et al.*, 2018) (Dugan *et al.*, 2020) (Noor *et al.*, 2020).

1.9 Background on Candidate Biomarkers

This section provides information on the candidate biomarkers identified during this study.

1.9.1 Caspases

Caspases exist as inactive pro-caspases until they are activated by another caspase as part of an activation cascade. Activated caspases must then dimerise to form the active site, which is forced by induced proximity. In apoptosis, executioner caspases cleave specific substrates leading to the activation of other pro-apoptotic proteins and the degradation of integral cellular structures such as cytoskeletal proteins. Given their role in inflammation and cell death, differential expression of several caspases is to be expected in a model of chronic pain.

Casp1 and *Casp4* are cysteine-aspartic acid protease (caspase) pro-enzymes belonging to inflammatory group 1 (Munday *et al.*, 1995). Caspases exist as zymogens, and activation of procaspase 1 and procaspase 4 is mediated by the inflammasome formation by cleavage (Martinon & Tschopp, 2004). Rat *Casp1* has a human orthologue and *Casp4* is represented by *CASP4* and *CASP5* in humans. In humans, caspases 1, 4, and 5 form part of the inflammasome,

an important innate component of the inflammatory response and apoptosis (Tournier *et al.*, 2007). As part of the inflammasome, caspase 1 activates inflammatory cytokines IL1β (Martinon *et al.*, 2002) and IL18 (A Lu *et al.*, 2016). Caspase 4 feeds into these pathways by activating caspase 1 (Akhter *et al.*, 2012).

The inflammasome is a multi-protein complex activated by the intrinsic or extrinsic pathways during the early stages of the inflammatory response (Lang *et al.*, 2018). The intrinsic pathway is triggered by internal cellular distress signals, such as damage-associated molecular patterns (DAMPs) from damage to genomic DNA or mitochondria (Tummers & Green, 2017). The damaged outer mitochondrial membrane allows the contents of the inter-membrane space to spill out into the cytosol, and cytochrome c is released (Figure 1.9). Cytochrome c is a haem group containing redox-active protein, and haem group switching between Fe^{2+} and Fe^{3+} oxidation states is a key part of the electron transport chain (Kranz *et al.*, 2009). Cytosolic cytochrome c binds to apoptotic protease activating factor 1 (APAF1) adaptor protein and facilitates the formation of an APAF1 heptamer, exposing the caspase recruitment domains (CARDs) to which caspase 9 molecules bind to (Zou *et al.*, 1999). Induced proximity of the caspase 9 molecules lead to its activation and the caspase cascade is triggered (Salvesen & Dixit, 1999).

The extrinsic pathway is triggered upon the activation of receptors on the cell surface, and the transduction of intracellular effector molecules (Creagh, 2014). For example, TNF receptors (a Death receptor subfamily) can be activated by TNFα during an inflammatory response. All death receptors contain a 'death domain' within the cytoplasmic tail, which in receptor activation allows adaptor protein recruitment (e.g. FADD). These adaptor proteins contain death-effector domains which recruit pro-caspase 8, which becomes activated by induced proximity and dimerization and forms initiator caspase 8. This activated complex comprising of death

receptors, FADD, and caspase 8 is known as the death-inducing signalling complex (DISC). Active caspase 8 goes on to cleave and activate executioner caspases (3, 6, and 7) (Figure 1.9).

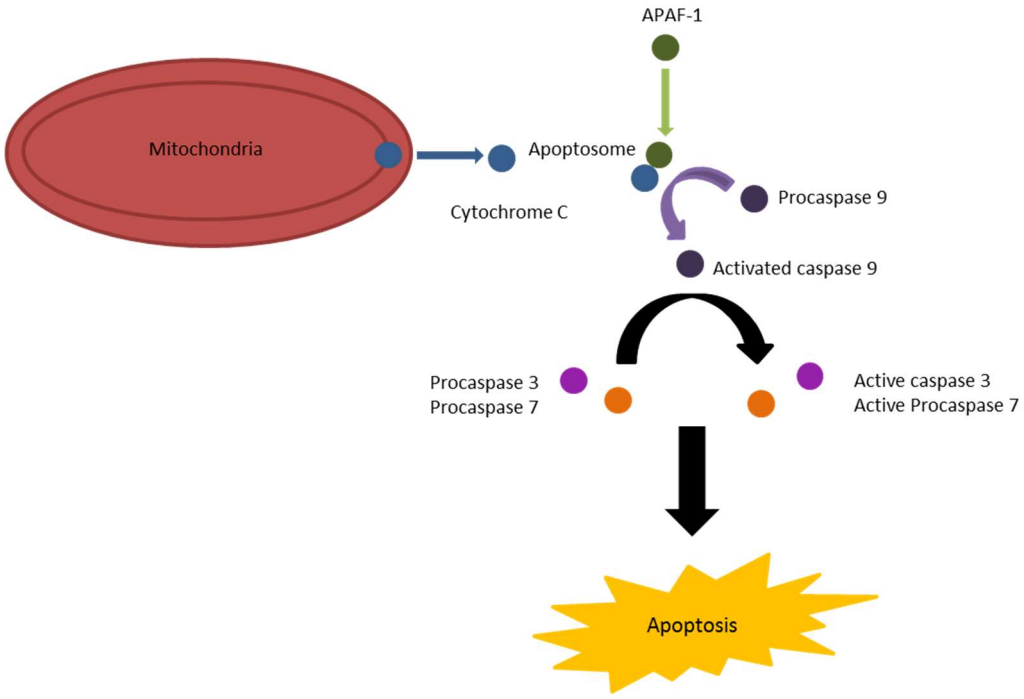


Figure 1.9: The Role of Cytochrome C in Apoptosis. Cytochrome c is a component of the electron transport chain anchored within the inner mitochondrial membrane. Cytochrome c is released into the cytosol under oxidative stress, and here it forms part of the apoptosome complex by binding to apoptotic protease activating factor 1 (Apaf-1) and procaspase 9. Procaspase-9 is activated and forms caspase 9, which in then activates caspases 3 and 7.

1.9.2 Inflammatory Mediators

Thioredoxin (TXN, sometimes denoted as Trx) is a class of two proteins (thioredoxin 1 and thioredoxin 2) that are fundamental to all life (Wollman *et al.*, 1988). These are oxidoreductase enzymes which reduce other proteins by cysteine thiol-disulfide exchange, a are essential for mammalian development (Nordberg & Arner, 2001). The thioredoxins are cytoprotective against oxidative stress (Yoshida *et al.*, 2005), and upon binding to apoptosis signal-regulating kinase 1 (ASK1) promotes its ubiquitination and degradation (Liu & Min, 2002). Thioredoxin

inhibition of ASK1 decreases release of the electron transport chain component cytochrome c from the mitochondria to the cytosol (Andoh *et al.*, 2002); both anti-apoptotic properties. The ASK1 apoptosis pathway is induced by oxidative stress, which may occur as part of the inflammatory response in neuropathic pain (Hattori *et al.*, 2009). TXN also promotes the binding of the transcription factor NF-κB and activator protein 1 (AP1) to DNA (Hirota *et al.*, 1997). These factors regulate transcription of an array of genes for various outcomes including cell survival and apoptosis (Gilmore, 2006) and are induced after brain injury to promote neuronal survival and repair (Pennypacker *et al.*, 2000).

CCR5 is expressed on the surface of macrophages, dendritic cells, eosinophils, T cells of the adaptive immune system, and microglia in the CNS, and facilitates chemotactic cell trafficking (Chtanova & Mackay, 2001, Waller & Sampson, 2018). It also promotes secretion of IL1β (Zhou *et al.*, 1998), and caspases 3, 8, and 9 (Ma *et al.*, 2006) (Ma *et al.*, 2005) indicating both pro-inflammatory and pro-apoptotic roles. To support this, *CCR5* upregulation has been reported in human rheumatoid arthritis, an inflammatory joint disease (Auer *et al.*, 2007) with which neuropathic pain is often reported (Ahmed *et al.*, 2014, Koop *et al.*, 2015).

Cluster of differentiation 4 (CD4) is a glycoprotein co-receptor expressed on the surface of T-helper (Th) cells, also known as CD4+ T cells (J Zhu & Paul, 2008). These cells assist the adaptive immune system in several functions, including antigen presentation, B cell antibody class switching, and cytotoxic CD8+ T cell maturation, as well as macrophage function in pathogen destruction (Taams *et al.*, 2005). These activities make CD4+ T cells are pro-inflammatory.

Romo1 protein reactive oxygen species modulator 1 (ROMO1) induces reactive oxygen species (ROS) production and has been linked to TNF-alpha-induced ROS production that in turn triggers apoptosis (JJ Kim *et al.*, 2010). *ROMO1* has previously been implicated as a diagnostic and

prognostic marker in lung and colorectal cancers (HJ Kim *et al.*, 2017, SH Lee *et al.*, 2017) and as a druggable target (Kim, Lee*, et al.*, 2018).

Fpr2 codes for the G-protein coupled receptor formyl peptide receptor 2 (FPR2) involved in the attenuation of the inflammatory response and limits tissue damage by neutrophil response attenuation (Martini *et al.*, 2016, Dakin *et al.*, 2014). FPR2 has several known ligands including annexin A1 and lipoxin A4. Annexin A1 is a Ca^{2+}-dependent phospholipid-binding protein which suppresses eicosanoid production and inhibits leukocyte adhesion in the acute phase of inflammation (Perretti & Dalli, 2009). Lipoxin A4 is a product of arachidonic acid and suppresses the expression of pro-inflammatory genes (Chandrasekharan & Sharma-Walia, 2015).

1.9.3 Other Genes of Interest

Four genes of interest, *A3galt2, Sh3bgrl3, Tmem88, and Plac8,* represent potential novel biomarkers due to their functionality not currently being fully understood, particularly with regards to pain mechanisms. Alpha 1,3-Galactosyltransferase 2 (A3galt2) is involved in the Globo Sphingolipid Metabolism and Glycosphingolipid biosynthesis pathways. A3galt2 synthesises the galactose-alpha (1,3)-galactose group on isogloboside 3 (iGb3) and catalyse the addition of galactose to iGb3 itself to form polygalactose structures. *ABO* is a paralog of A3galt2, which codes for the proteins of the human blood group system (Cid *et al.*, 2019), and *A3galt2* has implications for immunological compatibility in xenotransplantation (SE Kim, Kang*, et al.*, 2018). The iGb3 protein is recognised by natural killer (NK) cells (D Zhou *et al.*, 2004).

Sh3bgrl3 codes for the SH3-Domain-Binding Glutamate-Rich Protein Like 3 (SH3BGRL3), also known as tumour necrosis factor-alpha inhibitory protein, TIP-B1 (Berleth *et al.*, 2000). This protein is anti-inflammatory, and thioredoxin-like but cannot reduce other proteins, as it lacks the CXXC motif (Mazzocco *et al.*, 2002). TMEM88 is a known inhibitor of the Wnt/β-catenin canonical pathway (Figure 1.10), which is involved in neural development and plasticity in

embryogenesis and in adult brains (Chenn, 2008) and has been implicated in the production of hyperalgesia and allodynia in rat models of neuropathic pain (Zhang *et al.*, 2013).

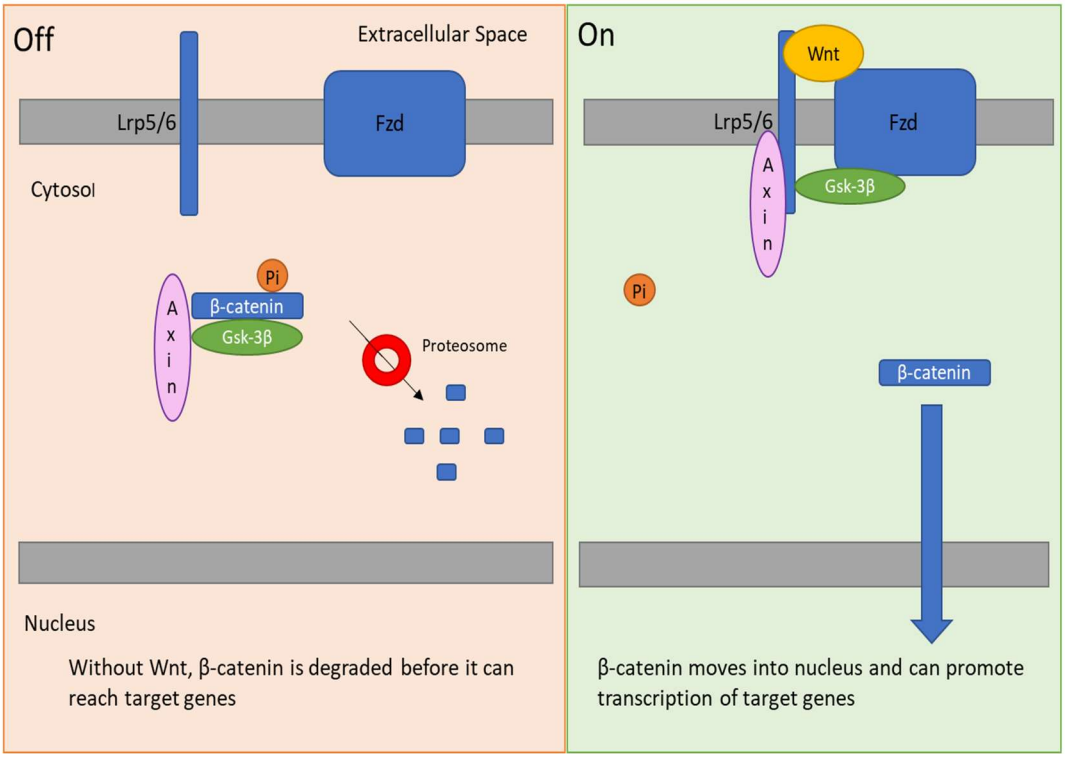


Figure 1.10: Wnt/β-catenin Canonical Pathway. In the absence of Wnt, β-catenin is phosphorylated and targeted for degradation by proteasomes. This inhibits transcription for genes which require β-catenin. In the presence of Wnt, β-catenin is dephosphorylated and can move into the nucleus to promote transcription of target genes. Therefore, Wnt is an essential factor in the transcription of β-catenin targeted genes. Fzd = Frizzled Receptors, GSK3β = Glycogen synthase kinase 3β.

Plac8 codes for the cysteine-rich placenta-specific 8 protein (also known as onzin), involved in differentiation of adipocytes, and nasopharyngeal cancer (Huang, *et al.*, 2019), and has a role in the regulation of autophagy. Recently a PLAC8 protein-motif has been described by (Cabreira-Cagliari *et al.*, 2018) to be conserved in a protein group of which there are three types: type I in mammals, fungi, and plants, and types II and III exclusive to plants. This PLAC8 motif-containing protein group is involved in a range of activities, including detoxification and infection response, and Ca^{2+} influx (Cabreira-Cagliari *et al.*, 2018).

1.9.4 MicroRNAs

MicroRNAs are small non-coding chains of approximately 18-22 nucleotides, which bind to messenger RNA (mRNA) of a target gene to block translation and protein synthesis (Olsen & Ambros, 1999). This reduces expression of the target gene and marks the mRNA for degradation (Bagga *et al.*, 2005). Differential expression of microRNAs has implications in disease, and even small changes to the microRNA profile have the potential to have knock-on effects with clinically relevant results. Simultaneous changes to several regulatory microRNAs for a target gene may also be indicative of its functional importance in a disease, e.g. neuropathic pain.

Table 1.9: MicroRNAs of Interest and Target Genes

MicroRNA	Targets	Target Function
mir29c	*Bace1, Mcl1*	Beta-secretase 1 is a transmembrane protease, involved in the formation of amyloid beta peptide from amyloid precursor protein in Alzheimer's disease pathology (Vassar *et al.*, 1999). MCL1 codes for the BCL2 family apoptosis regulator, an anti-apoptotic protein (Michels *et al.*, 2005).
mir34c	*Notch1 (Bae et al., 2012), Bcl2* (Slaby *et al.*, 2009),	The notch signalling pathway is highly conserved in mammals (Artavanis-Tsakonas *et al.*, 1999), and is involved in critical cell communication during growth and differentiation in many tissue types. In neuronal tissue, there is evidence for its role in the maintenance of neuronal stem cells (Aguirre *et al.*, 2010; Hitoshi *et al.*, 2002). *Bcl2* is a cell death inhibitor (Skommer *et al.*, 2010).
mir19b1	*Bace1 (Hebert et al., 2008)*	*See mir29c*
mir181b1	*Bcl2* (W Zhu *et al.*, 2010), *Nova1* (Zhi *et al.*, 2014), *E2f1* (Slaby *et al.*, 2009)	The *E2f*s are a family of transcription factors involved in regulation of cell cycling and apoptosis (Slaby *et al.*, 2009). Neuro-oncological ventral antigen 1 (target of hsa-*Mir181b1)* is an RNA binding protein which

		regulars alternative splicing and is exclusive to neurones (Zhi *et al.*, 2014).
mir Let 7a2	*Casp3, Casp8, (Tsang & Kwok, 2008) IL6 (Iliopoulos et al., 2009), E2f2 (Dong et al., 2010)*	Caspases 3 and 8 in humans involved in inflammatory processes
Mir1843b	*Card10*	CARD10, caspase recruitment domain family member 10 (Wang *et al.*, 2001)
hsa-Mir205	*Pten (Qu et al., 2012), IL24 (Majid et al., 2010)*	Phosphatase and tensin homolog (PTEN) is a tumour suppressor, involved in the regulation of cell cycling and apoptosis. IL24 is a pro-inflammatory cytokine with tumour suppressive effects.

Changes to *Mir29c* expression has been linked to psoriasis (Zibert *et al.*, 2010), Parkinson's disease (Pasinetti, 2012), and schizophrenia (Perkins *et al.*, 2007). *Mir34c* upregulation has been linked to Alzheimer's disease (Satoh, 2010, Zovoilis *et al.*, 2011), as is *mir19b1* downregulation via increased *Bace1* (Beta-secretase 1) activity. Beta-secretase 1 is a transmembrane protease, involved in the formation of amyloid beta peptide from amyloid precursor protein in Alzheimer's disease pathology (Vassar *et al.*, 1999). *Bace1* is also targeted by *miR29c* (Zong *et al.*, 2011), as well as the *Mcl1* gene, which codes for the anti-apoptotic protein BCL2 family apoptosis regulator (Michels *et al.*, 2005).

The notch signalling pathway is highly conserved in mammals and is involved in critical cell communication during growth and differentiation in many tissue types (Artavanis-Tsakonas *et al.*, 1999). In neuronal tissue, there is evidence for its role in the maintenance of neuronal stem cells (Aguirre *et al.*, 2010, Hitoshi *et al.*, 2002). *Mir34c* also targets *Bcl2*, a cell death inhibitor protein which promotes cell survival (Cleary *et al.*, 1986), which makes has implications for an anti-apoptotic role of *mir34c*.

It is reasonable to suggest that an altered microRNA expression profile may contribute to neuropathic pain pathology through pro-inflammatory and pro-apoptotic mechanisms. Changes in target gene expression are not necessarily reflected in the microRNA data because regulation is post-transcriptional. As particular microRNA profiles have already been recognised as unique to pathologies, including cancers and cardiovascular diseases (Bonci *et al.*, 2016, Min & Chan, 2015), there is evidence to support their potential use as biomarkers. CSF microRNA profiles have demonstrated use in identifying AD with 95.5% accuracy (Denk *et al.*, 2015), and lung cancer has been detected experimental using the plasma microRNA signature with 81% specificity (Sozzi *et al.*, 2014). MicroRNA-targeting drugs are being developed, including miravirsen, a short RNA molecule which targets miR-122 in the human liver, which despite concerns of inducing herpetic cancer (due to the protective role of miR-122), has demonstrated safety in chronic hepatitis C patients (van der Ree *et al.*, 2014) and the drug remains in Phase 2 clinical trials.

1.9.5 Olfactory Receptors

Olfactory receptors (ORs) are typically localised in sensory organs where they detect numerous ligands associated with odour within the compact cilia. ORs are Gs-coupled proteins activating adenylyl cyclase and increasing cyclic AMP levels, which targets an olfactory-specific cyclic nucleotide gated ion channel, allowing cation influx and depolarisation.

Expression of so-called 'ectopic' ORs and their downstream signalling molecules have been reported in human CNS neurons and other tissue types (Ferrer *et al.*, 2016). Olfactory Marker Protein (OMP), a plasma membrane protein which functions as a potassium-dependent sodium/calcium exchanger and a Ca^{2+}-ATPase, found in mature neurons of vertebrates and regulates OR expression (Pyrski *et al.*, 2007). OR expression is dysregulated in other neurological

diseases, including Parkinson's disease and Alzheimer's disease (Ansoleaga *et al.*, 2013, Grison *et al.*, 2014)

1.9.5 Histone proteins

Histone proteins package nuclear DNA in eukaryotic cells. DNA is wound around histone octamers to form nucleosomes, compacting the DNA. Histones exist in the forms H2A, H2B, H3, and H4 are core histones, and H1/H5 are linker histones (Bhasin *et al.*, 2006). Histone proteins bundle DNA into nucleosome units, the first component of DNA packaging in the nucleus (Figure 1.11). Histones are proteins which DNA coils around to tightly compart. Together, histones and DNA form the nucleosome, a key component of the chromatin structure. Histones play a key role in DNA accessibility, through post-translational modifications, such as acetylation and phosphorylation, to their structure known as histone code. These mechanisms alter the tightness of the chromatin and thus DNA accessibility and transcription. The packaging of DNA is a fundamental part of genome regulation, as access of transcriptional machinery to the DNA can be tightly controlled.

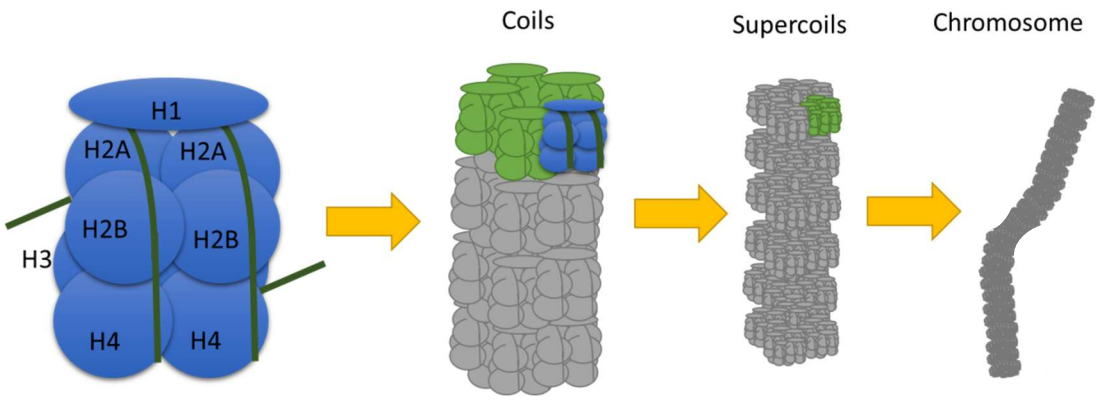


Figure 1.11: Histone Proteins and the Packaging of the Genomic DNA. Histone proteins H2A, H2B, H3, and H4 form octamers which the DNA wraps around to form the nucleosome complex. The H1 proteins sits outside the structure to keep it intact. These form the so-called "beads on a string" structure, which coils up twice to form a supercoil. The supercoil them folds several times over to form the recognisable chromosome structure of packaged DNA. These structures unravel to expose the DNA strand during transcription and replication (Verdone *et al.*, 2006).

Chemical modifications to histone proteins has a key role in gene expression regulation, altering access to DNA by transcription machinery. Acetylation and deacetylation of histone lysine residues are key gene regulation processes, carried out by the enzymes histone acetyltransferase (HAT) and histone deacetylase (HDAC). These processes are involved in gene expression changes in disease states, including inflammation (Barnes *et al.*, 2005), and altered activity of HAT enzymes and HDACs have been linked to asthma and chronic obstructive pulmonary disorder (Mroz *et al.*, 2007).

Histone acetylation occurs *in vivo* as a method of transient gene expression modification, induced in response to changes in the extracellular environment. Addition of the acetyl groups to histone tail lysine residues is catalysed by HATs (Robison & Nestler, 2011), and their removal by HDACs catalyse the removal of acetyl groups from histones (Kouzarides, 2007). Together, these enzymes tune gene expression; histone acetylation loosens the chromatin, whereas acetyl group removal causes the chromatin to tightly condense, making the DNA inaccessible to

transcription factors (Kuo & Allis, 1998). Thus, acetylation and deacetylation promote and restrict gene transcription and expression respectively (Bowman & Poirier, 2015). Dysregulated histone protein modification results in defective chromatin remodelling, DNA replication and repair, and epigenetic regulation of genes (Chen *et al.*, 2014). Histone deacetylation has been implicated as a critical step in the development of pain resulting from nerve-injury, with deacetylation inhibitors blocking chemokine and cyclo-oxygenase-2 upregulation and relieving pain (Khangura *et al.*, 2017, Danaher *et al.*, 2018). Sanna *et al.* describe the effect of the selective HDAC1 inhibitor, LG325, in dose-dependently reducing mechanical allodynia in mouse SNI (Sanna *et al.*, 2017). HDAC5 is implicated in both depression-related behaviours in rat models, and in the SNI model of neuropathic pain (Descalzi *et al.*, 2017).

Histone modification and release is also seen in other disease states. For example, in PD histone acetylation levels are higher in midbrain dopaminergic neurons than controls (Park *et al.*, 2016), and histones can be released by damaged cells can serve as DAMPs (Qaddoori *et al.*, 2018). Histones are also involved in DNA repair and the recruitment of repair proteins to sites of double stranded breaks (Uckelmann & Sixma, 2017), an important mechanism in cell health.

1.9.6 Ribosomal Proteins

Ribosomal proteins make up the ribosomal subunits involved in translation in protein synthesis and are highly conserved in all species. Differential expression of ribosomal protein genes occurs in organ development, for example RPL24 in ovary and testis in marine shrimp (Zhang *et al.*, 2007). Stress-specific changes to the expression of ribosomal proteins have been reported in plant roots (Wang *et al.*, 2013), and in 2015 Saisu *et al.* reported expression changes in ribosomal proteins in the amygdala of SNL mice. It is possible that the large-scale changes involved in the shift of an organism into a hypersensitive state induces or requires changes to transcriptional machinery such as ribosomes.

1.9.7 Pseudogenes

A high number of pseudogenes are differentially expressed in the SNL versus sham. Pseudogenes are segments of genes that are related to protein-coding genes with high sequence similarity, do not produce a protein (Tutar, 2012). This loss of function may be due to premature stop codons or frameshift mutations (Zheng *et al.*, 2007). In humans there are approximately twenty thousand pseudogenes in the genome, compared to twenty-seven thousand protein-coding genes (Han *et al.*, 2011). Pseudogenes however are not always non-functional and may perform some regulatory functions similar to that of other non-coding DNA segments such as small interfering RNAs (siRNAs). Some pseudogenes lack promoter regions and introns termed "processed pseudogenes", which have been incorporated into the chromosome from mRNA (Harrison *et al.*, 2005). Differential expression of pseudogenes in the SNL model may either have functional relevance in the pathophysiology of neuropathic pain or may result from other regulatory changes. In the microarray, changes to expression of pseudogenes are not reflected in their coding gene counterparts.

1.9.8 Transcription Factors

Transcription factors (TFs) represent an important mechanism for gene expression control, and common TFs amongst differentially expressed genes in a disease can be indicative of important mechanisms. These are proteins that bind sequence-specific segments of DNA or messenger RNA (mRNA) (Latchman, 1997). They bind to enhancer or promoter regions of DNA upstream of the gene, and they can either promote or block gene transcription (Gill, 2001). TFs achieve this by stabilising the binding of RNA polymerase to the DNA for transcription, or by blocking it. Coactivators and corepressors are accessory proteins recruited by the transcription factors which enhance or decrease transcription rates respectively (Xu *et al.*, 1999). Transcription factors are important for cellular response to environmental cues, as well as in cell

differentiation in development and gene expression changes during the cell cycle. Four TFs are of interest to this study, representing common upstream regulators of for our candidate biomarkers of neuropathic pain.

Pax-6 is active during embryonic development and neurogenesis (Heins *et al.*, 2002). It contains a paired box domain and a homeobox domain both of which act as transcription factors. This protein is important for neural development. Brn-2, a POU-III class protein involved in neuronal differentiation ensures Schwann cell development and myelination in the absence of Oct-6 (Jaegle *et al.*, 2003). It also enhances activation of genes regulated by corticotropin-releasing hormones (Ramkumar & Adler, 1999).

Mesoderm posterior protein 2 (MESP2) belong to the basic helix-loop-helix (bHLH) family of TFs, and controls Notch signalling pathways, which has been previously implicated in the establishment and maintenance of neuropathic pain (Yan-Yan 2012). Notch pathways are highly conserved proliferative signalling pathways involved in neurogenesis. MESP2 is principally involved in somitogenesis in embryonic development (Maroto *et al.*, 2012).

Homeobox transcription factor Gsx-2 (formerly Gsh-2) is expressed during CNS development (Pei *et al.*, 2011), and is active during the lateral ganglionic eminence (LGE) in the ventral telencephalon (Szucsik *et al.*, 1997), an area which later forms the basal ganglia, where it promotes the maintenance of LGE progenitors and prevents differentiation (Pei *et al.*, 2011). Gsx2 mutants exhibit a larger volume of oligodendrocyte progenitor cells in mice embryos (Chapman *et al.*, 2018). Gsx-2 is also expressed in mouse olfactory bulb stem cells (Mendez-Gomez 2012 (Mendez-Gomez & Vicario-Abejon, 2012) and is required for olfactory bulb neurogenesis (Waclaw *et al.*, 2009). As olfactory receptor expression is abhorrent in the SNL model, it is possible that Gsx-2 may be in part facilitating these changes. It is possible these TFs

are active in the neuropathic pain state as structural changes and plasticity occur subsequent to injury, and these factors may be (in part) facilitating such changes.

1.10 Aims and Objectives

This project aims to identify biomarkers of chronic neuropathic pain with potential for the development of improved diagnostic techniques and targeted pharmaceutical treatments. This may include novel proteins not previously linked to neuropathic pain, or proteins with known functions in either pain or inflammation, but not explicitly chronic neuropathic pain.

More specifically, this project aims to:

1. Identify candidate biomarkers, using differential gene expression in the ipsilateral dorsal horn tissue in SNL rat model of neuropathic pain versus sham-operated control. Consider fold change, significance level, and known biomarker function and interactions with other genes and pathways reported in the literature.
2. Validate the candidate biomarkers in a clinical cohort of neuropathic pain patients, by assessing differential gene expression by qPCR from whole blood samples, by comparison of patients and healthy controls without neuropathic pain.
3. Measure expression changes of 'validated' candidates at both gene and protein level in inflammatory cellular models relevant to the central nervous system or the immune system, to elucidate their response to inflammatory conditions for comparison to data from neuropathic animal models and patients.

Candidates which demonstrate differential gene expression in both the SNL model and clinical cohort could have a significant role in either the development or maintenance of the neuropathic pain state. This cross-species validation will provide strong evidence for a role in

the pathology of neuropathic pain. Though extensive functional analysis of the candidates will be required to assess their full potential as biomarkers for neuropathic pain, and will provide essential groundwork for the biomarker development pipeline into clinical tools, this project aims to fulfil the first stage of the pipeline by identifying candidate biomarkers. The goal of this work is to contribute to the identification of a biomarker or biomarkers which can be developed into a clinical tool for accurate diagnosis of neuropathic pain.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Animal Model Work

- Sprague Dawley rats supplied by Harlan, UK
- Rat Transcriptome Assay (RTA) 1.0 Arraychip (Affymetrix, Santa Clara, USA) was performed by AROS Applied Biotechnology (Aarhus, Denmark) on an Affymetrix GeneTitan instrument.

2.1.2 Clinical Work

- Patient questionnaire included the (Self-reported) Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS) (Bennett, 2001), Patient Health Questionaire-9 (PHQ-9), and Generalised Chronic Pain Scale (GCPS)
- Clinical samples collected at Seacroft Hospital, Leeds
- Control Samples Collected at the clinical facilities at University of Huddersfield Podiatry Clinic, Huddersfield
- PAXgene Blood RNA Tubes purchased from Beckton Dickinson and Company (BD) Diagnostics (Wokingham, United Kingdom)
- LC480 LightCycler and LightCycler® 480 SYBR Green I Master Roche Life Science (Basel, Switzerland)

2.1.3 Cell Culture

- Human Astrocytes 1321N1 purchased from Sigma Aldrich (St Louis, USA)
- SH-SY5Y, SK-N-SH, and THP-1 cells purchased from ATCC®

- Cell culture vessels including flasks (T25, T75, T175) from Corning® Sigma Aldrich (St. Louis, MO, USA), and plates (6 well, 12 well, 24 well, 96 well) from Starstedt (Nümbrecht, Germany)
- Cell culture media including Dulbecco's Modified Eagles Medium, Roswell Park Memorial Institute (RPMI) 1640, and Ham's F-12 Nutrient Mixture were purchased from ThermoFisher (Waltham, MA, USA)

In Vitro Work

- GeNorm Reference Gene purchased from PrimerDesign (Southampton, UK)
- Primers purchased from Eurofins (Luxembourg)
- iTaq Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
- CFX96 Bio-Rad Laboratories, Inc.
- Infinite® F50 Absorbance Microplate Reader and Magellan™ data analysis software, Tecan Life Sciences (Männedorf, Switzerland)

Western Blot

- FiveEasy Standard pH Meter Mettler-Toledo Ltd. (Leicester, United Kingdom)
- Millipore H_2O Purification System, Merck (Darmstadt, Germany)
- The following were purchased from Bio-Rad Laboratories, Inc.
    - Gel casts, plates, and well combs
    - Mini-PROTEAN Tetra Vertical Electrophoresis Cell
    - PowerPac Universal Power Supply
    - Transblot Turbo Transfer System
    - ChemiDoc XRS+ Imaging System
    - 4x Laemmli Sample Loading Buffer
    - Immun-Blot® PVDF Membrane

- Blot Absorbent Filter Paper
- Clarity Western ECL Blotting Substrates

2-Mercaptoethanol (Sigma-Aldrich)

SDS-PAGE Gels made in-house:

- 40% Acrylamine ThermoFisher
- 2% Bis solution Bio-Rad Laboratories, Inc.
- Tris Solution pH 8.8 and pH 6.8 made in-house: Tris Base (ThermoFisher, Waltham, MA, USA) plus Hydrochloric Acid and Sodium Hydroxide solutions (ThermoFisher) for pH change
- 10% SDS Solution Sigma Aldrich (St. Louis, MO, USA)
- TEMED Bio-Rad Laboratories, Inc. (Hercules, CA, USA)
- TBS-T: NaCl 137mM, KCl, 2.7 mM, Tris Base 19nM, Tween 20 (x20) ThermoFisher (Waltham, MA, USA)

Running Buffer made in-house: 144g Glycine, 30g Tris Base, 10g SDS, ThermoFisher (Waltham, MA, USA), Millipore H_2O adjust to 1 litre (Merck, Darmstadt, Germany)

Transfer Buffer made in-house: 3g Tris Base, 14.4 g glycine, 200 ml methanol ThermoFisher (Waltham, MA, USA), Millipore H_2O adjust to 1 litre (Merck, Darmstadt, Germany)

<u>Antibodies</u>

- Anti-ANXA1 Rabbit Polyclonal Antibody (ab137745) Abcam (Cambridge, UK)
- Anti-FPR2 Rabbit Polyclonal Antibody (STJ192622) St John's Laboratory (London, UK)
- Anti-OMP Rabbit Polyclonal Antibody (STJ13100243) St John's Laboratory
- Goat Anti-Rabbit Alexa 546 Fluorescent Secondary Antibody (A-11035) ThermoFisher (Waltham, MA, USA)

- Goat Anti-Mouse Alexa 647 Fluorescent Secondary Antibody (A-21245) ThermoFisher
- Horseradish Peroxidase (HRP) Conjugated Secondary Antibody Goat Anti-Rabbit (GtxRb-003-DHRPX) Immunoreagents Inc (Raleigh, NC, USA)
- Horseradish Peroxidase (HRP) Conjugated Secondary Antibody Goat Anti-Mouse (GtxMu-003-DHRPX) Immunoreagents Inc

ELISA Kits

- Human IL-6 DuoSet ELISA R&D Systems Inc., Bio-Techne (Minneapolis, USA)
- PLAC8 FineTest (Hubei, China)
- ROMO1 Elabscience (Houston, TX, USA)

2.2 Methods

2.2.1 Spinal Nerve Ligation (SNL) Model

The spinal nerve ligation (SNL) model of neuropathic pain in adult male Sprague Dawley (Harlan, UK) provided the spinal cord dorsal horn tissue for this project. Animal husbandry, surgery, and tissue harvest procedures were approved by the Animal Care and Research Ethics Committee, National University of Ireland (NUI), Galway, Ireland, and carried out under license from the Department of Health in the Republic of Ireland and in accordance with EU Directive 2010/63. Rats were housed singly with free access to food and water, under a controlled temperature (21 ± 2°C) and 12-hour light-dark cycling. After one week, animals underwent surgery, either L5 SNL (n=10) or sham (n=10) and maintained until 35 days post-surgery. Euthanasia by decapitation was performed and tissue was harvested from the spinal cord DH ipsilateral to the side of nerve injury was snap-frozen on dry ice and stored at -80°C. These preparations are matched for age and weight.

Establishment of the model, maintenance, behavioural tests, and dorsal horn tissue extraction were performed by collaborative colleagues in Prof. David Finn group at NUI, Galway. Behavioural tests include von Frey test, Hargreaves test, and acetone test, method as previously described in Moriarty *et al.,* 2016.

2.2.2 Clinical Cohort2.2.2 Recruitment and Blood Collection

Fifty-three adult neuropathic pain patients were recruited from Seacroft Leeds Teaching Hospital (Ethics NHS - 14/YH/0117). Questionnaires completed at time of blood collection included screening questions for exclusion criteria (fibromyalgia, cancer, multiple sclerosis, and diabetes patients were excluded), the pain assessment (self-reported) Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS), Patient Health Questionaire-9 (PHQ-9), and Generalised Chronic Pain Scale (GCPS) were completed by the patients. Data concerning the nature of the pain was also collected, including diagnosis, number of months since pain began, current medications, and comorbidities. Current relevant medications were categorised as i) anti-inflammatory drugs including non-steroidal anti-inflammatories (NSAIDs) and paracetamol, ii) anti-depressants, including tricyclic anti-depressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin and norepinephrine reuptake inhibitors (SNRIs), iii) anti-convulsants, and iv) opioid analgesics.

Sixty-five age and sex-matched controls were recruited from Huddersfield, UK. The control cohort also completed the questionnaire without the pain assessment. Informed consent was obtained for both cohorts prior to participation. Questionnaires were completed by participants themselves.

Blood samples from both cohorts were collected in PAXgene Blood RNA Tubes (BD diagnostics, Wokingham, United Kingdom) from the antecubital fossa using the standard phlebotomy technique. Blood collection was performed by trained phlebotomists Catherine McHugh, Laura

Clayton, and Kristen Hawkins at the University of Huddersfield. Samples were stored at -20°C for <24 hours prior to long term storage at -80°C.

2.2.3 Cell culture

Human Astrocytes 1321N1 (Sigma Aldrich, St Louis, USA) were cultured in Dubecco's Modified Eagle's Medium High Glucose supplemented with 10% FBS (Gibco™, ThermoFisher) and 10nM L-glutamine (Sigma Aldrich, St Louis, USA) at 37°C, 5% CO_2, 25% O_2. Astrocytes were plated in 6 well tissue culture plates (Starstedt) at seeding density $1x10^5$. After 24 hours, FBS supplement was reduced to 3% to slow further growth and inflammation was induced. Tumour necrosis factor alpha (TNFα) (400µM) and interferon gamma (IFNγ) (100µM) (ThermoFisher) were added to the media and cells were incubated for a further 48 hours.

SH-SY5Y (ATCC® CRL-2266™) were cultured in Dubecco's Modified Eagle's Medium High Glucose supplemented with 10% FBS (Gibco™, ThermoFisher) and 10nM L-glutamine (Sigma Aldrich, St Louis, USA) at 37°C, 5% CO_2, 25% O_2. Cytokine treatment included 100µM IFNγ for 24 or 48 hours.

SK-N-SH (ATCC® HTB-11™) were grown to 80% confluency in 6-well plates in DMEM media supplemented with FBS (10%) and 10µM L-glutamine. Once confluent, media was removed and replaced with low serum media (5% FBS) to inhibit proliferation. Retinoic acid was added at concentration 10µM. Negative controls included no addition of retinoic acid and the addition of a DMSO bolus for volume adjusted control.

THP-1 cells (ATCC® TIB-202™) were maintained in RPMI media supplemented with 10% Fetal Bovine Serum (FBS) and L-glutamine 10mM (all from ThermoFisher). Cytokine treatment included 100µM IFNγ for 24 or 48 hours.

The pilot mechanical stress test was performed on 1321N1 cells at 90% confluency. A 10ul pipette tip was used to scrape vertically and horizontally in a grid-like formation. Cells were photographed at x20 magnification 24 hours, 48 hours, 72 hours, and 96 hours EVOS XL Core Imaging System.

2.2.4 RNA Extraction

2.2.5.1. RNA Extraction of Rat Dorsal Horn Tissue

Total RNA extraction was performed by Dr David Buckley (McHugh group) at the University of Huddersfield using PAXgene Blood RNA Tubes using the Preserved Blood RNA Purification Kit II (Norgen, Biotek, ON, Canada) according to the manufacturer's instructions. RNA was treated with DNAse and purified on columns. RNA concentration was measured on a NanoDrop ND2000 ultraviolet–visible (UV) spectrophotometer (Labtech International Ltd, UK)

2.2.5.2. RNA Extraction of Whole Blood

Total RNA was extracted using the Preserved Blood RNA Purification Kit II (Norgen, Biotek, ON, Canada) according to the manufacturer's instructions. In brief, the RNA was treated with DNase (Thermo Fisher Scientific) and purified on columns. RNA extraction from clinical samples was performed by Catherine McHugh (McHugh Group) at the University of Huddersfield. Final RNA concentration was measured on a NanoDrop ND2000 UV spectrophotometer. Complementary DNA (cDNA) was prepared from the 500ng extracted RNA using the Verso cDNA Synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.2.5.3 RNA extraction from Tissue Monolayers

Adherent cells were washed twice with DEPC PBS and lysed on the culture dish using 1ml of TRI Reagent (ThermoFisher) per 10cm^2 of glass culture plate surface area. Cell lysate was mixed by

pipette to form a homogenous lysate and incubated at room temperature for 5 minutes. For suspension cells, the media containing the cells was centrifuged at 300g for 10 minutes. Media was removed and cells were resuspended in DEPC PBS. This process is repeated once before final centrifugation and resuspension in 400µl TRI Reagent and incubated at room temperature for 5 minutes.

Chloroform was added to the cells at a ratio 1:5 to TRI Reagent. Samples were shaken vigorously for 15 seconds and incubated for 15 minutes at room temperature. Samples were centrifuged at 12,000g for 15 minutes. The upper aqueous phase was transferred to a fresh 1.5ml tube containing isopropanol at 1:2 ratio to TRI Reagent, mixed by pipette, and incubated 10 minutes room temperature. Samples were then centrifuged at 12,000g for 10 minutes. Supernatant is removed by pipette and the RNA pellet is washed with 75% ethanol at 1:1 ratio to TRI Reagent, after the addition of which the tubes are centrifuged 7,500g, or 12,000g if RNA pellet floats, for 5 minutes. RNA pellet was air-dried for 10 minutes and then resuspended in DEPC H_2O (30-50µl depending of pellet size). To facilitate dissolution sample tubes were mixed in a shaking incubator at 55–60 °C for 15 minutes.

2.2.4.4 RNA Quality Control

RNA concentration of each sample was analysed by NanoDrop ND2000 UV Spectrometer and 5µl was run on a 1.2% agarose gel, prepared with DEPC TBE, to assess RNA integrity.

2.2.5 Affymetrix Microarray

RNA library was prepared by Dr. David Buckley and samples were posted to AROS Applied Biotechnology (Aarhus, Denmark) for RNA quality control and microarray on Rat Transcriptome Assay (RTA) 1.0 Arraychip (Affymetrix). Total RNA was labelled using an Ambion WT Expression kit (Life Technologies, Bleiswijk, The Netherlands) and hybridised to Affymetrix Rat

Transcriptome Array (RTA) 1.0 (Affymetrix, Santa Clara, CA, USA). Sample labelling, hybridization to chips, and image scanning were performed according to the manufacturer's instructions on an Affymetrix GeneTitan instrument by AROS Applied Biotechnology (Aarhus, Denmark). Quality control was performed using Affymetrix Expression Console and interpretation of data was facilitated by Affymetrix Transcriptome Analysis Console 2.0 (TAC2.0). The term 'transcript' is used here to refer to cDNA sequences on the microarray transcriptome chip and includes genes, microRNAs, and non-coding transcripts. Genes that belong to *Rattus norvegicus* are written according to the proper nomenclature e.g. '*Fpr2'*. Non-italised coded refer to the protein product of these genes: 'Fpr2'. When genes and proteins are discussed in the context of the work of others, the appropriate nomenclature will be used to refer to the candidate according to the species in which the experiment was performed.

2.2.6 DNase treatment and cDNA Synthesis

The following protocol was carried out on RNA from tissue monolayers and RNA extracted from clinical blood. Approximately 500ng RNA in DEPC H_2O (<4µl) is used for cDNA synthesis. DNase buffer x1 (0.5µl) and DNase (1U) is added and the sample is incubated at 37°C for 60 minutes. Thereafter 0.5µl x1 EDTA is added followed by 65°C incubation for 10 minutes. The Verso cDNA Synthesis Kit (ThermoFisher) was used for cDNA synthesis. To each sample 2µl x5 reaction buffer, 1µl dNTP mix (final concentration 500 µM each), 0.75µl (400 ng/µL) 300ng random hexamers, 0.25µl (500 ng/µL) 125ng anchored oligo dTs, and 0.5µl Verso enzyme (units not specified by kit), is added. Samples are then incubated 42°C for 60 minutes followed by 2 minutes at 95°C.

2.2.8 Quantitative Polymerase Chain Reaction

For clinical samples, qPCR was performed on the Roche LC480 system in a 96-well format. Reaction mix of 10µl per well was prepared with 1µl diluted 1:50 cDNA, 0.3µM forward and reverse primers and 5µl x2 Roche Mastermix containing SYBR Green I dye. Cycling conditions were as follows: one cycle of pre-incubation 95°C for 5 minutes, 45 cycles of amplification including 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C, followed by the melting curve protocol of 5 seconds at 95°C and 1 minute of 65°C, and finally cooling at 40°C for 30 seconds.

For cell culture samples, primers were designed for optimal performance using web-based Primer 3 software [primer3.ut.ee]. For the cell culture samples, qPCR was performed on the BioRad CFX96 Reaction mix of 10µl per well was prepared: 5µl cDNA, 0.3µM forward primer, 0.3µM reverse primer, and 5µl x2 iTaq Mastermix containing SYBR Green I dye. Cycling conditions were as follows: one cycle of pre-incubation 95°C for 5 minutes, 45 cycles of amplification including 10 seconds at 95°C, 10 seconds at 60°C, and 10 seconds at 72°C, followed by the melting curve protocol of 5 seconds at 95°C and 1 minute of 65°C, and finally cooling at 40°C for 30 seconds.

A GeNorm analysis was carried out on 6 samples to determine the most stable reference genes in the samples using qbase+ software (Biogazelle, Belgium). Data was then normalised to 2-3 reference genes with stability criteria (M value <0.5, CV <25%). Log normalised gene expression data was output from qbase+ for further data analysis discussed below. GeNorm analysis was performed on each cell line, THP-1, PMA-treated THP-1, SHSY5Y, and 1321N1.

2.2.9 Protein Extraction

Growth medium was removed from the cells by pipette aspiration. Adherent cells were washed twice with PBS. Suspended cells were transferred to an Eppendorf and spun for 10 minutes at

5,000g. Ice cold PBS (1ml) was added to the well and the cells were scraped from the well by pipette and transferred to an Eppendorf. For suspended cells ice cold PBS was added to the tube and the pellet was resuspended. Samples are then spun down at 1000 x g for 5 min.

Appropriate volume of RIPA Buffer (Sigma-Aldrich) (~50-200ul) and protease inhibitor (Sigma-Aldrich) (1ul in 100ul RIPA) was added to each sample, which was then incubated at 4°C) for 20 minutes. The samples are then centrifuged at 10,000 x g for 10 minutes. The supernatant was transferred to a fresh tube and Bradford Assay is performed. Proteins are stored at -80°C.

2.2.10 Protein Quantification

Bradford Assay was performed on 1ul of protein extract in a 96 well flat-bottomed plate with 200ul of Bradford Assay Reagent (BioRad). Standard curve protein samples were prepared using Bovine serum albumin (Sigma-Aldrich) 10mg/ml stock dilution within a 1mg – 62.5µg/ml range. Absorbance was measured using the Tecan Plate Reader at 595nm and Magellan™ data analysis software. Protein concentrations for each extract sample was then calculated using the standard curve readings.

2.2.11 Western Blot

Plasma and protein samples were prepared at 20µg in 15µl with 5µl 4x laemmli sample buffer (LSB) (BioRad) with β–mercaptoethanol (Sigma Aldrich) and boiled at 100^{o}C for 10 minutes. Protein samples were run on a 12% SDS PAGE separating gel and 6% acrylamide stacking gel by electrophoresis at 35mA with Precision Plus Protein Standard (BioRad). Proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane using the Transblot® Turbo™ (BioRad). The membrane was blocked for 1 hour with 5% Marvel dried skimmed milk powder in TBS-T blocking buffer at room temperature on a seesaw rocker.

Primary antibody was prepared in a 1:1000 dilution in 1% milk blocking buffer, added to membrane and incubated on ice overnight rocking. Fluorescent antibodies for the protein of interest (rabbit host) and reference protein B Actin (mouse host) (1:2000) are prepared in the same solution and incubated with the membrane simultaneously.

The membrane was then washed in six changes of TBS-T with 5-minute incubation per change. The membrane is then incubated for 1-hour rocking with the secondary antibody in a 1:2000 dilution in blocking buffer. TBS-T washing step is repeated with a final wash in TBS without Tween 20. ECL substrate solution is prepared 1:1 (BioRad) and membrane is imaged and quantified on Chemidoc Imaging System (BioRad) using the appropriate channel (e.g. Alexa 647).

For the enhanced chemiluminescence (ECL) method, the membrane is stripped of all antibodies and re-probed with the primary antibody for the reference protein. Membrane is washed TBS-T and incubated in Restore™ stripping buffer (Thermofisher) at room temperature for 30 minutes. Protocol is repeated from blocking step. Incubate with primary for the reference protein, wash, incubate with secondary antibody, wash, and imaged as described. Western blot quantification for both methods was performed on the Chemidoc Imaging System (BioRad) software, and normalised to the loading control in MS Excel.

2.2.12 Enzyme-linked Immunosorbent Assay

The Sandwich ELISA kit for ROMO1 (Elabscience, Houston TX USA) was used according to the manufacturer protocol. In brief, 100µl prepared standards (10-0.16ng/mL) and samples (500ng) was added to each well and plate was sealed and incubated for 90 min at 37°C. The samples and standards were then removed by inverting the plate on a paper towel, and 100µL Biotinylated Detection antibody was added per well for 1-hour incubation at 37°C with plate sealed. The plate was then aspirated and washed thrice with wash buffer, then 100µl HRP-Conjugate per well was

added and the plate was sealed and incubated for 30 min at 37°C. The plate was then aspirated and washed five times with wash buffer, and then 90µl of substrate reagent was added to each well for a 15-minute incubation at 37°C with plate sealed. Thereafter 90µL TMB Substrate is added to each well and the plate was sealed and incubated for 30 minutes at 37°C. Finally, 50µL Stop Solution was added to each well and the plate was read immediately at 450nm using the Tecan Magellan Infinite® F50 microplate reader.

The PLAC8 Sandwich ELISA kit (FineTest, Hubei, China) was used according to the manufacturer protocol. In brief, plate was washed twice, standards (1000-15.625pg/ml) and 500ng of protein samples were prepared and 100µL standard or sample was added to each well, plate was sealed and incubated for 90 minutes at 37°C. Biotin-labelled antibody 0.1 ml working solution added into above wells (standard, test sample & zero wells). Plate is sealed with a cover and incubated at 37°C for 60 min. Plate is aspirated and washed plates 3 times, with wash buffer incubating for 1 minute at room temperature with each wash. SABC Working Solution 100µL is added to each well, and plate is sealed and incubated for 30 minutes at 37°C. Plate is then aspirated and washed as before five times, and 90µL TMB Substrate is added to each well. Plate is then sealed and incubated for 30 minutes at 37°C. Finally, 50µL of Stop Solution is added and the plate is read at 450nm immediately.

2.2.13 Data Analysis

2.2.12.1 Statistical Analysis of Microarray Data

A Gene Level Differential Expression Analysis was executed in Affymetrix Transcriptome Analysis Console 2.0 (TAC2.0) (ThermoFisher Scientific). A One-Way Between-Subject ANOVA (unpaired) with the criteria (fold change $\geq +1.25$ and p value ≤ 0.05) applied. Alternative Exon Splice

Analysis was also executed in Affymetrix Transcriptome Analysis Console 2.0 (TAC2.0) (ThermoFisher Scientific).

Data was further analysed using QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) to identify. The *p* value of overlap between the dataset and existing literature finding is calculated in IPA® using a right-tailed Fisher's Exact Test where significance level threshold of ≤ 0.05 was applied. The IPA® database searches publications across all species, and the human nomenclature. From this analysis, genes of interest were selected according to the known protein function and interactions for testing in clinical samples by qPCR. Common transcription binding sites among genes of interest in *Homo sapiens* were identified using MatInspector™ software (Genomatrix, Munich). The core similarity value indicates degree of core sequence match for the transcription factor and gene. The core sequence is defined as the highest conserved positions in the transcription factor. The maximum value of 1 represents a full match.

2.2.12.2 Statistical Analysis of Clinical Samples

A GeNorm analysis was carried out on 12 samples to determine the most stable reference genes in the samples using qbase+ software (Biogazelle, Belgium). Data was then normalised to the reference genes *TOP1* and *YWHAZ,* which met the stability criteria (M value <0.5, CV <25%). Log normalised gene expression data was output from qbase+ for further data analysis discussed below. Normalised data was analysed using covariate analysis with Bonferroni correction and age and gender controlled for, in IBM ® SPSS Statistics Software (Armonk NY, USA) ($p \leq 0.05$) with 95% confidence interval. Graphs were made in GraphPad Prism 7 (GraphPad Software Inc., San Diego CA). To determine whether each differentially expressed gene may be contributing to nociceptive or neuropathic pain, the whole patient cohort was split according to their S-LANSS

score. High scores (≥12) are indicative of neuropathic pain and low scores (<12) are indicative of nociceptive pain. Samples sizes are as follows: n = 13 low score and n = 38 low score.

The data which met the appropriate criteria was analysed to investigate the possibility of gene expression changes in the administration of drug groups by one sample t-test, and patient questionnaire data was analysed by linear regression. using IBM ® SPSS Statistics Software (Armonk NY, USA).

2.2.12.3 Statistical Analysis of Cell Culture

Mean±SEM was calculated in Microsoft Excel. Kruskal-Wallis was performed on Western blot and qbase+ data with Dunn's correction in GraphPad Prism 7 (GraphPad Software Inc., San Diego CA). Graphs were created using GraphPad Prism 7.

Chapter 3: Gene Expression Profile of the Dorsal Horn Tissue in the Rat Spinal Nerve Ligation Model versus Sham-Operated Control

3.1 Introduction

Gene expression changes in an animal model of neuropathic pain could be indicative of which molecular pathways are active or inactive in the disease. Animal models of neuropathic pain have been developed since the 1970s, and modern surgical models involve peripheral nerve ligation to produce the neuropathic pain phenotype in rat or mouse. The L5/6 spinal nerve ligation (SNL) model in male Sprague Dawley rats, a popular choice for the study of neuropathic pain, was used to study differential gene expression in the dorsal horn (DH). Initially developed by (Kim & Chung, 1992), SNL involves ligation of L5 and L6 lumbar spine nerves under isoflurane anaesthesia. Sham-operated control rats underwent the same procedure without ligation to control for the effects of surgery. Behavioural tests are used to evaluate the success of the neuropathic pain model in producing the desired phenotype. These tests assess specific neuropathic pain symptoms including hot and cold sensitivity, allodynia, and hyperalgesia. Techniques elicit pain responses in the model and measure e.g. time to response, strength of response, and specific nocifensive behaviours such as licking. In this study the Von Frey assay for mechanical allodynia, the Hargreaves assay for heat sensitivity, and the acetone spray test for cold allodynia were used. Results of the behavioural tests for this dataset are owned by the collaborator of this work Prof. David Finn at National University of Ireland, Galway.

The manual Von Frey assay assesses mechanical sensitivity on the hind paw in response to pressure from Von Frey filaments (small nylon rods) of varying diameter. Pain responses (e.g. paw withdrawal and other nocifensive behaviours) are expected to occur at a lower pressure as

Von Frey filaments become narrower. In mechanical sensitivity the threshold for pain responses is lowered. The animal is elevated on a mesh platform and the filaments are inserted through the mesh onto the hind paw. This approach negates the risk of handling-induced stress which could influence results, though it is necessary to acclimatise the animal to the cage beforehand (Chaplan *et al.*, 1994). Natural grooming behaviours could be interpreted at false negative responses (e.g licking) and exploring behaviours could provide false positive responses (e.g. avoidance). There is also the possibility that rodents respond to the initial touch of the filament if it is not applied smoothly, or if it causes scratching. Responses could be difficult for less experienced experimenters to distinguish and this has implications for data quality. This can be improved by training and experience, or video recording for two-person validation. Alternative methods of measuring mechanical sensitivity in animal models include the electrical Von Frey fibre method, an automated system which applies a single filament to the hind paw over a range of pressure.

The Hargreaves assay allows quantification of heat threshold and thermal sensitivity using a high-intensity beam of light on the hind paw (Hargreaves 1988). Time to hind paw withdrawal is measured, and this is expected to decrease with increased sensitivity. Although a modified Hargreaves test has been developed which uses a glass floor to smoothly apply the heat source, it is not yet commercially available (Banik & Kabadi, 2013). Alternative tests for heat stimuli sensitivity in animal models include the tail flick test (which uses a light beam) (D'amour & Smith, 1941), hot plate test (Woolfe and Macdonald, 1944), and thermal probe test (Deuis & Vetter, 2016).

The acetone test involves exposure of the hind paw to acetone. It is used for measuring the threshold to cold stimuli and measures cold sensitivity (cold allodynia) (Vissers & Meert, 2005). Though there is concern that results of this test could be confounded by reactions to the smell

and sound of the acetone (Colburn *et al.*, 2007). However, this assay has been validated in several neuropathic pain models (Carlton *et al.*, 1994). Video recording is often required to accurately quantify nocifensive responses. A temperature preference test could also be used as a marker for thermal aversion (Moqrich *et al.*, 2005).

DH tissue was chosen for gene expression analysis because there is evidence for this area of the central nervous system (CNS) being a key player in the establishment of neuropathic pain (Ossipov *et al.*, 2010). The DH of the spinal cord is where peripheral nerves synapse with CNS neurones, and is an important area for pain modulation (Todd, 2010). The DH tissue is a grey area of the spinal cord (no myelinated axons), comprised of the termini of primary afferent neurons, interneurons, secondary projection neurones, satellite glial cells (SGCs), and microglia (Meneses *et al.*, 2017, Haring *et al.*, 2018). Further details of the role of the DH in neuropathic pain is discussed in Chapter 1, Section 1.4.4.

Global gene expression in the DH tissue was measured using the Affymetrix Rat Transcriptome Array (RTA). Microarray measures global gene expression measurement in one sample, which is useful for comparing gene expression across groups e.g. SNL model versus sham-operated control. Microarray exploits the hydrogen bonding of complementary nucleotides between two DNA strands. The microarray chip contains thousands of spots on its surface (Figure 3.1). Each spot contains 'probes', which are DNA oligonucleotides complementary to sequences within the target gene. The sample is prepared with fluorescent labelling, in this case cDNA prepared from the DH tissue. The closer the probe sequence complements its target in the sample, the higher the number of hydrogen bonds formed between the two cDNA strands. When the chip is washed weakly complementary sequences are broken and non-specific strands are removed; only strongly matched hybridisations remain. Probe-target hybridisations are detected and

quantified by fluorescent signalling as a measure of gene expression in the sample. The greater the fluorescent signal in each spot, the higher the gene expression in the sample.

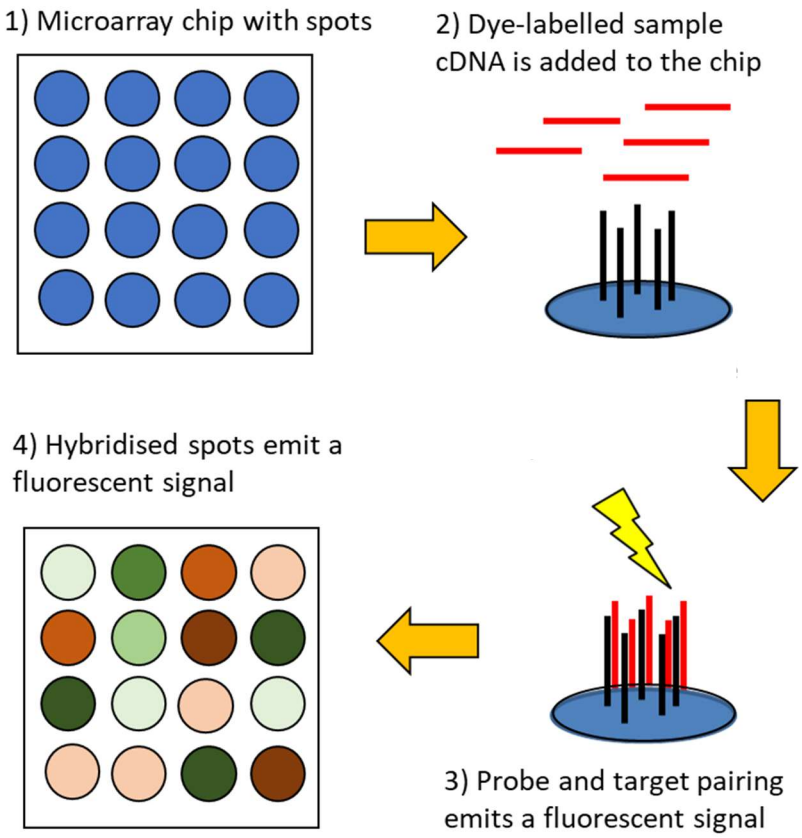


Figure 3.1: Principles of the DNA microarray. 1) The DNA microarray chip contains thousands of 'spots', each containing multiple nucleotide chains (probes) complementary to a target gene. 2) Sample cDNA is labelled with a fluorescent dye and added to the chip. 3) Transcripts in the cDNA sample bind to complementary probes. The higher the complementarity between the target and probe, the greater the number of hydrogen bonds, and the stronger the interaction between the two strands. The chip is washed to remove weakly bonded transcripts, leaving only transcripts strongly bound to their complementary probe. 4) The probe is then scanned, and the remaining transcripts fluoresce. The strength of the signal corresponds to the number of transcripts present, providing a quantitative measure of gene expression across the transcriptome in the sample.

The RTA is a traditional solid-phase array, where the spots are attached to a solid surface (chip). The RTA is an Exon Array, and probes are specific to exon splice sites in a target. Alternative splice analysis can be used to analyse changes to transcript ratios between groups. The relative intensity of these splice-specific probes allows the detection and relative quantification of different splicing isoforms in the transcriptome sample.

Bioinformatic analysis of the microarray data involved Affymetrix Transcriptome Analysis Console (TAC), Ingenuity Pathway Analysis (IPA®) (Qiagen, Hilden, Germany) to elucidate genes and pathways of interest (candidates), and Genomatrix MatInspector™ to identify common transcription binding sites between the genes identified. Candidates were selected from differentially expressed genes according to their function, known interactions from the literature, fold change, and level of significance.

Common transcription factor (TF) binding sites are of interest because TFs regulate gene transcription and have multiple, overlapping targets. It is possible that some differentially expressed genes result from a change in the expression and activity of a common upstream TF. Results from the TF binding sites analysis could represent common upstream pathways changed in the SNL DH tissue. Literary search of these common TF binding sites could help determine whether these represent a promising avenue for further research and help elucidate upstream mechanisms in neuropathic pain.

3.2 Results

3.2.1 Changes to Gene Expression Occurs in the Dorsal Horn after SNL

Of the total 68,842 transcripts in the RTA array, 1371 (1.99%) met our selection criteria (fold change ≥ ±1.25 and p value ≤ 0.05) (Figure 3.1). Of these 1371 genes, 1046 (76.29%) were

upregulated and 325 (23.71%) were downregulated (Figure 3.2). The full table of RTA results and quality control report is available at https://www.dropbox.com/sh/sc7je8e3n9o1cdm/AADalh6vSxhFDI2CCW2hxO6Ja?dl=0.

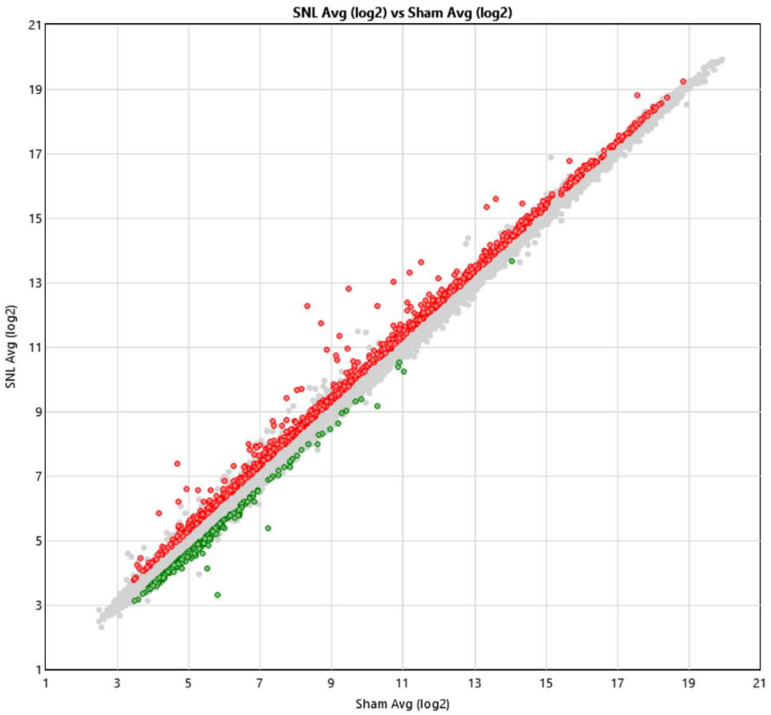


Figure 3.2: Scatterplot of gene expression changes in Sprague Dawley SNL versus sham. A total of 1371 genes passed the ±1.25 fold change and $p \leq 0.05$ filter. Of these, 325 transcripts are downregulated in SNL versus Sham) (23.71%), shown in green and 1,046 genes are upregulated in SNL versus sham (76.29%), shown in red. Genes which did not change expression are shown in grey. Image from Affymetrix Transcriptome Analysis Console (TAC) v4.0.

Of the 66,842 total genes in the Affymetrix RTA 1.0 microarray 10,838 (15.74%) are currently unassigned (unmapped) in the RTA library. A sample of unassigned genes was investigated using online databases and tools, including Aceview, UCSC genome browser, and Rat Genome Database (available at www.ncbi.nlm.nih.gov/ieb/research/acembly; www.ucsc.edu/; and rgd.mcw.edu/ respectively) and were found to be largely ribosomal or non-coding in origin and disregarded from the analysis.

Table 3.1: Differential Gene Expression Profile of Sprague Dawley SNL versus Sham Rats.

Group	Total	Passed Filter	Up-Regulated	Down-Regulated
Unassigned	10838	348	308	40
Coding	24753	327	275	52
Non-Coding	25625	302	137	165
Pseudogene	2026	138	136	2
Precursor microRNA	1626	97	73	24
Multiple Complex	2259	70	57	13
Small RNA transcripts	1324	45	23	22
Ribosomal	370	37	30	7
tRNA	21	7	7	0

Of the 1,046 upregulated genes 26.29% are coding, 29.45% are unassigned, and 13.1% are non-coding (Figure 3.3). Of the 325 downregulated genes 16% are coding, 12.31% are unassigned, and 50.77% are non-coding (Figure 3.3). Multiple complexes are transcripts that form part of a larger unit.

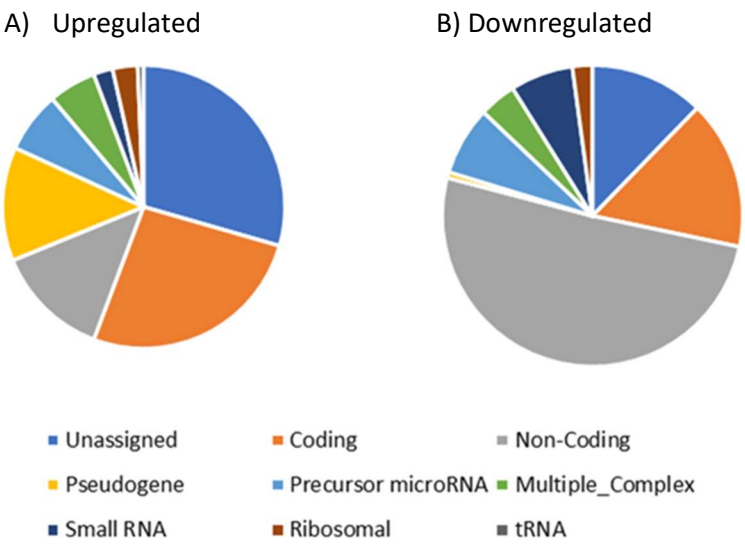


Figure 3.3: Differential Gene Expression Profile of Sprague Dawley SNL versus Sham Rats. A) Of the 1,046 upregulated genes 26.29% are known coding, 13.1% are non-coding, and 29.45% are unassigned. Of the remaining genes, 13% are pseudogenes, 2.2% are small RNAs, 2.87% code for ribosomal proteins, 5.45% of Multiple Complex genes, 6.98% are precursor microRNAs, and 0.67% are for tRNAs. **B)** 16% of the 325 downregulated genes are coding, and 50.77% are non-coding. Unassigned genes make up 12.31% of these and 7.38% are precursor microRNAs. 4% are part of multiple complexes. 0.62% are pseudogenes, 6.77% are small RNAs, 2.25% code for ribosomal proteins, and none of the downregulated genes are for tRNAs. Data output from Affymetrix Transcriptome Analysis Console (TAC) v4.0.

The top 20 upregulated genes (Table 3.2) demonstrate a range of different proteins including receptors, transcription factors, secreted chemokines, and zymogens. Candidates marked in blue were selected for network interaction analysis in IPA® based on their known function, to find known interactions between candidates in the literature.

Table 3.2: Top 20 upregulated gene expression changes in Sprague Dawley SNL versus sham-operated control

Transcript Cluster ID	Accession No	Gene Symbol	Gene Name	Fold Change	*P* value
TC4_KL567939v1_ random00000014.rn.1	NM_005523.5	*Hoxa11*	Homeobox A11	2.01	0.04
TC1500000377.rn.1	NG_007044.1	*Trav3d-3*	T-cell receptor alpha variable	1.95	0.02
TC1400000175.rn.1	NM_001130715.	*Plac8*	Placenta-specific 8	1.61	0.02
TC1700001790.rn.1	NM_021839.1	*Hist1h2a*	histone cluster 1, H2a	1.56	0.03
TC1500000736.rn.1	XM_017014657.1	*Anxa1*	*Annexin a1*	1.43	0.05
TC0800001253.rn.1	NM_022706.2	*Gabarapl2*	Gamma-aminobutyric acid receptor-associated protein-like 2	1.42	0.02
TC0700001918.rn.1	NM_001308639.1	*Hoxc11*	Homeobox C11	1.38	0.05
TC1700000666.rn.1	NM_022686.2	*Hist1h4b*	Histone cluster 1, H4b	1.38	0.02
TC0600000290.rn.1	NM_019150.1	*Ucn*	Urocortin	1.37	0.01
TC1300000004.rn.1	NM_001100983.1	*Myl6l*	Myosin, light polypeptide 6	1.37	0.00
TC0500002077.rn.1	NM_001109428.1	*Mrpl20*	Mitochondrial ribosomal protein L20	1.36	0.00
TC1400001453.rn.1	NM_001017496.1	*Cxcl13*	Chemokine (C-X-C motif) ligand 13	1.35	0.03
TC0800001851.rn.1	NM_053960.3	*Ccr5*	chemokine (C-C motif) receptor 5	1.35	0.04

TC0400001196.rn.1	NM_053289.1	*Reg3b*	regenerating islet-derived 3 beta	1.35	0.02
TC0800000013.rn.1	NM_053736.2	*Casp4*	Caspase 4	1.34	0.03
TC1800001603.rn.1	NM_012839.2	*Cycs*	Cytochrome C	1.33	0.03
TC1000003076.rn.1	NM_001128155.1	*Tmem88*	Transmembrane protein 88	1.33	0.02
TC0700000551.rn.1	NM_001277222.1	*Ndufa13*	NADH:ubiquinone oxidoreductase subunit A13	1.32	0.02
TC0300002143.rn.1	NM_001195490.1	*Romo1*	Reactive oxygen species modulator 1	1.32	0.01
TC0500001514.rn.1	NM_138524.2	*A3galt2*	alpha 1,3-galactosyltransferase 2	1.31	0.04

The top 20 downregulated genes are shown in Table 3.3 with candidates marked in blue. Of the twenty, ten are olfactory receptors, four are inflammatory mediators, and one is involved in inflammatory resolution.

Table 3.3: Top 20 downregulated gene expression changes in Sprague Dawley SNL versus sham

Transcript Cluster ID	Accession No	Gene Symbol	Gene Name	Fold Change	*P* value
TC0200002726.rn.1	NM_017110.1	*Cartpt*	CART prepropeptide	-1.44	0.01
TC2000000930.rn.1	NM_001000266.1	*Olr1671*	Olfactory receptor 1671	-1.40	0.02
TC0500001327.rn.1	NM_001000409.1	*Olr855*	Olfactory receptor 855	-1.39	0.01
TC0300001396.rn.1	NM_001000610.1	*Olr771*	Olfactory receptor 771	-1.38	0.02
TC2000000944.rn.1	NM_001000274.1	*Olr1690*	Olfactory receptor 1690	-1.37	0.05
TC0900001683.rn.1		*Defb49*	Defensin Beta 49	-1.36	0.02
TC0100004596.rn.1	XM_001073508.5	*Fpr2*	Formyl peptide receptor 2	-1.35	0.00
TC0500003477.rn.1	NM_001000412.1	*Olr867*	Olfactory receptor 867	-1.35	0.02
TCUn_KL568409v 100000010.rn.1	NM_001000177.1	*Olr172*	Olfactory receptor 172	-1.34	0.01
TC0800002334.rn.1	NM_001000789.1	*Olr1338*	Olfactory receptor 1338	-1.31	0.00
TC1200000934.rn.1	NM_001192005.1	*Cd209e*	CD209e molecule	-1.31	0.01
TC1700000686.rn.1	NM_146807.1	*Olfr1366*	Olfactory receptor 1366	-1.30	0.00
TC1400001946.rn.1	n/a*	*Rfwd2l1*	Ring finger and WD repeat domain 2-like 1	-1.30	0.05
TC2000000017.rn.1	NM_001000275.1	*Olr1688*	Olfactory receptor 1688	-1.29	0.02
TC0200000571.rn.1	NM_030945.3	*C1qtnf3*	C1q and tumour necrosis factor related protein 3	-1.28	0.01

TC1500000686.rn.1	NM_001037529.1	*Defb44*	Defensin Beta 44	-1.28	0.01
TC0700000081.rn.1	NM_001000068.1	*Olr1024*	Olfactory receptor 1024	-1.27	0.01

**Rfwd2l1 has since been withdrawn by the Rat Genome Database*

In addition to the top 20 upregulated and downregulated genes, four differentially expressed genes (Table 3.4) were also selected network interaction analysis in IPA® based on their known function, and some well-known interactions with other selected candidates. These are *Sh3bgrl3, Casp1, Cd4, Txn1,* and *Omp.*

Table 3.4: Additional Genes of Interest

Transcript Cluster ID	Accession No	Gene Symbol	Gene Name	Fold Change	*P* value
TC0500003800.rn.1	NM_031286	*Sh3bgrl3*	SH3 domain binding glutamate-rich protein like 3	1.30	0.008
TC0800000012.rn.1	NM_012762	*Casp1*	Caspase 1	1.27	0.046
TC0400003743.rn.1	NM_012705	*Cd4*	CD4 molecule	1.26	0.049
TC0500002825.rn.1	NM_053800	*Txn1*	Thioredoxin 1	1.28	0.003
TC0100006086.rn.1	NM_012616	*Omp*	Olfactory Marker Protein	-1.25	0.005

Seventy-nine microRNAs met the criteria (fold change ± 1.25, $p \leq 0.05$). Due to the high volume, six are shown and discussed based upon their known targets and relevance to neuropathic pain (Table 3.5).

Table 3.5 MicroRNA Expression Changes in Sprague Dawley SNL versus Sham

Array ID	Accession No	MicroRNA	Fold Change	*P* Value
TSUnmapped00000239.rn.1	NR_031835	*Mir29c*	1.31	0.039
TC0800000518.rn.1	NR_031800.1	*Mir Let 7a2*	1.25	0.016
TC0800002532.rn.1	NR_031848	*Mir34c*	-1.25	0.041
TC1300002249.rn.1	NR_031920	*Mir205*	-1.26	0.016
TC1500001090.rn.1	NR_031821	*Mir19b1*	-1.28	0.048
TC1300000383.rn.1	NR_031926	*Mir181b1*	-1.4	0.011

Below is a flowchart diagram which illustrates how the results of the microarray informed the selection of candidates for biomarkers and subsequent methods of investigation (Figure 3.4).

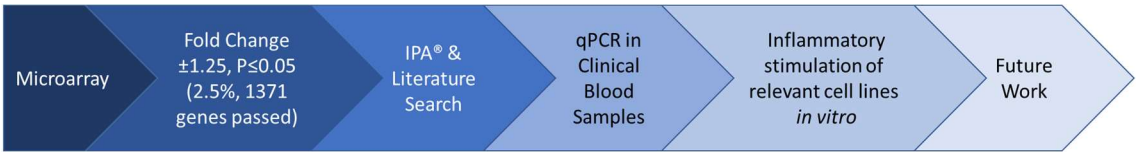


Figure 3.4: Flowchart diagram of Workflow. Microarray data was analysed, and the results were processed using Ingenuity Pathway Analysis (IPA®). Candidates were selected based on their fold change, interactions, and known function. Differential expression of candidates was then investigated in inflammatory models of relevant cells lines.

3.2.2 Ingenuity Pathway Analysis (IPA®) Software Elucidated Known Interactions Between Candidates

IPA® elucidates functional relationships between the differentially expressed genes in neuropathic pain (Figure 3.5). Twenty-two genes of interest were selected according to their known function and analysed against the IPA® literary database. Figure 3.5 shows interactions between these genes of interest that have been reported in the literature. Four additional genes involved in the caspase cascade (*Casp3, Casp8, Casp9,* and *Nlrp3).* Three components of the

Nalp3-inflammasome complex, formed during the innate inflammatory response (Broz 2016), was also included (*Casp1, Asc,* and *Nalp3*).

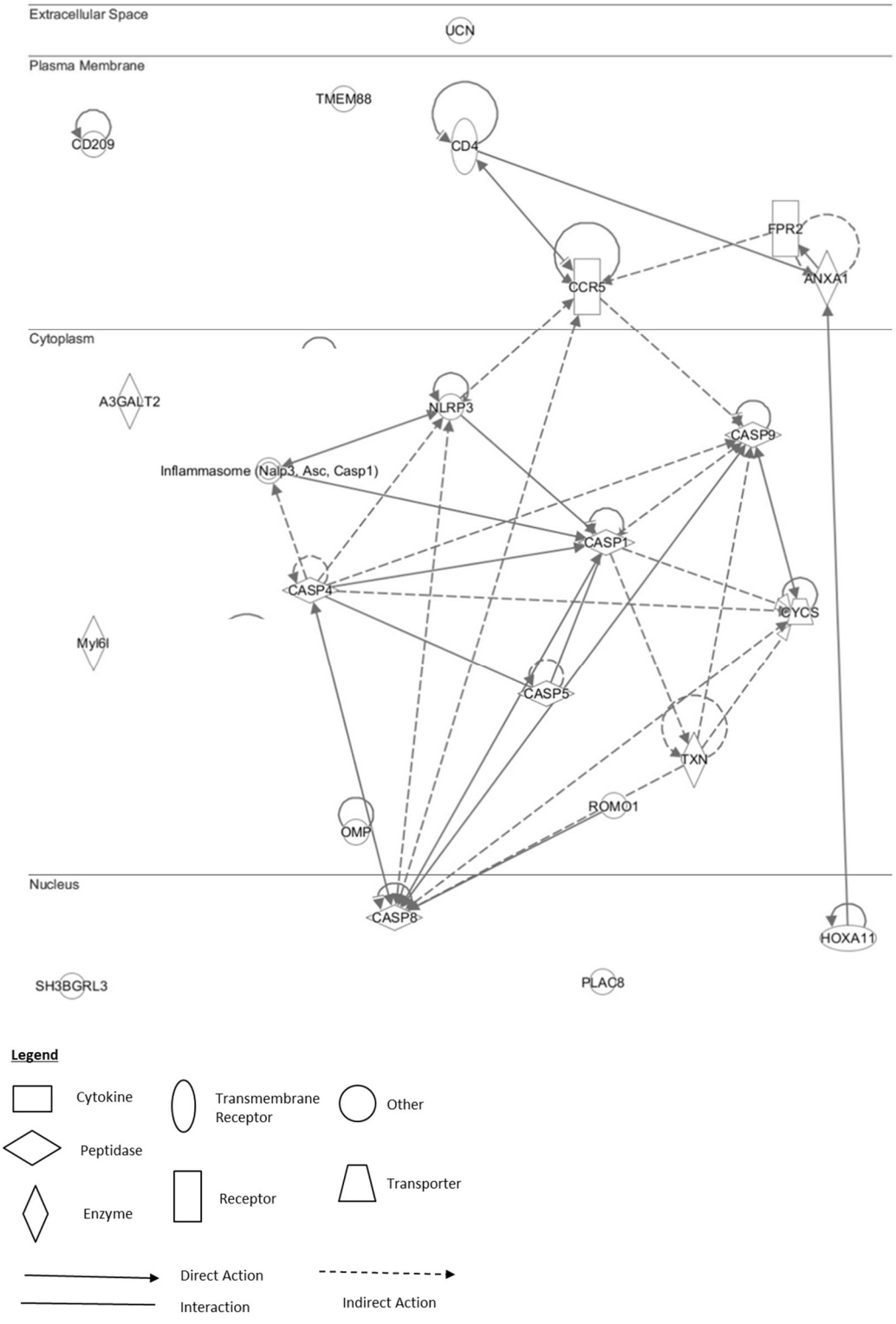


Figure 3.5: Literature mining of interactions between candidates from top rat array results in IPA®. Pathway analysis highlights interactions amongst differentially expressed genes and their associated protein products, to identify potential pathways and networks active in the neuropathic pain SNL model. Figure downloaded from IPA® Software, Qiagen. IPA® Figure Legend adapted from IPA® Software, Qiagen.

Seven of these genes (*Ucn, Cd209, Tmem88, A3galt2, Plac8, Sh3bgrl3*, and *My161)* have no known interactions with the other genes of interest found in the IPA® database. Most of these interactions are direct or indirect actions, shown by solid and broken lines respectively (Figure 3.5). None of the interactions are inhibitory except for the self-regulation of CASP8 (Wurstle et al., 2010). The schematic diagram in Figure 3.6 demonstrates the relationships between caspases 1 and 4, thioredoxin, and cytochrome c, with supporting evidence from *in vitro* experiments, as discovered through IPA® literature mining.

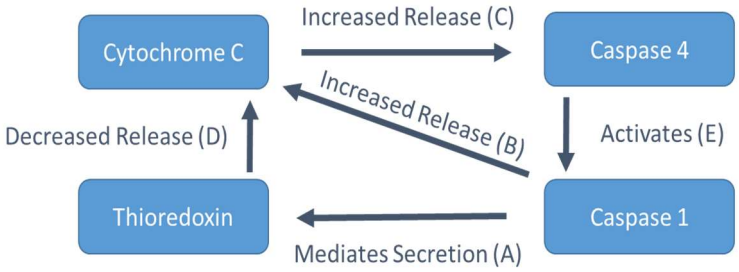


Figure 3.6: Interactions between the genes Cycs, Casp4, Casp1, and Txn1 and the proteins they code for in *in vitro* models. These genes are upregulated in the SNL rat within 1.27-1.34 range, *p* value < 0.05. Caspase 1 (*Casp1*) mediates thioredoxin secretion (A) (Sollberger *et al.*, 2012). Caspase 1 and caspase 4 (*Casp4*) facilitate migration of cytochrome c from mitochondria to the cytosol (B) (Pelletier *et al.*, 2006) (C) (Verkerk *et al.*, 2001), where thioredoxin reduces it (D) (Andoh *et al.*, 2002). Caspase 4 activates caspase 1 (E) (Akhter *et al.*, 2012). Adapted from figure provided by IPA® Software. Qiagen.

IPA® identified the top canonical pathways active in the neuropathic pain model (Figure 3.7). These are pathways which have the highest proportion of genes differentially expressed.

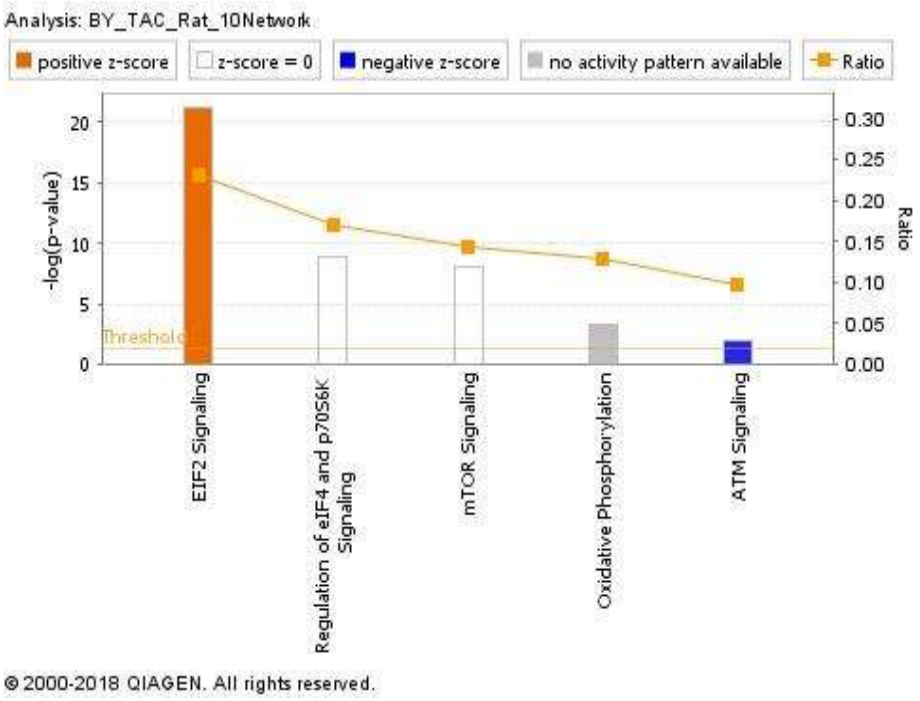


Figure 3.7: Top Canonical Pathways in Ingenuity Pathway Analysis (IPA). EIF2 Signalling is the pathway with the highest proportion of components activated (23%, 44 of 191). The remaining four pathways of the top five are *Regulation of eIF4 and p70S6K Signalling* (17.0% 24/141), *mammalian target of rapamycin (mTOR) Signalling* (14.4% 26/180), *Oxidative Phosphorylation* (12.8% 11/86)*, and Ataxia-telangiectasia mutated (ATM) Signalling* (9.8 % 9/92). Figure downloaded from IPA® Software, Qiagen.

The top five differential "molecular and cellular functions" in the SNL DH were reported in IPA® (Table 3.7). This identified processes which were most changed in the SNL compared to sham-operated control, and included gene expression, cell death and survival, cellular growth and proliferation, protein synthesis, and cell-to-cell interaction.

Table 3.6: Molecular and Cellular Function

Name	*P* value range	Number of Molecules
Gene Expression	2.28E-02 - 2.15E-06	147
Cell Death and Survival	2.71E-02 - 1.62E-15	86
Cellular Growth and Proliferation	2.82E-02 - 1.34E-05	85
Protein Synthesis	2.33E-02 - 2.15E-06	77
Cell-To-Cell Signalling and Interaction	2.82E-02 - 1.34E-05	49

Changes in physiological system developments are reported in IPA®. The top five most differentiated systems are listed in Table 3.7. This data highlights systemic changes to in the SNL model DH. The most changed system includes molecules involved in embryonic development, followed by the haematological system, connective tissue, immune cell trafficking, and the broadly terms “organismal functions”.

Table 3.7 Physiological System Development and Function

Name	*P* value	Number of Molecules
Embryonic Development	2.82E-02 - 1.53E-03	48
Haematological System Development and Function	2.82E-02 - 1.34E-05	44
Connective Tissue Development and Function	2.82E-02 - 2.58E-04	18

Immune Cell Trafficking	2.82E-02 - 2.24E-04	16
Organismal Functions	2.55E-02 - 1.12E-03	13

3.2.3 Four Interacting Candidates, *Casp1, Casp4, Cycs,* and *Txn1,* have at least two Common Transcription Factor (TF) Binding Sites

These common transcription factor (TF) binding sites represent potential mechanisms for simultaneous upregulation or downregulation of candidate genes. These were investigated using the MatInspector™ database and the *Rattus norvegicus* genome library. *Casp1*, *Casp4*, C*ycs,* and *Txn1* were found to have multiple common TF binding sites (Table 3.8).

Table 3.8: Common Transcription Factors for Rat Array Hits in IPA Network (Rat Genes)

Transcription Factor	**Core Similarity**			
	Casp1	*Casp4*	*Cycs*	*Txn1*
Mesoderm posterior 2	0.941	Not present	0.951	0.915
Homeobox transcription factor Gsx-2	0.966	Not present	0.950	0.951
Pax-6 paired domain binding site	0.891	0.786	0.893	0.967
Brn-2, POU-III protein class	0.913	0.955	0.918	0.957

3.3 Discussion

Comparative microarray analysis provided a differential gene expression profile for DH tissue in SNL rat model of neuropathic pain. Candidate genes were selected from the microarray data for further investigation in clinical neuropathic pain samples (Chapter 4) and *in vitro* cell culture (Chapter 5). Bioinformatic tools IPA® and MatInspector™ were used to investigate the function of candidates, uncover network interactions and changes to systems and pathways, and identify common TF binding sites.

3.3.1 Pathways Are Changed in the SNL Model

The top canonical pathways which are differentially expressed in the SNL DH are reported in IPA® (Table 3.7). The z-score is a measure of the number of standard deviations data lies from the mean (Figure 3.7). The positive z-score indicates that the pathway is likely activated, and inhibition of a pathway is indicated by a negative z-score (Subbian *et al.*, 2013). Eukaryotic Initiation Factor 2 (EIF2) Signalling is the pathway with the highest proportion of components activated and has a positive z score of 4.8 (Figure 3.7). EIF2 protein mediates being of tRNA to the ribosome during protein synthesis (Asano *et al.*, 2000). The upregulation of the EIF2 signalling pathway in the SNL DH agrees with the large increase in gene expression which is reported in Figure 3.2 and differential expression of 77 molecules involved in protein synthesis (Table 3.6).

Regulation of eIF4 and p70S6K Signalling, and mammalian target of rapamycin (mTOR) signalling pathways have a z score of 0 (Figure 3.7, p values < 0.001), which means they are neither activated nor inhibited in the SNL DH. It could be that the number of upregulated and downregulated genes is equal, therefore the signalling pathways are neither. Oxidative phosphorylation is also active (p = 0.005, Figure 3.7), which could be indicative of the high energy consumption occurring at cellular level in the SNL DH, perhaps during the extensive gene

expression and plastic changes as apoptosis and inflammation are induced as indicated in Table 3.6. Ataxia-telangiectasia mutated (ATM) signalling has a negative z-score (Figure 3.7), which means this pathway is inhibited or downregulated in the DH SNL. The ATM protein is a serine/threonine kinase activated in response to double-stranded DNA breaks (Lee & Paull, 2007) and facilitates apoptosis by phosphorylating pro-apoptotic proteins including those associated with tumour suppression (Rotman & Shiloh, 1998). Its downregulation could suggest that this pro-apoptotic pathway is suppressed (Figure 3.7), however it is not an essential pathway for DNA-damage induced apoptosis (Choy & Watters, 2018). As pro-apoptotic pathways are known to be activated in the SNL e.g. caspases are upregulated, it is likely other pro-apoptotic signals are active instead of ATM signalling, rather than the general suppression of apoptosis.

There are 147 genes involved in the gene expression process (including transcription and translation) that are differentially expressed in the RTA, and 77 involved in protein synthesis (Table 3.6). Eighty-six differentially expressed genes are represented in 'Cell Death and Survival', eighty-five in 'Cellular Growth and Proliferation', and forty-nine in 'Cell-To-Cell Signalling and Interaction'. This demonstrates the large number of changes and the dynamic response to the SNL injury, and the network of knock-on changes that likely occur in response to the changes in the intercellular signalling systems. These changes are to be expected in the SNL model given the vast changes that occur during the establishment of the neuropathic pain state, including differential gene expression (Figure 3.1), protein expression, and more generally tissue damage and repair, and neuronal plasticity.

Table 3.7 lists the top five physiological systems in which genes involved are differentially expressed. Forty-four genes in the haematological system, including immune cells involved in the inflammatory response are differentially expressed. Sixteen genes are involved in immune

cell trafficking, though these two categories are not independent of each other. This agrees with reports of vast gene expression changes in cells of the inflammatory response to tissue damage that has been reported neuropathic pain models and patients (Schomberg *et al.*, 2015).

Forty-eight differentially expressed genes are involved in "Embryonic Development" (Table 3.7), important for the physiological development of embryos in the womb, some of these genes essential to life (e.g. *Txn1*). Genes involved in embryonic development may have important functions in adults, such as apoptosis genes e.g. caspases. Similarly, eighteen differentially expressed genes are involved in "Connective Tissue Development" (Table 3.7). Thirteen genes are categorised as "organismal functions" (Table 3.7), a broad categorisation with limited number of candidates.

3.3.2 Differentially Expressed Genes Have a Wide Functional Range

3.3.2.1 Caspase Gene Expression

Increased expression of *Casp1* (fold change 1.27, $p = 0.046$) and *Casp4* (fold change 1.34, $p = 0.025$) is indicative of caspase activation in the DH of the SNL model, as part of the inflammatory response to the ligation injury. The protein products of these genes, caspase 1 and caspase 4, are both inflammatory enzymes and result in the activation of IL-1β (Thornberry *et al.*, 1992). They facilitate the inflammatory programmed cell death pathway (pyroptosis) in response to microbial infection (Man *et al.*, 2017).

Casp1 deficient mice have no defects in apoptosis but are more susceptible to viral infections and tumours (Kuida *et al.*, 1995). Caspase 1-dependent activation of IL-1β can also occur in response to P2X7-receptor activation, which is triggered by ATP (released from damaged cells e.g. during injury) (Burgh and Rothwell 2017). These pathways could actively contribute to tissue damage and the maintenance of the inflamed state, which is a key component of the initial

development stages of neuropathic pain. Abnormalities in the caspase cascade can cause disease, including cancer (given their role in apoptosis) (Ghavami *et al.*, 2014), lymphocyte defects (Puck & Zhu, 2003), and development (Juo *et al.*, 1998). The NLRP3-Caspase-1 signalling pathway has been implicated in brain injury and neuronal apoptosis (B Wang *et al.*, 2019), and inhibition of Casp1 can prevent brain injury in response to hypoxia (Dapaah-Siakwan *et al.*, 2019). Further, the NLRP3 inflammasome and IL-1β release has been associated with morphine-induced prolongation of neuropathic pain in chronic constriction injury (CCI) rat spinal cord (Grace *et al.*, 2016). *NLRP3* was subsequently investigated in the clinical cohort (Chapter 4).

Casp3 is essential for neuronal cell death and deficient mice have brain hyperplasia (Kuida *et al.*, 1996). Caspase 3 is also required for B-cell regulation (Woo *et al.*, 2003). *Casp8* is essential for survival (Juo *et al.*, 1998). Given the importance of these genes in development and adult health, caspases could not appear to be obvious suitable targets for pharmacological intervention for neuropathic pain, nevertheless caspase inhibitors have been designed and tested for an array of diseases of inflammation and apoptosis (including cancer) (MacKenzie *et al.*, 2010) but most compounds have failed at preclinical trial stage due to poor pharmaceutical properties (Noonan *et al.*, 2016). The upregulation of caspases in the DH tissue of the SNL is indicative of increased inflammation (through its contribution to IL-1β release) and apoptosis in the DH of rat SNL.

As caspases exist as inactive pro-enzymes and must be cleaved before they become functional (Shi, 2004). Therefore, caspase activity cannot be implied by gene or protein upregulation, as activity is dependent on cleavage. Bioluminescent detection assays are required to be certain of a change in caspase activity. The Promega Caspase-Glo® assay system, for example, involves addition of a pro-luminescent substrate to a cell sample, which following caspase cleavage releases amino luciferin to be digested by luciferase and release a luminescent signal. In the animal model itself this is not possible as tissues are harvested after death but could be used in

cell culture models of inflammation, or more sophisticated models designed to mimic neuropathic pain.

3.3.2.2 *Cycs* (Cytochrome C) and *Ndufa13*

Cycs codes for cytochrome c protein on the inner mitochondrial membrane (IMM). It is located between Complex III and Complex IV, involved in the electron transport chain (ETC) and ATP synthesis (X Jiang & Wang, 2004). Pro-apoptotic signals can trigger cytochrome c release from mitochondria into the cytosol. This activates a caspase cascade involving caspases 9, 3, 6, and 7 (Figure 1.9) (Ow *et al.*, 2008). Increased *Cycs* transcription precedes increased cytosolic cytochrome c release (Sanchez-Alcazar *et al.*, 2000). In the cytosol, Cytochrome c activates caspase 9 by promoting dATP binding to apoptotic protease-activating factor-1 (Apaf-1) and the formation of the multimeric complex, which processes procaspase 9 (X Jiang & Wang, 2000). *Cycs* is upregulated in the SNL model (fold change 1.33, p = 0.033) (Table 3.1), suggestive of increased cytosolic levels that would feed into apoptotic mechanisms. Cytochrome c overexpression in response to proapoptotic signalling, enhances caspase activation to promote cell death (Chandra *et al.*, 2002). There is evidence that cytochrome c migration is facilitated by caspases 1 and 4 (Pelletier *et al.*, 2006) (Vereker *et al.*, 2000), which are also upregulated in the SNL DH (Table 3.2, Table 3.4).

NADH:ubiquinone oxidoreductase subunit A13 (*Ndufa13*) is a subunit of NADH dehydrogenase (ubiquinone/Complex I) enzyme involved in the ETC. It is increased at mRNA level in the SNL DH (fold change = 1.32, p = 0.02). Complex I is the largest of the respirator complexes in the ETC, and subunit A13 is one of approximately thirty-one subunits that form the transmembrane region (Walker, 1992), and it is likely an increased function of the complex would require simultaneous upregulation of all the subunits. NADH dehydrogenase (ubiquinone) subunit 1 is also up-regulated (fold change 1.8) in the DH of CCI rat model (HC Moon & Park, 2017).

3.3.2.3 *Txn1, Sh3bgrl3,* and *Romo1*

Thioredoxin 1 (*Txn1*) is a highly conserved anti-apoptotic protein (J Lu & Holmgren, 2014). Upregulation of *Txn1* in the SNL DH (fold change = 1.28, $p = 0.003$) could function as a protective or fine-tuning feedback mechanism to control or counteract the pro-apoptotic properties of caspases 1 and 4 (Figure 3.6). Thioredoxin upregulation could also have a downstream effect on NF-κB and AP1 target genes by increasing transcription, and through this mechanism contribute to the neuropathic pain state by feeding through several pathways. The thioredoxin-like protein *Sh3bgrl3* (SH3 domain-binding glutamic acid-rich-like protein 3) is also upregulated (fold change 1.30, $p = 0.008$) (Table 3.4). SH3BGRL3 is a small protein 98% homologous to the SH3BGR protein proline-rich N-terminus (Egeo *et al.*, 1998). SH3BGR regulates cell migration and angiogenesis via the STAT3 pathway (Li *et al.*, 2016), and thus is implicated in development. Glutaredoxin and thioredoxin proteins are upregulated in cancers and facilitate tumour resistance to oxidative stress (Karlenius & Tonissen, 2010). SH3BGRL3 is also known as the TNF Inhibitory Protein B1 (TIP-B1) and protects from TNF-induced cytotoxicity (Henn *et al.*, 2001). The upregulation of both *Txn1* and *Sh3bgrl3* in the SNL of DH are two complementary findings, and though SH3BGRL3 cannot reduce other proteins (Mazzocco *et al.*, 2002), together provide strong evidence that anti-apoptotic and reductive mechanisms are activated in neuropathic pain.

Txn1 protein and the vasodilator nitric oxide together modulate caspase 8 activity and therefore the apoptotic pathway can be modulated before the caspase 3 is activated and apoptosis is completed (Sengupta *et al.*, 2010). Thioredoxin is a downstream target gene of the nuclear factor (erythroid-derived 2)-like 2 (NRF2) transcription factor, which induces transcription of antioxidant genes (Chen, Wu*, et al.*, 2017). NRF2 is induced after traumatic brain injury and could reduce neuronal damage and loss through the induction of thioredoxin (Hatic *et al.*, 2012). This

mechanism could be active in the SNL model to reduce oxidative stress and subsequent damage to the neurones.

There is evidence that oxidative stress is induced in the DH after SNL, with ROS scavenger phenyl-N-tert-butylnitrone reversing mechanical hyperalgesia (Yowtak *et al.*, 2011). Upregulation of *Romo1* in the DH tissue (Table 3.2) also supports this. ROS production is also seen in other neuropathic pain models, including L5 spinal nerve transection (SNT) and CCI. NADPH oxidase 2 (Nox2)-derived reactive oxidative species are also released by DH microglia after SNT (Kim *et al.*, 2010) and could be a strong driving force in the production of pro-inflammatory cytokines. CCI increases Nox2 activation and ROS production which can be attenuated by spinal sigma-1 receptor inhibitor BD1047 (Choi *et al.*, 2013). Induction of anti-oxidative proteins such as thioredoxin indicates that protective feedback mechanisms have been induced in the DH, 35-days after SNL.

3.3.2.4 N-formyl peptide receptor 2 *(Fpr2)* and Annexin A1 *(Anxa1)*

Decreased expression of *Fpr2* (fold change 1.35, p = 0.002), for the G-couple Formyl peptide receptor 2 (Fpr2), is indicative of active anti-inflammatory pathways. FPR2 is part of the inflammation attenuation response, and its ligand Annexin A1 (*Anxa1*) is upregulated (fold change 1.43, p = 0.047). The downregulation of miR-181b1 (fold change -1.40, p = 0.012) confirms this *Fpr2* expression inhibitory mechanism is not active in the SNL model, so *Fpr2* downregulation is caused by a different upstream mechanism. *Fpr2* downregulation also indicates that Annexin A1 is active in the SNL DH at a different receptor and pathway. Annexin A1 activation of Fpr2 interaction modulates neutrophil recruitment and activates neutrophil apoptosis, by suppressing phospholipase A2 and subsequent eicosanoid production (Ernst *et al.*, 2004) (Sugimoto *et al.*, 2016).

Annexins are induced by glucocorticoids (Peers *et al.*, 1993), and glucocorticoid-induced annexin 1 derived peptides are generated *in vivo* and inhibit PMN diapedesis and infiltration (Perretti *et al.*, 2002). Differential expression of *Anxa1* supports other evidence for active anti-inflammatory mechanisms in neuropathic pain, which has been reported by previous studies (Milligan *et al.*, 2005) (Luchting *et al.*, 2015). This is a good example of how multiple mechanisms of inflammation and resolution are activated or inhibited as an injury occurs and persists in neuropathic pain.

Annexin A1 is produced in CD4+ T cells, and its deficiency in T cells can exacerbate the inflammatory response of these cells (Yang *et al.*, 2013). *Cd4* is also upregulated in the DH of the SNL model (Table 3.4). It is possible that *Anxa1* upregulation is a consequence of increased infiltration of CD4+ T cells to the DH. Annexin A1 is relevant in other diseases and has previously been reported as a positive marker for high-risks HPV infections and associated penile carcinoma risk (Calmon *et al.*, 2013), and decreased expression correlated with breast cancer progression (Shen *et al.*, 2006). Annexin A1 has also been implicated in the regulation of positive and negative selection of T cell receptor repertoire (Paschalidis 2010), therefore its upregulation in SNL could have implications for the proportion of helper CD4+ to cytotoxic CD8+ T cells.

3.3.2.5 Alpha 1,3-Galactosyltransferase 2 (*A3galt2*)

A3galt2 upregulation (fold change 1.31, $p = 0.042$) is an interesting finding as its function is not fully understood. In some animals, including rats, the glycosphingolipid product of *A3galt2* isoglobotriosylceramide (iG3) is recognised by NK cells (Zhou *et al.*, 2004). Human NK cells are able to recognise foreign iG3, and this has implications in xenotransplantation research (Christiansen *et al.*, 2008). In humans, galactosyltransferase proteins catalyse the transfer of galactosidase, and the synthesis of polysaccharides (Campbell *et al.*, 1997). Therefore, this enzyme family has an important function (Kolbinger *et al.*, 1998). In the SNL rat model, it is

possible that *A3galt2* upregulation and subsequent increase in iGb3 levels could induce a response in the NK cells, which could then contribute to the inflammatory response and neuropathic pain development. A3GALT2 has no known interactions with the other candidates (Figure 3.5), and no other galactosidase-coding genes are differentially expressed in the cohort. The *A3galt2* expression change in the RTA could be an isolated phenomenon, however due to the large number of unassigned transcripts this is uncertain. It is possible A3GALT2 represents a novel biomarker for neuropathic pain, and additional experiments into the consequences of *A3galt2* knockdown (e.g by siRNA) could improve our understanding of this protein in both health and disease.

3.3.2.6 C-C chemokine receptor type 5 *(Ccr5)*

CCR5 is a chemokine receptor involved in immune cell trafficking, which is altered in the SNL DH (Table 3.7). CCR5 is expressed on the surface of leukocytes, primarily T cells (where is it a co-receptor for HIV entry) and macrophages (Wang, Liu*, et al.*, 2016). *Ccr5* is upregulated in the SNL DH (fold change 1.35, p = 0.04). It is possible that upregulation of *Ccr5* in the rat DH is due to infiltration of the tissue by white blood cells (including T cells, supported by increased CD4 Table 3.4) in response to inflammation. This could be measured by immunohistochemistry of white blood cell markers in SNL DH slices. *CCR5* expression in cortical neurones is exclusively expressed after stroke (Joy *et al.*, 2019), and CCR5 inhibition by anti-HIV drug Maraviroc has shown potential in stroke recovery, and natural loss-of-function *CCR5* allele *CCR5Δ32* carriers demonstrate better post-stroke outcomes (Joy *et al.*, 2019). Increased *CCR5* could contribute to damage in the SNL DH, and it could be useful to investigate the effect of a CCR5 blockade with maraviroc in a model of neuropathic pain. In 2018 Dutta *et al.* described a novel compound (MCC22) with CCR5 antagonist and μ opioid receptor agonist properties for the treatment of arthritis, an inflammatory chronic pain condition.

Down-regulation of the chemokine receptor CCR5 on dendritic cells is dependent on the induction of lipoxin A4, which is a product of arachidonic acid metabolism and a ligand of Fpr2 (Aliberti & Sher, 2002). Another ligand of *Fpr2*, *Anxa1,* is upregulated (Table 3.2), but there is no evidence that lipoxin A4 levels are altered in the SNL model as it is a by-product of arachidonic acid metabolism. However, as *Ccr5* is upregulated, lipoxin A4 induction unlikely occurs in the SNL DH.

3.3.2.7 *Cd4* (Cluster of differentiation 4)

Cd4 upregulation (fold change 1.26, $p = 0.049$) could be due to the infiltration of CD4+ T cells in the SNL DH tissue. This is reported by previous groups and associated with mechanical allodynia and thermal hyperalgesia (Cao & DeLeo, 2008, Moalem *et al.*, 2004). Kobayashi *et al.* reported T cell migration towards the injury site in mice partial sciatic nerve ligation (PSL) (Kobayashi *et al.*, 2015). Immunohistochemistry staining of the DH tissue could confirm this hypothesis, and immune cell infiltration can occur after the blood brain barrier is degraded in neuropathic pain (Lim *et al.*, 2014). Gattlen *et al.* reported cytotoxic CD8+ T cell infiltration of the spinal cord in rat Spared Nerve Injury (SNI) model (Gattlen *et al.*, 2016), which would remove damaged cells in response to tissue damage, and potentially contribute to neuropathic pain. However, in clinical neuropathic pain patients with chronic unspecified low back pain, flow cytometry analysis of blood samples shows a significant shift from pro-inflammatory Th17 T cells (reduced) to anti-inflammatory Treg cells (increased) (Luchting *et al.*, 2015). This paper reported the same changes in the adaptive system is seen in both nociceptive and neuropathic pain, and therefore changes to levels of T cell types could not be useful in neuropathic pain diagnosis but could be useful in treatment. It is possible that this anti-inflammatory shift is established in clinical chronic pain as a protective feedback mechanism, or as suggested by the authors could be induced by

the psychological experience of neuropathic pain, because an anti-inflammatory T cell shift has been reported in depression and stress (Torres-Harding *et al.*, 2008, Hong *et al.*, 2013).

3.3.2.8 *Tmem88* (Transmembrane protein 88) and *Plac8* (Placenta-specific 8)

Tmem88 codes for target transmembrane protein 88 (TMEM88), an inhibitor of the Wnt/β-catenin pathway, which mediates β-catenin-dependent transcription (Figure 1.10). *Tmem88* downregulation (fold change -1.33, $p = 0.016$) is indicative of Wnt/β-catenin pathway inhibition. In the SNL DH this could contribute to the plastic changes occurring in response to the prolonged injury. This coincides with the upregulation of *Plac8* in the SNL model (fold change 1.61, $p = 0.02$). *Plac8* downregulation has been shown to upregulate the Wnt/β-catenin canonical pathway. β-catenin dependent pathway Wnt pathway has been implicated in the generation of neuropathic pain (S Liu *et al.*, 2015). Given the role of Wnt and β-catenin in neuronal plasticity, both *Tmem88* and *Plac8* could contribute to such in neuropathic pain and represent opposing regulatory pathways active in neuronal remodelling. Some of the target of genes of β-catenin include other transcription factors and regulatory genes, including Achaete-Scute Family BHLH Transcription Factor 2 (ASCL2), Axin-related protein (AXIN2), and MYC (Herbst 2014). Therefore, changes to β-catenin have wider implication through knock-on effects through downstream target genes.

In clinical neuropathic pain, neuronal remodelling processed could be targeted for suppression to prevent disease progression. This may be useful in diseases such as diabetes or after injury, where the onset of neuropathic pain is predicable. This may not be useful for patients whose neuropathic pain is already clinically presented, as major neuronal remodelling may have already occurred.

3.3.2.9 Olfactory Marker Protein *(Omp)*

Olfactory marker protein (OMP) regulates olfactory receptor expression in mature olfactory neurones (AC Lee, He*, et al.*, 2011). Olfactory receptors are typically localised in sensory organs where they detect numerous ligands associated with odour, expression of so-called 'ectopic' olfactory receptors and their downstream signalling molecules (indicating function) have been reported in human CNS neurones (Ferrer *et al.*, 2016) and other tissue types including bladder and thyroid. OMP, despite previously reported to be exclusive to olfactory neurones, has been identified in bladder, thyroid, thymus, heart, and testis tissue (Kang & Koo, 2012). In the olfactory neurones, only one olfactory receptor type is expressed, and those expressing the same receptor converge (Ressler *et al.*, 1993). The olfactory system is involved in several behavioural processes in mammals, including mating and food preference (Baum & Cherry, 2015, Bhutani *et al.*, 2019, Ramos-Lopez *et al.*, 2019).

Omp downregulation (fold change -1.25, p = 0.005), and the downregulation of numerous olfactory receptors (Table 3.3) is reminiscent of reports of differential expression of olfactory receptors in the neurodegenerative diseases Parkinson's Disease (PD) and Alzheimer's Diseases (AD) (Mesholam *et al.*, 1998) (Bahuleyan & Singh, 2012, Zou *et al.*, 2016). OMP, a plasma membrane protein which functions as a potassium-dependent sodium/calcium exchanger and a Ca^{2+}-ATPase (Pyrski *et al.*, 2007). Given this function it is possible that its expression in neuronal tissue could have some implications for action potential propagation and therefore neuronal functioning. However, it is not clear whether the differential expression of *Omp* is occurring in the neurones, their surrounding cell types (e.g. satellite glial cells), or both. It is possible to determine the location of protein changes using immunohistochemistry on slices of the tissue of interest. This technique would only detect protein level changes, and if present, would confirm the translation of differentially expressed mRNA transcripts to protein production and

expression at the cell surface. However, for downregulated transcripts it could be assumed that the protein is also downregulated, as the transcript is required for protein translation. Although findings reported here could be indicative of increased ATP energy consumption, such as the increased expression of ETC components (Table 3.2), the downregulation of the ATPase OMP in the SNL DH indicates that is not a mechanism through which increased ATP is being consumed.

Ten olfactory receptor coding genes are among the top twenty downregulated genes (Table 3.3). Olfactory receptor knockdown has been reported in response to IFMs such as TNFα (Sultan *et al.*, 2011), which indicates inflammatory olfactory receptor dysregulation and would explain why there is a downregulation in the SNL rat DH. Olfactory receptor expression is dysregulated in PD and AD, and neuropathic pain is commonly reported in PD and to some extent in AD (Ansoleaga *et al.*, 2013, Grison *et al.*, 2014, Achterberg *et al.*, 2013) though dementia compromises patient capacity to communicate and difficulties with self-reporting in AD. Olfactory receptor upregulation has also been reported in bacterial infection, causing enhanced MCP-1 production and macrophages activation (Li *et al.*, 2013), indicative of a change in olfactory receptor expression in response to inflammation.

OMP knockout in mice reduces cholecystokinin (CCK) levels by 50% (Buiakova *et al.*, 1996). This could be significant with regards to the neuropathic pain phenotype, as the CCK receptor 2 (CCKR2) is involved in the processed of mechanical sensitivity and hyperalgesia (Kurrikoff *et al.*, 2004). Thus, it is reasonable to suggest that *OMP* downregulation could be induced as a feedback mechanism in neuropathic pain to reduce CCKR2 activation, thereby reducing hyperalgesia. Although CCK expression was not reported to change with SNL in our microarray data, 81.75% of the microarray hits are not yet annotated and will require further scrutiny and CCK could be among the 865 unassigned genes.

The downregulation of olfactory receptors and their regulatory protein OMP in the DH of the rat model in response to SNL injury could potentially occur as part of a downregulation of non-necessary genes, to reduce unnecessary transcription and energy expenditure. As the exact role of olfactory receptors and OMP in neuropathic pain is not yet clear, this represents an area of interest as it is reasonable to suggest that olfactory receptor expression profile changes could provide a useful indicator of disease progression or prognosis in neuropathic pain if properly developed.

3.3.2.10 Reactive Oxygen Species Modulator 1 *(Romo1)*

ROMO1 is a modulator of ROS and can reduce ROS-mediated damage to cells and tissues (YM Chung *et al.*, 2006). *ROMO1* was upregulated in the SNL DH (fold change = 1.32, p = 0.01) and likely contributes to the oxidative stress in the SNL model (Kim *et al.*, 2010). Though ROS is not exclusive to neuropathic pain, *ROMO1* upregulation could be a characteristic of neuropathic pain. This could make it an attractive druggable target to attenuate ROS after injury to slow down or prevent neuropathic pain development. However, Romo1 knockdown enhances apoptosis (Gupta *et al.*, 2012), which could risk further unwanted tissue damage. In another study, the mitochondria-targeting drug Mitoquidone reduced *Romo1* expression (fold change -1.24) in male Sprague Dawley rats and inhibited neuronal death driven by oxidative stress after subarachnoid haemorrhage (Zhang *et al.*, 2019). *Romo1* could therefore contribute to maladaptive oxidative stress changes in CNS injury, such as SNL. Research on ROMO1 protein has focussed on its role in tumour proliferation and its implication in poor prognosis and survival (Kim *et al.*, 2017, Lee *et al.*, 2017).

3.3.2.11 Other Upregulated Genes

3.3.2.11.1 Homeobox A11 *(Hoxa11)* and Homeobox C11 *(Hoxc11)*

Homeobox proteins are TFs which primarily regulate morphogenesis during embryonic development (Corsetti *et al.*, 1992), though specific homeobox proteins have been implicated in disease states in adults, including atherosclerosis (Dunn *et al.*, 2015), and angiogenesis (Gorski & Walsh, 2000). The HOX subset of homeobox genes make up a major class of factors and are classified into one of four clusters (A, B, C, D) (Garcia-Fernandez & Holland, 1994). HOXA genes are critical for normal lung and cardiac development in mice (Di-Poi *et al.*, 2010). *HoxA5* overexpression can inhibit inflammation (Lee, Park*, et al.*, 2011).

In the SNL DH tissue, *Hoxa11* and *Hoxc11* are upregulated (fold change = 2.01, p = 0.04; fold change = 1.38, p = 0.05). The upregulation of *Hoxa11* and *Hoxc11* suggests that these genes are active in the DH of the SNL, though the implications for this are unclear. However, Habib *et al.* reported a point mutation in the Zinc finger homeobox 2 gene *ZFHX2* causes defective pain sensitivity, as downstream regulated genes involved in pain mechanisms are downregulated (Habib *et al.*, 2018), upregulation would feed into pain pathways. Further investigation into the role of Hox proteins into pain mechanisms and inflammatory could help elucidate their role in neuropathic pain. However, given the wider implications for Hox role across many different systems, particularly in embryonic development, it is unlikely they will represent suitable candidates for drug targeting. As with the TFs elucidated from the MatInspector™ analysis, understanding of common TF binding sites and pathways upstream and downstream of the candidates can improve our understanding of neuropathic pain mechanisms, and this is essential for drug development.

3.3.2.11.2 T-cell receptor alpha variable 3d-3 *(Trav3d-3)*

The *Trav3d-3* gene codes for the T-cell receptor alpha variable protein (Ohga *et al.*, 1990), which was upregulated in the SNL DH (fold change = 1.95, p = 0.02). It is amongst the highest fold changes in the whole transcriptome and could be indicative of an increase in T-cell activity. This

supports the increase in *Cd4* also seen (Table 3.2). Decreased *Trav3d-3* in CD4+ T-cells is associated with PD (Saunders *et al.*, 2012), and dysregulated T-cells have been implicated in the neuroinflammation component of several neurodegeneration diseases, including MS, AD, and PD (Sommer *et al.*, 2017). The increase of chemokines *Cxcl13* and receptor *Ccr5* also supports the presence of lymphocyte trafficking, which could contribute to the increased presence of T-cell markers (Shechter & Schwartz, 2013).

3.3.2.11.3 Gamma-aminobutyric acid receptor-associated protein-like 2 *(Gabarapl2)*

Gamma-aminobutyric acid receptor-associated protein-like 2 is upregulated in the SNL DH tissue (fold change = 1.42, p = 0.02). This is an important protein in neurotransmission, as it mediates interactions of neurotransmitter receptors and the cytoskeleton, allowing for clustering of receptors at synapses (Walsh & Kuruc, 1992). Its upregulation could be indicative of an increase in neurotransmitter trafficking and release, which may contribute to increased synaptic activity involved in neuropathic pain (Osikowicz *et al.*, 2013). GABARAPL2 is also essential for autophagosome formation and disintegration after lysosome fusion (Szalai *et al.*, 2015), making it an important part of the immune system response to infection.

3.3.2.11.4 Urocortin (*Ucn)*

Urocortin is a neuropeptide belonging to the corticotropin-releasing factor (CRF) family. *Ucn* is increased in the DH tissue of SNL model (fold change = 1.37, p = 0.01) and is involved in the stress response (Skelton *et al.*, 2000). It is closely related to CRF, an analgesic which mediate the stress response and induces opioid release (Schafer *et al.*, 1994). The increase in urocortin in the SNL DH could be part of an endogenous analgesic feedback mechanism to reduce the chronic pain.

3.3.2.11.5 Myosin light polypeptide 6 *(Myl6l)*

Myl6l gene codes the "Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle like" protein, involved in muscle contraction, and is upregulated in the rat SNL DH (fold change = 1.37, $p < 0.00$). It is possible that *Myl6l* dysregulation could occur as a consequence of neuropathic pain.

3.3.2.11.6 C-X-C motif ligand 13 *(Cxcl13)*

Cxcl13 is upregulated in the SNL DH (fold change = 1.35, $p = 0.03$). This gene codes for the C-X-C motif ligand 13, CXCL13, also known as B lymphocyte chemoattractant, which is indicative of increased B cell trafficking signal to the DH (Legler *et al.*, 1998, Ansel *et al.*, 2002). However, a simultaneous upregulation of its B cell receptor CXCR5 is not seen on the microarray, but it could be that B cells had not yet reached the site at the 35-day timepoint. This gene may also be among the unassigned genes in the RTA. It is possible that the upregulation is due to movement of CD4+ T cells into the DH tissue, as indicated by the upregulation of CD4+. *Cxcl13* upregulation supports the theory that immune cells could be migrating into the CNS. *Cxcl13* upregulation was reported in DRG tissue in the SNL model (Strong *et al.*, 2012) and observed under inflammatory chronic pain conditions in DRG and spinal cord (Wu *et al.*, 2016, Wu *et al.*, 2019). Its knockdown with si-RNA can attenuate neuropathic pain (BC Jiang *et al.*, 2016). CXCL13 contributes to allodynia and production of pro-inflammatory cytokine production in a painful diabetic neuropathy mouse model (Liu *et al.*, 2019). Increased CXCL13 and its receptor CXCR5 in the anterior cingulate cortex (ACC) has been linked to conditioned place aversion in an SNL model (Wu *et al.*, 2019).

3.3.2.11.7 Regenerating islet-derived 3 beta *(Reg3b)*

Regenerating islet-derived 3 beta is a lectin which protects against bacterial infections of the intestines (Ferrara *et al.*, 2011). It is also expressed in the spinal cord and is increased in the DH of the SNL model (fold change = 1.35, $p = 0.02$). Lectins are proteins which bind to carbohydrate groups, and function in biological recognition (Rutishauser & Sachs, 1975). Given the anti-

bacterial role of *Reg3b* it is likely active during the innate immune response and associated with inflammation. LaCroix-Fralish *et al.* performed a meta-analysis on microarray studies of pain and found that four out of fourteen neuropathic pain rat model studies reported an upregulation of *Reg3b*, supporting our finding (Table 3.2) (Lacroix-Fralish & Mogil, 2009). However, none of the six studies of inflammatory pain reported this change, suggesting differential *Reg3b* expression could be exclusive to neuropathic pain. There could be microarray studies of inflammatory pain that do report an upregulation of *Reg3b* that this meta-analysis missed. In 2014 Dawes *et al.* reported Reg3b upregulation in the DRG after inflammation was induced by damage by ultraviolet light (Dawes *et al.*, 2014) suggesting its upregulation in the SNL is likely a result of the inflammatory component triggered by the ligation injury.

3.3.2.12 Other Downregulated Genes

3.3.2.12.1 Cocaine- And Amphetamine-Regulated Transcript Prepropeptide *(Cartpt)*

Cocaine- And Amphetamine-Regulated Transcript (CART) is a neuropeptide involved in the reward system, stress response, and appetite (Zhang, Han*, et al.*, 2012), as well as the anxiety response, fear behaviour, and addiction (Dandekar *et al.*, 2008). CART is named after its inhibitory effect on dopamine-mediated locomotor activity induced by cocaine and amphetamine (Upadhya *et al.*, 2012). This is likely a feedback mechanism to dampen the response (Rakovska *et al.*, 2017). CART prepropeptide *(CARTpt*) transcript was downregulated in the SNL model (fold change = -1.44, p = 0.01). This reduction in CART could be part of a pain dampening response, through a reduction in a dopamine inhibitory mechanism. In addition to the reward mechanisms dopamine is antinociceptive via dopamine 2 receptors (Hagelberg *et*

al., 2004). Dopamine receptor activity mediates neuronal plasticity which contributes to chronic neuropathic pain (Megat *et al.*, 2018), and dopamine inhibition ablates chronic pain in animal models (Wang, Shen*, et al.*, 2016, Zhou *et al.*, 2017). An experimental triple reuptake inhibitor LPM580098, which inhibits dopamine, attenuates neuropathic pain in SNL mice (Li *et al.*, 2019). Currently SSRIs and SNRIs are used in clinical neuropathic pain treatment but dopamine blocking anti-depressant medications are not (Obata, 2017). Dopaminergic modulation in the hypothalamus is involved in hyperalgesic priming, a plasticity mechanism in chronic pain (Megat *et al.*, 2018). Hyperalgesic priming can be reversed by lesion in dopaminergic neurones (Megat *et al.*, 2018). In humans, conditions of dysregulated dopamine such as PD and depression are associated with pain hypersensitivity (Jarcho *et al.*, 2012). In the context of this mechanism CART reduction could be contributing to pain in the SNL. CART contributes to cognition, and its downregulation agrees with the observed reduced cognition in chronic pain patients (Bharne *et al.*, 2016, Bushnell *et al.*, 2013).

As discussed in Chapter 1, Section 1.3.5 chronic pain has huge psychological consequences for the patient. Pain induces a stress response and stimulates reward pathways when relief is achieved (Becker *et al.*, 2012). These are impaired in rat models of neuropathic pain (Ozaki *et al.*, 2002), and it is possible the reduction in *Cartpt* is involved in this impairment. CART is also an endogenous psychostimulant (Kuhar *et al.*, 2002), producing similar behaviours to that of cocaine and amphetamine in rats, hence its name. CART is regulated by several hormones, including CCK (de Lartigue *et al.*, 2007), which is reduced by *OMP* knockout (Buiakova *et al.*, 1996). Due to the synergistic nature of the relationship between CART and CCK, it would be possible that OMP reduction would also decrease CART, and the results reflect this (Table 3.3).

3.3.2.12.2 Defensins

Two defensins *Defb44* and *Defb49* were downregulated in the SNL model (fold change = -1.28, p = 0.01; fold change = -1.36, p = 0.02). Defensins are small host defence cationic proteins rich in cysteine residues, released by immune system cells (neutrophils, macrophages, granulocytes, natural killer cells) to attack bacterial membranes, where they insert and form pores and break down the membrane (Ganz, 2003). Beta defensins are also expressed in epithelial cells, and their release in the intestinal tract is known to contribute to irritable bowel disease (Cobo & Chadee, 2013), an autoimmune disease.

3.3.2.12.3 Cluster of Differentiation 209e *(Cd209e)*

Cd209e codes for the Cd209e transmembrane receptor expressed on dendritic cells and macrophages, which is also known as Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN). In the SNL model DC-SIGN is decreased (fold change = -1.31, p = 0.01). It is involved in the recognition of pathogen-associated molecular patterns and induces phagocytosis in the innate immune response (McGreal *et al.*, 2005). It is also involved in the endothelial rolling interactions of CD4+ T cells (Pohlmann *et al.*, 2001). CD4 is upregulated in the SNL model, and as these expression changes were measured in the DH, it is possible that the downregulation of *Cd209e* occurs after CD4+ T cells have migrated into the tissue and Cd209e receptor no longer performs its chemo-attractive function. *Cd209e* is upregulated in chronic asthma and allergen exposure (Di Valentin *et al.*, 2009). It is unlikely that DC-SIGN is involved in any inflammatory response in the active in the neuropathic pain model as it is decreased. Its downregulation could result from feedback mechanisms activated after, in this instance, 35 days post-injury) to attenuate the inflammatory response. The simultaneous downregulation of other inflammatory mediators including *Cxcl13*, and the upregulation of anti-inflammatory mechanisms such as *Anxa1*. Similarly, *C1qtnf3,* which codes for Complement C1q tumour necrosis factor-related protein 3, is downregulated (-1.28, p = 0.01) in the DH SNL.

3.3.2.12.4 Differential Expression of Pseudogenes and Unassigned Genes

Of the 1,046 upregulated genes, 136 are pseudogenes, and could be altered in addition to its parent gene due to sequence similarity (i.e. simultaneous targeting by transcription factors). It is possible that changes to pseudogene expression could have unknown functional consequences in SNL pathophysiology. Similarly, 275 upregulated genes are classified non-coding, and the changes to their expression could also lack functional consequences, though this cannot be certain. A large proportion of the differentially expressed genes in the microarray dataset are unassigned in the RTA library (Figure 3.3). This is comprised of 29.45% of upregulated genes, and 12.31% of the downregulated genes. This is problematic because these differentially expressed genes cannot be considered. A subset of the unassigned genes was investigated and were found to be ribosomal or non-coding in origin (*data not shown*).

3.3.3 Candidate Interactions

3.3.3.1 Caspases, Thioredoxin, and Cytochrome C

Figure 3.5 represents a network of interactions between four gene products curated by IPA®. In human primary keratinocytes, Caspase 4 is necessary for Caspase 1 activation, according to the caspase cascade mechanism of zymogen activation (Akhter *et al.*, 2012). Caspase 4 activation, a pro-apoptotic signal, causes the release of cytochrome c from mitochondria in human tonsil-cultured plasma cells (Pelletier *et al.*, 2006), and this would trigger apoptosis (Figure 1.9, Chapter 1). Inhibition of Caspase 1 decreases cytochrome c release in rat hippocampus neurones (Vereker *et al.*, 2000), indicating that caspase 1 also facilitates its release from the mitochondria into the cytosol. Thioredoxin 1, a fundamental anti-apoptotic protein, decreases cytochrome c translocation to the cytosol (Andoh *et al.*, 2002). Caspase 1 mediates secretion of Thioredoxin 1

from human keratinocytes (Sollberger *et al.*, 2012) and could represent a feedback mechanism for caspase 1 medicated cytochrome c apoptosis. In the array these four genes were upregulated (Table 3.2, Table 3.4) (range in fold change 1.27 to 1.34). The opposing facilitative and inhibitory effects on cytochrome c release by the caspases and thioredoxin respectively are in competition. Cytochrome c upregulation precedes apoptosis (Chandra *et al.*, 2002), and *Cycs* upregulation in the SNL could be indicative of cytochrome c protein mitochondrial release and apoptosis in the DH. The inhibitory effect of thioredoxin on cytochrome c release could therefore be negated by the caspases. Casp1 is a pro-inflammatory cytokine and can induce pyroptosis by gasdermin D activation (Mascarenhas *et al.*, 2017). Pyroptosis is programmed cell death in highly inflammatory circumstances, and a full inflammatory response is required for it to occur (Bergsbaken *et al.*, 2009). Conversely, thioredoxin is an anti-apoptotic protein and has the opposite effect on cytochrome c release. In the context of neuropathic pain, these interactions could represent a feedback loop to fine-tune inflammatory and apoptotic mechanisms, as part of neuronal remodelling (Rumpf *et al.*, 2011).

CD4 and CCR5

IL16 is a chemoattractant for cells expressing CD4, a key inflammatory mediator, released during inflammatory response to the site of injury (Cruikshank *et al.*, 2000). Pro-IL16 is activated by casp3 (Zhang *et al.*, 1998), which is not identified in the RTA. CCR5 is a co-receptor for the IL16-CD4 interaction (Lynch *et al.*, 2003). IL16 interaction with CD4 reduces CCR5-induced migration (Van Drenth *et al.*, 2000), possibly via the STAT6 pathway which is activated in peripheral blood mononuclear cells (PBMCs) and the human monocytic cell line THP-1 (Liu *et al.*, 2007). Glycoprotein gp120 binding to both CD4 and CCR5 receptors is critical for HIV entry into the T cells (Deng *et al.*, 1996, Chen *et al.*, 1997, Misumi *et al.*, 2001), and IL16 is therefore an inhibitor of HIV replication. CCR5 and CD4 are physically associated on the surface of T cells (Platt *et al.*,

1998). CCR5 is also required for casp8 dependent apoptosis of CD4+ T cells (Algeciras-Schimnich *et al.*, 2002). The simultaneous upregulation of *Cd4* and *Ccr5* in the DH of the SNL model of neuropathic pain, indicates that these chemo-attractant pathways are activated, likely part of the inflammatory response to the ligation.

3.3.3.2 FPR2 and CCR5

Lipoxin A4, a ligand for FPR2 produced in the arachidonic acid metabolism pathway, can induce the downregulation of CCR5 expression of dendritic cells via the FPR2 pathway (Aliberti & Sher, 2002). In the SNL model the opposite is seen, *Fpr2* is downregulated and *Ccr5* is upregulated (Table 3.2, 3.3), so it is unlikely that this inhibitory mechanism is active in neuropathic pain. *Ccr5* and other chemoattractant pathways are activated in the SNL DH (Table 3.7). It could be that *Fpr2* is downregulated to allow *Ccr5,* and other components of the inflammatory response e.g. neutrophil activation, to be prolonged.

3.3.3.2 HOXA11 and ANXA11

HOXA11 protein induces a decrease in ANXA1 transcription in kidney cell lines (Valerius *et al.*, 2002). *Hoxa11* was increased in the SNL model (2.01 fold change, $p = 0.04$), as was *Anxa1* (1.43 fold change, $p = 0.05$) so it appears this mechanism is not present in the DH of the SNL model. It could be that this interaction does not occur in the DH (e.g. if the proteins are compartmentalised), or there are other pathways that counteract this effect.

3.3.4 Differentially Expressed MicroRNAs Have Downstream Effects on Gene Expression

MicroRNAs (miRNAs) are a type of small RNAs, along with small interfering RNAs (siRNAs), and small nucleolar RNA (snoRNAs), which are non-coding nucleotide chains with important post-transcriptional regulatory functions. MiRNAs block translation of their complementary mRNA transcripts by base-pair binding (Macfarlane & Murphy, 2010). Dysfunctional miRNA expression can lead to distinct pathologies (Gandla *et al.*, 2017), including neuropathic pain conditions (Pan

et al., 2018, Tramullas *et al.*, 2018, Zhang *et al.*, 2020). An increased expression of a miRNA is indicative of knockdown of its target genes at mRNA, whereas a decreased miRNA expression suggests that the given inhibitory mechanism is not active. It is important to note that mRNAs are targeted by multiple miRNAs, and miRNAs have multiple targets (Hashimoto *et al.*, 2013), therefore as an individual miRNA function could considered, it cannot be assumed to be an isolated change as several miRNAs could be acting at the same mRNA. The miRNA profile is critical to the understanding of upstream regulatory mechanisms active in neuropathic pain. Several miRNAs were differentially expressed in the SNL model versus sham (Table 3.5). Two miRNAs were upregulated and four downregulated in SNL versus sham (Table 3.5).

MiRNA dysregulation has been reported by several groups in spinal cord injury (SCI) models. Nakanishi *et al.* reported increased *miR-223* and decreased *miR-124a* in spinal cord RNA from SCI mice (Nakanishi *et al.*, 2010). Microarray analysis of rat T8 level spinal cord reported a marked decrease in miRNAs in a seven-day SCI model (Yunta *et al.*, 2012). Von Shack *et al.* (2011) reported differential expression of sixty-two miRNAs in the DRG of rat SNL, and 59 of these were downregulated. Schwann cells in the mouse sciatic nerve distal segment crush injury model indicated that *Mir34a* and *Mir140* have a regulatory role in peripheral nerve regeneration, targeting *Notch1* and *Egr2* respectively (Viader *et al.*, 2011). *Egr2* (Early growth response 2) is a transcription factor and master regulator of myelination (Kipanyula *et al.*, 2013). In a rat lingual nerve injury model, miR-138 is upregulated versus sham controls, and is negatively correlated with behavioural change (time spent drinking) at day 3 post-injury (Tavares-Ferreira *et al.* 2019). In the same study, miR-29a was downregulated in lingual nerve neuromas of patients with higher pain versus patient group with lower pain, according to Visual Analogue Scale score (Tavares-Ferreira *et al.* 2019).

Several of the miRNA expression changes in SNL DH reported here (Table 3.5) have previously been linked to neurodegenerative diseases with psychological symptoms such as PD (Pasinetti, 2012), AD (Satoh, 2010, Zovoilis *et al.*, 2011), and schizophrenia (Perkins *et al.*, 2007) (Table 4.1), and there are some overlap in miRNA targeting such as *BACE1*, which is important for amyloid beta peptide formation in Alzheimer's disease pathology (Vassar *et al.*, 1999) (as discussed in Section 1.9.4).

Changes to *mir29c* expression has been linked to inflammatory diseases e.g. psoriasis (Zibert *et al.*, 2010), and neurodegenerative disease e.g. PD (Pasinetti, 2012). It has also been linked to and schizophrenia (Perkins *et al.*, 2007). Reports of *Pten* downregulation in the CCI rat model of neuropathic pain (Huang *et al.*, 2015) is consistent with *mir29c* upregulation reported here (fold change 1.31, p = 0.039, Table 3.5). Phosphatase and tensin homolog (PTEN) is a tumour suppressor, involved in the regulation of cell cycling and apoptosis (Song *et al.*, 2012), targeted by *mir29c* (Tumaneng et al., 2012). Pten is anti-apoptotic and its downregulation indicates activation of pro-apoptotic mechanisms. *Mir29c* also targets *Mcl1* mRNA, which codes for the anti-apoptotic protein BCL2 family apoptosis regulator (Michels *et al.*, 2005). Together these findings suggest *mir29c* upregulated would promote apoptosis. *Bace1* is also targeted by *miR29c* (Zong *et al.*, 2011), so *miR29c* upregulation here suggests this AD mechanism may not be active.

Pten is also targeted by microRNA205 (Qu *et al.*, 2012). *Mir205* also targets interleukin 24 which is a pro-survival and pro-proliferation interleukin with tumour suppressive effects. (Majid *et al.*, 2010). However, unlike *mir29c, mir20*5 is downregulated (fold change -1.26, p = 0.016, Table 3.5), and this inhibitory mechanism for the protein expression of these anti-apoptotic and protective mediators is not active in SNL DH. This a good example of two miRNAs which target the same mRNA having opposed differential expression in the same disease model.

Let7a2 targets the inflammatory caspases mRNAs *Casp3 and Casp8,* (Tsang & Kwok, 2008), *IL6* (Iliopoulos *et al.*, 2009), and *E2f2* (Dong *et al.*, 2010). *Let 7a2* expression was increased (fold change = 1.25, p = 0.016, Table 3.7), which suggests reduced translation of its target mRNAs. *Let7a* activity will downregulate the pro-apoptotic caspases 3 and 8 and the inflammatory cytokine IL6 (see Table 1.2). BCL2 (B-cell lymphoma 2 protein) is an apoptosis regulator located at the outer mitochondrial membrane (Radha & Raghavan, 2017). Increased inhibition of apoptosis via *Bcl2* expression is also indicated by reduced *mir34c* and *mir181b1* (Zhu *et al.*, 2010). However, there is a concurrent increase in pro-apoptotic genes *Casp1* and *Casp4* (Table 3.2, Table 3.4). These gene expression changes with conflicting pro-apoptotic and anti-apoptotic functions could represent feedback or fine-tuning mechanisms in the DH SNL and contribute to neuronal remodelling during disease progression after ligation.

Members of the Notch family are targeted by *mir34c*. The notch signalling pathway is involved in critical cell communication during growth and differentiation in many tissue types (Hitoshi *et al.*, 2002). It is highly conserved in mammals (Artavanis-Tsakonas *et al.*, 1999). In neuronal tissue, there is evidence for its role in the maintenance of neuronal stem cells (Aguirre *et al.*, 2010) (Hitoshi *et al.*, 2002). The Notch signalling pathway is critical in neuronal plasticity and inflammation (Artavanis-Tsakonas *et al.*, 1999) which are both relevant to neuropathic pain development. Downregulation of mir34c (-1.25, p = 0.041) decrease the inhibition of translation for *Notch1,* as well as and *Notch2* (Garofalo & Croce, 2011)*, Notch3* (Q Ji *et al.*, 2009)*, and Notch4* (Yu *et al.*, 2012) in humans. This is indicative of abnormal cell-cell communication in damaged or inflamed tissue (Hitoshi *et al.*, 2002), consistent with reports that DH Notch activation is necessary in mechanical allodynia in neuropathic pain models (Xie *et al.*, 2015). Specifically, Xie *et al.* found that mechanical allodynia did not develop with early inhibition of the notch signalling pathway which was reversed with late inhibition. *Mir34c-5p* has been identified as an important

miRNA in nociception, with key targets including the genes for the calcium channel *Cav2.3*, purine receptor *P2rx6*, and the opioid receptors *Oprd1* and *Oprm1* (Gandla *et al.*, 2017). The same study reported knockdown of $Ca_v2.3$ in the DRG causes hypersensitivity in mice, and *Mir34c-5p* upregulation would increase pain via this mechanism, but also conversely prevent knockdown of the opioid system receptors and increase opioid mediated pain attenuation. *Mir34c* is downregulated, and therefore *Mir34c* mediated $Ca_v2.3$ knockdown (which is not identified in the RTA) cannot be contributing to hypersensitivity in the SNL.

Mir34c upregulation has been linked to AD (Satoh, 2010, Zovoilis *et al.*, 2011), as is *mir19b1* downregulation (fold change -1.28, $p = 0.048$, Table 3.5) via increased BACE1 activity. Aberrant expression of the *mir19b1*-targeted E2F transcription factors involved in regulation of cell cycling and apoptosis (Slaby *et al.*, 2009), contribute to neuronal cell death in PD (Hoglinger *et al.*, 2007), and could occur in the SNL model as part of the subsequent neural plasticity and remodelling that is associated with neuropathic pain (Zhu *et al.*, 2018). E2F also negatively regulates *mir19b1* expression, which exemplifies the complexity of microRNA and target interaction (Woods *et al.*, 2007). E2F is also knocked down by *mi181b1*, which is downregulated in the SNL DH (fold change -1.4, $p = 0.011$).

Mir181b1 downregulation (Table 3.5, fold change -1.4, $p = 0.011$) could promote apoptosis via reduced *Nova1* inhibition (Neuro-oncological ventral antigen 1) (Zhi *et al.*, 2014). NOVA1 is an RNA binding protein which regulars alternative splicing and is exclusive to neurones (Zhi *et al.* 2014). *Mir181b1* also targets *Bcl2* (Zhu *et al.*, 2010) and E2f1 (Slaby *et al.*, 2009). The E2fs are a family of transcription factors involved in regulation of cell cycling and apoptosis (Slaby et al., 2009). This microRNA also targets FPR2 (Pierdomenico *et al.*, 2015), and so inhibits inflammation resolution via this receptor pathway.

As in the changes to inflammatory mediators (Table 3.2, Table 3.3), the changes to miRNAs demonstrate changes to pro- and anti-inflammatory mechanisms, as well as apoptosis regulatory mechanisms. These miRNAs could themselves represent a biomarker profile for neuropathic pain. Guo *et al.* implicated five serum miRNA biomarkers for prostate cancer risk (Guo *et al.*, 2018). In 2011 Von Schack *et al.* performed a microRNA expression analysis on the DRG tissue of a rat SNL model. This study also reported a downregulation of *miR34c, miR181b*, and *miR19b* in both L4 and L5 SNL (von Schack *et al.*, 2011), supporting the results in the SNL DH (Table 3.5). This study also reported downregulated *Let7a*, whereas here an increase was found (Table 3.5). These could demonstrate DH tissue-specific changes to the miRNA profile in SNL.

Simultaneous upregulation of conflicting pro- and anti-apoptotic mediators is also reported in Table 3.1. This could be a characteristic of chronic neuropathic pain where feedback mechanisms are activated to inhibit the overstimulation of damaging apoptotic pathways to reduce neuronal cell damage. Such chronic stimulation of inflammatory and pro-apoptotic pathways is difficult to resolve due to the vast mediators and pathways which contribute to these effects.

Potential methods for future research into the role of these miRNAs in neuropathic pain include measuring microRNA expression in clinical samples of neuropathic pain to validate the changes to specific microRNA expression in a human sample. Such validation would indicate a cross-species validation and provide evidence for its role and the importance of its target knockdown in clinical neuropathic pain. It is also possible that the microRNA profile changes over disease time course, given the heterogeneity of clinical neuropathic pain, and such data has the potential for biomarker research.

The clinical application of circulating microRNA measurement has long been an attractive prospect in biomarker research across many pathologies (Faraldi *et al.*, 2018). Application of

miRNAs has recently shown promise. Injection of *miRNA340-5p* in CCI rats attenuated neuropathic pain symptoms by decreasing inflammatory mediator levels including IL-1β, TNF-α, and IL-6 (Gao *et al.*, 2019). Similar results were reported for *miRNA28-5p* (Bao *et al.*, 2018), *miRNA183-5p* (Shi *et al.*, 2018), and *miRNA124* (Grace *et al.*, 2018). The miRNAs reported here (Table 3.5), particularly miRNAs decreased in the SNL, could be effective in attenuating neuropathic pain. Injection of microRNA candidates in SNL models and the dose-dependent effects on neuropathic pain symptoms (measured by behavioural tests), could be used to further investigate the role of these miRNAs in neuropathic pain.

3.3.5 There is Disruption to Expression of Histone Proteins

Two histone proteins, H2a and H4 (fold change 1.56 and 1.38, $p = 0.03$ and $p = 0.02$ respectively), are differentially expressed in the RTA (Table 3.2). Though an increase in histone protein expression is reported here it cannot deduce whether there is a change to histone modification. Histone protein increase has been reported in proliferative cells in response to oestrogen treatment (Zhu *et al.*, 2009), and non-nuclear histone 1 has been reported in the neurones and astrocytes of AD patients (Bolton *et al.*, 1999). It is possible that histone protein expression changes reported here, which result from the SNL injury, could have subsequent effects on the transcription of other genes. Dysregulated histone production could occur downstream of transcription factors involved in the neuropathic pain phenotype.

3.3.6 There is Disruption to Expression of Ribosome Proteins

Thirty-seven ribosomal proteins are differentially expressed at mRNA level in the RTA, thirty upregulated and seven downregulated (Table 3.1). Additionally, mitochondrial 39S ribosomal protein L20 is coded for by *Mrp120*, which is upregulated in the SNL model (fold change = 1.36, $p < 0.00$). Ribosomal proteins are the assessor protein components for the rRNA complexes responsible for translation of mRNA into protein (Fatica & Tollervey, 2002). There are seventy-

nine ribosomal proteins (Zhou *et al.*, 2015). Changes to the gene expression of ribosomal proteins in the SNL model could have wider implications for translation of other genes. Chemical modification of the rRNA component of ribosomes can result in dysregulated protein synthesis and contributed to disease states including cancer (Natchiar *et al.*, 2017) and haemological disorders (Shenoy *et al.*, 2012). Ribosomal proteins have extra-ribosomal functions including tumour suppressive, development, and immune system (X Zhou *et al.*, 2015). S19 can reduce the pro-inflammatory actions of macrophage migration inhibitory factor (MIF) (Filip *et al.*, 2009) (Lv *et al.*, 2013). S15 is involved in degeneration in PD (Martin *et al.*, 2014) and L22 is involved in T cell development (Stadanlick *et al.*, 2011).

It is possible that the upregulation of ribosomal proteins is a characteristic of the general upregulation of genes in the SNL model, or their dysregulation could contribute to the neuropathic pain state. Dysregulated ribosomal expression has been reported in AD patients, with an increase in 18S and 28S mRNA compared to healthy elderly controls (Rasmussen *et al.*, 2015). Changes to ribosomal protein expression in SNL DH could be tissue specific, as suggested by Rasmussen *et al.* (2015). If changes to the ribosomal proteins profile in clinical blood samples from neuropathic pain patients could elucidate a unique signature, it might be useful in diagnosis.

3.3.7 Changes to Common Transcription Factors Could be an Upstream Regulator

The common transcription factors (TFs) found in the rat (Table 3.10) represent potential mechanisms for the simultaneous upregulation of the genes presented in Figure 3.5. TFs, also known as sequence-specific DNA-binding factors, are proteins which control transcription rate of associated genes by binding to TF binding sites and initiate increased or decreased gene expression. The core similarity value indicates degree of core sequence match for the

transcription factor and gene. The core sequence is defined as the highest conserved positions in the transcription factor. The maximum value of 1 represents a full match.

Given the important role of TFs in transcription, many TFs are tumour suppressors or oncogenes including p53 and STAT family TFs (Baumgart *et al.*, 2013). Other diseases involving dysregulated TFs include the neurodevelopmental disorder Rett syndrome (Moretti & Zoghbi, 2006), and immunodysregulation polyendocrinopathy enteropathy X-linked (or IPEX) syndrome (van der Vliet & Nieuwenhuis, 2007). There are no known explicit links of these TFs to neuropathic pain, but several either have additional targets that have been implicated in neuropathic pain or are involved in neural development.

Many TFs, including these common TFs (Table 3.8) are involved in regulating gene expression which drives embryonic development. Embryonic development is the top differentially expressed system (Table 3.7), and it is possible that these TFs could be contributing to some of the changes. Mesoderm posterior 2 (*Mesp2*) is important for somitogenesis in embryonic development (Q Liang *et al.*, 2015). *Mesp2* regulates Notch signalling (Morimoto *et al.*, 2005), for which the upstream miRNA *mir34c* is downregulated in the SNL (Table 3.5). The homeobox transcription factor *Gsx-2* is also involved in embryonic development, including fates of olfactory bulb interneurons (Waclaw *et al.*, 2009). Similarly, *Pax-6* is known as a master control TF for the development of sensory neurons during embryonic development (van Heyningen & Williamson, 2002). The CNS specific POU-III protein class TF *Brn-2* is required for embryonic neural development (Fujii & Hamada, 1993). The common function of these TFs in neural development suggests that changes to the neuronal systems may be occurring in the DH after SNL, which supports notions of neuronal remodelling in neuropathic pain. These common TFs could provide a potential avenue for further research into neuropathic pain, and comprehensive understanding of neuropathic pain specific changes to the TF profile would provide invaluable

insight to neuropathic pain processed on a molecular level, and given their function in neuronal development, physiological changes to the CNS.

3.3.8 Strengths and Limitations of the Methodology

Spinal Nerve Ligation Model and Behavioural Tests

The SNL model was maintained for 35 days to replicate chronic neuropathic pain conditions. The sham-operated group controls for the anaesthetic and surgical operation procedure. Three behavioural tests validated the SNL model, including Von Frey assay, Hargreaves assay, and the acetone spray test (Buckley *et al.*, 2017). These tests have strengths and weaknesses, as do the alternative methods of validating neuropathic pain in models (as discussed in Chapter 1, Section 1.7, see review (Deuis *et al.*, 2017) but are well-established behavioural tests. Therefore, the results of these behaviour tests suggest that gene expression changes are a result of the SNL itself as the control used in this study is sham-operated, and control rats are a subjected to all aspects of the procedure including anaesthesia. This strengthens the argument that the gene expression changes are relevant to the neuropathic pain model and not from the operation incisions and injury. Further, the 35-day model is considered chronic and is routinely used in research (Fletcher & Bao, 1996) (Tahawi *et al.*, 2001). This strengthens the validity of the results reported here.

Rodent models for neuropathic pain are widely used as a surrogate for human models across all research disciplines. The availability of full genome data for rat species, as well as for lower-order species e.g. the fruit fly *Drosophilia melanogaster* and nematode worm *Caenorhabdits elegans* allows these to be utilised in the study of human disease (Strynatka *et al.*, 2018), as conserved genes have the same function in different organisms (Lander, 2011). Despite fundamental physiological differences between humans and surrogate models, they are invaluable to the study of specific molecules and pathways in the context of a living system. In

neuropathic pain, rat models are useful to consider the complex interactions of multiple systems, including the PNS and immune components in disease aetiology, CNS pain modulation in chronic states, cognitive processing of the pain experience, and the induction of behaviours.

DNA Microarray

Microarray chips contain multiple distinct probes for each transcript, and there can be variability between each probe signal. Guanine and cytosine (GC) content of a probe is the most important factor for signal intensity (Leparc *et al.*, 2009). Normalisation is required to account for technical variation (such as that which occurs during labelling and chip hybridisation) in microarray data, which does not reflect biological differences between the samples.

RNA-Seq represents an alternative though more expensive method for global gene expression analysis. Both methods are highly reproducible, and results are comparable with high correlation (Chen, Sun*, et al.*, 2017), but RNA-Seq is better at detecting low abundance transcripts, and differentiating between genetic variants (Zhao *et al.*, 2014). Both have limitations but these are better understood for microarray as it is a well-established, commonly used technique for decades, whereas RNA-Seq is relatively new. RNA-Seq is widely considered superior to microarray, as it sequences the whole transcriptome and is not limited by prior knowledge. Microarray chips contain probes comprised of known DNA sequences which bind to complimentary mRNA sequences in the sample to produce a fluorescent signal, whereas RNA-Seq employs next-generation sequencing to quantify the mRNA in the sample. This technique also means RNA-Seq data can be used to discover novel mRNAs, and new information can be analysed from the original dataset; for microarray new sequences would have to be incorporated onto the chip, and the samples rerun. Sequences changes and polymorphisms can also be explicitly identified by RNA-Seq, but not by microarray. However, RNA-Seq requires a large amount of computational processing power and highly specialised infrastructure, as well

as expertise to analyse the data. Microarray data by comparison is relatively easy to analyse, which is why (as well as cost) it was chosen over RNA-Seq for this study. However, RNA-Seq may overcome the problem of large number of unassigned genes from the RTA.

None of the genes passed the FDR cut-off of $p = 0.05$ and most have small fold changes (<1.5) however small gene expression changes can have subsequent physiological consequences. For example, a 23% decrease (i.e. -1.23 fold change) in GAD27 mRNA is thought to be a functional component of schizophrenia (T Hashimoto *et al.*, 2008). Large FDR cut-offs are a common phenomenon for microarray with small sample sizes (Pawitan *et al.*, 2005). This limitation could be part of the reason why there has been a recent shift away from microarray technologies and a move towards RNA-Seq.

Gene expression changes indicated by the microarray data can only tell us about the individual gene in isolation. Taken together these changes can determine whether pathways and interactions are active or inactive, such is the function of the IPA® analysis (Figure 3.5). Although the interactions uncovered by IPA® analysis could be unrelated to neuropathic pain, published findings are critical to understanding the function of candidate genes. Receiver operative characteristic (ROC) curve analysis, which will evaluate how useful the selection of parameters could be in the identification of a disease state. In Chapter 4, ROC curve analysis is used to evaluate gene expression changes in clinical samples in the identification of neuropathic or inflammatory pain. These follow-up analyses enrich the microarray data and are essential to evaluate the candidates as potential biomarkers of neuropathic pain.

Circadian Rhythm

Circadian rhythm and cell cycle have an effect of gene expression and must be considered for analyses. Twenty-four-hour cycles of circadian rhythm exist to control physiological processes in

plants, insects, and animals (Vitaterna *et al.*, 2001). The biological processes subject to circadian rhythm are adjusted to external cues including light and temperature. The so-called master clock in the suprachiasmatic nucleus in the hypothalamus controls mRNA expression in the peripheral circadian processes (Sukumaran *et al.*, 2010).

Gene expression changes are therefore the fundamental to the circadian rhythm cycle. Cyclic gene expression should not be factor in the study as SNL and sham-operated animals were sacrificed within a short time (35-days). There should also be very little to no variation in circadian rhythm, as all the animals were subjected to the same environmental cues in identical living conditions, except for the *ad libitum* availability of food and water which could result in individual differences in feeding behaviour. However, for clinical data the effect of circadian rhythm cannot be controlled for due to individual differences in subject's waking hours, eating habits, and other biological activities. This will be a source of variation within the clinical cohort which is essentially absent in the animal model dataset.

3.4 Conclusion

This data indicates which systems are in increased or decreased in the SNL DH tissue. Long term transcriptional changes to essential machinery (e.g. ribosomes and histones) are reported, in addition to predictable changes to inflammatory and apoptotic processes as described by previous studies. The gene expression changes in the DH tissue of the rat SNL model are representative of systemic changes that shift towards a neuroinflammatory phenotype. Inflammatory mediators and apoptotic proteins are altered at the mRNA level, with largely in pro-inflammatory and pro-apoptotic transcripts upregulated (e.g. caspases and *Romo1*). There is also strong evidence for an increase in immune cell trafficking. Concurrently a decrease in some anti-inflammatory mechanisms (e.g. *Fpr2* and *Cartpt*) is reported, alongside an increase in

others with potentially protective function (e.g. *Anxa1* and *Txn1*). These changes at the DH, a critical area of pain modulation, in a 35-day SNL model provide a snapshot of the physiological changes that occur following nerve ligation as neuropathic pain develops.

The differentially expressed genes highlight the pathways which are active and inhibited during these physiological changes. The changes which are unique to neuropathic pain could be exploited for an improved diagnostic test, or if appropriate, pharmacological targets for treatment or symptom management. Unique changes represent potential biomarkers, and the development of potential biomarkers (candidates) into clinically useful biomarkers requires further investigation to fully elucidate their function in both health and neuropathic pain and evaluate their usefulness in diagnosis and drug targeting. This is the first step into the investigation into potential biomarkers for neuropathic pain. The results from the microarray data form the basis of this study. Fifteen genes of interest were selected based on their fold change and function and analysed in blood samples of clinical neuropathic pain patients, discussed in Chapter 5.

Chapter 4: Gene Expression Analysis of Neuropathic Pain Patients

4.1 Introduction

A subset of the genes of interest, identified in the dorsal horn (DH) of the rat spinal nerve ligation (SNL) model (Chapter 3), were subsequently measured in whole blood samples from neuropathic pain patients. Subset genes were selected based on their known function, fold change, and functional interactions with other candidates, as elucidated by Ingenuity Pathway Analysis (IPA®). This approach allowed the candidates to be first selected in a highly relevant tissue type from a model of neuropathic pain, dorsal horn (DH) tissue, which is unavailable from patients, and subsequently validated in the heterogeneous clinical disease cohort of neuropathic pain.

Fifty-three adult neuropathic pain patients were recruited from Seacroft Leeds Teaching Hospital (Ethics NHS - 14/YH/0117). Questionnaires were completed by the patients at time of blood collection included the (self-reported) Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS), Patient Health Questionaire-9 (PHQ-9), and Generalised Chronic Pain Scale (GCPS). Screening questions for exclusion criteria (history of multiple sclerosis, fibromyalgia, and diabetes) was also included. Clinical data including diagnosis, number of months since pain began, current medications, and comorbidities was also collected by questionnaire for trend analysis. Sixty-five age and sex-matched 'healthy' controls were recruited from the University of Huddersfield (UK) and completed the same questionnaire omitting the S-LANSS pain assessment. Variable scores were analysed by linear regression to elucidate trends and identify neuropathic pain characteristics that could be relevant for the development of improved diagnosis tools.

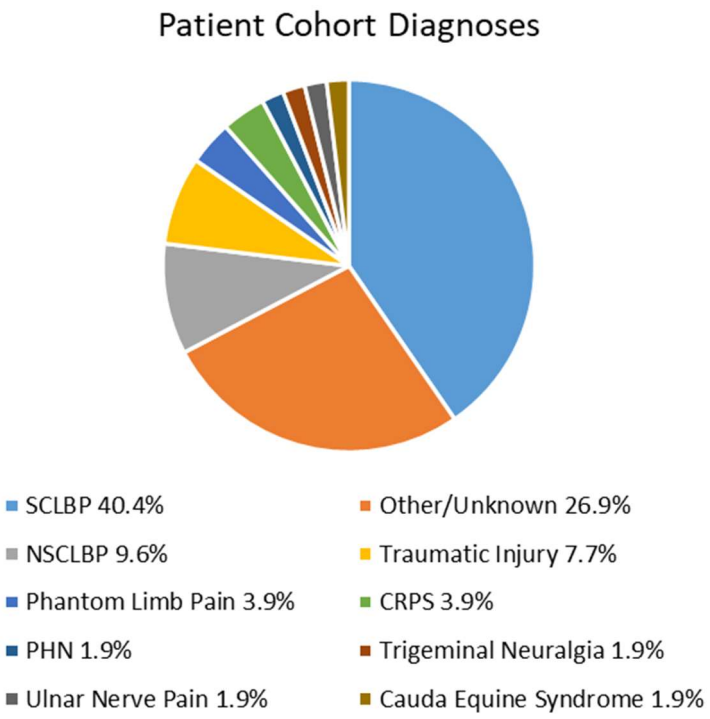


Figure 4.1: Patient Cohort Diagnoses. Specific Chronic Lower Back Pain (SCLBP) is the most common diagnosis in the clinical cohort (40.4%). 26.9% of patient as classified as other or did not have a clear cause for their chronic pain. Similarly, Non-specific Chronic Lower Back Pain (NSCLBP) accounts for almost 10% (9.6%). 7.7% of patients had pain resulting from a traumatic injury, 3.9% of patients had pain resulting from an amputation, and 3.9% has Complex Regional Pain Syndrome (CRPS). One patient had post-hepatic neuralgia (PHN 1.9%), one patient had Trigeminal Neuralgia (1.9%), one patient had ulnar nerve pain in the writs and one patient had Cauda Equine Syndrome (1.9%).

Figure 4.1 shows the diagnosis categories for the patient cohort. Specific Chronic Lower Back Pain (SCLBP) and Non-specific Chronic Lower Back Pain (NSCLBP) are chronic pain syndromes (three months or more) of the lower back. Specific CLBP is a general heterogeneous diagnosis which encapsules several origins including musculoskeletal pain (Fourney *et al.,* 2011) (Russo *et al.,* 2018). Non-specific CLBP is a diagnosis where the cause is unknown. Complex Regional Pain Syndrome (CRPS) is a pain condition which worsens over time but remains concentrated to one

area (Urits *et al.* 2018). Cauda Equine Syndrome (1.9%) is caused by damage to the nerve bundle at the base of the of the spinal cord (Todd 2017).

Gene expression changes in whole blood of clinical neuropathic pain patients could be indicative of genes and proteins involved in the underlying neuropathic pain pathophysiology. For this study gene expression was performed by quantitative real-time polymerase chain reaction (qPCR) to quantify gene expression. Gene expression of candidates from the whole blood samples in the neuropathic pain patient was compared to that of the healthy control cohort to identify differentially expressed genes. PCR is performed using cDNA synthesised from the sample RNA. Amplification is monitored and measured as it occurs throughout the reaction using a double-stranded DNA (dsDNA)-binding fluorescent dye (Figure 4.2). Data is output as a Cq value (also referred to as Ct value); this is the cycle number at which the fluorescent signal exceeds baseline threshold. This information is proportional to the amount of gene template in the cDNA sample. A lower Cq value indicates higher gene expression as fewer amplification cycles are required to suppress threshold fluorescence.

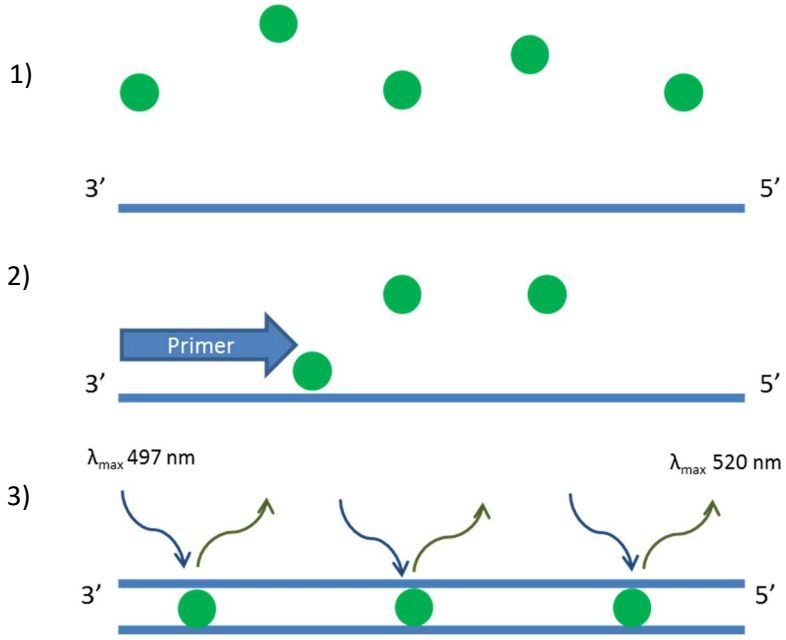


Figure 4.2: SYBR Green I dye mechanism during a quantitative PCR amplification cycle. The fluorescent dye molecules bind to double-stranded DNA and emit a fluorescent signal. 1) Single-stranded DNA and free fluorescent SYBR Green I molecules in the qPCR mix. 2) Primer anneals to the single DNA strand. 3) During extension, and double-stranded DNA synthesis, dye molecules bind to the double-stranded DNA and emit fluorescent wavelengths. The intensity of the resulting fluorescent reading is proportional to the amount of dsDNA within the sample, which increases with each PCR cycle.

Biomarkers could be used in combination for a diagnostic test, which can be evaluated in terms of their sensitivity or specificity. For example, in the instance where test value increases with disease severity, the appropriate disease threshold for diagnosis is unclear. If the threshold is too high, specificity is high and milder disease cases could be missed (sensitivity is low). Conversely if the threshold is too low false positive diagnoses will occur (high sensitivity with low specificity). Receiver operating characteristic (ROC) analysis is used to evaluate the usefulness of a combination of candidate biomarkers in the indication of whether a patient case is neuropathic or inflammatory in nature, based on the S-LANSS score. The ROC analysis allows the trade-off between specificity and sensitively to be visualised.

The S-LANSS Questionnaire assesses clinical pain, and patient is given a score based on their answers to questions about the nature and severity of their pain (Bennett 2001). LANSS is a widely used tool in neuropathic pain diagnosis, but an obvious limitation of the S-LANSS questionnaire is its subjective nature and cannot provide an objective account of the patient's physiological pain. Chronic pain cases which score ≥12 are classified neuropathic in origin, and cases which score <12 are considered non-neuropathic/inflammatory in origin, according to the questionnaire guidelines for use (Bennett 2001). In this study, patients diagnosed with neuropathic pain were included, and any previous S-LANSS scores were not considered for recruitment.

The International Association for the Study of Pain (IASP) defines neuropathic pain as "pain caused by a lesion or disease of the somatosensory nervous system". It is not necessarily in the presence of chronic inflammation, though patients often present with it at the site of nerve injury. Inflammation occurs during the injury process and it has been difficult to elucidate exactly if and how inflammatory mediators can contribute to the production and maintenance of neuropathic pain (Kuffler 2020, Hung *et al.*, 2017). For example, in traumatic injuries where peripheral nerves are damaged, inflammatory processes will be activated at the site to control tissue damage, protect against infiltration pathogens, and begin healing. Given the destructive nature of pro-inflammatory mechanisms (e.g. apoptosis) it is possible that destruction of the nerve endings that precedes neuropathic pain is caused, in part, by inflammatory mediators. For this study, S-LANSS score was used to distinguish cases of chronic pain likely of neuropathic origin, to those of non-neuropathic/inflammatory origin. Gene expression changes across the two groups could indicate their role in chronic pain, and their relevance to clinical neuropathic pain. ROC curve analysis was performed on the differentially expressed genes in triplicates, based on their known function, in the high and low scoring S-LANSS scoring categories. This

evaluates whether the combination of gene expression changes can be used to predict the S-LANSS score category and identify potential combinations of biomarkers for future use in diagnostic tests.

Other variables of the pain experience are quantified by participants by appropriate questionnaires. The Patient Health Questionaire-9 (PHQ-9) is used to monitor depression and response to anti-depressive treatment. It asks the individual about their behaviour and the impact of their mood on daily activities in the past two weeks (Kroenke *et al.*, 2001). Depression is often reported amongst chronic pain patients (Dhanju *et al.*, 2019), and anti-depressant medications are often used to treat neuropathic pain. Scoring the patients with the PHQ-9 mood at the point of blood collection is useful as it gives an indication of their general emotional state, which can be influenced by anti-depressant medications often prescribed to neuropathic pain patients.

The State-Trait Anxiety Inventory (STAI) is a questionnaire tool used to measure state anxiety and trait anxiety in scores STAI-Y1 and STAI-Y2 respectively. Anxiety is defined by feelings of unease, apprehension, and stress (Sydeman *et al.* 1994), and is often present in chronic pain patients (Yalcin & Barrot, 2014). State anxiety refers to the temporary anxiety induced by specific threatening situations, and activation of the autonomic nervous system (Renner *et al.*, 2018). Trait anxiety refers to the ongoing feeling of stress and discomfort during typical day-to-day activities. The questionnaire includes forty questions (twenty STAI-Y1, twenty STAI-Y2) quantifying state and trait anxiety separately. STAI was used in this study as a comprehensive measure of anxiety in neuropathic pain patients and to investigate if there is a relationship between anxiety and other variables such as duration of neuropathic pain and S-LANSS score in our cohort. STAI data was only collected for a subset of patients (n = 22) as it was added to the questionnaire after the first thirty samples had been collected.

The Graded Chronic Pain Scale (GCPS) is indicative of pain intensity and disability severity (Von Korff *et al.*, 1992). The higher the score, the more intense the pain and severe the pain-associated disability. It was included in our study as an indicator of the impact of neuropathic pain on patient quality of life and physical disability. The GCPS contains seven questions designed to measure the severity of an individual's pain and how it interferes with daily life.

Gene expression amongst the neuropathic pain cohort was analysed according to the following medication groups: i) Anti-depressants ii) Anti-convulsants iii) Anti-inflammatories, and iv) Opioids. Patients were assigned to the medication group if they were prescribed, and presumed to be taking, the medication at the time of blood collection. Medication data was also collected by patient questionnaire, and later verified using their medical history, though some uncertainty with regards to the duration for previous medications remains. However, in this study only current medications were considered, and the length of time the patient had taken a given medication was not considered. Medication data was not collected for the control group.

4.2 Results

4.2.1 Gene Expression Changes in Clinical Neuropathic Pain Patients vs Controls

Fourteen genes were measured by qPCR in the clinical samples. Additionally, *CASP5* was measured as in humans *CASP4* and *CASP5* both represent orthologues of rat *Casp4* (Table 4.1).

Table 4.1: Gene Expression Changes in SNL Rat Model (Microarray) and FDR-adjusted Neuropathic Pain Patients (Whole Cohort) vs. Controls

Gene	Fold Change in Rat	*P* Value	Clinical Fold Change	*P* Value
A3GALT2	1.31	0.0421 (*)	1.37	0.018 (*)
ANXA1	1.43	0.0477 (*)	1.10	0.106
CASP1	1.27	0.0459 (*)	1.10	0.024 (*)
CASP4	1.34	0.0252(*)	1.10	0.045 (*)
CASP5	n/a	n/a	1.57	<0.001 (***)
CCR5	1.35	0.0403 (*)	1.23	0.033 (*)
CD4	1.26	0.0499 (*)	1.11	0.104
CYCS	1.33	0.0334 (*)	1.08	0.164
FPR2	-1.35	0.0024 (**)	1.33	0.001 (***)
OMP	-1.25	0.0048 (**)	1.03	0.654
PLAC8	1.61	0.0246 (*)	1.08	0.127
SH3BGRL3	1.30	0.0085 (**)	-1.19	<0.001 (***)
ROMO1	1.32	0.0136 (*)	-1.08	0.468
TMEM88	1.33	0.0167 (*)	1.56	<0.001 (***)
TXN1	1.28	0.0034 (**)	1.03	0.554

Analysis of covariance (ANCOVA) of gene expression in controls versus neuropathic pain patients showed differential expression of *A3GALT2*, *CASP1*, *CASP4*, *CASP5, CCR5, FPR2,* and *TMEM88,* which is significantly increased in neuropathic pain, whereas *SH3BGRL3* is significantly decreased in neuropathic pain patients versus controls (Table 3.1, Figure 4.3).

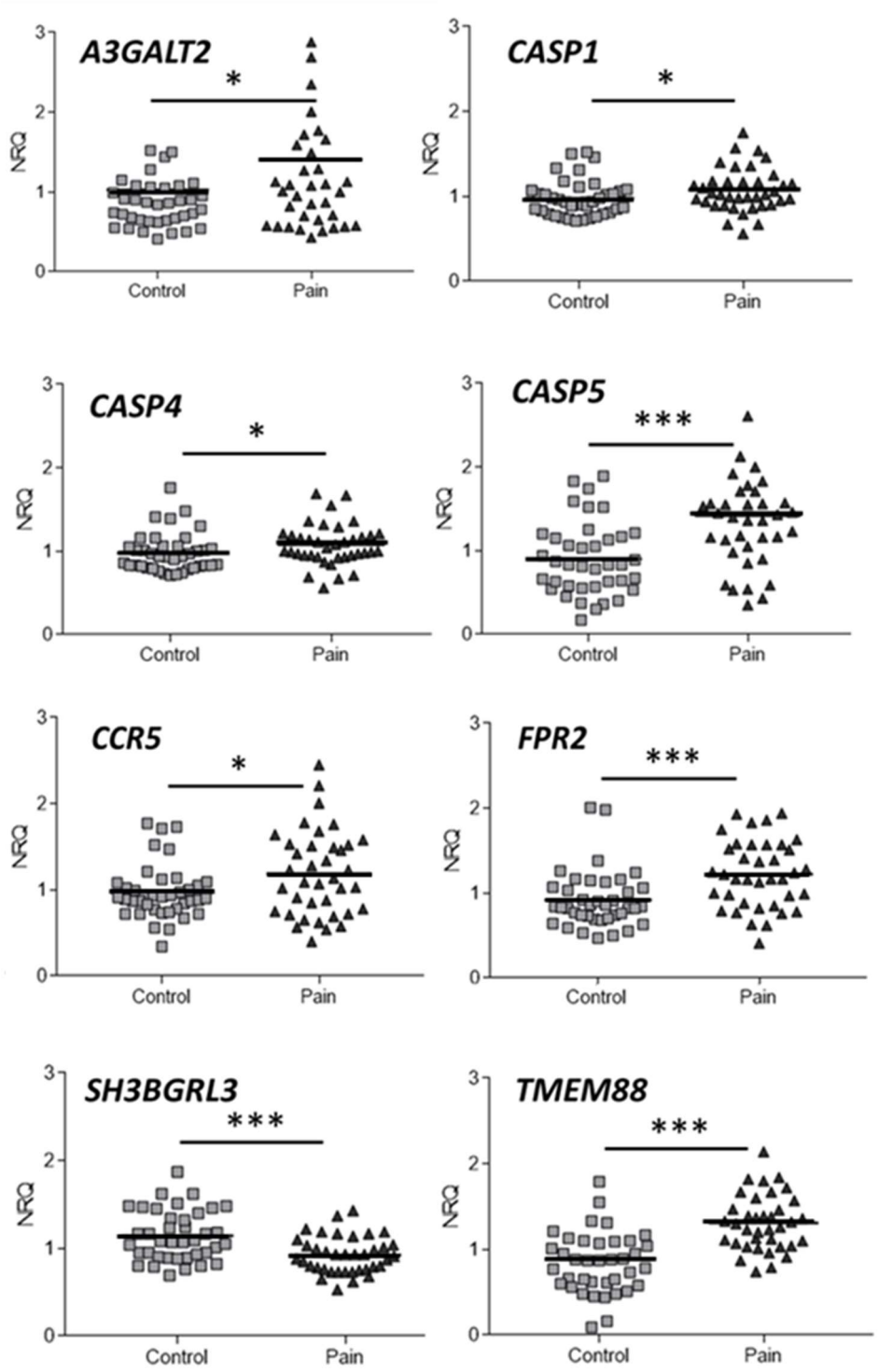


Figure 4.3: Expression changes in whole blood samples from neuropathic pain patients versus healthy controls. Significant changes in *A3GALT2* (Control 1.03±0.13, Pain 1.41±0.16), *CASP1* (Control 0.98±0.03, Pain 1.07±0.04), *CASP4* (Control 0.98±0.04, Pain 1.08±0.04), *CASP5* (Control 0.90±0.07, Pain 1.42±0.11), *CCR5* (Control 0.97± 0.05, Pain 1.19±0.08), *FPR2* (Control 0.92±0.05, Pain 1.22±0.06), *SH3BGRL3* (Control 1.14±0.05, Pain 0.92±0.03), *and TMEM88* (Control 0.88±0.07, Pain 1.38±0.08) expression were observed in qPCR analyses of clinical samples. Data was analysed

by covariate analyses in SPSS with gender and age controlled for, and Bonferroni correction. False discovery rate corrected for. * = $p < 0.05$, *** = $p < 0.001$. NRQ = Normalised Relative Quantity.

Four additional genes, *CASP3, CASP8, CASP9,* and *NLRP3* were also measured in whole blood samples (Table 3.2) to further elucidate the caspase pathways actively involved in clinical neuropathic pain. *CASP8* and *CASP9* were found to increase in clinical neuropathic pain (fold change 1.79 $p<0.001$, and 1.67 $p<0.001$ respectively).

Table 4.2: Expression on genes associated with caspase cascade in pain patient cohort versus controls.

Gene	Clinical Fold Change	*P* value
CASP3	1.03	0.650
CASP8	1.79	<0.001 (***)
CASP9	1.67	<0.001 (***)
NLRP3	1.01	0.848

4.2.2 Gene Expression in High vs Low S-LANSS Scoring Patients

The neuropathic pain cohort was divided into two groups according to low non-neuropathic/inflammatory S-LANSS score (≤12) and high neuropathic S-LANSS score (≥12). Gene expression was analysed between each of the two groups and the control cohort. None of the differentially expressed genes in neuropathic pain cohort are exclusively differentially expressed in the low S-LANSS scoring group. *TMEM88* is differentially expressed in both groups (versus control) (Figure 4.4) and *A3GALT2* is not differentially expressed in either of the groups. Genes differentially expressed in the high S-LANSS score group are *CASP1, CASP4, CASP5, CCR5, FPR2,* and *SH3BGRL3* (Figure 4.4). Genes differentially expressed in the low S-LANSS score group but not in the whole neuropathic pain cohort (versus controls) are *ROMO1* and *PLAC8* (Figure 4.4).

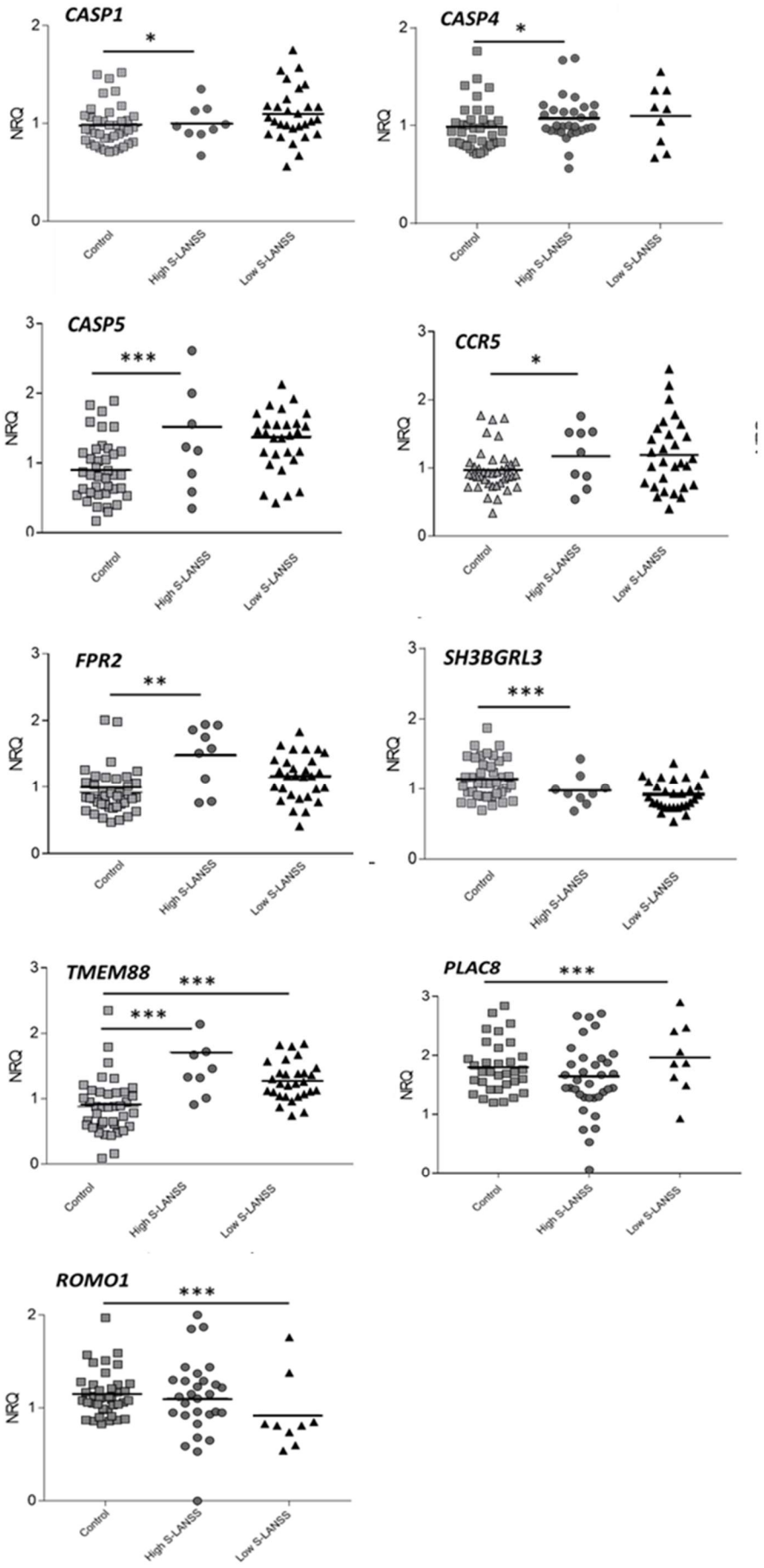

CASP1
*
CASP4
*
CASP5
***
CCR5
*
FPR2
**
SH3BGRL3
***
TMEM88
***
***
PLAC8
***
ROMO1
***
NRQ
Control
High S-LANSS
Low S-LANSS

Figure 4.4: Genes with differential expression in the high S-LANSS and low S-LANSS scoring patients. *CASP1* (Control 0.98±0.03, High S-LANSS 1.14±0.06, Low S-LANSS 0.96±0.07), *CASP4* (Control 0.98±0.04, High S-LANSS 1.16±0.06, Low S-LANSS 0.91±0.07), *CASP5* (Control 0.90±0.07, High S-LANSS 1.63±0.17, Low S-LANSS 0.99±0.16), *CCR5* (Control 0.90±0.05, High S-LANSS 1.26±0.12, Low S-LANSS 1.11±0.12), and *FPR2* (Control 0.92±0.05, High S-LANSS 1.17±0.09, Low S-LANSS 1.31±0.16) were increased in the high S-LANSS scoring group versus control but were not significantly different between low scoring S-LANSS group and control. *TMEM88* was increased in both S-LANSS groups versus control (Control 0.88±0.07, High S-LANSS 1.30±0.07, Low S-LANSS 1.49±0.32). *PLAC8* is increased in low scoring patients versus control (Control 1.68±0.28, High S-LANSS 1.30±0.06, Low S-LANSS 1.62±0.14) ($p<0.001$) and *ROMO1* is decreased (Control 1.15±0.04, High S-LANSS 1.15±0.10, Low S-LANSS 0.93±0.10) ($p<0.001$). Data was analysed by covariate analyses in SPSS with gender and age controlled for, and Bonferroni correction. False discovery rate corrected for. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. NRQ = Normalised Relative Quantity.

4.2.3 Gene Expression in Medication Groups

A3GALT2 expression is reduced in patients taking opioid medication (n=31) versus patients not taking opioid medication (n=22) ($p < 0.001$). *ROMO1* is increased in patients taking anti-convulsants (n=23, $p = 0.049$) and decreased in NSAIDs (n=20, $p = 0.031$). The medication groups analysed do not modify (or have no effect on) the expression of the differentially expressed genes (neuropathic pain vs control) *CASP4, CCR5, FPR2*, *SH3BGRL3*, and *TMEM88*.

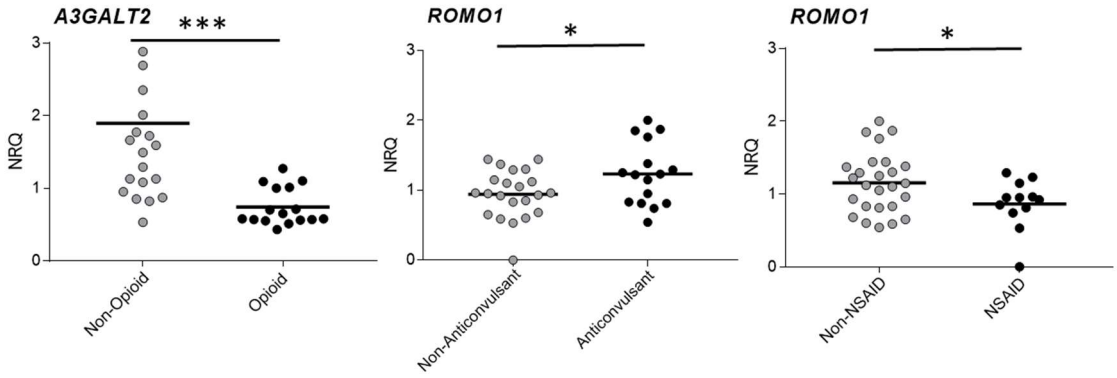


Figure 4.5: Medication and Gene Expression Changes. *A3GALT2* and *ROMO1* are differentially expressed in patients taking certain types of medication (*A3GALT2* expression with opioid medication Control 1.89±1.08, Opioid 0.74±0.26, *ROMO1* Control 0.97±0.10, Anti-convulsants 1.14±0.01, *ROMO1* Control 1.07±0.02, NSAIDs 1.05±0.1). Data was analysed by covariate analyses in SPSS with gender and age controlled for, and Bonferroni correction. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. NRQ = Normalised Relative Quantity.

4.2.4 Receiver operating characteristic (ROC) Curve Analysis

Six combinations of three candidates were analysed by receiver operating characteristic (ROC) curves to evaluate how well they could test for either low or high S-LANSS score category. Of these triplicates, one combination of candidates was a good test for S-LANSS score (Table 4.3). Two others showed promise with an area under curve between 0.5-1 but were not significant (Table 4.3). Though neuropathic pain is the interest of this project, tests for inflammatory pain origins are useful to determine the role of inflammation in a patient's pain condition.

Table 4.3: Receiver Operating Characteristic Analysis Results

Combination	Area	*P* value	Conclusion
A3GALT2, SH3BGRL3, TMEM88	0.314	0.96	Not useful as area is < 0.5, Not Significant
CASP1, CASP4, CASP5	0.713	0.057	Potential as a test for inflammatory pain
FPR2, SH3BGRL3, TMEM88	0.625	0.264	Not Significant
FPR2, CCR5, CD4	0.705	0.066	Potential as a test for inflammatory pain
FPR2, ANXA1, PLAC8	0.782	0.013	Useful as a test for inflammatory pain
SH3BRL3, TMEM88, PLAC8	0.162	0.003	Not useful as area is < 0.5

The combination of *FPR2, CCR5,* and *CD4* expression changes is a good predictor for inflammatory pain (Area 0.705, $p = 0.066$), as is the combination of *FPR2, ANXA1,* and *PLAC8* (Area = 0.782, $p = 0.013$). There is no evidence that in combination, *A3GALT2, SH3BGRL3,* and *TMEM88* can predict the presence of neuropathic pain (low S-LANSS score), with an area < 0.5 (0.314) and $p = 0.96$. Caspases 1, 4, and 5, were tested in combination for their ability to test for inflammatory pain (low S-LANSS score) but is not significant (Area = 0.713, $p = 0.057$). *FPR2, SH3BGRL3,* and *TMEM88* cannot predict inflammatory pain (Area = 0.625, $p = 0.264$), neither can the combination *SH3BRL3, TMEM88,* and *PLAC8* (0.162) ($p = 0.003$).

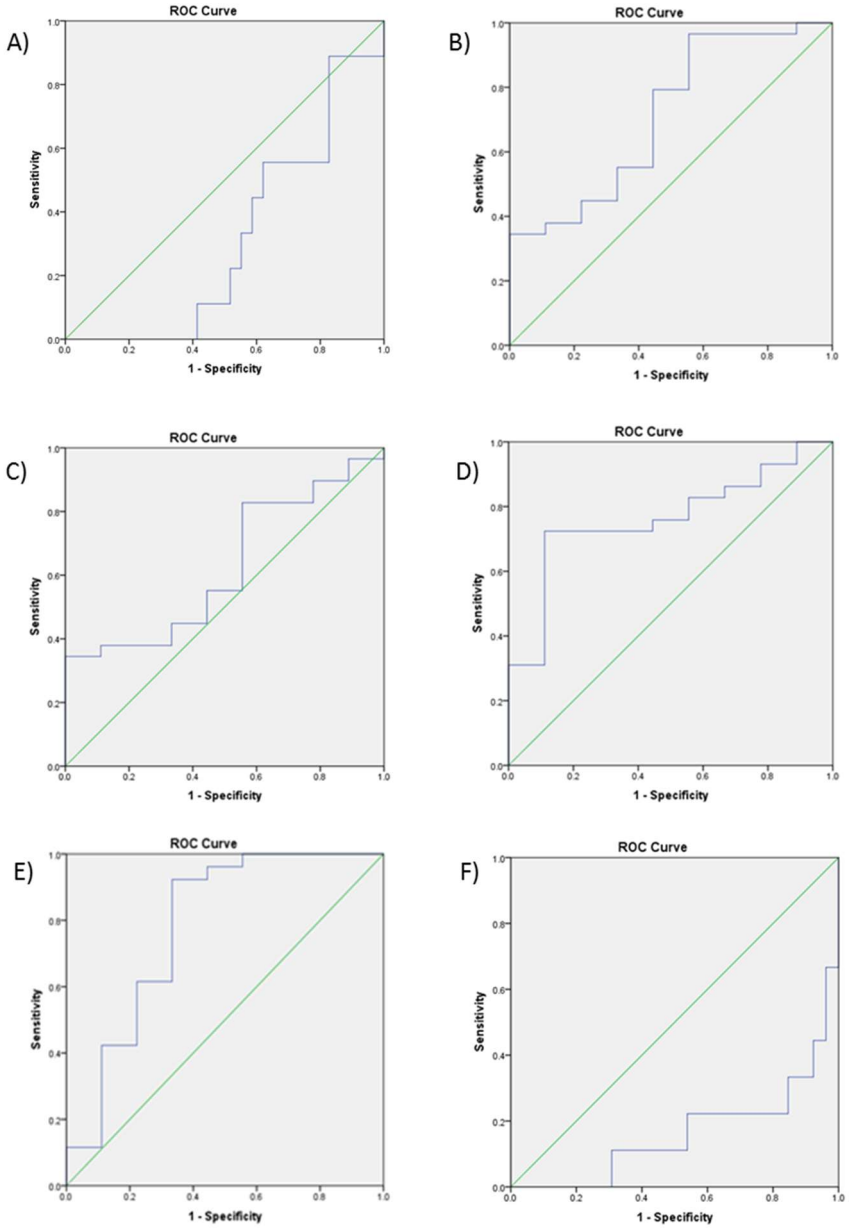


Figure 4.6: Receiver Operating Characteristic Analysis of Candidate Combinations Test for S-LANSS Category. *A) A3GALT2, SH3BGRL3,* and *TMEM88* Area = 0.314, *p* = 0.96. B) *CASP1*, *CASP4*, and *CASP5* (Area = 0.713, *p* = 0.057). C) *FPR2, SH3BGRL3, TMEM88* (Area = 0.625, *p* = 0.264). D) *FPR2, CCR5, CD4* (Area 0.705, *p* = 0.066). E) *FPR2, ANXA1, PLAC8* (Area = 0.782, *p* = 0.013). F) *SH3BRL3 TMEM88 PLAC8* (Area = 0.162, *p* = 0.003).

4.2.5 Linear Regression

Due to the assumptions for linear regression not being met by most of the patient questionnaire variables (Section 4.3.4), a limited selection could be analysed. Four notable results have been found in our cohort (Figure 4.7). These are GCPS and PHQ-9 score ($r^2 = 0.366$, $p < 0.001$), STAI-Y1 score for state anxiety and PHQ-9 score ($r^2 = 0.299$, $p = 0.002$), STAI-Y2 for trait anxiety and PHQ-9 score ($r^2 = 0.478$, $p < 0.001$), and STAI-Y1 and STAI-Y2 ($r^2 = 0.628$, $p = 0.009$).

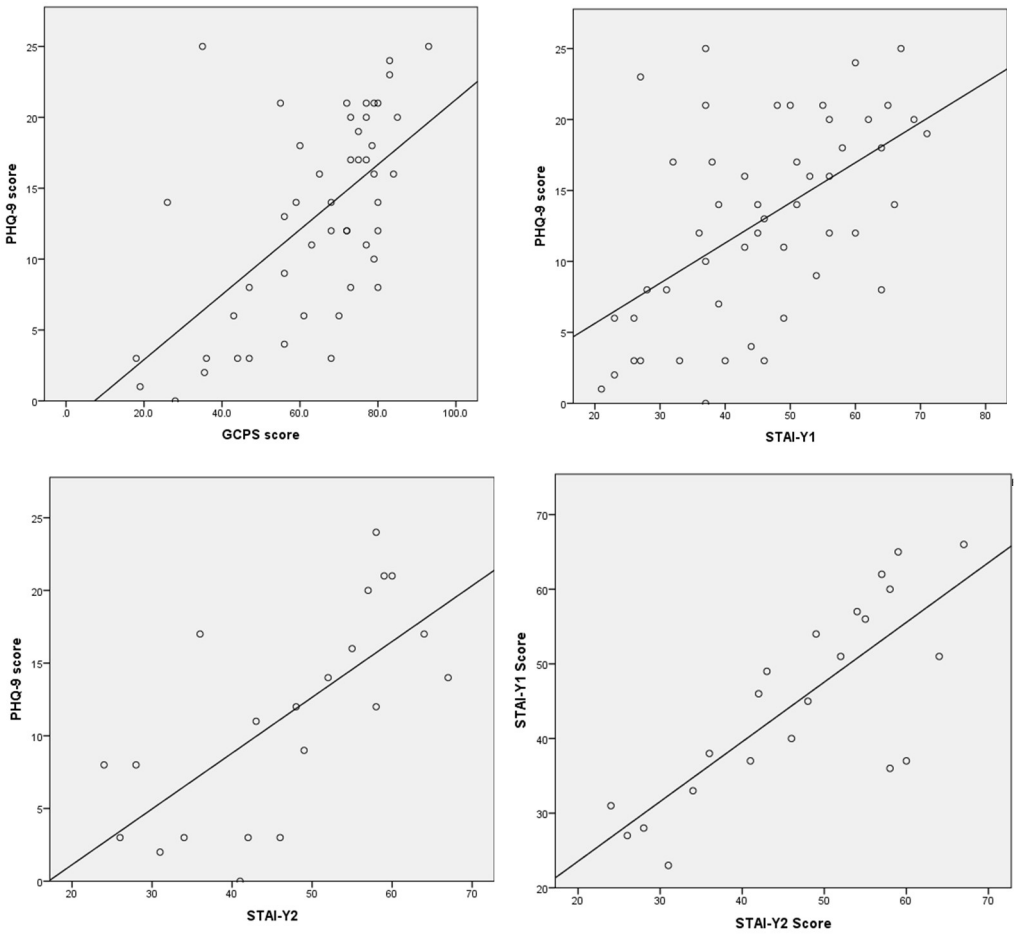


Figure 4.7: Linear Regression Trends in Patient Cohort Data. PHQ-9 depression score has a positive relationship with GCPS ($r = 0.617$, $p < 0.001$), STAI-Y1 score for state anxiety ($r = 0.652$, $p = 0.002$), and STAI-Y2 for trait anxiety ($r = 0.813$, $p < 0.001$) in our cohort. STAI-Y1 and STAI-Y2 are also significantly positively related ($r = 0.555$, $p = 0.009$).

4.3 Discussion

Expression of candidate genes, selected from the rat DH microarray dataset and IPA® analysis, were analysed in the clinical whole blood samples from a cohort of neuropathic pain patients (according to diagnosis) compared to non-neuropathic 'healthy' control cohort, by qPCR. Other parameters, including questionnaires for depression and anxiety, were measured in the self-reported questionnaire and relationships were elucidated, as well as candidate expression changes within medication groups.

4.3.1 Translational Results

Eight genes which are differentially expressed in the rat SNL DH microarray, are also differentially expressed in the clinical cohort. These are *A3GALT2, CASP1, CASP4, CASP5, CCR5, FPR2, SH3BGRL3,* and *TMEM88* (Table 4.1). The rat SNL model was validated using three behavioural tests and provided a neuropathic pain model with low variability. However, the results are limited in its relevance to clinical neuropathic pain. Clinical neuropathic pain patients provide a more biologically relevant sample set, but due to the higher degree of variability among a patient cohort, there is a greater chance for the influence of external variables on gene expression. Genes that are differentially expressed in the rat SNL model could be involved in the production or maintenance of neuropathic pain, and were flagged for further investigation in clinical blood samples from neuropathic pain patients. Differential expression of genes in the clinical samples is more relevant for clinical applications such as biomarker development or drug targeting.

The increase in *A3galt2* in the rat SNL DH (fold change 1.31, $p = 0.042$) was replicated in the clinical blood samples *A3GALT2* (fold change 1.37, $p = 0.018$). The possible role of A3GALT2 in neuropathic pain is difficult to deduce from published findings as there are limited studies into its function. Though its role is not obvious, its differential expression in both the SNL model and

clinical cohort indicates it could have an important role in the pathology of neuropathic pain and could provide a potential candidate as a biomarker for improved diagnostic tests.

The caspases *CASP1* and *CASP4* are increased in the clinical cohort. The human orthologue to *Casp4*, *CASP5,* was also increased. These are inflammatory caspases involved in the production of pro-inflammatory cytokines (Sollberger *et al.*, 2014; Vigano *et al.*, 2015). In addition, *CASP8* and *CASP9* were also increased (fold change 1.79 p <0.001 and 1.67 p <0.001 respectively). *CASP8* upregulation conflicts with the upregulation of the miRNA *Let7a2* in the SNL model (Chapter 3, Section 3.2, and Table 3.5). These caspases are involved in apoptosis (Tummers & Green, 2017, Zou *et al.*, 1999). The *CASP3* gene, the protein product of which is acted upon by caspase 8, which was not significantly changed in either the SNL model or clinical cohort. *CCR5,* the chemokine receptor, was upregulated in both the SNL model (fold change 1.35, p = 0.040) and clinical samples (fold change 1.23, p = 0.033). The validation of these differential gene expression changes in an animal model and clinical neuropathic pain patient cohort suggests they may have an active role in neuropathic pain.

FPR2 is upregulated in the pain patient cohort (fold change 1.33, p = 0.001), which was the opposite direction of the fold change in the SNL model (-1.35, p < 0.01). Similarly, *SH3BGRL3* was decreased in the clinical cohort (-1.19, p < 0.001), which was upregulated in the rat SNL DH. The discrepancy in direction of change in the rat and clinical samples could be due to several factors. The rat transcriptome was extracted from the DH of the nervous system, whereas the clinical samples were extracted from whole blood. This means that the transcriptomes of the two sample sets were extracted from different tissues, neuronal and blood. Regardless of the direction of change, the differential expression of *FPR2* and *SH3BGRL3* in both rat and human is indicative of either their involvement in or sensitivity to changes in neuropathic pain. Given their protective role in anti-inflammation and anti-apoptosis, they are likely carrying out such roles in

response to prolonged tissue damage in the neuropathic pain phenotype. The direction of change was not the same for both molecules (*FPR2* was decreased in the SNL DH but increased in the clinical cohort, whereas the opposite was true for *SH3BGRL3*). It is possible that other protective mechanisms were active at different points of disease progression. *TMEM88was* upregulated in the rat DH (fold change 1.33, $p = 0.017$) and in the clinical cohort (fold change 1.56, $p < 0.001$). This suggests that its product, transmembrane protein 88 is important in neuropathic pain, which could be due to its inhibition of the neural plasticity pathway Wnt/β-catenin. This could represent a protective fine-tuning mechanism in the neural remodelling that occurs in chronic neuropathic pain, as discussed in Section 1.9.3.

4.3.2 S-LANSS Score Categories

The S-LANSS questionnaire is used in the diagnosis of neuropathic pain. A score of 12 or higher is indicative of neuropathic pain, and lower scores are indicative of nociceptive pain. By separating the neuropathic pain cohort into high and low scoring S-LANSS cases, gene expression between these groups could be indicative of whether the gene is involved in inflammatory or neuropathic processes. Genes differentially expressed in the high S-LANSS score group are *CASP1, CASP4, CASP5, CCR5, FPR2,* and *SH3BGRL3* (Figure 4.3). All are increased in high S-LANSS versus control, except for *SH3BGRL3* which is reduced in this group. This could be indicative of their roles in the neuropathic pain, as oppose to initial injury and associated inflammatory processes which could precede or contribute to the chronic pain state. The exclusivity of three caspases genes *CASP1, CASP4,* and *CASP5*, as well as *CCR5,* in the high-scoring S-LANSS neuropathic pain patients is surprising given the role of these proteins in inflammation.

FPR2 is an anti-inflammatory protein which could only be activated in chronic neuropathic pain as an inflammation-limiting mechanism, not active in non-neuropathic chronic pain. However, the thioredoxin-like gene *SH3BGRL3* is downregulated in the high-scoring (neuropathic) S-LANSS

patients. *TMEM88* is increased in both groups (versus control), which indicates its involvement in both neuropathic and non-neuropathic pain (Figure 4.3). The role of TMEM88 protein in chronic pain is not clear from published data, but it is a known inhibitor of the Wnt/β-catenin canonical pathway (Ma *et al.*, 2017). This It is possible that its role in chronic pain is multifaceted and contributes to non-neuropathic pain through another mechanism not yet identified.

In addition to the eight genes differentially expressed in neuropathic pain, *PLAC8* and *ROMO1* are also increased in the low S-LANSS scoring patients (Figure 4.4), indicative that this change occurs in non-neuropathic chronic pain. *PLAC8* could be involved in neuronal plasticity via its regulation of the Wnt/β-catenin canonical pathway. This does not explain why *PLAC8* upregulation is only significant in the low scoring S-LANSS pain patients.

The increase in *ROMO1,* reactive oxygen species modulator 1, indicates that its role in clinical chronic pain is exclusive to inflammatory mechanisms involved in pain, and could not be involved in the neuropathic pain mechanism. This contradicts the finding in the rat DH, where *ROMO1* is increased in the SNL versus sham, which indicates this gene expression change is not due to the inflammatory response to the surgery. It could however occur as part of an inflammatory response to the SNL itself (which did not occur in sham). It is also possible that due to the heterogeneity of disease within the patient cohort, inflammation was also variable, and a change in *ROMO1* could not be detected. As the clinical samples were collected from blood rather than the DH, it is possible that the gene expression changes are different in the two tissue types as part of the disease.

4.3.3 Receiver operating characteristic (ROC) Analysis

As the ROC analysis was performed on the S-LANSS category, the results indicate how well the candidate combinations can predict the S-LANSS category score (i.e low or high). Therefore, the validity of the combination in predicting the true origin of the chronic pain (i.e. neuropathic or

inflammatory) depends on the validity of the S-LANSS method. S-LANSS has limitations (section 4.3.7) and it is not possible to truly evaluate the validity of test in its ability to diagnose neuropathic pain.

The ROC curve visualises the trade-off between the specificity (x axis) and sensitivity (y axis) of a test, and the origin represents 0% for both. The bottom left of the plot represents the extreme scenario where 100% of the time the test gives a false negative for the disease, and a true negative for the control (sensitivity is at 0%, and specificity is at 100%). Conversely the top right represents the opposite extreme, where 100% of the time the test gives a false positive for the controls, and true negative for the disease (sensitivity is at 100%, and specificity is at 0%). The diagonal dotted line represents the thresholds where the fraction of false positives equals the fraction of true positives. The solid curve on which the data is plotted demonstrates trade-off at each threshold. The best threshold can only be determined by an experienced person who can appropriately evaluate the consequences of a false positive result versus a false negative result.

Two combinations of three genes represent good predictors for inflammatory pain (S-LANSS score ≤12). These are *FPR2, CCR5,* and *CD4* pain (Area 0.705, $p = 0.066$) and *FPR2, ANXA1,* and *PLAC8* (Area = 0.782, $p = 0.013$). None of the combinations tested were good predictors of neuropathic pain (S-LANSS score ≥12), but these candidate triplicates could be developed to assess the inflammatory profile of a patient during diagnosis and complement other diagnostic tools. The ROC curve results are indicative of the role of these candidates in inflammatory pain, which for genes with known inflammatory functions (*FPR2, ANXA1,* and *CCR5*) is not surprising. The function of *PLAC8* is not well established at this time, but these results suggest its role in neuropathic pain could be linked to the anti-inflammatory role of the FPR2 and ANXA1 interactions in patients with S-LANSS score ≤12.

4.3.4 Linear Regression Analysis of the Neuropathic Pain Patient Cohort

Linear regression was used to analyse the relationship between two variables and identify whether any could be used as a predictor for another and therefore have clinical implications for neuropathic pain. This is distinct from correlation which describes a relationship between two variables in terms of the degree to which a change in one variable changes the other, and the direction of that change.

Linear regression analysis requires several assumptions to be met. These include the following:

- The relationship is linear within the data range
- Scatter around the line of best fit (regression line) is of Gaussian distribution
- The data is homoscedastic, meaning that the residuals (distance between the points and the regression line) is the same, or corrected for by differential weighting
- Data points are independent of each other
- X and Y values are independent of each other – one is not used to produce the other in the first instance
- X values are precisely known

Few of the variables met these criteria and linear regression analysis could only be performed on a small subset of data. Transformation of data to fit these assumptions is not recommended, as alternative approaches to linear regression can be used on these data. These include non-linear regression and logistical regression. Similarly, a low R^2 value represents a small linear relationship and does not explain non-linear relationships that could exists between the variables.

Reported here are the r values, within the range -1 to 1, which describe the relationship between X and Y, where 0 represents no linear relationship and -1 and 1 represent perfect relationships

(with no outliers) in opposite directions. R^2 is the coefficient of determination and indicates the percentage of the variance which is produced by the linear model. The remaining variance is due to other factors which could be biological, or due to errors in study design.

The graphs are equally as important to the interpretation of the linear regression analysis as R^2 and *p* values (Figure 4.7). The slope of the graph demonstrates how much Y will change with a 1-unit change of X. The higher the value, the greater the change of Y when X changes by 1 unit, and the further away from horizontal the line is. When interpreting the data is it also important to consider that the predictions could not be useful outside of the actual data range measured by the study.

The GCPS is indicative of pain intensity and disability severity, and in this cohort is a predictor of PHQ-9 score for depression ($r = 0.617$, $p < 0.001$, Figure 4.7). The higher the GCPS score the more intense the pain and severe the pain-associated disability. The PHQ-9 questionnaire tool is used to monitor depression and response to anti-depressive treatment. The R^2 is 0.46, so 46% of the variance in PHQ-9 can be accounted for by this linear regression model (i.e. variance in GCPS score) and the remaining 54% of variance is due to other factors. This relationship between GCPS and PHQ-9 is not surprising as the association between chronic pain and depression is well established (Sheng *et al.*, 2017).

Variance in PHQ-9 is also influenced by variance in the two anxiety measures STAI-Y1 and STAI-Y2 ($r = 0.652$, $p = 0.002$ and $r = 0.813$, $p < 0.001$ respectively, Figure 4.7). The r values are relatively high for these parameters. These indicate that in this cohort 65.2% of the variance in PHQ-9 occurs in response to variance in STAI-Y1, and 81.3% of PHQ-9 variance occurs in response to variance in STAI-Y2. This is unsurprising as the overlap between depression and anxiety is well-documented (Goodwin, 2015). Trait anxiety (STAI-Y2) variance in this cohort influences state variance STAI-Y1 ($r = 0.555$, $p = 0.009$, Figure 4.7). This is expected as the two anxiety states

are not independent of each other and patients with trait anxiety are likely to exhibit state anxiety in response to transient situations (Xie & Karan, 2019).

In the case of linear regression, the assignment of X and Y to the two respective variables was carefully considered, and it analyses the accuracy to which X could be used to predict Y. Therefore, X and Y are not interchangeable and will give a different result if switched around as it will be performing a different test (that is, the accuracy to which Y could be used to predict X). For example, in the case of GCPS and PHQ-9, X was assigned to GCPS and Y was assigned to PHQ-9 to test whether pain intensity (measured by GCPS score) could predict depression severity (measured by PHQ-9 score) in patients.

Linear regression analysis did not identify any relationships between S-LANSS score and other questionnaire parameters in our cohort, including PHQ-9 score (for depression), or STAI Y-1 and Y-2 (anxiety). This contradicts published findings which described depression and anxiety as important comorbidities of chronic pain (Sheng *et al.*, 2017), though the relationship between the disease states is not well understood and it remains unclear how they contribute to each other. It is possible a larger cohort is required to identify a relationship. Further, in this cohort, age and gender have no effect on S-LANSS score. The logic behind testing for this effect is that some studies suggest a gender different in pain perception (Gear *et al.*, 1996, Jeong *et al.*, 2020).

4.3.5 Medications

Expression of the candidates was tested in four medication groups, anti-depressants (n = 28), anti-convulsants (n = 23), anti-inflammatories (NSAIDs, n = 20), and opioids (n = 31). Only *A3GALT2* and *ROMO1* were significantly different in medication groups; *A3GALT2* in the opioid group, and *ROMO1* in the anti-convulsant and anti-inflammatory groups. As *A3GALT2* was increased in the neuropathic pain patients (Figure 4.5), a decreased expression in those taking opioids suggests that opioids bring *A3GALT2* expression back towards control levels. As the

function of A3GALT2 protein is not fully understood, it is difficult to identify potential pathways by which opioid medication could reduce its expression. This could be a novel area of research of interest in neuropathic pain pathways and opioid use.

ROMO1 expression increased in patients taking anti-convulsants (p = 0.049) and decreased in the anti-inflammatory medication group (p = 0.031) (Figure 4.5). Anti-convulsant gabapentinoid medications are commonly used treatment in neuropathic pain, in addition to epilepsy. Anti-convulsant medications dampen neuronal firing to reduce the occurrence of seizures. In neuropathic pain, the mechanism of action of anti-convulsants is less well-defined, but likely reduces the abhorrent firing of pain signals by inhibiting calcium currents. Inflammation and oxidative stress are important components of epilepsy (Vezzani *et al.*, 2011), and anti-convulsants can reduce inflammation and oxidative stress in rat models of epilepsy (Mao *et al.*, 2017). Oxidative stress reduction in anti-convulsant use may be mediated by the induction of endogenous protective mechanisms e.g. ROMO1.

Anti-inflammatories are used in a range of pain conditions, not exclusive to neuropathic pain, but a decrease in *ROMO1* expression is unsurprising in patients taking anti-inflammatory drugs given the role of ROMO1 protein in oxidative stress regulation (Shyamsunder *et al.*, 2015). The anti-inflammatory effects of medications may act on feedback mechanisms and reduce the activity of endogenous anti-inflammatory pathways such as molecules which protect from oxidative stress. As this group includes over-the-counter medications such as paracetamol and ibuprofen, it is impossible to be sure the information provided is accurate, unlike prescribed medications, as there is no detail of this in the medical records.

4.3.6 Considerations

Rat Dorsal Horn and Clinical Whole Blood Samples

Possible reasons for the discrepancy in results from the rat model and clinical neuropathic pain (aside from species differences) includes the duration of neuropathic pain and tissues analyses. The model was maintained for 35 days prior to tissue harvest, and neuropathic pain duration in patient varies greatly with a range of 577 months (mean 94.5 ± 14). The DH is an important area for pain modulation, which is why it was selected for gene expression analysis in the rat SNL model. By comparison, qPCR of cDNA obtained from whole blood samples examines gene expression in the nuclei of peripheral white blood cells. DH tissue cannot not be extracted from living humans, and whole blood samples provide the best alternative as a source of obtainable gene expression profile by a minimally invasive procedure for a biomarker test. Cerebrospinal fluid (CSF) would also provide a good source of genetic material for a similar study and has been used in other studies of neuropathic pain (Lind *et al.*, 2019, Backryd *et al.*, 2017) but CSF extraction is an invasive procedure. Genetic testing of patient whole blood samples would likely form the basis for the robust diagnostic test for neuropathic pain, the development of which is the long-term aim of the work presented here. The changes in expression of these genes in the nuclei of peripheral immune cells from the clinical whole blood samples likely reflect the active state of the immune system. Peripheral nerve injury and inflammation facilitates the breakdown of the blood brain barrier (BBB) (Echeverry *et al.*, 2011, DosSantos *et al.*, 2014) which allows infiltration of pro-inflammatory immune cells otherwise prevented in health (Cao & DeLeo, 2008) and potential leakage of extracellular proteins from the CNS into the bloodstream. However, changes in gene expression within the cells of the CNS (e.g. neurons, astrocytes, and microglia) will not be detectable in whole blood samples at the mRNA level unless there is cell damage and subsequent leakage of mRNA. Gene expression changes in the CNS could be

measurable by proteomics, as protein products of genes could be exported into extracellular space in the CNS and leak through the disrupted BBB and into the bloodstream. Quantitative PCR measures gene expression, which does not necessarily translate into protein expression and function. To be sure that the changes in gene expression is representative of functionality, protein assays would have to be performed.

Rat SNL blood samples would have complemented this work and provided an insight into the correlation between CNS and blood biomarkers based on leakage through the BBB. However, this tissue was not available to us and could not be used for this study. This could be a consideration when designing future studies, as it would potentially strong evidence for candidate potential for a role in neuropathic pain and its translation to clinical research.

The rat SNL cDNA microarray is limited to n=8 for both SNL and sham-operated control. The false discovery rate for (FDR) is the proportion of false positives reported in the results. For the Affymetrix RTA none of the genes passed the false discovery rate (FDR) cut-off of p = 0.05. However small gene expression changes can have subsequent physiological consequences (Narita *et al.*, 2006), and large FDR cut-offs are a common phenomenon for microarray with small sample sizes (Pawitan *et al.*, 2005). Differential expression of several candidates by qPCR in clinical neuropathic pain whole blood samples supported the expression changes reported in the RTA. The analysis performed on the clinical data was FDR-corrected, therefore the gene expression changes are valid.

A differential gene expression analysis of the rat SNL blood samples would be an ideal sample set to bridge the gap between the rat DH and clinical blood samples. This data would give valuable insight into correlations between differential gene expression between DH and circulating blood and would be very informative for the interpretation of the clinical dataset. Unfortunately this tissue was not available for this project.

qPCR Limitations

SYBR qPCR uses a fluorescent dye that indiscriminately binds to double-stranded DNA. SYBR produces a signal for all double-stranded DNA products including non-specific products and primer dimer. This potential cause of false positive signals can be accounted for by using the appropriate controls, melt curve analysis, and running products on a gel to determine their size. *In silico* PCR was used to predict whether a non-specific PCR product was likely to be amplified by a given primer set. The melt curve analysis was used to eliminate wells containing a product with a higher or lower melting temperature (Tm) than expected from the desired product, or with similar Tm to control wells. Dye-based PCR requires post-PCR processing such as this, as well as normalisation to reference genes. No universal strategy of normalisation or reference genes exists for qPCR, and this can have a profound effect of gene expression analysis if the most appropriate normalising factors are not selected (Faraldi *et al.*, 2018). For this study, qBase+ software was used to determine the most stable reference genes for the whole blood samples, and to normalise the Cq values of the genes of interest against two reference genes.

There are alternative forms of qPCR to SYBR, which are more expensive but provide a more accurate quantification. TaqMan® uses a fluorogenic probe specific to the PCR product region on the template, which contains a dye label at the 5' end and a quencher at the 3' end. The quencher prevents the signal from being emitted by absorbing the fluorescence by Förster resonance energy transfer (FRET). The probe is bound to the template and flanked by the unlabelled primers. Taq polymerase has 5´–3´ exonuclease activity, and as the polymerase reaction proceeds the probe is degraded. The release of the fluorophore from the quencher allows the signal to be read and increased in proportion to the amount of the targeted amplicon.

Digital PCR (dPCR) follows the same principles of SYBR qPCR but segregates the transcripts in oil droplets. In principle the whole fluorescent signal is broken down from one into thousands of

individuals signals. A sample is fractionated into 20,000-35,000 droplets, and each droplet will either contain the template DNA or not. In droplets containing template an isolated PCR will occur and a signal will be produced. In empty droplets no PCR can proceed, and no signal is produced. This is the basis for the digital (or binary, 1 or 0) signal. The distribution of target molecules across the droplets is calculated using Poisson statistics, and with the results used to calculate starting concentration of target (Majumdar *et al.*, 2017).

Digital PCR has been celebrated as the next-generation qPCR technique (Cao *et al.*, 2016, Salipante & Jerome, 2019), and there are several benefits of dPCR over qPCR. Quantitative PCR provides a relative quantification and requires a standard curve, whereas dPCR provides absolute quantification of template in a sample. This also removes the need for calibration and ddCt analysis (Hindson *et al.*, 2011), though the appropriate controls are required to confirm the reaction has been successful. The partitioning of the sample provides precise template measurements, and this allows small fold differences to be picked up that otherwise would be missed by qPCR (Pinheiro *et al.*, 2012). There are different systems for dPCR available. Some systems are expensive and require specialist thermocyclers and chips that can increase costs, whereas others can be performed in standard thermocyclers and well plates. The smaller reaction volumes used by dPCR could also be an additional cost benefit, and the possibility of multiplexing (simultaneous quantification of multiple TaqMan probe targets in a single reaction) can reduce set-up time and reagent use. Digital PCR can use SYBR or TaqMan, with comparable precision and reproducibility (Miotto *et al.*, 2014). TaqMan and digital PCR methods were not used for this study due to high costs associated with their use. TaqMan is not a cost-effective option for high throughput. They could provide a more accurate method of cDNA quantification if they were available.

S-LANSS Criticisms

The pain experience of the patient was self-reported using the S-LANSS questionnaire, and each case was assigned a score based on their answers about the nature and severity of their pain. Scores of ≥12 are classified neuropathic (M Bennett, 2001), but in this study, patients with a clinical diagnosis of neuropathic pain were included regardless of their S-LANSS score. This is because as with all questionnaire-based methods, the S-LANSS is subjective and could not provide a true reflection of the patient's physiological pain. It is therefore possible that non-neuropathic pain patients, misdiagnosed as neuropathic cases, are present in the clinical dataset. This is likely to explain the opposite direction of differential gene expression of two genes (*SH3BGRL3* and *TMEM88*) in the clinical samples versus the SNL model, though as the gene expression analysis results are unchanged when the neuropathic pain group is divided according to S-LANSS score (Figure 4.4).

The splitting of patients into neuropathic and inflammatory pain groups according to S-LANSS score is problematic due to the limitations of the S-LANSS questionnaire. It is possible patient scores do not reflect the true clinical nature of their condition and as such group patients this way might be arbitrary. However, due to the lack of empirical assessments available for diagnosis of chronic pain patients, there is currently no better alternative for patient grouping than clinical questionnaires.

In addition to the S-LANSS, questionnaires available for the diagnosis of neuropathic pain include the *Douleur Neuropathique en 4* questions (DN4), Neuropathic Pain Questionnaire (NPQ), and painDETECT (May & Serpell, 2009). Tools vary in their format and assess different aspects of the pain experience such as quality, location, exacerbating factors, record data by a graded or binary scoring system, analysis of facial expressions, or with vocal descriptors (Dansie and Turk 2013). DN4 is sensitive and specific but requires clinical examination for part of the assessment (VanDenKerkhof *et al.*, 2018). NPQ has been criticised for not being sensitive enough for

diagnosis (Catley *et al.*, 2013). PainDETECT would provide a suitable alternative to S-LANSS in this study, given its self-reporting format and grading system which is more accurate than the yes/no binary format of other questionnaires. S-LANSS was selected as it is a well-established tool in the diagnosis of neuropathic pain, however, its limitations as a self-report questionnaire forms part of the need for more objective diagnostic tools in neuropathic pain, such is the aim of this project.

Comments on Questionnaire Elements

The GCPS was selected as a measure of pain severity and associated disability. It has the advantage of being relatively short (seven questions) compared to similar but more comprehensive questionnaires such as the Roland Morris Disability Questionnaire (twenty-four questions). The compromise is the reduced depth and description of patient pain experience. For the purpose of this study, the shorter questionnaire was selected as it formed one section of a larger questionnaire and longer questionnaires generally have a reduced response rate (Rolstad *et al.*, 2011). The STAI tool was selected as a comprehensive measure of both state and trait anxiety, with its self-reporting format and scoring system. A limitation of the inclusion is its length, which comprises forty questions. The questionnaire could be time-consuming for some patients, and this could compromise the validity of patient responses if they become bored or frustrated. Despite its length, the Likert scale system (where patients provide answers using a 4-point scale) makes the questionnaire relatively straightforward to complete.

Levis *et al.* performed a meta-analysis on the accuracy of PHQ-9 for major depression detection (MDD) and reported that the tool was comparable to semi-structured interviews, but could be less specific for younger patients and therefore produce variable results across subgroups (Levis *et al.*, 2019). Manea *et al.* reported that PHQ-9 is not sensitive in MDD (Manea *et al.*, 2015). Both studies suggested a cut-off at a score of ten, however as the numerical score itself was used in

this study as a measure of depressive mood, this recommendation was therefore not considered.

Other questionnaires used for the diagnosis of depression include the Structured Clinical Interview for DSM-IV-Axis-I Disorders (SCID-I), Mini International Neuropsychiatric Interview (MINI), Primary Care Evaluation of Mental Disorders (PRIME-MD), and Hospital Anxiety and Depression Scale (HADS). SCID-I is a thorough exam performed by a mental health profession, lasting up to two hours (Zawadzki *et al.*, 2015). This was unfeasible and the self-reported PHQ-9 was selected because it took minutes to complete without the need for a mental health professional. Similarly, MINI requires an interview to be performed by a clinician and was therefore deemed unsuitable (Sheehan *et al.*, 1998). PRIME-MD is used for the evaluation of common mental disorders and was therefore more extensive than necessary for this study (Spitzer *et al.*, 1994). HADS would have been a suitable alternative to both the PHQ-9 and STAI components, as it scores both depression and anxiety by a short questionnaire without the need for a clinical professional. However, in 2015 Pettersson *et al.* performed a systematic review of these and others, and concluded that "only the SCID-I, MINI and PHQ-9 with a cut-off score of 10 fulfilled the minimum criteria for sensitivity and specificity", and that there was insufficient evidence for the PRIME-MD and HADS. PHQ-9 therefore provided the most suitable tool for depression scoring for this study, with its straightforward administration and supporting evidence (Pettersson *et al.*, 2015).

There is a debate in the published literature about the role of personality traits, such as neuroticism (a higher than average tendency to exhibit negative feelings, including anxiety), in the development of chronic pain (Fishbain *et al.*, 2006). Whether certain personality traits increase an individual's chance of developing chronic pain, or whether chronic pain exacerbates certain emotional states and exhibited associated behaviours is not known. The well-

characterised phenomenon of pain catastrophising describes the influence of a personality trait in the exacerbation of chronic pain (Van Damme *et al.*, 2002).

Neuropathic pain manifests through a range of symptoms of varying severity from patient to patient. A quantitative sensory testing (QST) study of clinically diagnosed neuropathic pain patients found that different aetiologies presented different symptoms (Maier *et al.*, 2010). For example, only 39% of peripheral nerve injury cases presented thermal sensitivity, and allodynia is most common in Postherpetic neuralgia patients compared to other aetiologies (Maier *et al.,* 2010). In addition to the problems this poses to clinical diagnosis according to outward symptoms, this suggests that the relevance of surgical animal models of neuropathic pain may be limited to specific aetiologies. For surgical models of L5 and L6 nerves , the endpoint hypersensitivity of the hind limb in response to a mechanical or thermal stimulus, thus limiting the application of the model to neuropathic pain types in which these occur (Calvo *et al.*, 2019). Other types of behavioural change assays may be more clinically relevant, including force swim test (Yankelevitch-Yahav *et al.,* 2015) or elevated plus maze tests could be used to assess anxiety or depression-like symptoms induced in neuropathic pain models (Pellow *et al.,* 1985). Further, pain behaviours can differ between inbred strains of homologous animal models (Lariviere 2010), which suggests the response profile may be specific to each strain's genetic background, further limiting their relevance to genetically diverse populations of clinical neuropathic pain patients. Strain-specific genetic background also influences axonal regeneration, plasticity, and immune response to ligation (Calvo 2019). In a recent review, Calvo *et al.* (2019) suggest that the use of "genetically heterogenous outbred mice" could address this problem. This is relevant for the study of diseases with high variability in diverse populations caused by genetic differences, such as neuropathic pain. Alternatively, multiple strains could be used to account for strain differences in genetic background. Further, neuropathic pain research in genetically

heterogenous populations of animal models can be useful in mapping response variability to specific alleles, in addition to the study of gene expression levels (Recla *et al.*, 2019).

Medications

Here only medications prescribed for the patient at the time of sample collection were considered. No previous medications were considered due to the insufficient data on dose, and date prescribed, and duration of medication use. The effect of individual medications could not be investigated due to limited sample size, with a small number of patients taking one drug. Dosage for current medications was also disregarded due to insufficient data collection as the information was given by patients themselves and many chose to omit this information, and not always available in their medical history. Dosage is therefore a source of variation in the medication analysis. To fully investigate the effect of dosage of medications on gene expression in neuropathic pain patients a larger cohort with comprehensive medication data needs to be collected, and such is beyond the scope of this study. Medication data for controls were not collected, so it is possible that some effects of medication on gene expression (independent of neuropathic pain) was missed.

Circadian Rhythm

Blood was taken from patients at various times during the day, so inevitably circadian rhythm is a confounding factor for gene expression analyses among the clinical samples. Variation in local environment amongst the clinical samples is likely to be high. These will including light/dark cycle depending on individual sleeping patterns and area of living, and possibly some anomalies which could have a more acute effect on gene expression e.g. jet lag (Vosko *et al.*, 2010). Aside from the effect of disrupted or abnormal circadian rhythms in some patients, circadian rhythm

could impact gene expression itself. The implications for this, is the effect of circadian rhythm changes might mask neuropathic pain associated gene expression changes.

Chronic Inflammatory versus Neuropathic Pain

Chronic pain is defined as pain that persists for at least twelve weeks irrespective of treatment. Different types of pain can be diagnosed as part of a disease diagnosis e.g. arthritis or fibromyalgia, and neuropathic pain is common in multiple sclerosis patients. In neuropathic pain, inflammatory mediators have an important role in the establishment and maintenance of the disease state, and teasing out mechanisms exclusive to neuropathic pain is difficult. Similarly, osteoarthritis (OA) is referred to as non-inflammatory arthritis but is treated with NSAIDs and corticosteroids to reduce inflammation and pain. This is because some inflammation can occur in OA as cartilage breaks down in the joint. A study into the function of biomarkers in neuropathic pain could include OA and rheumatoid arthritis patients to provide non-neuropathic/non-inflammatory pain and inflammatory pain cohorts respectively, for comparison against a neuropathic pain cohort. This strategy could help elucidate biomarkers exclusive to neuropathic pain.

4.3.7 Potential Biomarker and Drug Targeting Candidates

The literary evidence presented here suggests that these biomarkers of neuropathic pain could be clinically relevant as disease indicators in pain assessment and diagnosis and might represent drug targets for an improved pharmacological management. However, for these biomarkers, which demonstrate critical roles in the production and maintenance of neuropathic pain, to be of clinical value they need to be objectively measurable and/or druggable. Druggability is the measure of a candidate's affinity binding with which it provides a therapeutic effect for the patient (Cheng *et al.*, 2007).

If a biomarker is suitable for pharmaceutical targeting, then its expression or action could be altered by medication. Thus, there is potential for clinical use where neuropathic pain could be prevented, halted, or even reversed. For patients this could mean improved quality of life. However, it is important that the ability to perceive and process physiologically relevant nociceptive pain remains intact. This represents an avenue for future research with implications for evidence-based medicine.

Establishment of a universal scale for the clinically relevant biomarkers in neuropathic pain could provide a more reliable, replicable, and accurate system for pain assessment. Diagnosis of neuropathic pain could be improved by detection and measurement of a specific biomarker in human blood or urine. This could also provide implications for disease severity or progression. The development of individual pain management programs, involving pharmacological treatments and physical therapies, according to a patient's biomarker and disease indicator profile will likely improve response rates and in turn relieve the social and economic burden of chronic neuropathic pain. This work implicates several genes and their proteins for further investigation in their role in neuropathic pain. These candidates include *A3GALT3, CASP1, CASP4, CASP5, CCR5, FPR2, SH3BGRL3*, and *TMEM88*.

4.4 Conclusion

Several of the differentially expressed genes presented in this chapter have been selected for further scrutiny *in vitro* to characterise their function under inflammatory conditions in different cell lines. The role of candidates could be in clinical neuropathic pain will be investigated to help further assess their potential as biomarkers for diagnosis or drug target development.

Differentially expressed genes in the rat SNL model of neuropathic pain which also change in the clinical whole blood samples in neuropathic pain patients represent candidates for further

investigation *in vitro.* The differentially expressed genes were first identified in an animal model of neuropathic pain and subsequently corroborated by transcriptome analysis in a clinical cohort of patients. These results are from two different species, providing strong evidence for the role of these candidate genes in the neuropathic pain phenotype. In addition to this, gene expression was measured in two different tissue types from different systems (central nervous system and blood circulatory system). This emphasises the importance of these candidates in the disease as the gene expression changes occur at both in central nervous tissue involved in pain processing and signalling, and in blood cells in the immune system involved in inflammation and tissue repair after injury.

Chapter 5: Investigation of Cellular Pathways in *In Vitro* Models of Neuroinflammation

5.1 Introduction

5.1.1 Aims of *In Vitro* Work

There are currently no recognised cell culture models for the investigations of mechanisms involved in the development of neuropathic pain symptoms. In the absence of sophisticated *in vitro* models of neuropathic pain, mRNA and protein expression changes were measured in relevant cell lines in response to pro-inflammatory conditions. Given the role of inflammatory mediators (IFMs) in neuropathic pain, pro-inflammatory cytokine-treated cell culture models can provide valuable insight into the function of candidates under inflammatory conditions. Four cell lines and two inflammatory cytokines were used to produce three *in vitro* inflammatory models to analyse gene and protein expression changes of candidates, and investigate their functional role in neuronal, glial, and immune cell types. Gene expression changes to candidates in the *in vitro* models using quantitative/real-time PCR (qPCR), and protein level changes were measured using Western blot and enzyme-linked immunosorbent assays (ELISAs).

Potential avenues for the development of a multi-cell type *in vitro* model which is more relevant to neuropathic pain physiology is also discussed. Commercially available immortal cell lines were used in this project as a surrogate for primary cell lines for practical reasons (discussed further in section 5.3). The cell lines selected were 1321N1 astrocytoma (ECACC 86030402) (Ponten & Macintyre, 1968), SH-SY5Y neuroblastoma (ECACC 94030304) (Biedler *et al.*, 1973), and monocytic THP-1 (ECACC 88081201) (Tsuchiya *et al.*, 1980) cell lines. All three cell lines are human in origin as these will be most relevant to clinical neuropathic pain. Together, the gene and protein expression changes in these cell lines under inflammatory conditions may help

elucidate the functional changes and interactions of candidates across several types of cell lines relevant to neuropathic pain.

5.1.2 Astrocytes and 1321N1 Astrocytoma

The 1321N1 cell line was used to represent a glial cell line, as glial cells carry out activities essential for brain function, such as maintaining the optimal external environment and providing physical support and nutrients to neurones in the brain (Sofroniew & Vinters, 2010). Glial cells are found throughout the central and peripheral nervous systems, and support neurones both structurally and in their physiological function (Jessen, 2004).

There are several glial cell types, including microglia, oligodendrocytes, and astrocytes in the central nervous system (CNS), and Schwann cells and satellite glial cells in the periphery. Microglia cells perform a similar function to macrophages in the circulatory system and scavenge the CNS, including pruning unnecessary synapses, engulfing pathogens, and removing debris from damaged cells (Lee & Chung, 2019). These activities are essential for healthy brain function. Oligodendrocytes and Schwann cells are closely associated with neurones and provide the myelin sheaths necessary for fast nerve conduction (Bhatheja & Field, 2006). Astrocytes are star-shaped cells closely associated with neurones. Astrocytes carry out essential functions to maintain the optimal environment for neurones, and for endothelial cells at the blood brain barrier (BBB) (Abbott *et al.*, 2006), such as providing nutrients and controlling the extracellular ionic balance (Table 5.1).

Under certain conditions, astrocyte can contribute to some disease states, including cytokine activation in chronic pain (Sofroniew & Vinters, 2010). Astrocytes exist in a non-reactive state and can become activated (shift to the reactive state, known as astrogliosis) under certain physiological circumstances *e.g.* in the presence of a lesion or ischaemia (Bush *et al.*, 1999). Astrogliosis is well-characterised in neurodegenerative diseases including Alzheimer's disease,

Parkinson's disease, and multiple sclerosis. During astrogliosis the number of astrocytes increases, and their functions move away from microenvironmental maintenance towards neural tissue and BBB repair, inflammation control, and scar formation (Table 5.1) (Barres, 2008, Sofroniew, 2009). Additionally, as non-reactive astrocytes can modulate neuronal signals at the synapse (Lee & Chung, 2019) they may also have a key role in the establishment of chronic neuropathic pain states (Zhang *et al.*, 2017, Choi *et al.*, 2019).

Table 5.1: Astrocyte Function in Health and Reactive Astrogliosis

Non-Reactive Astrocytic Functions	Reactive Astrocytic Functions
Uptake of K+ ions, water, neurotransmitters (e.g. glutamate, glycine), glucose and water from blood vessels (Simard & Nedergaard, 2004, Zador *et al.*, 2009)	Loss of function – no longer promotes survival of neurones, cannot perform phagocytosis or maintain synapses (Liddelow *et al.*, 2017)
Release of energy substrates, transmitter precursors, and growth factors from blood vessels at the BBB (Abbott *et al.*, 2006)	Scar formation in tissue injury repair – structural support and limits migration of infiltrating inflammatory immune cells and isolates lesions (Fawcett & Asher, 1999)
Regulation of extracellular ion concentration (Katsura *et al.*, 1994)	Production and release of glutathione to reduce oxidative stress (Chen *et al.*, 2001)
Control of blood flow through release of dilatory factors (e.g. nitric oxide, prostaglandin) and vasoconstrictors (e.g. arachidonic acid) (Gordon *et al.*, 2007)	Uptake of excess excitatory glutamate (Bush *et al.*, 1999)
Synaptic function and maintenance (Perea *et al.*, 2009)	Damaged BBB repair (Weidenfeller *et al.*, 2007)

For reviews see Sofroniew & Vinters, 2010, Bush et al., 1999 and Miller, 2018

The human astrocytic cell line 1321N1 was selected as the glial cell line for the *in vitro* investigation into neuropathic pain, derived from a sub-clone from a parent malignant glioma line. In addition to the inflammatory model, a mechanical stress test pilot was performed to determine whether it was possible to cause enough damage to induce the reactive astrocyte phenotype using 10µl pipette tips. This would be an ideal alternative to direct use of

inflammatory cytokines and would provide a more relevant model for research into neuropathic pain caused by neuronal tissue injury. Reactive astrocytes conduct a critical protective role in traumatic brain injury via several functions which isolate the lesions and promote tissue repair (Table 5.1) (Burda *et al.*, 2016), and analysis of gene expression changes would provide insight into the injury-induced astrocytic response.

5.1.3 Neurones and SH-SY5Y Neuroblastoma

As mature neurones do not proliferate, primary neurones are notoriously difficult to culture (Gordon *et al.*, 2013), though not impossible. Secondary neuronal cell lines, like many commercially available immortalised cells, are derived from tumour lineages. The immortal neuronal cell line SH-SY5Y was selected as a suitable surrogate for primary cell neurones, after preliminary tests were also carried out on the SK-N-SH neuroblastoma cell line, from which SH-SY5Y were derived (Biedler *et al.*, 1973). SK-N-SH cell line is derived from bone marrow biopsy from a four-year old girl with neuroblastoma (Biedler *et al.,* 1973).

SH-SY5Y are a widely used model for neuronal function, including Parkinson's disease (Xicoy *et al.*, 2017) and more complex psychiatric disorders (Iwata, 2018). SH-SY5Y are undifferentiated neuronal cells, characteristic of immature neurones which proliferate rapidly, aggregating in clumps (Pahlman *et al.*, 1984). SH-SY5Y cells lack the myelin sheath, but express adrenergic receptors and dopaminergic markers (Shipley *et al.*, 2016). Loss of neuronal markers has been described in higher passages (Kovalevich & Langford, 2013), therefore passage number <25 was used for this project.

Undifferentiated SH-SY5Y was selected as the neuronal model for this preliminary work so that propagation could continue, and biological repeats could be derived from the same cell vial at different passages. Differentiated SH-SY5Y represents an ideal subsequent model to follow on from this work. Differentiated SK-N-SH itself was also considered for the neuronal cell model

and was used for pilot experiments on protein using sandwich ELISA (see section 5.1.8). The six-day treatment step with retinoic acid, which was necessary for the differentiation, added an extra source of variability. Due to practicality issues and time constraints, the three biological repeats did not contain any technical repeats, and for these reasons this was not repeated for the full experiment.

5.1.4 Monocytes and the THP-1 monocytic cell line

Finally, an immune system component and monocytic cell line THP-1 was selected. These cells were originally derived from a young male acute monocytic leukaemia patient (Tsuchiya *et al.*, 1980). Candidate expression changes in THP-1 cells may have implications in and immune infiltration of the CNS in neuropathic pain, following the breakdown of BBB. Monocytes are leukocytes which during an immune response can migrate from the circulation into tissues and differentiate in macrophages (Kratofil *et al.*, 2017).

Peripheral monocytes differentiate into macrophages when they migrate into the tissue and this migration is facilitated by IFMs in the local environment (Eming *et al.*, 2007). Macrophages are an important component of the innate immune response, engulfing cellular debris from damage and invading pathogens (phagocytosis). The macrophage phenotype and function depend on the IFM profile (Figure 5.1), previously described as M1 and M2 (IFNγ and IL4/IL13 induced respectively) type macrophages (Mills, 2012). This two-dimensional classification of macrophage phenotypes has since been deemed insufficient (Alvero *et al.*, 2012) though it is currently still used throughout the literature (Huang *et al.*, 2019).

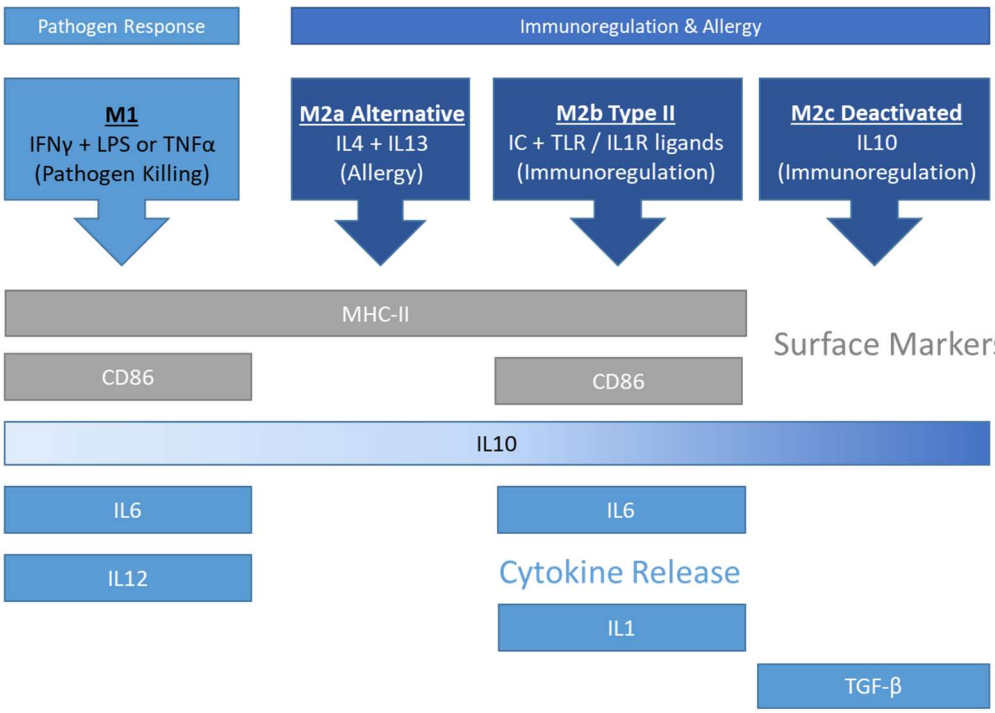


Figure 5.1: Classification of M1 and M2 Macrophages. The classical M1 macrophage is involved in pathogen response. Induced by the pro-inflammatory cytokines IFNγ and TNFα, the M1 type expresses major histocompatibility complex (MHC) II, and CD86 surface markers. IL10 is released by all types, but in very low concentrations in M1, and high concentrations in M2c. Pro-inflammatory IL12 and IL1 are released by M1 and M2b respectively, and IL6 is released by both types.

THP-1 cells can be activated by numerous infectious agents, including bacterial liposaccharide (LPS) (Schildberger *et al.*, 2013), phorbol-12-myristate-13-acetate (PMA) (Daigneault *et al.*, 2010) and IFNγ. IFNγ was used to activate THP-1 in the model as it is a cytokine released by activated T-helper cells (Romagnani, 1992), and induces the production of pro-apoptotic cytokines in THP-1 cells including caspase-8 and monocytic attractant protein-1 (MCP-1), also known as chemokine (C-C motif) ligand 2 (CCL2) (Inagaki *et al.*, 2002). LPS-activation would be less appropriate as it would mimic inflammation in response to infection. Further, there is

evidence for IFNγ mediated microglial activation in an animal model of neuropathic pain (Tsuda *et al.*, 2009), which suggests that IFNγ may mediate common immune activation effects in neuropathic pain, and thus makes it a more suitable candidate for THP-1 activation in our model. THP-1 cells were also differentiated into the monocytes cell line using PMA, an established method of monocyte to macrophage differentiation (Murao *et al.*, 1983, Schwende *et al.*, 1996) (Daigneault *et al.*, 2010).

5.1.5 Models of Inflammation

IFNγ and Tumour Necrosis Factor α (TNFα) were used either independently or in combination to produce the inflammatory phenotype in the three cell lines. IFNγ acts at the Interferon gamma receptor 1 (IFNγR1) (expressed in all human cell types) and 2 (IFNγR2), and activates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway to induce production of pro-inflammatory cytokines in the target cell (Figure 5.2), including TNFα in astrocytes (IY Chung & Benveniste, 1990). IFNγ is an important component in other neurodegenerative diseases including multiple sclerosis and autoimmune encephalomyelitis (Arellano *et al.*, 2015).

TNFα is released by stimulated monocytes and macrophages (Jovinge *et al.*, 1996). TNFα activates its receptors TNFR1, found in most tissue types, and TNFR2 found primarily in immune system cells where it induces the production of pro-inflammatory cytokines in many cell types, including monocytes, T-cells, and fibroblasts (Heinhuis *et al.*, 2011). TNFR1 is constituently expressed, whereas TNFR2 is induced by inflammatory conditions in response to injury (Parameswaran & Patial, 2010). TNFα is an initiator for neuropathic pain (Milligan *et al.*, 2001) (Ishikawa *et al.*, 2013), and is expressed by both neurones and glial cells (Xu *et al.*, 2006) (Ohtori *et al.*, 2004). TNFα can induce the reactive astrocyte phenotype (Liddelow *et al.*, 2017), which *in vivo* is released by activated microglia.

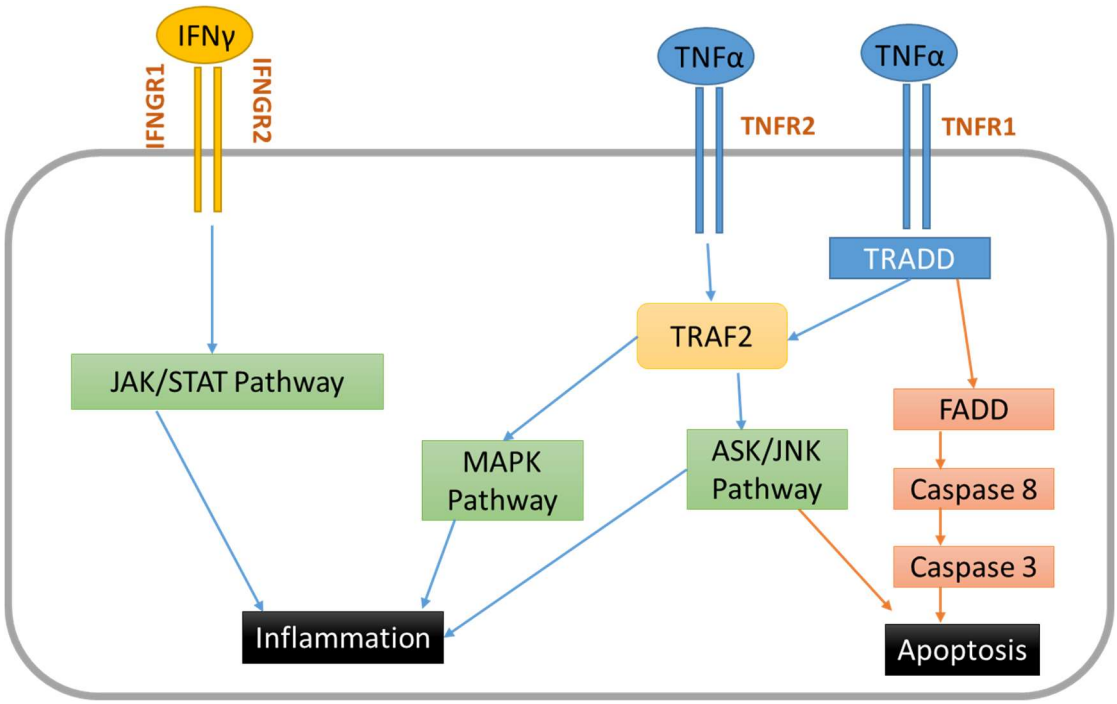


Figure 5.2: Intracellular secondary messenger pathways of interferon gamma (IFNγ) and tumour necrosis factor (TNFα). Upon activation, the IFNG Receptors dimerise and activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. TNFR activation triggers several pathways. TNFR1 is associated with the including TNF receptor-associated factor 2 (TRAF2) and the Apoptosis signal-regulating kinase (ASK)/ c-Jun N-terminal kinase (JNK) pathway, and also the mitogen-activated protein kinases (MAPK) pathway to induce inflammatory cytokine production by promoting transcription. Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD) also triggers the apoptotic caspase cascade via Fas-associated protein with death domain (FADD) (Benveniste & Benos, 1995).

Interleukin 6 (IL6) and interleukin 1β (IL1β) were measured by sandwich ELISA as a marker of inflammation. IL6 is a pro-inflammatory cytokine released from neurones in response to TNFα (Hung 2017) and has been implicated in both inflammatory and neuropathic pain. There is evidence that IL1β is a critical component in the development of neuropathic pain (Wolf *et al.*, 2006, Honore *et al.*, 2006), and neuropathy patients have elevated cerebrospinal fluid levels of IL1β (Backonja *et al.*, 2008). This makes IL1β a pro-inflammatory cytokine relevant to neuropathic pain.

The tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine the quantity of viable cells able to metabolise the dye after 24 and 48-hour cytokine treatment (Figure 5.5). In this colorimetric assay, the dye is metabolised by the oxidoreductase enzymes in viable cells to formazan (purple colour), and the shift in absorbance post-treatment is proportional to the change in cell viability. This assay was not performed on SK-N-SH as they were not used for the stimulation experiments.

5.1.7 Gene Expression Changes

Gene expression changes in the cell lines after cytokine treatment for 24-hours and 48-hours was performed by RNA extraction and qPCR. Changes to expression after cytokine treatment will identify candidates sensitive to inflammatory conditions, which either may be activated in the initial stages of neuropathic pain when inflammation occurs or be more involved in inflammatory pain cases than neuropathic. The differential gene expression profile for each candidate in the SNL model of rat, blood samples from neuropathic pain patients, and across the three cell lines will then be considered to assess its possible role in the neuropathic pain phenotype.

5.1.8 Protein Level Changes

5.1.8.1 Protein Quantification

Changes to the candidates were measured at protein level using the Western blot and ELISA techniques. Proteins were extracted from the samples and quantification was performed by Bradford Assay (Bradford, 1976). This method uses a colorimetric protein assay and spectroscopy to measure the protein concentration in a sample against a bovine serum albumin standard curve at concentrations 1mg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, and 0µg/ml. Coomassie Brilliant Blue G-250 dye in the Bradford reagent exists in an ionic blue state, and a red brown cationic state. When protein in a sample is added to the reagent, the reagent

shifts from its red form to its blue form. This colorimetric change is visible to the naked eye, and the deeper the blue colour can give a comparative qualitative measure of the amount of protein across the samples. The Coomassie dye forms strong noncovalent bonds to the proteins, including van der Waals forces to the protein carboxyl group and ionic interactions between positive amino groups and the dye's negative charge. These interactions stabilise the dye in its anionic form and allow a spectrometer reading to be taken. The unbound brown-red form has absorption at 465nm, whereas the anionic blue form has absorption at 595nm. The proportion of the two absorption spectrums in each sample allows the amount of protein to be quantified.

5.1.8.2 Western Blot

Both fluorescent and enhanced chemiluminescence (ECL) techniques for visualisation were used in this study and selected based on which worked best for each given antibody (Figure 5.3). In the ECL method, the membrane is first probed for the protein of interest and then stripped of all antibodies before being probed for a reference protein. Reference proteins are required to control for loading error and normalisation when analysing differences in treatment groups. For the fluorescence method, the protein of interest and reference protein can be probed for simultaneously, but for these to be distinguished from each other it is necessary that the two primary antibodies are from different species, and the secondary antibodies used must absorb light at different wavelengths.

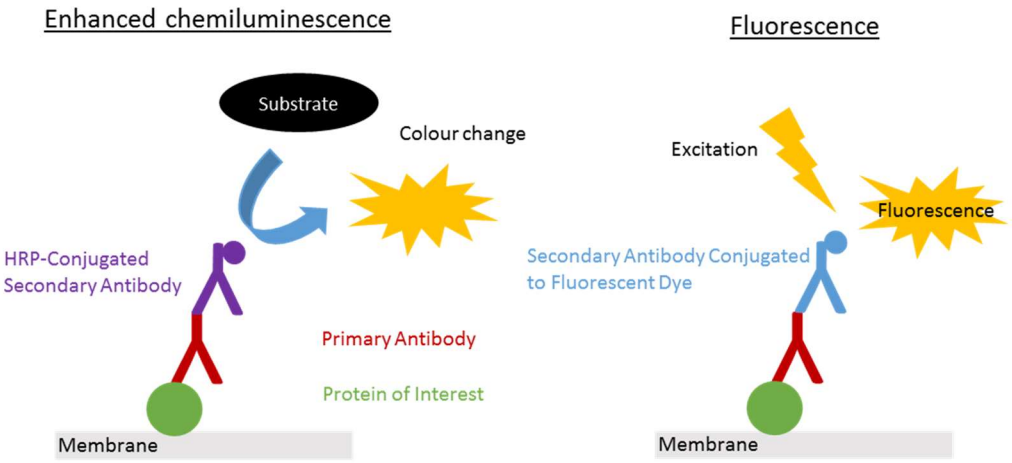


Figure 5.3: Schematic of Western blot probing methods. After the protein samples have been run on a gel and transferred onto a nitrocellulose membrane, the target protein of interest is probed for visualisation with a primary antibody, which in turn is probed by a secondary antibody specific to the primary antibody host, either by enhanced chemiluminescence (ECL) or fluorescence. For ECL, the secondary antibody is conjugated to horseradish peroxidase (HRP) enzyme, and the substrate is added. For the fluorescence method, a secondary antibody conjugated to a fluorescent dye is used, and the output is detected upon excitation of the dye, and the signal is read at the location of protein and antibody complex.

ELISA

ELISA was used to quantify protein expression of ROMO1 and PLAC8 for the pilot study in 1321N1 astrocyte, neuronal SK-N-SH, and THP-1 monocytes (Figure 5.4). Upregulation or downregulation of these genes was observed in the neuropathic pain model (Chapter 3) but no change was observed in the clinical neuropathic pain cohort (Chapter 4). Despite this, ROMO1 and PLAC8 were investigated in a short pilot study to evaluate changes under inflammatory conditions, given the known function of ROMO1 during oxidative stress, and the potential novelty of the role of PLAC8 in neuropathic pain. The ELISAs used for these experiments were chromogenic sandwich ELISAs. The kits purchased included a 96-well plate pre-treated with the capture antibody for the protein of interest (antigen). A blocking reagent provided in the kit was used to block non-specific binding sites prior to the addition of the samples. An antibody was

added to the wells and is bound to the antigen. The antigen becomes 'sandwiched' between the two antibodies. A third antibody conjugated to e.g. HRP specific to the Fc region of the second antibody was added to the wells. The reaction was stopped using stop solution to prevent saturation of the signal prior to imaging.

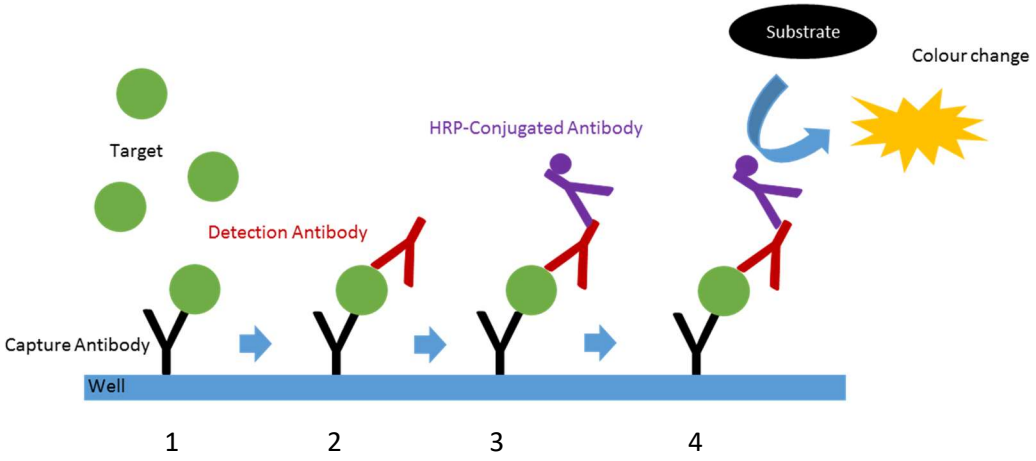


Figure 5.4: Schematic of sandwich ELISA method. 1) The sample is added to the ELISA plate and the target protein binds to the capture antibody on the well surface. Proteins not bound are then washed away. 2) The detection antibody is added to the wells and binds to the target. The target protein is 'sandwiched' between the two antibodies. 3) A third antibody conjugated to horseradish peroxidase (HRP) enzyme binds to the detection antibody. 4) A substrate mix is added to the wells and allowed to develop for a short period, as the HRP digests the substrate and causes a visible colour change, which is quantified by a spectrometer.

5.2 Results

5.2.1 Cytokine Stimulation Tests

5.2.1.1 Cell Viability Assays

To assess cell viability, tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed on the cell lines SH-SY5Y, 1321N1, and THP-1 (non-treated and PMA-treated) (Figure 5.5, Table 5.2).

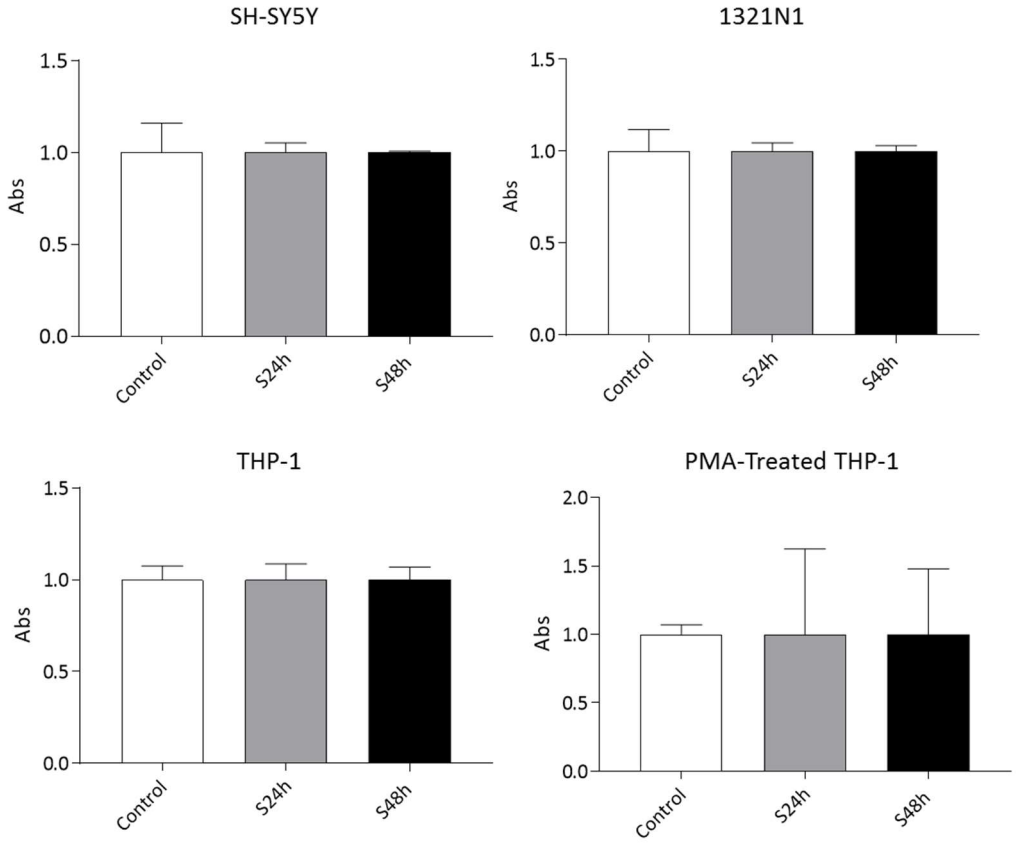


Figure 5.5: MTT Assay Results for SH-SY5Y, 1321N1, and THP-1 (non-treated and PMA-treated). Metabolism of MTT dye to formazan was not significantly altered after 24-hour (S24h) and 48-hour (S48h) cytokine treatment. SH-SY5Y p = 0.4678, 1321N1 p >0.999, THP-1 p = 0.4219, PMA treated THP-1 p >0.999. N = 3 (Technical Repeats). Abs = Absorbance.

Table 5.2: Mean±SEM for MTT Assay of Cell Culture Stimulation Models

	Mean ± SEM		
Experiment	**NS**	**S24h**	**S48h**
SH-SY5Y	0.99 ± 0.09	1 ± 0.03	1 ± 0.003
1321N1	1 ± 0.07	1 ± 0.03	1 ± 0.02
THP1	1 ± 0.04	1 ± 0.05	1 ± 0.04
THP1 PMA	1 ± 0.04	1 ± 0.36	1 ± 0.28

MTT assay results were not significantly different after 24- or 48-hour cytokine treatment in any of the three cell lines, plus PMA treated THP1 cells, indicating that the treatment had no significant effect on cell viability.

5.2.1.2 1321N1 Astrocytoma Cytokine Stimulation Test

The 1321N1 astrocytoma cells are star-shaped with full cell bodies. Changes in cell shape were observed after 48-hour stimulation with TNFα and IFNγ as the cells became elongated and larger (Figure 5.6).

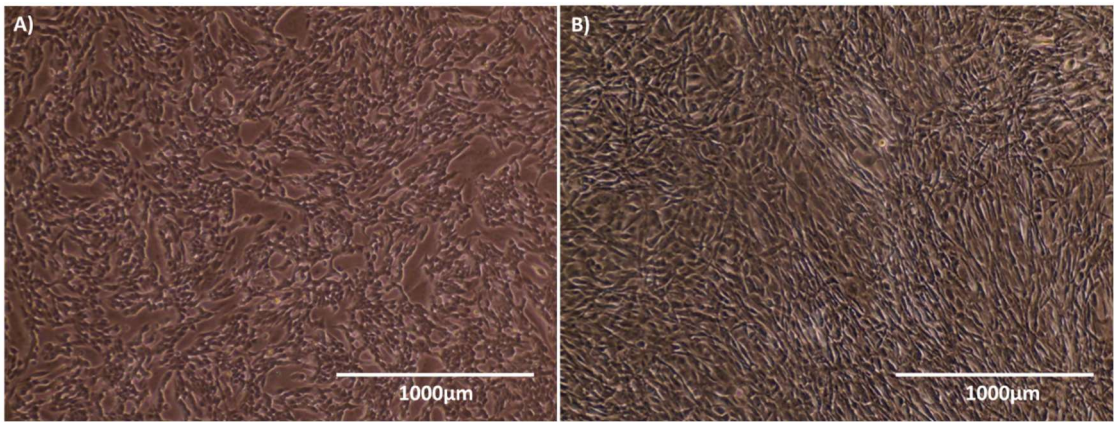


Figure 5.6: Morphology of Non-Stimulated (A) and Stimulated (B) Astrocytes. Astrocytes stimulated with 100µM IFNγ 400µM TNFα become elongated. Astrocytes were stimulated at 80% confluency and grown for a further 48-hours before imaging. Cells imaged at 100% confluency. Image taken on EVOS XL Core Cell Imaging System (x10 Magnification).

IL1β release from the 1321N1 astrocytoma model was measured by sandwich ELISA and increased after 48-hour cytokine stimulation only. IL1β was significantly increased in stimulated 1321N1 cells after 48-hour treatment versus control (p = 0.0065) but was not significantly different after 24-hour treatment. (Figure 5.7).

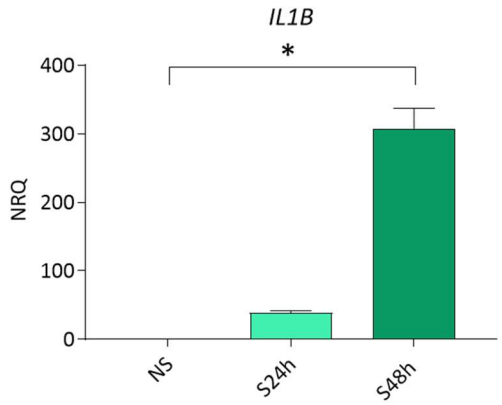


Figure 5.7: IL1β Release in Pro-inflammatory Cytokine Treatment of 1321N1. IL1β significantly increased after 48-hours of stimulation with TNFα and IFNγ (S48h) (214.80±57.34) versus non-stimulated (NS) (1±0.23) (p = 0.036) but was not significant compared to 24-hour stimulation (S24h) (34.30 ± 6.73) (p = 0.377) NRQ = Normalised relative quantity.

5.2.1.3 SH-SY5Y Cytokine Stimulation Test

Prior to cytokine stimulation the neuronal-like cells have plump cell bodies with thin extensions (Figure 5.8). After 48-hour stimulation with IFNγ and TNFα SH-SY5Y the extensions become elongated and cell bodies become enlarged.

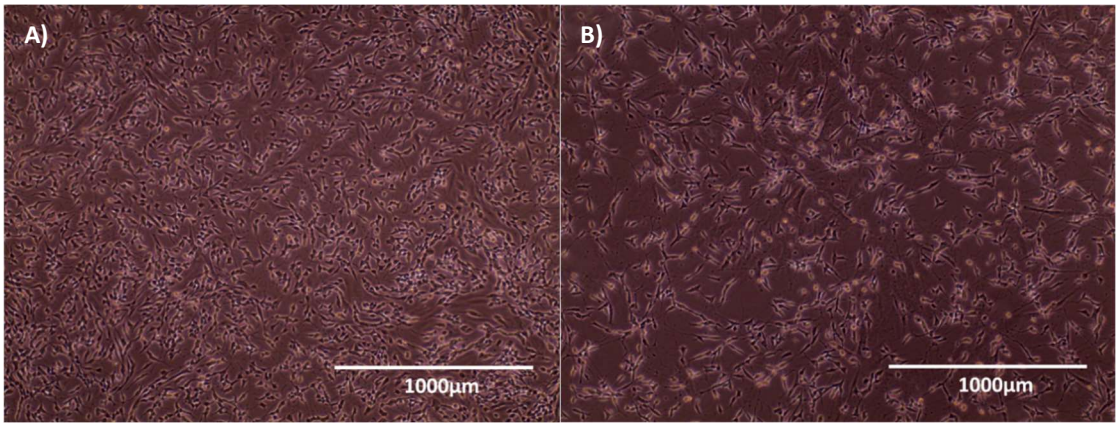


Figure 5.8: Microscopy Images of Cytokine Stimulated SH-SY5Y. Undifferentiated SH-SY5Y have a plump body with short, thin extensions. After 48-hour cytokine treatment, neuronal-like cell bodies become enlarged and extensions more elongated. Image taken on EVOS XL Core Cell Imaging System (x10 Magnification).

IL1β release was measured in SH-SY5Y by qPCR, after 24-hour and 48-hour stimulation by cytokines. No change was observed after 24-hour cytokine stimulation. IL1β release is increased after 48-hour stimulation (S48h) (Figure 5.9) compared to both control (NS) ($p < 0.001$) and 24-hour treatment of cytokines (S24h) ($p < 0.001$).

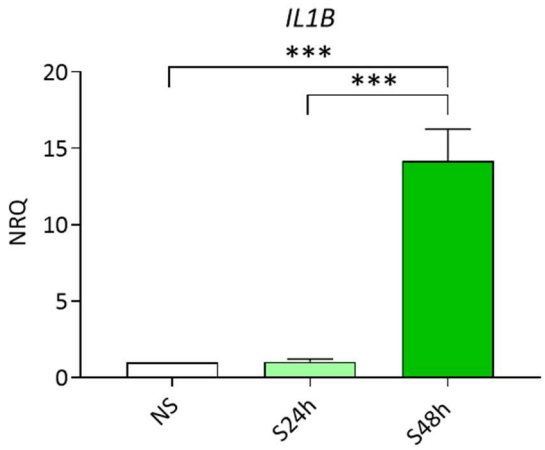


Figure 5.9: IL1β Release in Pro-inflammatory Cytokine Treatment of SH-SY5Y. Interleukin 1β was significantly increased in the cytokine stimulated SH-SY5Y after 48-hour stimulation (14.18±0.98) versus non-stimulated (NS) (p = 0.014) and after 24-hour stimulation (S24h) (1.05±0.09) (p = 0.01) versus non-stimulated (1±0.26). NRQ = Normalised relative quantity.

5.2.1.4 THP-1 Monocyte Cytokine Stimulation Tests

THP-1 cells are monocytic suspension cells which after PMA treatment become adherent, larger in diameter, and begin to lose their round shape (Figure 5.10).

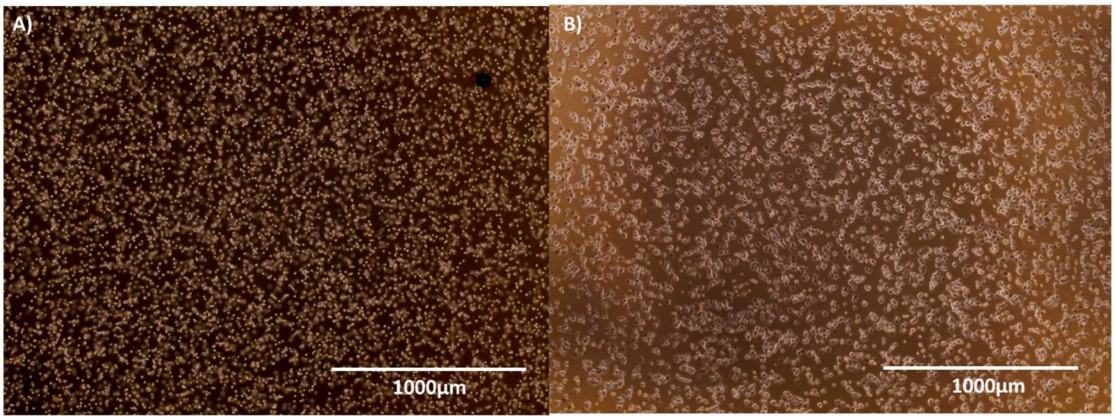


Figure 5.10: Microscopy Images of Non-Treated and PMA-Treated THP-1. Non-treated THP-1 cells are small, round, and suspended in the media. After PMA treatment THP-1 cells become attached to the bottom of the well and become larger, losing their defined round shape. Image taken on EVOS XL Core Cell Imaging System (x4 Magnification).

IL6 release in the THP-1 model was measured by sandwich ELISA (Figure 5.11, Table 5.3) in response to cytokine (IFNγ) treatment over four concentrations, 50, 100, 150, 200 µM.

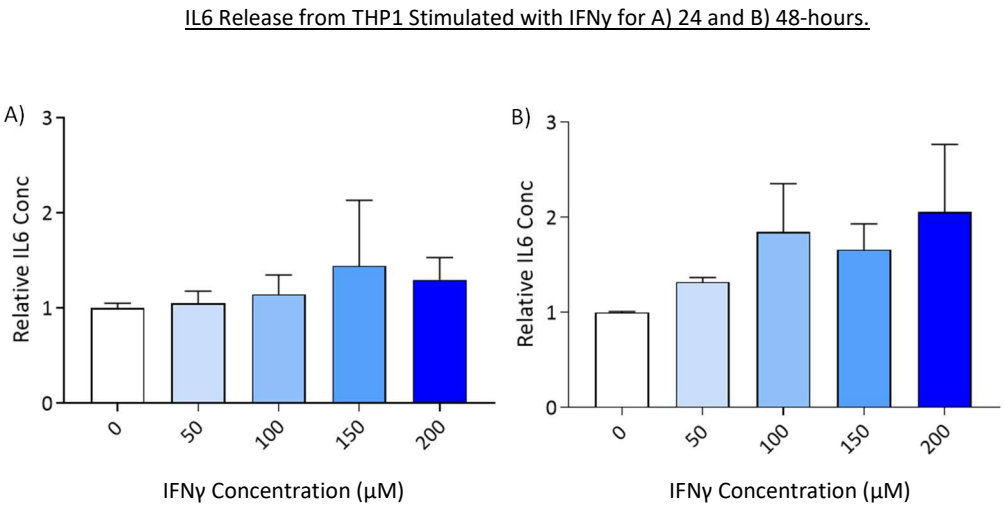


Figure 5.11: IL6 Release from THP1 Stimulated with IFNγ for 24 and 48-hours. A) IL6 release from THP-1 cells after 24-hours (S24h) of cytokine stimulation was non-significant p = 0.4977. B) After 48-hours of cytokine stimulation (S48h) the increase in IL6 release is significant p = 0.0238 (One-Way ANOVA). After Dunn's multiple comparisons the results are not significant, with p values > 0.0514. N=3.

Table 5.3: Mean±SEM for IL6 Release in THP1 Stimulated with IFNγ for 24 and 48-hours

	Mean ± SEM	
Cytokine Concentration (μM)	**S24h**	**S48h**
0	1 ± 0.04	1 ± 0.01
150	1.05 ± 0.07	1.32 ± 0.03
100	1.14 ± 0.12	1.84 ± 0.03
150	1.44 ± 0.40	1.66 ± 0.16
200	1.30 ± 0.14	2.05 ± 0.41

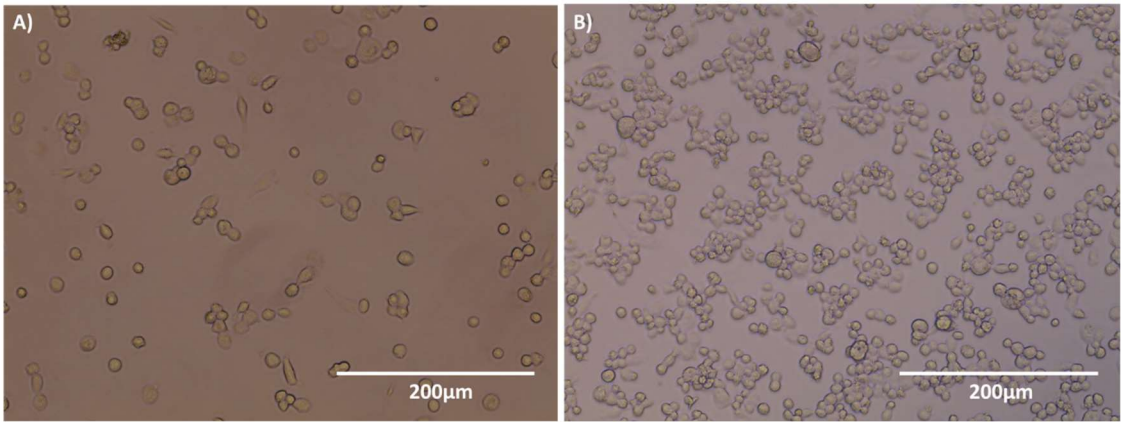


Figure 5.12: Microscopy Images of A) PMA-Treated Non-Stimulated and B) Stimulated 24-hours with IFNγ THP-1. The PMA treated THP-1 cell lines become granulated after 48-hour cytokine treatment. Image taken on EVOS XL Core Cell Imaging System (x20 Magnification).

5.2.2 Pilot Stimulation Tests

5.2.2.1 PLAC8 and ROMO1 ELISA

Protein expression levels of PLAC8 (Figure 5.13) and ROMO1 (Figure 5.14) were measured in a pilot model in cytokine stimulated (non-differentiated) THP-1 monocytes and (differentiated) SK-N-SH cells.

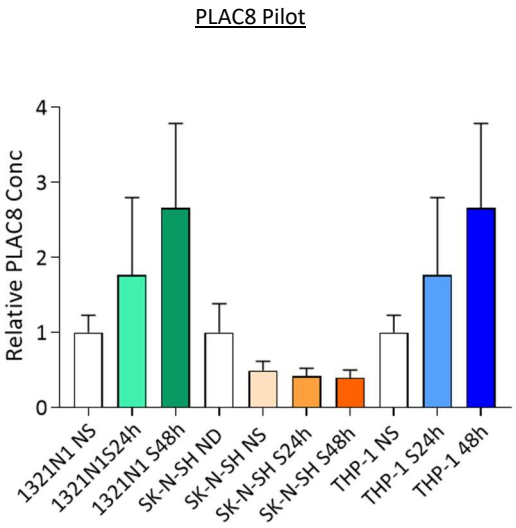


Figure 5.13: PLAC8 ELISA Results. Astrocyte (1321N1) stimulation increased PLAC8 release but with high variability between the replicates (n = 3) and are not significant (p = 0.196). PLAC8 decreases slightly in differentiated SK-N-SH cells with cytokine stimulation but are not significant (p = 0.242, n=2). Though PLAC8 shows a slight decrease in THP-1 cells with cytokine stimulation, results have wide error bars and are not significant (p = 0.1964 n = 3).

PLAC8 shows an upward trend in both 1321N1 cells and undifferentiated THP-1 cells after stimulation with IFNγ (Figure 5.13). Across the three pilot models there is a common trend of an initial increase in ROMO1 levels which then decreases after 48-hour cytokine stimulation (Figure 5.14).

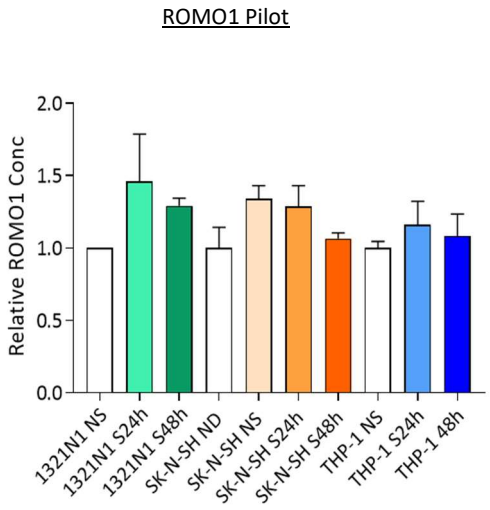


Figure 5.14: ROMO1 ELISA Pilot Results. In 1321N1 there is an increase in ROMO1 protein expression after 24-hours of cytokine stimulation (S24h) and subsequent decreases after 48-hours (S48h) (p = 0.336, n = 3). ROMO1 is increased after differentiation (RA treatment) and then decreases with cytokine stimulation (p = 0.1679, n = 2). In THP1 there is only a slight variation of ROMO1 protein levels (p = 0.629, n = 3).

5.2.2.2 Olfactory Marker Protein (OMP) Western Blot

OMP levels were measured in the pilot samples by western blot (Figure 5.15), with highly variable expression in the 1321N1 astrocytes (n=3). OMP levels are decreased in the THP-1 cell lines after cytokine stimulation and increased in SK-N-SH with differentiation and cytokine stimulation, though these changes are not significant (n=3, Figure 5.15).

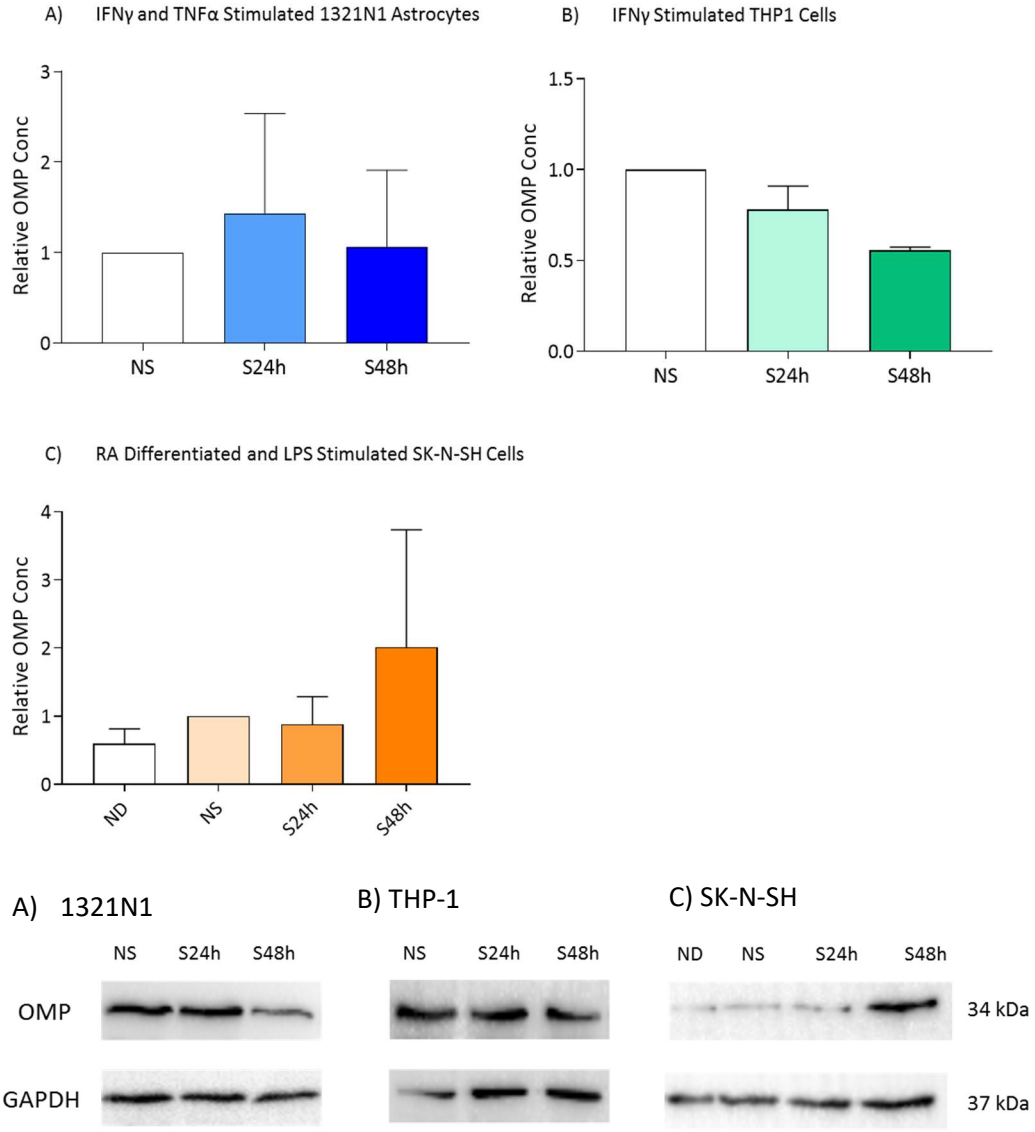


Figure 5.15: OMP Western Blot on pilot group of stimulated cell lines. OMP displayed an upward trend in expression at the protein level in stimulated 1321N1 astrocytes after 24-hours (S24h) and returns to control levels after 48-hours (S48h) (n = 3). This difference was not significant (p = 0.200) due to high variation. In THP-1 OMP expression is trending downwards with stimulation but is not significant (p = 0.150) (n = 3). In SK-N-SH cells OMP protein levels are low when non-differentiated, and do not change when differentiated with retinoic acid, and with additional stimulation with LPS for 24-hours. After 48-hours of stimulation the OMP level trends upwards, but with large variation (n = 3) this is not significant (p = 0.741).

Table 5.4 Mean±SEM of OMP Western Blot on pilot group of stimulated cell lines.

Experiment	Mean ± SEM
1321N1 NS	1±0.056
1321N1 S24h	0.78±0.13
1321N1 S48h	0.56±0.02
THP1 NS	1±0.12
THP1 S24h	1.43±1.10
THP1 S48h	1.06±0.84
SK-N-SH ND	0.60±0.22
SK-N-SH NS	1±0.15
SK-N-SH S24h	0.88±0.41
SK-N-SH S48h	2.01±1.72

5.2.3 Astrocytoma Stimulation

5.2.3.1 Gene Expression Changes in Cytokine-Stimulated 1321N1

Five candidate genes were measured at gene expression level in the 1321N1 astrocytoma cell line. Three genes, *ANXA1, FPR2,* and *OMP* were not significant across any treatment groups. Two genes, *PLAC8* and *SH3BGRL3* were significantly decreased in the inflammatory astrocytes (Figure 5.16). *SH3BGRL3* gene expression was decreased after 48-hours treatments with pro-inflammatory cytokines. *PLAC8* was significantly decreased after both 24- and 48-hour treatment, the gene expression was not changed between the treatment intervals, and the decreased expression is maintained.

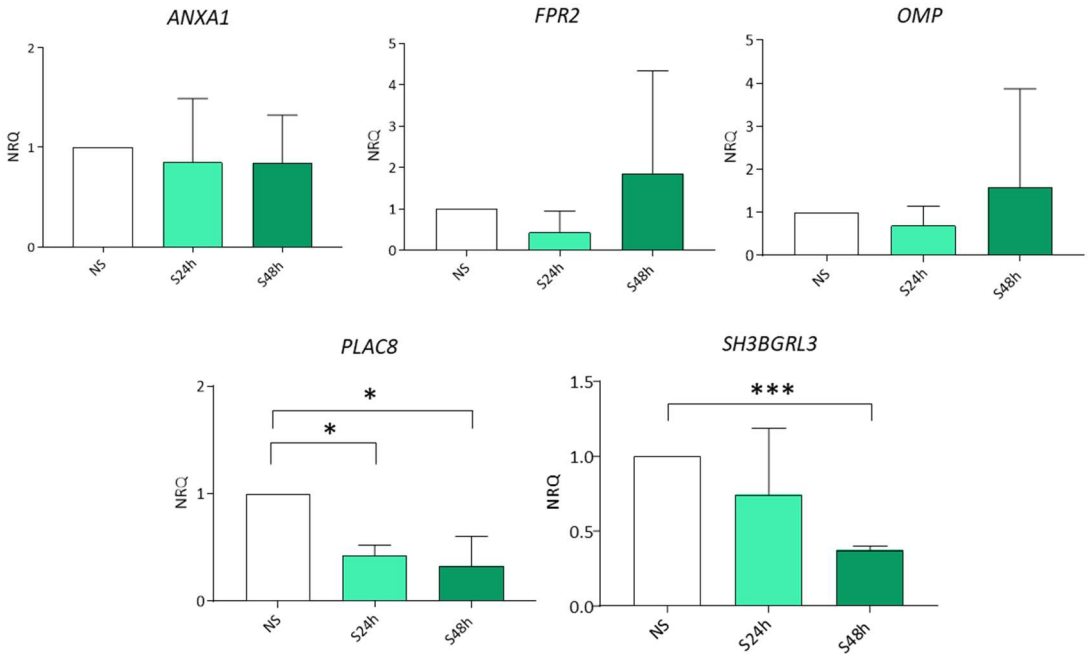


Figure 5.16: Gene expression changes in cytokine stimulated 1321N1 astrocytoma cells. Gene expression changes in the stimulated 1321N1 astrocytes showed that *SH3BGRL3* was decreased after 48-hour (S48h) treatments with pro-inflammatory cytokines (p = 0.0464). *PLAC8* was significantly decreased after both 24- (S24h) and 48-hour treatment (S48h) (p = 0.0500), and gene expression was not different between the S24h and S48h though the decreased was maintained. No changes were seen in the expression of *ANXA1, FPR2*, or *OMP*.

Table 5.5. Gene expression changes in cytokine stimulated 1321N1 astrocytoma cells (Mean±SEM)

	Mean ± SEM				
Experiment/Gene	*ANXA1*	*FPR2*	*OMP*	*PLAC8*	*SH3BGRL3*
1321N1 NS	1 ± 0.33	1 ± 0.32	1 ± 0.38	1 ± 0.34	1 ± 0.21
1321N1 S24h	0.64 ± 0.42	0.53 ± 0.23	1.02 ± 0.38	0.44 ± 0.04	0.58 ± 0.24
1321N1 S48h	0.84 ± 0.48	1.84 ± 1.44	1.59 ± 1.32	0.33 ± 0.16	0.37 ± 0.02

None of the candidate genes measured was increased in the 1321N1 astrocytoma cell line after cytokine treatment, though ANXA1 was differentially expressed at the protein level.

5.2.3.2 Protein Expression Changes in Cytokine-Stimulated 1321N1 Astrocytoma Cells

ANXA1 is increased after 48-hour cytokine stimulation at protein level in the 1321N1 astrocytoma cell line, measured by Western blot (Figure 5.17). No change is seen in ANXA1 expression at the 24-hour treatment interval.

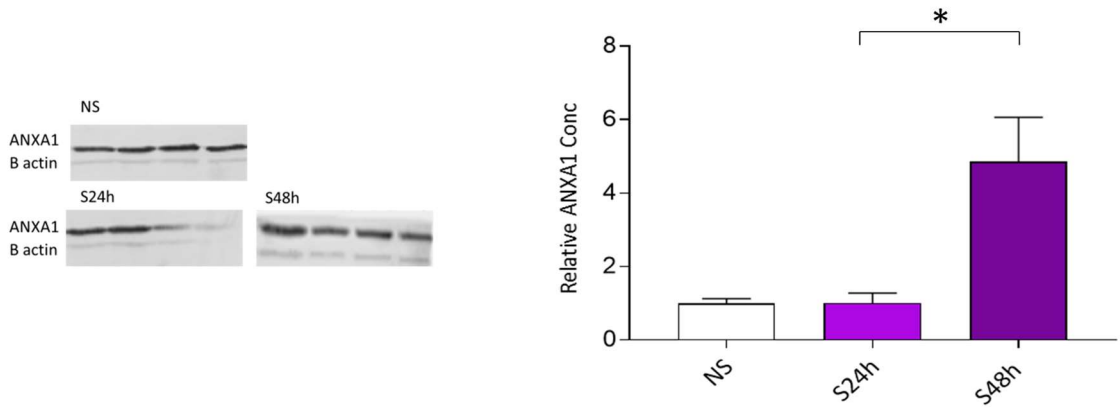


Figure 5.17: Annexin A1 Expression Changes at Protein Level in Cytokine-Stimulated 1321N1 Astrocytoma. Annexin A1 protein expression levels were not significantly changed after 24-hours of cytokine stimulation (S24h) or 48-hour treatment (S48h) but was increased between these intervals (p = 0.427). Mean ± SEM ANXA1 S24h 1.00 ± 0.13 S48h 4.88 ± 0.61.

5.2.4 Gene Expression Changes to Cytokine-Treated SH-SY5Y Neuroblastoma

Several genes demonstrated differential expression in the SH-SY5Y neuronal cell line after 48-hour stimulation which was significantly different to both non-stimulated control and 24-hours stimulation (Figure 5.18, Table 5.6). None of the candidates demonstrated decreased expression. *ANXA1, CASP4, PLAC8, ROMO1, SH3BGRL3,* and *TXN1* all do not change after 24-hour stimulation (S24h) but are all increased significantly after 48-hour stimulation (S48h).

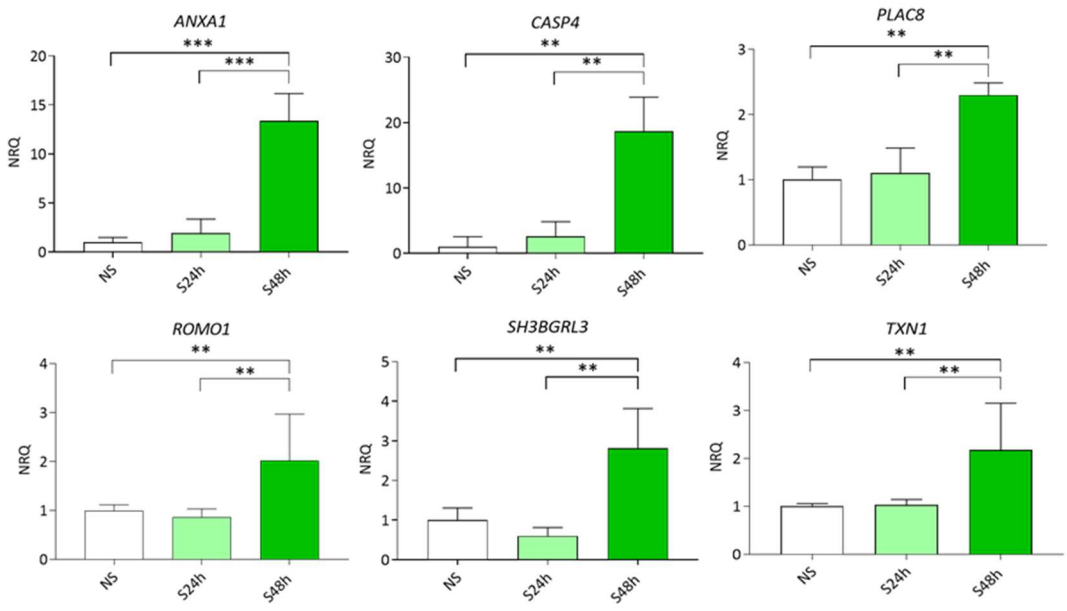


Figure 5.18: Gene Expression changes in cytokine stimulated SH-SY5Y. Seven genes are increased in the 48-hour cytokine stimulated SH-SY5Y cells (S48h) compared to the non-stimulated (NS) cells (n=3), and six of these are also significantly increased in S48h versus 24-hour stimulation (S24h). These are *ANXA1* (p <0.001), *CASP4* (p = 0.005), *PLAC8* (p = 0.005), *ROMO1* (p = 0.005), *SH3BGRL3* (p = 0.01), *TXN1* (p = 0.005).

Table 5.6. Gene expression changes in cytokine stimulated SH-SY5Y cells (Mean±SEM)

	Mean ± SEM					
Experiment/Gene	*ANXA1*	*CASP4*	*PLAC8*	*ROMO1*	*SH3BGRL3*	*TXN1*
SH-SY5Y NS	1 ± 0.28	1 ± 0.91	1 ± 0.11	1 ± 0.07	1 ± 0.13	1 ± 0.03
SH-SY5Y S24h	1.93 ± 0.83	2.61 ± 1.30	1.10 ± 0.22	0.87 ± 0.09	059 ± 0.09	1.03 ± 0.07
SH-SY5Y S48h	13.38 ± 1.60	18.68 ± 3.01	2.30 ± 0.11	2.02 ± 0.55	2.58 ± 0.40	2.18 ± 0.56

5.2.5 THP-1 Monocyte Cytokine Stimulation

5.2.5.1 Gene Expression Changes in the Cytokine and PMA-Treated THP-1 Monocytes

Six genes show differential gene expression across the five treatment groups (Figure 5.19, Table 5.7). *FPR2, OMP, ROMO1,* and *TXN1* were not changed at gene expression level. *ANXA1* was increased after PMA treatment (p = 0.014) and in PMA-treated monocytes after 24-hour stimulation (PMA S24h) (p = 0.0242). *CASP5* was decreased after cytokine stimulation for 24-

hours (p = 0.0022) and 48-hours (p = 0.0024), and after PMA treatment (p = 0.004) and PMA treatment and 24-hour stimulation (PMA S24h) (p = 0.0075). *CCR5* and *PLAC8* are decreased after PMA treatment (p = 0.029, 0.008 respectively) and *SH3BGRL3* was increased after PMA treatment and 24-hour cytokine stimulation (PMA S24h) (p = 0.031).

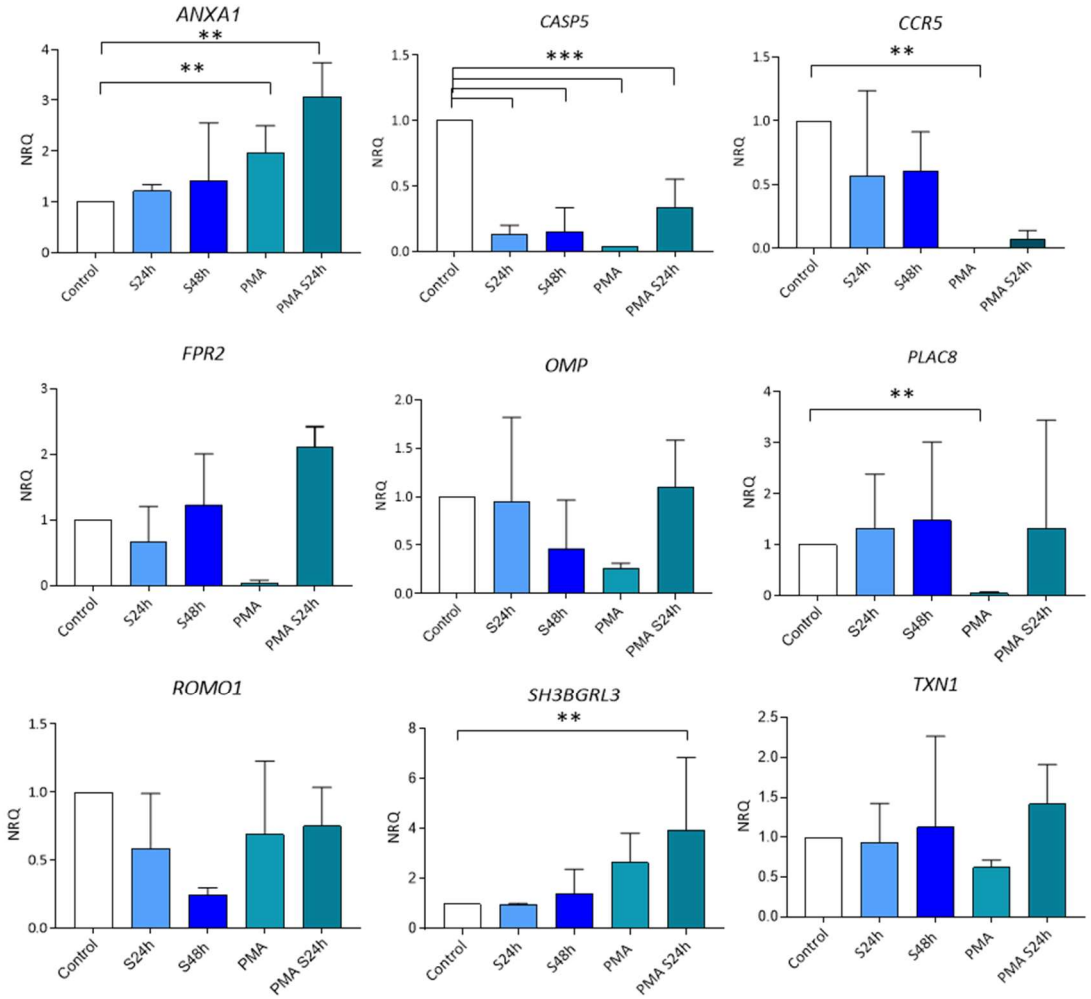


Figure 5.19: Gene Expression Changes in Cytokine Stimulated and PMA Treated THP-1 Monocytes. *FPR2, OMP, ROMO1,* and *TXN1* were not changed at gene expression level. *ANXA1* was increased after PMA treatment (p = 0.014), and increased in PMA-treated monocytes after 24-hour stimulation (p = 0.0242). *CASP5* was decreased after cytokine stimulation for 24-hours (p = 0.0022) and 48-hours (p = 0.0024), PMA treatment (p = 0.004), and PMA treatment and 24-hour stimulation (p = 0.0075). *CCR5* is decreased after PMA treatment (p = 0.029). *PLAC8* is decreased after PMA treatment (p = 0.008). SH3BGRL3 was increased after PMA treatment and 24-hour cytokine stimulation (p = 0.031).

Table 5.7. Gene expression changes in cytokine stimulated THP-1 cells (Mean±SEM)

Experiment /Gene	Mean ± SEM								
	ANXA1	*CASP5*	*CCR5*	*FPR2*	*OMP*	*PLAC8*	*ROMO1*	*SH3BGRL3*	*TXN1*
THP-1 NS	1 ± 0.22	1 ± 0.38	1 ± 0.12	1 ± 0.34	1 ± 0.29	1 ± 0.10	1 ± 0.19	1 ± 0.11	1 ± 0.11
THP-1 S24h	2.77 ± 1	0.30 ± 0.09	0.56 ± 0.19	0.62 ± 0.18	1.11 ± 0.47	1.31 ± 0.47	0.61 ± 0.09	2.20 ± 0.7	0.82 ± 0.09
THP-1 S48h	1.12 ± 0.14	0.75 ± 0.34	0.59 ± 0.19	0.98 ± 0.06	0.57 ± 0.31	0.79 ± 0.19	0.47 ± 0.08	1.46 ± 0.46	0.77 ± 0.16
THP-1 PMA NS	2.36 ± 0.70	0.05 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.22 ± 0.05	0.16 ± 0.09	0.63 ±0.14	3.56 ± 1.36	0.89 ±0.34
THP-1 PMA S24h	3.30 ± 0.31	0.97 ± 0.47	0.36 ± 0.23	1.09 ± 0.47	0.83 ± 0.046	0.85 ± 0.43	0.83 ± 0.12	4.30 ± 2.02	1.48 ± 0.39
THP-1 PMA S48h	8.06 ± 1.62	0.53 ± 0.04	0.28 ± 0.16	0.45 ± 0.17	0.56 ± 0.10	0.78 ± 0.31	1.03 ± 0.09	11.72 ± 2.7	1.27 ± 0.07

5.2.5.2 Protein Expression Changes in the Cytokine/PMA-Treated THP-1 Monocytes

Protein expression changes in the THP-1 model were measured by Western blot (Figure 5.20). ANXA1 was increased in PMA-treated monocytes after 48-hour stimulation ($p = 0.0338$), though protein levels for THP-1 (not PMA treated) were not included as signals could not be obtained

by western blot. The ANXA1 receptor FPR2 was altered at protein level between the following groups: S48h vs PMA (p = 0.0371), and S48h vs PMA and S24h (p = 0.285).

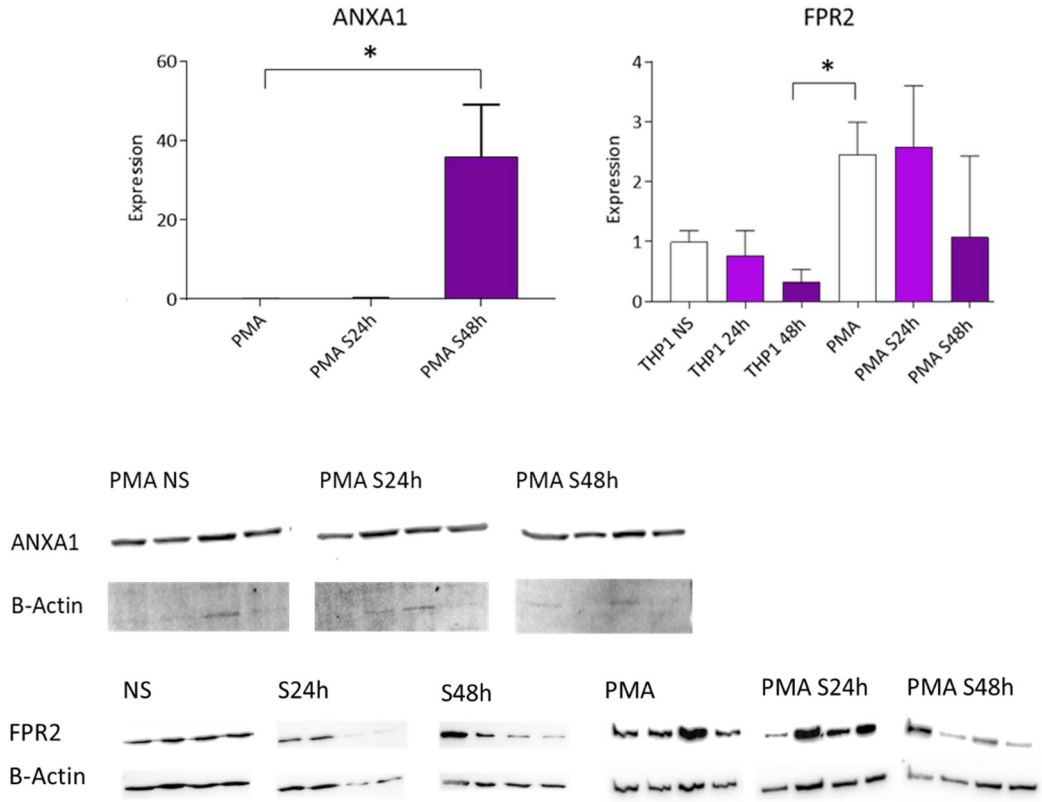


Figure 5.20: Protein Expression changes in ANXA1 and FPR2 in THP-1 stimulation models. ANXA1 was increased in PMA-treated monocytes after 48-hour stimulation (p = 0.0338). Mean±SEM ANXA1 PMA S24h 0.24±0.09, ANXA1 PMA S48h 33.16±6.07. FPR2 was altered at protein level between S48h vs PMA (p = 0.0371). FPR2 PMA S24h Mean±SEM 2.45±0.27, FPR2 PMA S48h 1.08 ± 0.68.

5.2.6 Preliminary Mechanical Stress Test in 1321N1 Astrocytes

A pilot mechanical stress test was performed on an astrocytic cell line (Figure 5.21). The stress test was insufficient to cause damage to individual cells, as desired to reproduce the cellular damage and pyroptosis required for a preliminary neuropathic model (Figure 5.21) and the astrocyte population began to recover within the first 24 hours.

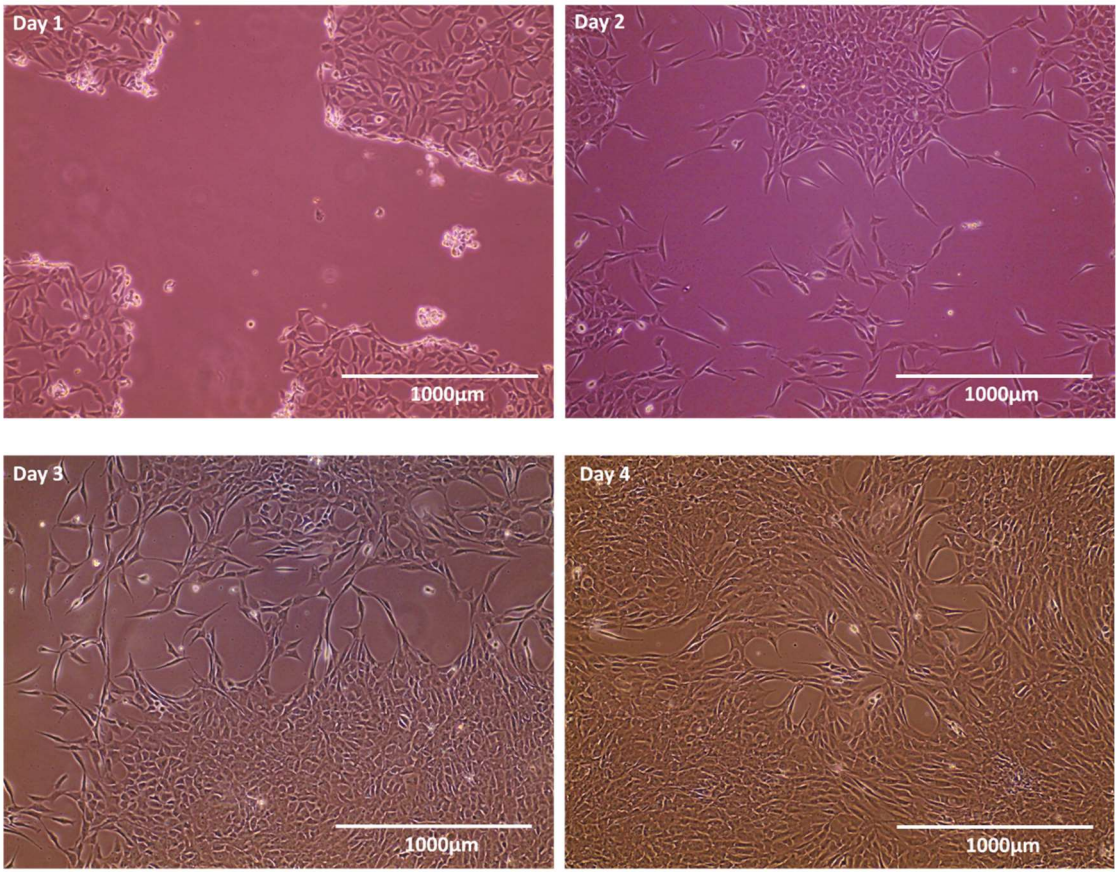


Figure 5.21: Mechanical Stress Test and Recover in the 1321N1 Astrocytes. Minimal damage to individual cells is observed, and recovery of the cellular population is seen from Day 1 (n=1) which continues through to Day 3. Cells which grew in the space are elongated in comparison to the surrounding cells outside of the damage area where cell density is higher. Image taken on EVOS XL Core Cell Imaging System (x20 Magnification).

5.3 Discussion

5.3.1 Cytokine Stimulation Tests

The IL1β qPCR and IL6 ELISA test results on the inflammation models confirmed the gene expression and release of these pro-inflammatory cytokines at protein level respectively. For SH-SY5Y and 1321N1 cells, 100µM IFNγ and 400µM TNFα were used according to established lab group protocol. IL1β mRNA expression levels confirmed sufficient activation of cells after stimulation (Figures 5.7, 5.9). IL6 protein, produced and released by THP-1 cells (Neuner *et al.*, 1991), was measured by ELISA in the cell media after cytokine treatment. After 24-hours of cytokine stimulation, IL6 release was not significant. IL6 release from THP-1 cells after 48-hours of IFNγ stimulation was highest with 200ng/ml, though 100ng/ml shows a similar increase. For this reason, 100µM was selected as the IFNγ concentration for the *in vitro* model (Figure 5.11).

5.3.1.1 Pilot Results

PLAC8 and ROMO1 proteins were not significant in the clinical cohort (Chapter 4, Table 4.1), but the known functionality of ROMO1 in ROS production and the novelty of PLAC8 for neuropathic pain research make them ideal candidates for a pilot study. Non-differentiated THP- monocytes and differentiated SK-N-SH were selected as monocyte and neuronal model respectively. None of the results of either the PLAC8 or ROMO1 pilot ELISAs was significant (Figure 5.13, Figure 5.14). For PLAC8 there is large standard error across replicates, and therefore we could not draw any conclusions as to how PLAC8 expression at the protein level may change in cell lines treated with pro-inflammatory cytokines. For ROMO1 the standard error was smaller, and the data demonstrated an interesting pattern across all three models which is a slight upward trend after 24-hours cytokine stimulation (and after differentiation in SK-N-SH) followed by a decreasing trend after 48 hours.

As the sample size was three biological replicates, each composed of three technical replicates, these results are valid, and representative of the true effect of inflammatory stimulation of non-differentiated THP-1 cells. For SK-N-SH cells the sample size was 2 (1 for ND) (Table 5.8), and repeats would be required to make any definitive conclusions. Given the results from the pilot, PLAC8 or ROMO1 protein levels were not tested in differentiated (PMA-treated) THP-1 cells, 1321N1 astrocytes, or in SH-SY5Y cell lines though gene expression levels were measured by qPCR.

Table 5.8: Pilot Model Replicates

THP-1 (n = 3)	SKNSH (n = 2) *(n = 1)
Non-stimulated (NS)	*Non-differentiated & non-stimulated (ND)
24-hours with IFNγ	6-day differentiation with Retinoic Acid, no stimulation (NS)
48-hours with IFNγ	6-day differentiation + 24-hours with LPS
-	6-day differentiation + 48-hours with LPS

OMP expression was highly variable across the replicates in the pilot 1321N1 astrocytes stimulated with IFNγ and TNFα (Figure 5.15). The error was smaller in the THP-1 pilot and OMP was decreased when stimulated with IFNγ (100µM) at both 24- and 48-hour intervals. In SK-N-SH cells OMP protein levels were low when non-differentiated, differentiated with retinoic acid 10µM and stimulated with LPS 100µM for 24-hours. After 48-hours of stimulation the OMP level increased (Figure 5.15) and this was evident in both the quantitative graph and the blot images. The levels of GAPDH did not reflect these patterns, which indicates that the difference in OMP levels between the samples was not fully attributable to differences in protein loading on the SDS-PAGE gel. OMP was downregulated in the microarray analysis of DH tissue from SNL rats versus sham (fold change -1.25, p = 0.005, Chapter 3 Table 3.3). This was consistent with the western blot findings (Figure 5.15). There was a decrease in OMP expression in all three cell lines

after 48-hour incubation with inflammatory cytokines, though the difference was not significant. This is true also for the THP-1 model (Figure 5.19) (not significant), but OMP was increased in the PMA-induced macrophage model (not significant). An increased number of biological replicates (>3) may be required to identify a true significant change. *OMP* was also measured at the mRNA level in SH-SY5Y cell model instead of the SK-N-SH model. Due to the time required for differentiation of SK-N-SH differentiation, and the increased opportunity for issues such as contamination (in which the whole sample is discarded and re-established) after the pilot, the non-differentiated SH-SY5Y cell line was chosen as the neuronal model. Changes to the olfactory receptor expression profile have been reported in neurodegenerative disease by several groups (Bahuleyan & Singh, 2012), as discussed in Section 3.3.2 and remains a target of interest in these areas.

5.3.1.2 1321N1 Astrocyte Stimulation

PLAC8 and *SH3BGRL3* were decreased following cytokine stimulation in 1321N1 (Figure 5.16). It is interesting to note that for *PLAC8* this is unexpressed considering the (non-significant) trend observed in the protein expression in the 1321N1 pilot study (Figure 5.13). The decrease in *SH3BGRL3,* an anti-apoptotic TXN1-like protein, agrees with the rat DH SNL from the microarray, but not with the clinical dataset (Table 4.1). This could be due to the relative homogeneity in rat models and cell models compared to the heterogeneity in the clinical cohort due to genetic variations and drug intake. ANXA1 is also increased at protein level after 48-hour stimulation (Figure 5.17), as it is in the rat DH SNL and clinical blood samples (Table 4.1), which indicates that an anti-inflammatory mechanism has been induced in the 1321N1 astrocytoma in response to the prolonged additive cytokine stimulation. This agrees with the rat SNL and clinical blood data.

Astrocyte can become reactive in response to several triggers in injury and disease (Table 5.9). These include TNFα and IFNγ released from neighbouring cells during an inflammatory response, and treatment with these cytokines might trigger astrogliosis. GFAP is a commonly used measurement of astrogliosis *in vitro* (Sofreniew, 2009). However, the 1321N1 cell line is GFAP negative, which means it could not be used to confirm the induction of a reactive state, nor measure the magnitude of activation. The use of a GFAP positive astrocyte cell line e.g. U-251MG (human astrocytoma) to investigate candidate behaviour during reactive astrogliosis represents an ideal future experiment discussed future in Section 5.3.5. Inflammatory conditions have been sufficiently induced as IL1β production was significantly increased in IFNγ and TNFα treated 1321N1 (Figure 5.7).

Table 5.9: Astrocyte Reactivity Triggers

Signals from Neighbouring Cells (astrocytes, microglia, infiltrating leukocytes)	Cell Damage and Death	Disease
Pro-inflammatory cytokines including IL6, IFNγ, TNFα, tumour growth factor TGFβ, IL1β, and IL10 (Levison *et al.*, 2000, Stellwagen & Malenka, 2006, John *et al.*, 2003)	ATP (Sofroniew, 2009)	Amyloid-β from neurodegenerative diseases (Forman *et al.*, 2005, Simpson *et al.*, 2010)
NH^{+}_{4} waste from neuroexcitation (Norenberg *et al.*, 2009)	Reactive oxygenated species (ROS) and Nitric oxide (NO) (Swanson *et al.*, 2004)	Liposaccharide and other pathogen-associated molecular patterns from infections (Farina *et al.*, 2007)

(Sofroniew & Vinters, 2010, Sofroniew, 2009)

5.3.1.3 SH-SY5Y Stimulation

Seven genes which vary in function were upregulated in the SH-SY5Y model after 48-hour stimulation with cytokines. These changes were observed after 48-hour stimulation, but not after 24-hour stimulation, which indicates these changes might require either time (e.g. 48-

hours) after initial stimulation or the accumulation of two doses of cytokines. Pro-apoptotic *CASP4* was increased (Figure 5.18), as it was in the rat and clinical datasets (Table 4.1). The pro-inflammatory *ROMO1* was increased (Figure 5.18), as it was in the rat dataset, though this change did not occur in the clinical cohort (Table 4.1).

The anti-inflammatory and anti-apoptotic mediators *ANXA1, SH3BGRL3,* and *TXN1* were also upregulated (Figure 5.18), which agreed with the changes in rat DH SNL, and for *ANXA1* and *TXN1* this finding also in line with the clinical data (Table 4.1). These seemingly conflicting functions may result from either the active fine-tuning of a response to the stimulation or represent the widespread changes across different mechanisms. *PLAC8* was increased after 48-hour stimulation, which supports its increase at mRNA level in both the rat SNL and clinical neuropathic pain datasets. This may imply that the increase in *PLAC8* in the neuropathic model and clinical patients occurs in response to the inflammatory component of the disease.

5.3.1.4 THP-1 Stimulation

In the THP-1 monocyte inflammatory model, unlike in the 1321N1 and SH-SY5Y models, changes to the anti-inflammatory mediator *SH3BGRL3* (up-regulated), and the pro-inflammatory *CCR5* and pro-apoptotic *CASP5* were complementary (down-regulated) (Figure 5.19). This indicates that a protective phenotype could be induced in response to cytokine stimulation. Some significant changes were seen between the monocyte and macrophage phenotypes, including *ANXA1* and *CASP5* which were increased and decreased respectively after PMA treatment, and PMA plus cytokine treatment (Figure 5.19). *PLAC8* was also decreased after PMA treatment. These gene expression changes, therefore, occurred after monocyte differentiation into macrophages. *ANXA1* and its increased activity in the macrophage phenotype imply that anti-inflammatory mechanisms are induced after differentiation. The absence of a concurrent increase in its receptor *FPR2* does not suggest that *ANXA1* is acting via this mechanism on the

astrocytoma cells but may be activated to induce that pathway in other target cell types *in vivo*, e.g. glucocorticoid receptors (Perretti & D'Acquisto, 2009). *SH3BGRL3* was increased in the PMA treated THP-1 cells after 24-hour cytokine stimulation versus control, which suggests that it is upregulated in the macrophage phenotype under inflammatory conditions. Given the anti-inflammatory *SH3BGRL3* role, this may be upregulated to attenuate the inflammatory response of the 1321N1 astrocytoma cells.

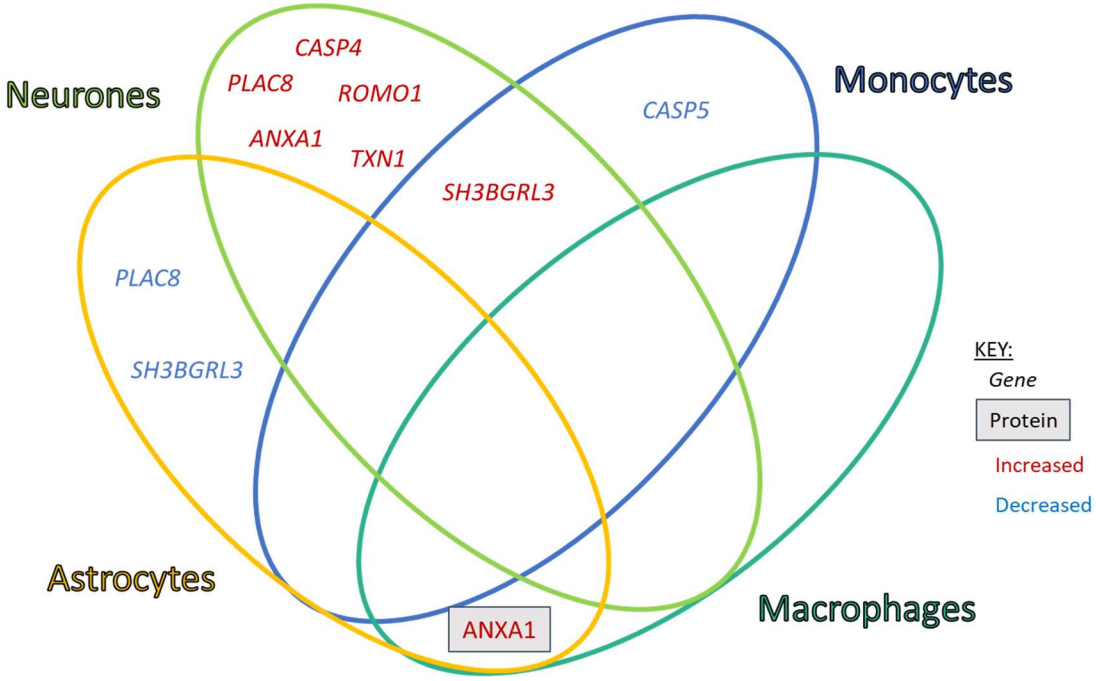


Figure 5.22: Schematic of the inflammatory model findings across three cell lines. Gene and protein expression of several candidates were altered across the four inflammatory models.

Together the changes to candidate gene and protein expression can help piece together a better understanding of what may be happening under inflammatory conditions *in vivo* (Figure 5.22). There was little overlap of changes in candidate genes across the inflammatory models, and most of the significant changes were seen in the in neuronal model SH-SY5Y neuroblastoma cell

line, with six upregulated candidate genes under inflammatory conditions. This could suggest that the changes in response to inflammatory conditions are largely mediated by neuronal cells. However, a more comprehensive study is required for a better understanding of these interactions.

Some candidates were differentially expressed in different directions. For example, *SH3BGRL3* was decreased in 1321N1 astrocytoma, but increased in SH-SY5Y and monocytes. Similarly, PLAC8 was decreased in 1321N1 and increased in SH-SY5Y. Though little is known about PLAC8 function, the PLAC8 protein motif 'OmFCR' is involved in detoxification and response to infection (Cabreira-Cagliari *et al.*, 2018), and its differential expression may be linked to a fine-tuning of this function during the response to the pro-inflammatory cytokines. Its potential influence on Ca^{2+} influx may explain its upregulation in the SH-SY5Y cells, as Ca^{2+} influx is a key event in synaptic transmission and neurotransmitter release (Sudhof, 2012). SH3BGRL3 protein function is also poorly understood, but its potential role in the inhibition of TNFα may explain its differential expression across three inflammatory models. Its decrease in 1321N1 may imply that this anti-inflammatory mechanism is not active in stimulated astrocytes, whereas it is active in SH-SY5Y and THP-1 when stimulates. There are several mechanisms which may complement or compensate for this pathway in these models, and without a comprehensive understanding of the protein's functionality and activity levels, it is not possible to make conclusion about its role here. Increased protein levels of ANXA1 was seen in the astrocytoma cell line and macrophage model, but not at mRNA level. *ANXA1* was increased at mRNA level in the neuroblastoma line, and this could not be measured at protein level by western blot due to methodical difficulties.

Of the caspases, *CASP4* was increased in SH-SY5Y and *CASP5* was decreased THP-1 monocytes. These genes carry out similar roles in the induction of cell death, so it is interesting to observe opposing differential expression across different cell lines. These findings suggest that THP-1

monocytes may reduce the expression of pro-apoptotic caspases as their main function is to respond to inflammation by transforming into the macrophage phenotype to clear up debris and pathogens. Therefore, the induction of monocyte apoptosis in the early stages of inflammatory response would inhibit macrophage function. The neuronal-like SH-SY5Y cells may be upregulating the pro-apoptotic caspases in preparation for organised cell death during the inflammatory response. Neurones do not have an active role in the inflammatory response in the same way that monocytes and macrophages do as part of the innate immune system.

5.3.1.5 Mechanical Stress Test

A quick mechanical test was performed on 1321N1 astrocytes to determine whether it would have been practical to perform a full experiment. The results showed that mechanical damage by scraping with a pipette tip was insufficient to damage individual cells, and the population recovery was evident after 24-hour (Figure 5.21). As 1321N1 does not express GFAP it could not be measured to confirm whether reactivity had been induced. It may be possible to create damage to the cells with a finer blade, and the use of a GFAP-positive cell line would allow reactivity to be measured by GFAP expression by qPCR or Western blot.

In vivo, mechanical stress can be induced by trauma and injury, and is relevant to neuropathic pain as it is damage to the nervous system which triggers the cascade of events in neuropathic pain establishment. A mechanical stress test in a neuronal model, such as SH-SY5Y, would also be relevant to periphery injuries. Further, as reactive astrocytes act on neurones, and function to protect neuronal tissue after injury, a co-culture of mechanical stress may be useful for future investigations into CNS injury.

5.3.2 Considerations

5.3.2.1 EVOS XL Core Cell Imaging System

The EVOS XL Core microscope used to capture the cell culture images (Figures 5.6, 5.8, 5.10, 5.12, 5.21) has a 3.1-megapixel camera to display images on a monitor (rather than traditional eyepieces) and cannot capture minute detail in the morphology. It is possible that higher resolution images may be useful in identifying finer details, though these images were suitable for this project. The use of other phenotype screening methods such as flow cytometry may also negate the need for high definition images of cell morphology changes in future experiments.

5.3.2.2 Cell Lines

The neuronal and astrocytic cell lines used in this study are commercially available immortal cells lines, derived from cancer patients, have a cancerous phenotype. This allows them to perpetually proliferate. This can result in clumps and mounds of cell growth at the undifferentiated stage. Cells in such high-density populations may behave differently in terms of signalling than cells in lower density areas, therefore introducing a source of variability that should be avoided by reducing cell density and passage number.

The tumour-derived origin of the cells used in this study must be recognised as a limiting factor in their validity as surrogate models for *in vivo* tissue. Cancerous cell lines by nature have abnormal physiology, such as altered apoptosis pathways, which allows them to divide and survive indefinitely (Cree, 2011). This may confound results and compromise the validity of results and how relevant they may be to the clinical disease. Despite these considerations, immortal cell lines provide a good *in vitro* model of physiological systems and can complement *in vivo* work where primary tissue culture is not feasible. Cells derived from tumour lineages do however have the advantage of minimal variability between cultures and are widely used *in vitro* for the study of many diseases (Lucey *et al.*, 2009).

Astrocytes in the adult CNS are not well-characterised in terms of their subsets (in the non-reactive phenotype), which are believed to exist and perform specific functions (Zhang & Barres, 2010), particularly in adults (Miller, 2018). Evidence for astrocytic heterogeneity is not surprising given their interaction with different cell types in different states (Molofsky *et al.*, 2013), (John Lin *et al.*, 2017, Liddelow *et al.*, 2017). A lack of understanding of astrocyte heterogeneity limits interpretation of results as unique populations (e.g. distinct cell lines) may produce results specific to that population (Goursaud *et al.*, 2009), which cannot be generalised to adult astrocytes *in vivo.* It is important to consider that in neuropathic pain astrocyte subsets may have specific pathological functions. To fully understand the role of astrocytes in neuropathic pain, first astrocytic functionality in different brain regions in health must be characterised.

Reactive astrocytes have been described as two distinct subsets termed A1 and A2 (Liddelow *et al.*, 2017). A1 astrocytes are associated with neurodegenerative disease and cause death of neighbouring neurones and oligodendrocytes, and A2 are neuroprotective (Miller, 2018). These populations have distinct markers, including increased C3, Gpp2, and Psmbb8 in A1, and high Clcf1, Ptx3, and Cd14 in A2 (Miller, 2018), which could be measured *in vitro* to assess the phenotype being induced under experimental conditions, or located by IHC in brain slices of neuropathic pain models or patients.

SH-SY5Y cells can spontaneously differentiate into an epithelial phenotype, which compromises its suitability as a neuronal cell line if the ratio of epithelial to neuronal cell types becomes too large (Kovalevich & Langford, 2013). Loss of neuronal characteristics is also reported in later passages, including loss of neuronal markers, and for this reason, SH-SY5Y cells were not used beyond passage twenty-five. In future studies, phenotypic markers should be quantified for each replicate to validate the neuronal model. SH-SY5Y can be differentiated into a neuronal phenotype using treatment with the vitamin A-derivative retinoic acid in low serum or serum

free media (Pahlman *et al.*, 1984). The differentiated cells extend neurites into the open spaces around them, mimicking the morphological features of primary neurones. Their growth slows down as they begin to express the mature markers including neuronal specific nuclear protein NeuN and beta III tubulin. For future investigations differentiated SH-SY5Y may give insight into mature neuronal behaviour in an inflammatory model.

Though primary cells do not proliferative, if attainable they can be used for short *in* vitro investigations. For example, Kaewpitak et al., cultured mouse trigeminal ganglion cell populations in vitro for two days to assess lipopolysaccharide (LPS) activation, and found that LPS induced an excitatory response on both neuronal and sensory neurons via TLR4- and TRPA1-dependent pathways (Kaewpitak et al., 2020). A more relevant *in vitro* approach than immortal cells lines would be primary dorsal horn cells from rat, and these could be used in a similar way to Kaewpitak *et al.* to assess the response to inflammatory stimulation. An additional investigation could involve samples from candidate knockout rats and differences in response to inflammatory stimulation.

Treatment of THP-1 cells with PMA is widely used to induce a macrophage phenotype similar to that of primary human macrophages (Lund *et al.*, 2016). Lipopolysaccharide (LPS) treatment of PMA-differentiated THP-1 is a well-established method of activating the macrophages and inducing TNFα and superoxide release (Lund *et al.*, 2016). LPS is derived from bacteria, and so was not considered for this experiment to increase its relevancy to sterile neuropathic pain. Instead, IFNγ was used induce the M1 phenotype (Chanput *et al.*, 2010).

5.3.2.3 Protein Concentration

The RIPA buffer used to extract protein from the cell samples contains SDS detergent, which is a disruptive contaminant of the Bradford assay reagent (Noble & Bailey, 2009). SDS contamination causes spectrometry shift during the reading of samples, causing inaccurate

reading and quantification of protein. This shift is dependent on SDS concentration. Critical micelle concentration (CMC) refers to the concentration of a detergent at which it forms micelle structures. Below CMC, SDS binds to the protein and inhibits its binding to the dye, and 465nm readings would be disproportionately low. Above CMC, SDS binds and stabilises Coomassie in its neutral green form, causing an equilibrium shift and an increase in 595nm absorbance independent of protein concentration. Therefore, protein concentration readings may be inaccurate, with implications for loading for lowly expressed proteins or sub-optimal antibodies.

5.3.2.4 Western Blot and ELISA

Both fluorescent and ECL antibodies were used in this study (Figure 5.4) as some primary antibodies worked better with one or the other. Both are highly sensitive and follow similar protocols with the additional step in ECL of substrate addition is performed immediately before signal detection. Western blot is a relatively affordable way of quantifying protein compared to ELISA, which requires only a specific antibody for the protein of interest in addition to the common Western blot reagents. ELISA requires kits including the antibody for the protein of interest either precoated on the solid phase e.g. 96-well plate or in a vial for pre-incubation. ELISA is a high throughput method, quicker than Western blot, but has a higher chance of false positives (Pruslin *et al.*, 1991). Western blot was used in this study to measure protein expression changes of candidates on a small scale because of reduced cost. Western blot was possible for the small number of experimental parameters, three for 1321N1 and SH-SY5Y, and six for THP-1 with repeats n = 4 for each. Western blot was also more suitable as it requires less sample than ELISA, therefore low protein concentrations in sample extractions from cell culture could still be used.

5.3.3 Potential Avenues for Further Investigation

5.3.3.1 Improving Methods

Improved characterisation of the inflammatory models by quantification of surface makers, for example CD14 expression on PMA-treated THP1 cells with flow cytometry, would definitively determine whether the desired phenotype has been induced. This would also allow a real-time profile of differentiation to be obtained as it happens. It would also be possible to use flow cytometry to measure surface expression of candidates e.g. FPR2, and measure expression in the inflammatory models across time. Intracellular proteins can also be measured by flow cytometry, though this requires addition steps of fixing and permeabilising the cells, which can reduce sensitivity (McKinnon, 2018).

5.3.3.2 Alternative Commercial Immortalised Cell Lines

NT2 (NTera) represents alternative neuronal cell lines to SH-SY5Y. NT2 is a human neuronal cell line, and like SH-SY5Y can be differentiated into mature neurones by retinoic acid (Gonzalez-Burguera *et al.*, 2016). The rat cell line PC12, which upon treatment with nerve growth factor differentiates into a neuronal phenotype (Greene, 1978). These alternative species cell lines could be used to develop the co-culture model where two cell types of the same species are used. A good candidate for DNA transfection, PC12 may be suitable for further experiments which investigate the effects of candidate upregulation by plasmid transfection in rat species. In a more complex model, transfected cells either (transient or stable) may be later used in a co-culture model. Results from the inflammatory astrocyte model (Figure 5.16) could subsequently be investigated in an immortalised microglial cell line (such as the Red Fluorescent Immortalised Human Microglial Cells, Innoprot), with potential for development of a co-culture model.

5.3.3.3 Primary Cell Culture

Primary cell cultures are taken directly from living tissue and subsequently established *in vitro* (also referred to as *ex vivo*) without modification. These are desirable as they have not been manipulated (i.e. by immortalisation), most closely represent in the tissue from which it is derived and exhibit normal anatomy and function. However, primary cell cultures are challenging to prepare and culture, and their experimental application is limited as they are not immortalised. Primary cells eventually die *in vitro*, whereas cell lines can proliferate almost infinitely, though cell lines change in morphology and physiology in later passages and are subject to genetic drift (Hughes *et al.*, 2007). This genetic drift introduces variability in cell lines, but primary cells from different donors are highly variable in their response to e.g. inflammatory cytokines, though it can be argued that this makes them more relevant to true responses in a heterogeneous population of patients. Primary cells take longer to grow *in vitro* than cell lines. Other disadvantages of primary cell cultures include the heterogeneity of the cultures (as tissues in living animals are composed of many cell types). Whilst this may be desirable for a cell culture model that mimics the real-life composition of brain tissue, it may present practical problems in terms of maintenance, as well as when evaluating gene expression changes in the individual cell types. Separation of cell types is necessary for a homogenous population by immunocytochemistry and flow cytometry, targeting the specific cell type markers. There is also a high cost associated with tissue sampling, cell type isolation, and *in vitro* environment optimisation and establishment of primary cells (compared to commercial cell lines). For these reasons, primary cell culture was beyond the scope of this preliminary study, and due to time and financial constraints, it was not considered. However, primary cells represent a potential avenue for investigations in the follow-up work to this study of cell lines. They represent an alternative to *in vivo* experiments but allows research on human tissue without complications

and higher costs associated with in man studies and may be more suitable for a 3D cell culture model designed to simulate a living construct.

5.3.4 Future Work

Differentiated SH-SY5Y cells would be an ideal model for measurement of gene and protein expression levels of candidates and may provide a more suitable model of neuronal activity under inflammatory conditions than the undifferentiated SH-SY5Y. For the astrocyte model, GFAP-negative 1321N1 cells were used due to availability. However, measurement of GFAP in a GFAP-positive cell line e.g. primary cell cultures, could be used for future astrocytic models of inflammation to detect and quantify the amount or ratio of reactive astrogliosis and the effect on candidate expression and function. If successfully induced, changes to candidate gene expressions could be helpful in determining their role in astrogliosis. Monocytes can also differentiate into dendritic cells when treated with interleukin (IL) 4 and granulocyte macrophage colony-stimulating factor (GM-CSF) (Sallusto *et al.*, 1995), and this may represent a possible line of enquiry in future models investigating neuropathic pain *in vitro.*

5.3.4.1 Functional Investigation of Candidates

As protein upregulation does not imply increased protein activity if the gene product is a zymogen, as in the case of the caspases. For this reason, the activity levels of the protein caspases should also be investigated. Activity of other candidates could be investigated using siRNA knockdown or drug inhibition, which would help us to deduce the precise activities of the candidates in the inflammatory models.

5.3.4.2 Pilot Co-Culture Model Experiment

In attempt to create a neuropathic cell model, co-culture of monocytic cells from the immune system and a neuronal cell lines, could be used to mimic the infiltration of immune cells into the

CNS after BBB breakdown in neuropathic pain. This preliminary model is comprised of an adhesive layer of neuronal cells on the bottom of a well, and monocytes in suspension physically separated by an insert with porous membrane (Figure 5.23) which allows cytokines to pass through.

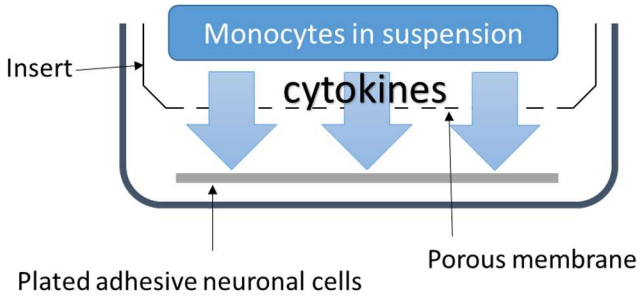


Figure 5.23: Schematic of Co-Culture Model Design for the Development of an In Vitro Model of Neuropathic Pain. Porous inserts physically separate the monocytic cell line (e.g. THP-1) from the neuronal cell line (e.g. SH-SY5Y) and their respective media, but still allows movement of cytokines produced by the monocytes to pass through the membrane and stimulate the neuronal cells on the bottom of the well.

THP-1 cells can be used for the monocytic cells in suspension, and the adhesive cell line SH-SY5Y could provide the neuronal model in the bottom of the well.

The samples in the co-culture model would be as follows:

- SH-SY5Y cells in supplemented DMEM and Ham's F12 media 1:1 mix as the control sample.
- SH-SY5Y cells in supplemented media with IFNγ would allow us to determine the effect of IFNγ on SH-SY5Y cells.
- SH-SY5Y cells in supplemented media with inserts containing THP1 supplemented RPMI (i.e. non-activated THP1 cells).
- SH-SY5Y cells in supplemented media with inserts containing THP1 in supplemented RPMI with IFNγ added 24 hours prior (i.e. pre-activated THP-1 cells). This is the experimental sample.

- SH-SY5Y cells in supplemented media with inserts containing THP1 in supplemented RPMI with IFNγ. This is to test whether THP1 activation can induce an immediate effect on SH-SY5Y cells.
- SH-SY5Y cells in supplemented media with inserts containing THP1 activated by IFNγ 24 hours prior in fresh RPMI.
- SH-SY5Y cells in 1:1 supplemented SH-SY5Y media and supplemented RPMI (from THP1+IFNγ after 24 hours). This is to isolate and evaluate the effect are due to the pro-inflammatory cytokines released into the media during the initial 24 hours post-activation.
- SH-SY5Y cells in supplemented media and insert with fresh supplemented RPMI to identify any effect of the RPMI media on SH-SY5Y gene expression.

As these cell types may require different media, inserts can be used to separate the two cell populations; and avoids cross-contamination of the samples during extraction. The inserts of choice have pores 0.4µm in diameter (THP-1 cells have a diameter of 11µm), which allows cytokines and other proteins (<0.4µm) released from the THP-1 cells to be able to transverse the membrane and reach the SH-SY5Y cells. The density of pores ($2x10^6$ pores per cm^2) allows maximum transport of proteins across the membrane (Figure 5.23). The manufacturer recommends 100-400µl working volume per and 800-1600µl per well were considered during design and 800µl of media was used per well and 400µl was used per insert.

Three genes indicative of the inflammatory state of THP-1, (*MCP1*, *GCH1*, and *CASP8*) could be measured in the THP-1 samples to validate response to cytokine treatment. Monocyte chemoattractant protein-1 (MCP1), also known as chemokine (C-C motif) ligand 2 (CCL2) is secreted by monocytes to attract immune cells during an inflammatory response (Carr *et al.*, 1994) (LL Xu *et al.*, 1996). Increased CCL2 expression in the dorsal horn is also associated with neuropathic hypersensitivity (Jeon *et al.*, 2009). GTP cyclohydrolase I (coded for by *GCH1*) is an enzyme involved in tetrahydrobiopterin (BH_4) synthesis pathway, a key co-factor in

neurotransmitter synthesis (Hatakeyama *et al.*, 1991). BH_4 production is increased in macrophages under inflammatory conditions and is directly involved in pain pathways (Latremoliere *et al.*, 2015). Genetic variants of *GCH1* carry a higher risk of neuropathic pain development (Nasser & Moller, 2014, Veluchamy *et al.*, 2018). Caspase 8 is an executioner caspase that facilitates apoptosis (Tummers & Green, 2017), and in its uncleaved form can regulate IL1β (Tian *et al.*, 2020, Man *et al.*, 2013). Further, caspase 8 expression is lower in THP-1 derived macrophages than in THP-1, so a reduction in *CASP8* would be indicative of the induction of a macrophage phenotype (Yoshino *et al.*, 2018).

Alternatively, to SH-SY5Y cells, the hybrid cell line F11 could be used to represent a neuronal population with dorsal root ganglion (DRG) characteristics if used with a rat or mouse monocytic or macrophage cell line, e.g. mouse WEHI-3 monocytes, mouse RAW 264.7 macrophages, or rat R2 macrophages. F11 is a commercially available somatic cell hybrid of the NT18TG2 mouse neuroblastoma cell line and embryonic rat DRG, with the genomes of both species retained.

The model can be further developed using a mixture of neuronal and glial cell lines, moving towards a valid representation of cell populations in the brain. For example, astrocytic cell lines such as 1321N1 could be used with SH-SY5Y, though a suboptimal compromised media would have to be used for both cell lines. SH-SY5Y cells grow in a 1:1 media mix of DMEM and Ham's. Both 1321N1 astrocytoma and SK-N-SH cell lines are cultured in DMEM media (supplemented with 2mM Glutamine + 10% Foetal Bovine Serum (FBS)), which would make co-culture suitable. However, the required differentiation step for SK-N-SH to display neuronal phenotype makes this cell line an unsuitable candidate for co-culture in the same physical space with another cell line. Retinoic acid treatment would also affect the second cell line and induce unwanted effects.

It is possible to perform the treatment prior to the addition of the second cell line, though it would be necessary to ensure SK-N-SH does not reach confluence too soon.

5.3.5 Conclusion

Despite the drawbacks of immortal cell lines, this investigation using the inflammatory *in vitro* model of physiological systems and complements the *in vivo* work (Chapter 3) and clinical work (Chapter 4) and lays the groundwork for extensive *in vitro* investigations into the function of candidate biomarkers, as outlined in Section 5.3.5. The mRNA and protein expression changes to the candidate biomarkers in the three immortal cell lines, 1321N1 astrocytoma, SH-SY5Y neuroblastoma, and monocytic THP-1, under pro-inflammatory cytokine stimulation, gives us valuable insight into their response to inflammatory conditions. This is relevant to inflammation that occurs in the early stages of neuropathic pain during and immediately after injury.

Chapter 6: General Discussion and Future Perspectives

6.1 Candidate Biomarkers of Neuropathic Pain

The development and maintenance of neuropathic pain is a dynamic process with gene expression and protein activity changing over time after injury (Kim *et al.*, 2009). It is possible that these processes are similar in most cases of neuropathic pain, but some differences are likely as it has many different physiological causes including physical trauma and disease (e.g. diabetes and multiple sclerosis). The heterogeneity of neuropathic pain is the main the reason why the discovery and development of empirical biomarkers and drug targets has not yet been achieved. Despite this, it is possible that common gene expression patterns, tissue or timepoint specific, may exist between cases with different underlying causes.

The results for expression changes of candidates in the rat SNL DH, clinical cohort of neuropathic pain patients, and three inflammatory cell models are presented in Table 6.1. All candidates were measured in the cell models using qPCR, but for some candidates no data could be obtained (Section 5.2). Data for expression changes presented in Table 6.1 are for gene expression (mRNA measured by qPCR) unless otherwise stated (ie. “protein”).

Table 6.1: Summary of Results

Candidate (Human nomenclature)	Gene Expression Change				
			Inflammatory Cell Culture Models (Human)		
	Rat SNL DH (35 days)	Clinical Blood	SH-SY5Y (Neurocytoma)	1321N1 (Astrocytoma)	THP-1 (Monocyte vs. Macrophage)
A3GALT2	Upregulated	Upregulated	No data	Not Significant	No data
ANXA1	Upregulated	Upregulated	Upregulated	Upregulated (protein)	Upregulated
CASP1	Upregulated	Upregulated	No data	No data	No data
CASP4 (CASP5)	Upregulated	Upregulated	Upregulated	No data	Downregulated
CCR5	Upregulated	Upregulated	No data	No data	Downregulated
FPR2	Downregulated	Upregulated	No data	Not Significant	Upregulated
OMP	Downregulated	Not Significant	No data	Not Significant	Not Significant
ROMO1	Upregulated	Not Significant	Upregulated	Not Significant	Not Significant
SH3BGRL3	Upregulated	Downregulated	No data	Downregulated	Upregulated
TMEM88	Upregulated	Upregulated	No data	No data	No data
TXN1	Upregulated	Not Significant	Upregulated	No data	Not Significant

PLAC8	Upregulated	Not Significant	Upregulated	Downregulated	Downregulated

Immune cell trafficking is clearly an active process in the SNL DH, with changes to several relevant genes, and this was elucidated by IPA® analysis of the whole microarray dataset (Chapter 3, Table 3.5). Some anti-inflammatory mediators are upregulated in the SNL DH, including Annexin A1 (*Anxa1)*, Reactive Oxygen Species Modulator 1 (*Romo1),* and Thioredoxin 1 (*Txn1)*. Conversely Formyl-Peptide Receptor 2 (*Fpr2*) is downregulated. It is possible that the acute inflammatory and chronic neuropathic pain phenotypes do not have distinct biomarker profiles. Instead, the transcriptome may change over time in response to the initial insult and neuronal damage. This may also explain why *FPR2* and *SH3BGRL3* are differentially expressed in different directions in different models. The difference in tissue type is also relevant (SNL DH and clinical blood samples were tested). Different tissue types are likely to exhibit different transcriptome profiles, and only brain-derived mRNAs and proteins which can pass through the blood brain barrier (BBB) are detectable in blood samples. Without functional evaluation of the BBB in the clinical cohort, its permeability cannot be assumed to be compromised.

Eight genes were differentially expressed in both the SNL DH and clinical blood samples. There are *A3GALT2, ANXA1, CASP1, CASP4 (CASP5), CCR5, FPR2, SH3BGRL3,* and *TMEM88* (Table 6.1). All except *FPR2* and *SH3BGRL3* were upregulated in both datasets (Table 6.1). *FPR2* was downregulated in the SNL DH and upregulated in the clinical samples. Conversely, *SH3BGRL3* was upregulated in the SNL DH and downregulated in the clinical samples.

The results from the inflammatory cell culture models gives partial insight into changes to candidate genes at RNA and/or protein level in three cell types; neurones (SH-SY5Y neurocytoma), astrocytes (1321N1 astrocytoma), and monocytes/macrophages (THP-1). Interestingly, increased levels of Annexin A1 was detected in all three models (Table 6.1). A comprehensive dataset was not obtained for all candidates in the cell culture models ("No Data", Table 6.1). This was due to poor qPCR data collection, and poor antibody efficiency in Western

blotting techniques. These techniques could be optimised further, but this requires a time-consuming trial and error approach.

Seven of the candidates highlighted in this work have well-defined roles in inflammatory and anti-inflammatory pathways. These are *ANXA1, CASP1, CASP4 (CASP5), CCR5, FPR2, TXN1, and ROMO1.* Though they may have active roles in the development and maintenance of neuropathic pain, they are likely to be involved in the inflammatory processes which contribute to the neuropathic pain phenotype. Therefore, it is unlikely that they are exclusively involved in neuropathic pain and may also have a role in chronic inflammatory pain pathology.

A3GALT2 is represents an interesting novel candidate as its function is not well described in the literature. It is upregulated in both the SNL model and clinical cohort, but not changed in the inflammatory cell models, which might suggest that it has a role specific to the neuropathic changes and is not part of the inflammatory process which occur before and concurrent to the establishment of neuropathic pain. *A3GALT2* is not significant in either the high-score or low-score S-LANSS groups when the clinical cohort is split. It is possible the groups are too small (n) to identify a change in *A3GALT2* expression.

ANXA1 represents an anti-inflammatory mediator which is upregulated across both models of neuropathic pain, and in the inflammatory models. It is unlikely that the role of *ANXA1* is exclusive to neuropathic mechanisms, and its upregulation likely occurs as part of a feedback loop to limit inflammation and tissue damage. Its upregulation across the five models also suggests that this response is not time limited, but may be induced early (cell models are 48-hours of inflammatory treatment) and could be sustained for prolonged periods post-injury (as in the 35-day rat model and clinical cohort). It has limited potential as a biomarker for neuropathic pain but could be evident of a continuous state of both inflammatory and anti-inflammatory mechanisms in the disease state. It is possible that other inflammatory mediators

not identified here may change over time post-injury (or between acute to chronic states) which may be useful as biomarkers for diagnosis of neuropathic pain. Its receptor FPR2 is differentially expressed in the SNL model (downregulated) and in the clinical cohort (upregulated) but given the differences in direction of expression changes its role in neuropathic pain is less clear.

The caspases *CASP1* and *CASP4/5* do not represent ideal biomarkers because they are involved in the terminal caspase cascades of the apoptotic pathways which occur as part of normal cell turnover in all tissues, and can be upregulated in response to infection or tissue damage. Further, their activities are widespread and vital to cell health which makes them unsuitable for pharmaceutical targeting in neuropathic pain treatments.

CCR5 is upregulated in the neuropathic pain model and clinical blood samples, but is downregulated in the inflammatory models. This may suggest that its upregulation is more relevant to neuropathic pain mechanisms rather than exclusively inflammatory conditions. Therefore, upregulation of *CCR5* represents a good potential biomarker for neuropathic pain. This could also support the theory that inflammatory mechanisms are key factors in the neuropathic pain state.

For three candidates, *OMP, SH3BGRL3,* and *TMEM88* there is a loose link in the literature to neurological plasticity and pain pathways, but their exact role not known. For *A3GALT2* and *PLAC8* the role in neuropathic pain is unclear, and they represent potentially novel candidates in the development and maintenance of neuropathic pain. Further investigation into the functionality of these five gene may reveal distinct roles in chronic neuropathic pain.

SH3BGRL3, similar to *FPR2*, is differentially expressed in both models of neuropathic pain but in different directions. It is upregulated in the SNL model and downregulated in the patient cohort. This makes the role of *SH3BGRL3* in neuropathic pain unclear, but given that its functionality is

not yet well-characterised in the literature, *SH3BGRL3* represents a novel candidate for further investigation. It is also *TMEM88* likely represents the most promising biomarker of neuropathic pain biomarker, with a potential role in neuronal plasticity. It is upregulated in both the animal models and patient, and is not differentially expressed in the inflammatory models.

It is important to recognise that this study does not identify proteins which are already well-established as having significant roles in neuropathic pain (Table 1.8) including TRPV1, voltage-gated sodium channels, and purogenic receptors. It is possible that they are amongst the unassigned gene in the Affymetrix RTA 1.0. They were not measured in the clinical cohort. TRPV1 has been previously implicated in studies of SNL (Jiang *et al.*, 2013, Hudson *et al.*, 2001), as has $Na_v1.7$ (Li *et al.*, 2019) and purogenic receptors (Kage *et al.*, 2002). It crucial that clinical biomarkers demonstrate consistent results across neuropathic pain studies and is well characterised across different etiologies and models.

6.2 Future Work

According to the biomarker development pipeline, it is essential that function of potential biomarkers is thoroughly investigated to accurately evaluate their activities in healthy individuals and disease patients. This knowledge will allow the appropriate use of biomarker measurement in clinical settings, and may have implications for drug targeting and pharmaceutical development.

MicroRNAs

MicroRNAs were measured in the RTA 1.0 microarray (Chapter 3, Table 3.5), but differential expression changes were not investigated further in the clinical cohort. However, the role of these microRNAs in neuropathic pain is a potential avenue for further investigation given the implications for altered protein production. The stem-loop reverse transcription (RT)-based

TaqMan MicroRNA assay is a commonly used method (Chen 2011). MicroRNA quantification is possible using fluorescence dyes with appropriate sample preparation (Raymond 2005, Sharbati-Tehrani 2008). Modified PCR dye kits are available for microRNA targets. Specific microRNA isolation protocols are required, and can be done with trizol and chloroform precipitation (Zununi *et al.,* 2016), or with specialised isolation kits e.g. Ambion mirVana™. Specialised kits include the incorporation of poly-A tails to the microRNA molecules to increase stability (Rissland *et al.*, 2017).

Clinical Work

A complete medication dataset could elucidate the effect of currently recommended neuropathic pain treatments on the disease transcriptome. Medication data would also be required for control patients. This is necessary to elucidate the precise effects of medications on potential neuropathic pain biomarkers, and patient response to treatment (e.g. pain improvement or adverse effects). It is possible that control cohort participants (non-neuropathic controls) could be taking medications recommended for neuropathic pain, as they are primarily used in the treatment of other diseases e.g. anti-convulsant medications in epilepsy, anti-depressant medications in mental health disorders.

It is difficult to collect comprehensive and accurate data on patient medication. In addition to the information provided by questionnaires (completed by patients), a qualitative sensory test element could be included to provide a quantitative measure of hypersensitivity. This may also be useful for a time-course experiment to analyse severity over time or patient response to a treatment. A larger patient cohort would increase statistical power and allow a more comprehensive analysis of S-LANSS scores in neuropathic pain, and the effect of medications (current and previous) on candidate expression.

Hypoxia and Oxidative Stress

In addition to co-culture models discussed in Chapter 5, changes to candidate expression and function in hypoxia and oxidative stress conditions would give some insight into how they might behave after injury in neuronal and astrocytic cells (Hosseini & Abdollahi, 2013, Sagalajev *et al.*, 2018, Shinozaki *et al.*, 2005). This would complement the inflammatory models by providing a sterile model of stress relevant to neuronal injury.

Mechanistic Study of Candidates *In Vitro*

Overexpression and knockout of candidates would give us a clearer idea of their role in different cell lines, and how they interact with downstream processes, by investigating subsequent effects on other genes and proteins in a cell line. By repeating such experiments in several cell lines (e.g. SH-SY5Y, 1321N1, and THP-1) would allow us to determine which activities are cell specific and which activities are performed in more than out cell type. Transfection of cell lines with plasmids containing candidates would provide a model of overexpression. Knockdown of candidates can be achieved by pharmaceutical inhibitor of the protein or by short-inhibiting RNA (siRNA) knockdown of the mRNA. Such work is essential for the candidates which have an unclear role and no well-defined role in inflammation or pain, including *A3GALT2* and *PLAC8*.

6.3 Perspectives and Conclusions

This study has highlighted the complicated nature of chronic neuropathic pain and the multitude of interwoven pathways – neurological, inflammatory, and apoptotic – that contribute to the development and maintenance of the neuropathic pain state. The results from this study help clarify the effect on candidate gene expression and protein levels in relevant cell models under inflammatory conditions. The change may be exclusive to inflammatory conditions, or also relevant to neuropathic mechanisms. Though neuropathic and inflammatory pain are often

described as discrete pain conditions, neuropathic pain by processes of tissue damage, involves inflammation at least in the initial stages (Ellis & Bennett, 2013). Twelve novel genes and their protein products are highlighted as potential candidate biomarkers for neuropathic pain, based on their differential expression in both an animal model of neuropathic pain and in a clinical cohort of patients.

It is likely an improved diagnostic test for neuropathic pain will be comprised of several components, including a pain experience questionnaire, measurement of known clinical biomarkers of chronic pain, and measurement biomarkers specific to neuropathic pain pathology. These can then be used throughout subsequent treatment regimens to monitor patient response.

The impact of an improved diagnostic approach and efficacious neuropathic pain specific treatments will be great for both individual patients and society. Diagnostic tests which are specific to neuropathic pain can provide a definitive and timely diagnosis and will reduce patient distress by reducing the delay for an appropriate treatment. Improved treatment regimens would have a profound impact on patients with neuropathic pain in its early stages by preventing or limiting development of more severe symptoms or pain chronicity. An effective treatment would help patients with existing or later stage neuropathic pain by relieving symptoms. It would also relieve neuropathic pain symptoms for patients with multiple sclerosis and diabetes and could have a knock-on effect on concurrent psychological illnesses including depression and anxiety in patients. A cure may be possible if neuropathic pain pathways involved in its development can be reversed, or healthy physiology can be restored. Nevertheless, with improved symptoms management, patients will have greater quality of life, with more independence and less reliance on carers, and an improved ability to perform daily tasks. It will relieve the strain on healthcare, and the economy could greatly benefit from reduced loss in

workdays to neuropathic pain. Effective non-opioid analgesia also has the potential to relieve the current opioid crisis in North America (Gruss *et al.*, 2019; LaPietra & Motov, 2019).

There is a large amount or work needed to be done to further investigate the role of the candidates reported here and evaluate their potential either as neuropathic pain biomarkers or as targets for pharmaceutical treatments. However, the confirmation of their differential expression in both an animal model of neuropathic pain, and a clinical cohort provides strong evidence for their role in the pathophysiology of the condition. The improvement of neuropathic pain diagnosis and treatment would greatly benefit both individuals and society, and therefore this research is highly relevant and has the potential to improve many lives.

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