Two methods of biomarker discovery: applications in neuropathic pain and pharmacotherapy

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Abstract

Biomarkers have potential utility in the treatment of pain as diagnostics and for quantification of drug efficacy and safety. A qualified biomarker will capture overlapping disease mechanisms and will be responsive to treatment. The necessity for these strict requirements renders it difficult to discover new biomarkers, particularly one that is reliable, practical and non-invasive, and simple for routine utilisation. This thesis demonstrates that two approaches may be useful to overcome these challenges: bottom-up and top-down biomarker discovery and development. Current animal models of neuropathic pain are inadequate to develop biomarkers as they only cover 'no pain' and 'high pain': not the heterogeneity that exists between these extremes. Therefore, a novel rat model of graded neuropathic pain was developed by advancing the existing chronic constriction injury model. Sciatic nerve and subcutaneous chromic gut sutures were varied, resulting in 'dose-dependent' behavioural allodynia. Allodynia was correlated with microglial activation marker expression in the ipsilateral lumbar dorsal horn of the spinal cord, suggesting that changes in behaviour are associated with disease mechanisms. A literature review of the pathophysiological mechanisms of pain, filtered by the criterion for accessible biomarkers, revealed that the peripheral immune system was the ideal target for the bottom-up approach. As such, the graded model was then used to explore peripheral immune mechanisms in order to begin the process of construct validation of potential neuropathic pain biomarkers. It was demonstrated that peripheral immune cells significantly contribute to chronic constriction injury-induced allodynia, as adoptive transfer of splenocytes or peripheral blood mononuclear cells from high pain donors to low pain recipients potentiates allodynia. Intrathecal transfer of high pain immune cells to low pain recipients potentiated allodynia, confirming that infiltrating immune cells are not passive bystanders, but actively contribute to nociceptive hypersensitivity in the lumbar spinal cord. The graded transcriptome of dorsal horn of the ipsilateral lumbar spinal cord was compared with that in the blood, identifying chemokines and transcription factors as potential blood-borne biomarkers of neuropathic pain. The top-down approach

explored the utility of saccadic eye movements as an objective, functional biomarker of sedation, an adverse effect associated with opioid treatment of pain. This study compared the interaction between sleep deprivation and opioids on opioid-naïve with opioid-tolerant participants. The naive-participant study evaluated the effects of sleep deprivation alone, morphine alone and the combination; the tolerant-participant study compared day-to-day effects of alternate-daily-dosed buprenorphine and the combination of buprenorphine on the dosing day with sleep deprivation. Psychomotor impairment was measured using saccadic eye movements, other oculomotor measures and an alertness visual analogue scale (VAS). Saccadic eye movements demonstrated an additive interaction between acute opioids and sleep deprivation, however the nature of the interaction between chronic buprenorphine and sleep deprivation remained unclear. This study revealed greater saccadic eye movement, but not VAS impairment in tolerant versus naive participants, suggesting that chronically dosed patients may not become tolerant to the sedative effects of opioids. These findings open up a number of new opportunities for pain biomarker development within the peripheral immune system, identify potential pain biomarker candidates, as well as further validating saccadic eye movement analysis as a biomarker of sedation. This thesis highlights that bottom-up and top-down approaches are appropriate methods for biomarker discovery and development.

Declaration

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Statement of Authorship

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Mr. Grace had a major input in the experimental design, performed most surgeries, most behavioural testing, tissue collection immunohistochemistry imaging and densitometry, statistical analysis and graphical presentation of the data collected, and prepared the manuscript for submission.

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Mr. Grace had a major input in the experimental design, recruited all participants, conducted all experimental procedures, some statistical analysis and all graphical presentation of the data collected, and prepared the manuscript for submission.

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Mrs. Gentgall was responsible for the clinical trial logistics and provided editorial assistance.

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Prof. Rolan was involved in the experimental design, contributed to the data interpretation and preparation of the manuscript.

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Abbreviations

5-HT	5-hydroxytryptamine/ serotonin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid
ASIC	Acid-sensing Ion Channel
ATP	Adenosine triphosphate
AVAS	Alertness visual analogue scale
BDNF	Brain derived neurotrophic factor
BK	Bradykinin
BOLD	Blood oxygenation level dependent
CB	Cannabinoid
CCI	Chronic constriction injury
CGRP	Calcitonin gene related peptide
CIP	Compact integrated pupillograph
CNS	Central nervous system
COX	Cyclooxygenase
CSF	Cerebrospinal fluid
CSGAAS	Cardiff saccades generating and analysis system
DA	Dark Agouti
DLF	Dorsolateral funiculus
DRG	Dorsal root ganglion
DSST	Digit symbol substitution test
EBN	Excitatory burst neuron
ECF	Extracellular fluid
EOG	Electro-oculography

EW	Edinger Westphal
FEF	Frontal eye field
fMRI	Functional magnetic resonance imaging
GABA	γ-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GKO	Gene knockout
IASP	International Association for the Study of Pain
IBN	Inhibitory burst neuron
IFN	Interferon
IL	Interleukin
IN	Internuclear neuron
i.p.	Intraperitoneal
i.t.	Intrathecal
LC	Locus coeruleus
LDI	Laser Doppler imaging
LIP	Lateral intraparietal area
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
medRF	Medullary reticular formation
MHC	Major histocompatibilty complex
MVN	Medial vestibular nuclei
N	Neuronal
NA	Noradrenaline
NFκB	Nuclear factor κB

NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NNT	Number needed to treat
NO	Nitric oxide
NOS	Nitric oxide synthase
NPH	Nuclei prepositus hypoglossi
NRS	Normal rat serum
OIH	Opioid induced hyperalgesia
OPN	Omnidirectional pause neurons
PAG	Periaqueductal grey
PAMP	Pathogen-associated molecular patterns
PAT	Pupil adaptation test
PBMC	Peripheral blood mononuclear cell
PET	Positron emission tomography
PG	Prostaglandin
PLR	Pupil light reflex
PNL	Partial nerve ligation
PNS	Peripheral nervous system
PO	Postoperative
PPRF	Paramedian pontine reticular formation
PSV	Peak saccadic velocity
QST	Quantitative sensory testing
ra	Receptor antagonist
rCBF	Regional cerebral blood flow

ROS	Reactive oxygen species
RPD	Resting pupil diameter
RVM	Rostral ventromedial medulla
S	Subcutaneous
S1, 2	Somatosensory cortex, primary, secondary
SC	Superior colliculus
SD	Sprague Dawley
SEF	Supplementary eye fields
SEMs	Saccadic eye movements
SG	Substantia gelatinosa
SNL	Spinal nerve ligation
SSRI	Selective serotonin reuptake inhibitors
TASK	Tandem of P domains in a Weak Inward rectifying K^+ channel-related acid-sensitive K^+
TCA	Tricyclic antidepressant
T_H	Helper T cell
TLR	Toll like receptor
TNF	Tumour necrosis factor
TREK	Tandem of P domains in a Weak Inward rectifying K^+ channel -related K^+ channel
trkA	Tyrosine kinase receptor A
TRPA	Transient receptor potential subfamily A
TRPV	Transient receptor potential vanilloid
VAS	Visual analogue scale

Chapter 1. Introduction

There are currently many new technologies and tools available for predictive human models of disease and clinical outcomes, diagnosis, treatment and disease monitoring, and drug discovery and development. These include mathematical modelling, biosimulation, systems biology and biomarkers, which are invariably interrelated. Despite considerable interest, a biomarker of pain has remained elusive and thus the subject of this thesis is highly topical. Before delving into the mechanisms of pain and identifying targets for biomarker discovery and development, some brief definitions, as well as the issues surrounding biomarker validation, will be presented.

1.1. Biomarkers

1.1.1. Definitions and classification framework

The NIH Biomarker Working Group (2001) has removed some of the confusion surrounding terminology and roles of biomarkers by describing them as 'a characteristic that can be measured and evaluated as an indicator of normal biologic processes, pathologic processes or pharmacologic responses to therapeutic intervention'. The idea biomarkers can be used to detect pathology as well as pharmacological efficacy is implicit in this definition. The latter has been further developed by Danhof and colleagues (2005) who proposed a 7 point mechanistic framework that identifies biomarkers on the causal path between drug administration and effect (figure 1-1). This framework will be further discussed using depression and SSRI (selective serotonin reuptake inhibitor) treatment as an example of these biomarker types.

A type 0 biomarker refers to genotype and/or phenotype as a determinant of the drug response. This may be related to either a factor in the disposition of the drug that determines the target exposure (i.e., the expression of a specific enzyme or transporter) or a factor determining the response directly (i.e.,

the expression of a specific receptor). E.g. the genotype of serotonin reuptake transporters, as this corresponds to the basal levels of 5-hydroxytryptamine/ serotonin (5-HT) available.

Figure 1-1. Schematic representation of the mechanistic classification of biomarkers.

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A type 1 biomarker refers to the concentration of the drug and/or a drug metabolite. Drug concentrations in blood are probably the most widely used biomarkers. The target site concentration is ultimately the most useful biomarker of this class, however free target site concentrations may not be readily accessible, as with drugs acting in the central nervous system (CNS) like SSRIs.

A type 2 biomarker refers to the target occupancy. In theory, drug effects may occur at different degrees of target occupancy. Information on the relationship between target occupancy and response is therefore important for the prediction of *in vivo* concentration-effect relationships and for the understanding of intra- and inter- individual variability in drug response. E.g. competitive SSRI binding studies using positron emission tomography (PET).

A type 3 biomarker refers to quantification of the target site activation. According to receptor theory, this target site activation is determined by the intrinsic efficacy of the drug in combination with the level of receptor expression in the target tissue. E.g. measurement of quantitative electroencephalography frequency changes in response to SSRI administration.

A type 4 biomarker refers to physiological measures in the integral biological system. An important feature of type 4 biomarkers is that the biomarker response is often influenced by *in vivo* homeostatic

control mechanisms. E.g. symptoms of increased serotonin such as increased body temperature, sweatiness and/ or tremor.

Type 5 biomarkers are parameters that characterize, in quantitative manner, disease processes. E.g. acutely this may be manifested as increased serotonin in the cell body and inhibition of the rate of neuron firing by 5-HT autoceptors; chronically there may be down-regulation of 5-HT autoceptors and disinhibition of serotonin resulting in the delay in treatment response.

Finally, type 6 biomarkers are clinical scales. E.g. mood scales.

The NIH Biomarker Working Group defines clinical endpoints as "a characteristic that reflects how a patient feels, functions, or survives" (2001). Surrogate endpoints are intended to substitute for clinical endpoints (Wagner, 2009), however, Rolan (1997) highlights that these cases are extremely rare. Thus, the classification of a surrogate refers to a subset of highly validated biomarkers.

A model 'is an experimental system used in drug development to simulate some aspects of the disease of interest in which the effects of the drug are to be examined' (Rolan, 1997). In some cases a model may fit under the definition of a biomarker when it simulates key aspects of a disease state and is responsive to treatment (type 4 and 5 biomarkers). These models and their contribution to the study of neuropathic mechanisms have been thoroughly reviewed by Klein and colleagues (2005) and commonly applied examples include capsaicin and the cold pain test. These models can be used in healthy volunteers, or to challenge chronic pain patients in order to stimulate and quantify altered nociceptive processing and treatment response.

The relationship between biomarkers, surrogate endpoints and models is represented as a Venn diagram in figure 1-2.

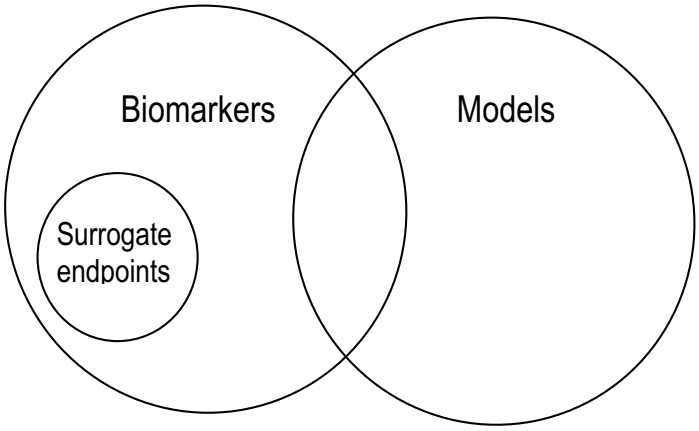

Figure 1-2. The relationship between biomarkers, surrogate endpoints and models.

Surrogate endpoints are subsets of biomarkers, whilst models intersect with biomarkers as some models may fit under the definition of a biomarker ((biomarkers ⊇ surrogate endpoints) and biomarkers ∩ Models).

1.1.2. Biomarker utility

Biomarkers have a number of key uses, including detection and quantification of disease progression, drug discovery and development, and quantification of drug efficacy and safety.

Diagnostic biomarkers have a role in clinical practice, such as diagnosis of pathophysiological states (e.g. prostate-specific antigen for the diagnosis of prostate cancer). They have particular utility when pathophysiological responses cannot be observed directly, like Alzheimer disease, where diagnosis based directly on neurodegenerative processes in the brain is not accessible, but phosphorylated τ and β-amyloid levels are measured in cerebrospinal fluid (CSF) are instead used as surrogates. Another clinical application of biomarkers is in the quest for personalised medicine. Heterogeneity to treatment response, as outlined below in the context of pain treatment, is a major clinical problem. However if biomarkers can identify populations that will respond to certain therapies, then they can be used to alter drug prescribing patterns through targeted therapy. This application of biomarkers may reduce the number needed to treat (NNT) by ensuring that the individual patient is treated with the appropriate drug

and reducing adverse affects. In addition to the improved safety profile due to a lower NNT, specific biomarkers may also be developed to monitor adverse events (Rolan et al., 2007; Wagner, 2009; Sistare et al., 2010).

Biomarkers have an important role to play in redefining traditional drug discovery (Colburn, 2003), as increasing costs of clinical drug development and increased market competitiveness render it more desirable to obtain an early answer to the likely clinical and commercial success of a novel compound (Rolan, 1997; Chizh et al., 2008; Lathia et al., 2009; Woodcock, 2009). The potential role of biomarkers in drug development is to confirm primary pharmacology and to extrapolate mechanism based modelling from a) *in vitro* test systems to the *in vivo* situation; b) *in vivo* animal studies to humans; c) healthy volunteers to patients; and d) the prediction of intra- and inter- individual variability in drug effects (Colburn, 2003; Colburn and Lee, 2003). Overall, biomarkers are useful to explore potential clinical effects of a drug, to obtain insights into disease mechanisms, to examine effects of control systems and to understand dose-concentration-response relationships. Within clinical drug development, biomarkers can potentially lower costs by rapid determination of the therapeutic potential of a novel compound, decreased cohort size due to reduced variability and enriched trial designs (Moore et al., 1998). These applications may ensure that an efficacious drug in a targeted population is clinically and commercially successful (Chizh et al., 2008; Woodcock, 2009).

Biomarkers have been sought in a broad range of research fields such as cancer (Petrik et al., 2006; Ransohoff, 2009), CNS diseases (Blumberger et al., 2008; Kaddurah-Daouk and Krishnan, 2009) and inflammatory diseases like osteo- and rheumatoid arthritis (van den Broek et al., 2008; Huang and Wu, 2009). There is great clinical need for biomarkers for neuropathic pain and the safety of neuropathic pain pharmacotherapy (Chizh et al., 2008).

1.1.3. Validation and qualification

In order for a biomarker to be useful, it should be clinically relevant, such that it is linked to biological and clinical endpoints; sensitive and specific to treatment effects; reliable; practical and non-invasive; and simple for routine utilisation without the need for expensive equipment, skilled operators or extensive time commitment (Lesko and Atkinson, 2001; Lee et al., 2006). Many of these factors are addressed through the process of validation and qualification.

Validation and qualification are key issues that address how reliable a biomarker is for the intended application and hence how confidently the data can be used for advanced clinical or business decision making (Lee et al., 2006; Wagner, 2009; Goodsaid and Papaluca, 2010; Sistare et al., 2010; Warnock and Peck, 2010). Validation refers to performance assessment of the analytical methods, whereas clinical qualification is the evidentiary and statistical process linking biologic, pathologic and clinical endpoints (Lee et al., 2006). The most rigorous standards of biomarker validation and qualification have been advocated by Fleming and DeMets (1996), stipulating that a biomarker must be correlated with the true clinical outcome and must fully capture the net effect of treatment on a clinical outcome. An additional criterion established by the International Conference on Harmonisation's 'Statistical Principles for Clinical Trials' (1998) was biological plausibility of the relationship. It is also highly important that a biomarker is standardised, such that it is reliable over time, and between subjects, investigators and laboratories. Consistency in results from multiple studies will then increase the strength of evidence to inform individualised treatment. It has been emphasised that validation and qualification is not binary, that is, valid or invalid, but rather a continuous variable between the two extremes (Rolan, 1997; Lee et al., 2006; Woodcock, 2009). Therefore, validation and qualification is comprised of three levels: criterion (gold-standard and treatment response), construct (evidence of a shared mechanism between the biomarker and clinical endpoint), face (biological plausibility) validity.

1.1.3.1. Criterion validity

Criterion validity is a measure of how well the test agrees with the gold standard (the 'criterion') (Rolan, 1997). It examines the strength of the statistical correlation under basal conditions and, to be a useful guide, the biomarker's ability to quantitatively predict changes in clinical outcome after several interventions of different types. Rolan (1997) and Lathia and colleagues (2009) are aware of the difficulties of this requirement, in that it is not feasible to develop drugs in the absence of a well-validated biomarker. However a biomarker cannot be properly validated without demonstrating responsiveness to drugs with different mechanisms. So while criterion validity is established using prior data (Rolan, 1997), it has little utility when it comes to the innovation of new biomarkers. A further challenge for criterion validation for biomarker of neuropathic pain is that many treatments target symptoms rather than the underlying disease mechanisms. A mechanistic biomarker, therefore, may not detect treatment response to current symptomatic therapies.

1.1.3.2. Construct validity

Construct validity requires support for the assumption being made, that is the scientific evidence of a shared mechanism between the clinical outcome and the biomarker (Rolan, 1997). This is probably the most important aspect of validation, but can be the hardest to measure. This level of validation is very important when discovering new biomarkers and will, in effect, become the criterion for further biomarker research. Lathia and colleagues (2009), whilst concurring that this level of validation is fundamental, highlight that it may be flawed when the clinical endpoint is subjective and/ or highly variable (e.g. stroke, Alzheimer disease and osteoarthritis) as the new biomarker may capture the true disease burden, resulting in a poor statistical correlation with the clinical endpoint.

1.1.3.3. Face validity

Face validity refers to the biological plausibility of the biomarker. It is possible that a certain biomarker may have good face validity, but not necessarily better construct or criterion validity. For example a driving simulator may have better face validity than a computerised tracking test, but may not have better construct or criterion validity. Lathia and colleagues (2009), however, contest this recommendation of plausibility as an absolute validation method on the grounds that it does not appear to discriminate between the successes and failures, as biomarkers are always thought plausible at the time of use. They also contend that the inverse is true, in that molecular biomarkers identified by complex mathematical and statistical methods will not have initial face validity.

1.1.4. Two methods of biomarker discovery

Before biomarkers can be validated and qualified, they must first be identified as candidates. This thesis will demonstrate two methods of biomarker development in the context of pain and pain pharmacotherapy: bottom-up and top-down discovery and development. However, as a valid and qualified biomarker will encompass overlapping mechanisms rather than relying on a single protein or gene, it is necessary to present some background information in order to understand where mechanism-based biomarkers can be identified within the pathophysiology of neuropathic pain. To this end, I will begin by reviewing the mechanisms underlying neuropathic pain and pain pharmacotherapy, as they are currently understood, before identifying leads within these pathways for pain biomarker development.

1.2. Pain

The International Association for the Study of Pain (IASP) defines pain as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Merskey and Bogduk, 1994), and thus, pain is a multidimensional experience (Neugebauer et al., 2009) (figure 1-3).

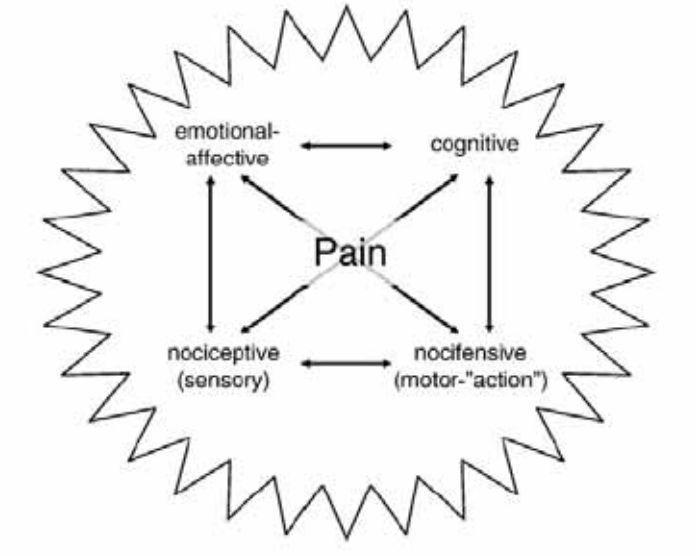

Figure 1-3. The components of the multidimensional pain experience.

The emotional-affective, cognitive, nociceptive and nocifensive components interact to form the multidimensional experience of pain. The reciprocal relationships are indicated by arrows. Reprinted from Brain Research Reviews, Vol. 60, Neugebauer et al., Forebrain pain mechanisms, 226-42, © 2009, with permission from Elsevier.

Acute pain, such as that following trauma or surgery, serves a biological purpose because it signals injury or disease and subsides as healing progresses. It is useful and adaptive to warn the individual of danger and the need to escape or seek help. However, when pain lasts longer than three months or beyond the time when an acute injury would be expected to have healed, the patient's presentation becomes more complex, often with more psychological features such as anxiety, depression and/or altered sleep patterns (Castro and Daltro, 2009; Neugebauer et al., 2009), as alluded to in the IASP definition. Pain at this stage is said to be 'chronic' and probably not directly related to any ongoing injury.

An Australian survey found that 17% of males and 20% of females suffer from chronic pain (Blyth et al., 2001), which commonly includes low back pain, arthritis and recurrent headache. Chronic pain comes to dominate the life and concerns of the patient and often also family, friends and other caregivers, leading to a campaign from the World Health Organisation and IASP to raise awareness for chronic pain as a specific health care issue and a disease in its own right. In addition to the severe erosion in quality of life, chronic pain imposes severe financial burdens on many levels, including costs of healthcare and welfare; job absenteeism; and loss of income. A recent report from Access Economics (2007) calculated this cost at over $34 billion annually, ranking it the 3rd most expensive health problem in Australia.

Diagnostic terminology for pain is being replaced with mechanistic descriptors to more accurately describe the condition (Woolf et al., 1998; Woolf, 2004; Finnerup and Jensen, 2006). For example, the term 'cancer pain' is replaced with, and broken down to, tissue pressure from the tumour, blocked blood vessels causing poor circulation, bone fracture from metastases, infection, inflammation, adverse effects from cancer treatment, and neuropathy. These terms describe the mechanisms, which may in turn allow for targeted treatments. Therefore, on an aetiological and mechanistic basis, the following types of pain can crudely be distinguished, although there is considerable overlap within these mechanisms: nociceptive; chronic inflammatory; and neuropathic. In the absence of a neurological disorder or peripheral tissue abnormality, the concept of a fourth pain category has been introduced, supported by the existence of an abnormal central operation of inputs leading to pain hypersensitivity (e.g. fibromyalgia, complex regional pain syndrome types I and II) (Talley and Spiller, 2002; Desmeules et al., 2003; Banic et al., 2004; Harris et al., 2007; Verdu et al., 2008; Costigan et al., 2009b).

1.2.1. Nociceptive pain

Nociceptive pain arises from mechanical, chemical or thermal irritation of peripheral sensory nerves (e.g. after surgery or trauma or associated with degenerative processes such as osteoarthritis), and is typically described as sharp and well localized (Goucke, 2003). This type of pain is adaptive, alerting the

individual to danger and protecting against further tissue damage by the milieu of pro-inflammatory mediators that sensitise nociceptors, receptors that detect noxious stimuli, resulting in a reduced threshold and increased responsiveness (Woolf and Ma, 2007), known as peripheral sensitisation (Woolf and Salter, 2000). Nociceptive pain resolves as the original injury heals.

A pertinent example of the protective role of pain is that of patients with hereditary sensory and autonomic neuropathy type 4 due to mutations in the nerve growth factor trkA receptor, resulting in a failure of embryonic nociceptor survival (Verpoorten et al., 2006). This mutation results in congenital pain hyposensitivity so that patients burn and chew their tongues and lips without detecting damage, and consequently lose the tips of their fingers and damage their joints.

1.2.2. Inflammatory pain

Inflammation is associated with *rubor* (redness), *calor* (heat), *tumor* (swelling), *dolor* (pain) and *functio laesa* (loss of function). Acute inflammation serves a useful role as an immediate and early response to injury, which is designed to deliver leukocytes to the site of injury. If the injury is not resolved, or in chronic inflammatory diseases such as tophaceous gout, chronic inflammation ensues, resulting in mononuclear cell invasion; tissue destruction which is largely directed by the inflammatory cells; and repair involving angiogenesis and fibrosis (Mitchell and Cotran, 2003). Chronic inflammation can result from viral infections (e.g. Hepatitis C), persistent microbial infections (e.g. peptic ulcers caused by *H. Pylori*), prolonged exposure to potentially toxic agents (e.g. asbestosis of the lungs) and autoimmune diseases (e.g. rheumatoid arthritis or multiple sclerosis). The pain associated with inflammation is caused by inflammatory mediators such as prostaglandins (PGs), bradykinin and cytokines. Many current pain therapies mainly target PG synthesis by inhibiting the precursor cyclooxygenase (COX) enzymes.

1.2.3. Neuropathic pain

Neuropathic pain is classified around the unifying feature of a lesion, damage or disruption to the peripheral nervous system (PNS) due to trauma, compression, tumour invasion, ischemia, inflammation, metabolic disturbances, nutritional deficits, cytotoxic agents and degenerative agents (Woolf, 2004); or to the CNS due to spinal cord injury, stroke, or multiple sclerosis (Ducreux et al., 2006). Neuropathic pain is currently classified on the basis of these aetiologies or the anatomical distribution of the pain. Although this classification has some use for the differential diagnosis of the neuropathy, it offers no framework for clinical management of the pain, as it does not capture the manifestation of maladaptive plasticity (Woolf and Mannion, 1999; Finnerup and Jensen, 2006; Costigan et al., 2009b). The diagnosis of neuropathic pain would benefit greatly from a mechanism-based approach, described above, beyond aetiological or disease-based clusters, anatomical, or symptomatic classification. However, the application of this approach is currently limited by the lack of appropriate tools, a gap that may be filled by biomarkers.

Neuropathic symptoms are usually spontaneous and typically described in terms of unpleasant, abnormal sensations such as ‘pins and needles’, ‘burning’ or ‘shooting pain’ (dysaesthesia) and are associated with exaggerated nociceptive states, such as pain in response to non noxious stimuli (allodynia), increased pain in response to noxious stimuli (hyperalgesia), or increased pain in response to repetitive stimuli (hyperpathia) (Goucke, 2003). Some of the pathophysiological mechanisms underlying the generation and maintenance of neuropathic pain are well understood, although this knowledge is incomplete. Current evidence suggests that mechanisms of peripheral and central sensitisation, ectopic activity of primary afferents and glial activation underlie neuropathic pain (Roza et al., 2003; Woolf, 2004; Scholz and Woolf, 2007; Costigan et al., 2009b). Opioids, anticonvulsants and antidepressants are currently the most widely administered analgesics for neuropathic pain, however they are only partially effective in treating these symptoms.

1.3. Pain transmission

René Descartes can be credited with the first documented attempt to understand pain, with his theory of pain transmission through a single channel in the skin to the brain. This simplified, mechanical scheme was published in 1664 in the *Treatise of Man*. The specificity theory dominated pain study and treatment for the next 330 years, proposing that a specific pain pathway carries messages from a pain receptor in the skin to a pain centre in the brain, which implies that simply the cutting of this pathway will result in complete analgesia. Many clinical cases demonstrate that this is not the case, but that damage to nerves can often result in exacerbation of painful symptoms, leading to central, unremitting pain. Ronald Melzack and Patrick Wall (1965) intensely disputed this theory by proposing the gate control theory. This rejuvenated the field of pain study and treatment and led to further investigation into the phenomena of spinal sensitisation and central nervous system plasticity. Research of recent years has shown pain is not modality specific, but rather that pain processing is an integrated matrix that occurs at the peripheral, spinal and supraspinal sites.

The following discussion will summarise the current conceptual and mechanistic understanding of pain transmission and is organised according to the peripheral to spinal to supraspinal signalling cascades that follow a peripheral nociceptive stimulus, incorporating changes to this system that are induced by neuropathic pain.

1.3.1. The peripheral nociceptive pathway

Fibres that innervate regions of the head and body arise from cell bodies in the trigeminal and dorsal root ganglia (DRG) respectively. These fibres are called nociceptors, a term coined by Sherrington (1906) over a century ago to describe the nerves responsible for detecting noxious stimuli. The nociceptor has four major functional components 1) the peripheral terminal which transduces external stimuli and initiates action potentials, 2) the axon that conducts the action potentials, 3) the cell body

that controls the identity and integrity of the neuron in the DRG, 4) the central terminal which forms the presynaptic element of the first synapse in the sensory pathway in the CNS (Woolf and Ma, 2007) (figure 1-4). Nociceptors can be categorized into three main groups anatomically and functionally: Aα- and Aβ-nociceptors are large diameter, myelinated fibres, detecting innocuous stimuli and do not contribute to pain; Aδ-fibres are medium diameter, thinly myelinated, rapidly conducting fibres; C-fibres are small diameter, unmyelinated slow conducting fibres (Julius and Basbaum, 2001).

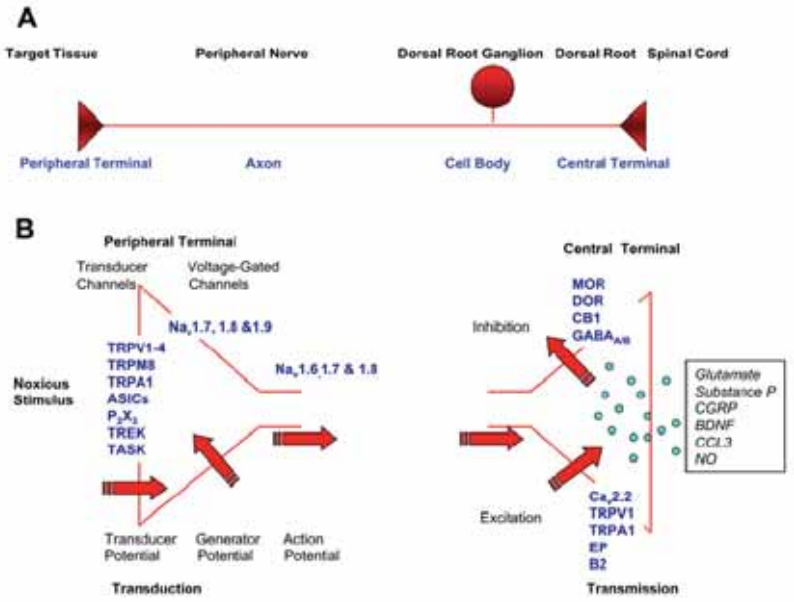

Figure 1-4. The nociceptor.

(A) The operational components of the nociceptor include 1) the peripheral terminal which transduces external stimuli and initiates action potentials, 2) the axon that conducts the action potentials, 3) the cell body that controls the identity and integrity of the neuron in the DRG, 4) the central terminal which forms the presynaptic element of the first synapse in the sensory pathway in the CNS. (B) Transduction is mediated by ion channels, which depolarize the peripheral terminal activating voltage dependent Na+ channels and an action potential. Transmission occurs in response to Ca^{2+} influx at the central terminal releasing glutamate and other synaptic modulators, subject to excitatory and inhibitory influences. B2: bradykinin receptor; BDNF: brain derived neurotrophic factor; CB1: cannabinoid receptor; CGRP: calcitonin gene related peptide; DOR: dynorphin opioid receptor; EP: PG receptor; GABA: γ-aminobutyric acid receptor; MOR: mu opioid receptor; NO: nitric oxide. Additional acronyms explained in Table 1-1. Reprinted from Neuron, Vol. 55, Woolf and Ma, Nociceptors - Noxious Stimulus Detectors, 353-64, © 2007, with permission from Elsevier.

1.3.1.1. Detection of noxious stimuli by ion channels

Nociceptors express many ion channels that detect noxious stimuli and are summarised in Table 1-1.

Table 1-1. Nociceptor ion channels.

TRPV1-4: transient receptor potential vanilloid types 1-4; TRPA1: transient receptor potential ankyrin transmembrane proteins type 1; trk A: tyrosine kinase A; ASIC: Acid Sensing Ion Channel; ATP: adenosine triphosphate; TREK: Tandem of P domains in a Weak Inward rectifying K^+ channel -related K^+ channel; TASK: Tandem of P domains in a Weak Inward rectifying K^+ channel-related acid-sensitive K^+; 5-HT: 5-hydroxytryptamine.

Channel	Modality/ ligand	Reference
TRPV1-4	Noxious heat	(Caterina et al., 1999; Caterina and Julius, 2001; Guler et al., 2002; Peier et al., 2002b; Xu et al., 2002)
	Acid	(Caterina and Julius, 2001)
	Lipoxygenase products	(Hwang et al., 2000; Shin et al., 2002; Lee et al., 2005)
	Inflammatory mediators	(Numazaki and Tominaga, 2004)
TRPM8	Cooling	(Peier et al., 2002a)
TRPA1	Cold	(Story et al., 2003)
	Mechanical pain	(Corey et al., 2004)
trkA	Nerve growth factor	(Moalem and Tracey, 2006)
ASIC	Acid	(Waldmann et al., 1997)
$P2X_3$	ATP	(Burnstock and Wood, 1996)
TREK	Heat, cold temperature, mechanical pain	(Noel et al., 2009)
TASK	Acid	(Cooper et al., 2004)
$5\text{-}HT_3$	Serotonin	(Bedford et al., 1998; Woolf and Ma, 2007)
$Na_V1.6, 1.7, 1.8, 1.9$	Membrane depolarisation	(Lee et al., 2005)

When these ion channels are activated, portions of the sensory neurons are depolarised which activate the voltage-gated Na^+ channels, resulting in rapid depolarisation of the membrane and generation of an action potential (Lee et al., 2005) (figure 1-4). Excessive mechanical or thermal stimuli cause acute pain, but the persistence of such pain after the stimulus has been removed, or the pain resulting from inflammatory or ischaemic changes in tissues, generally reflects an altered chemical environment in pain afferents. The main groups of substances that stimulate nociceptors are kinins, PGs and

substances released from damaged cells or tissues, such as 5-HT. Detailed discussion of these substances can be found in Appendix A.

1.3.1.2. Peripheral immune cells

Immune cells are present at the peripheral site of injury and are responsible for the release of a number of immune mediators that have an increasingly recognized role in nociception due to their direct and/or indirect action on nociceptors (Watkins and Maier, 2002) (Figure 1-4). It has also been demonstrated that immune cells infiltrate the CNS under neuropathic conditions (See section 1.3.2.7.).

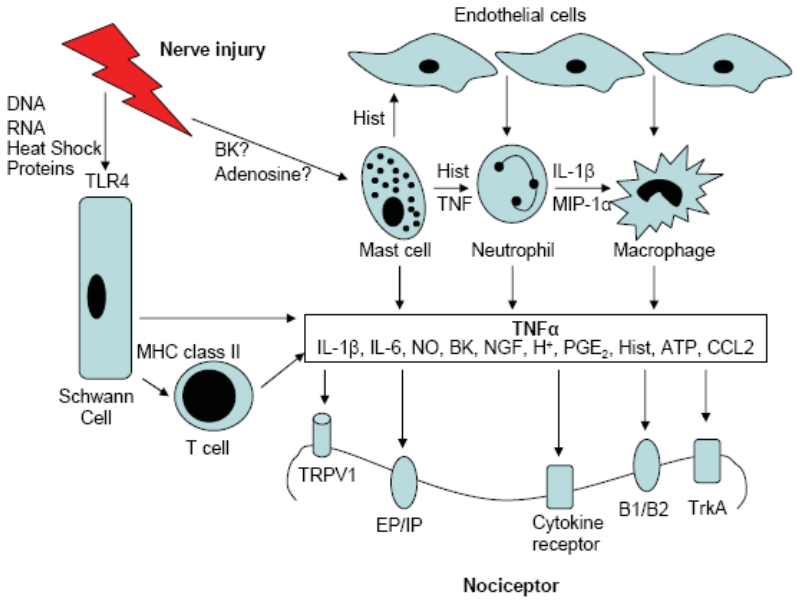

Figure 1-5. Peripheral nerve damage induces activation of resident immune cells as well as recruitment of inflammatory cells to the nerve.

Injury of a peripheral nerve initiates an inflammatory cascade in which mast cells residing in the nerve are the first to be activated. They release mediators such as histamine (hist) and tumour necrosis factor (TNF) α, which sensitize nociceptors and contribute to the recruitment of neutrophils and macrophages. Mediators released by neutrophils assist in recruitment of macrophages. Both neutrophils and macrophages in the nerve produce and secrete a variety of mediators that can further sensitize the nociceptor. Nerve injury also initiates Schwann cell de-differentiation and the release of algesic mediators including proinflammatory cytokines, nerve growth factor (NGF), PGs and ATP. These initial events promote the recruitment of T cells, which can secrete a variety of cytokines depending on their subtype. BK: bradykinin; IL: interleukin; MHC: major

histocompatibility complex; NO: nitric oxide. Adapted from Marchand et al. (2005), Moalem and Tracey (2006) and Thacker et al. (2007).

1.3.1.2.1. Peripheral immune cells: Mast cells

Mast cells are not only critical effector cells in allergic disorders but are also important initiators and effectors of innate immunity (Galli et al., 2005). There is a resident population of mast cells in the peripheral nerve, and these mast cells are degranulated at the site of a nerve lesion (Olsson, 1967; Olsson, 1968; Zuo et al., 2003). It is currently unclear as to how mast cells are activated by nerve injury, but it could be due to increased levels of adenosine or bradykinin (McLean et al., 2000; Sawynok et al., 2000). The granules released contain mediators such as histamine, leukotrienes, proteases, cytokines and chemokines, several of which are capable of sensitising or activating neurons directly, as well as initiating an inflammatory cascade by increasing endothelial permeability and recruiting neutrophils and macrophages to the lesion site (Lum and Malik, 1994; Gaboury et al., 1995; Metcalfe et al., 1997; Malaviya and Abraham, 2000; Zuo et al., 2003; Galli et al., 2005; Moalem and Tracey, 2006; Austin and Moalem-Taylor, 2010). Activation of mast cells may also cause secretion of mediators without overt degranulation by synthesis of lipid mediators such as PGD_2 or by transcription, translation and secretion of a wide range of cytokines and chemokines (Mekori and Metcalfe, 2000; Theoharides and Cochrane, 2004) (figure 1-5).

1.3.1.2.2. Peripheral immune cells: Neutrophils

Neutrophils are an essential part of the innate immune system. Their cytoplasm contains granules and secretory vesicles that can release a wide range of microbicidal effector molecules including bactericidal proteins and cytokines, proteinases and reactive oxygen species (ROS) (Witko-Sarsat et al., 2000; Faurschou and Borregaard, 2003). Neutrophils are not observed in normal nerves but are found in significant numbers in the endoneurium of the injured sciatic nerve, following mast cell-induced migration from small blood vessels into inflamed tissue (Perry et al., 1987; Clatworthy et al., 1995;

Gaboury et al., 1995; Perkins and Tracey, 2000; Zuo et al., 2003). Perkins and Tracey (2000) demonstrated that preventative, rather than curative, depletion of circulating neutrophils reduced the development of thermal hyperalgesia. Thus, neutrophils may be important during the early stages of neuropathic pain development by their release of chemokines and defensins which are chemotactic for macrophages, as well as contributing to inflammatory hyperalgesia by release of proinflammatory cytokines and lipoxygenase products, which activate TRPV1 (Levine et al., 1985; White et al., 1990; Scapini et al., 2000; Witko-Sarsat et al., 2000; Lee et al., 2005). It has also been demonstrated that neutrophils invade the DRG after nerve injury where it is likely that they promote excitability of the DRG (Morin et al., 2007; Shaw et al., 2008). Neutrophils release CCL3 (MIP-1α) and interleukin (IL)-1β that recruit macrophages (Witko-Sarsat et al., 2000; Perrin et al., 2005).

It should also be noted that neutrophils may have an anti-inflammatory and anti-nociceptive role. MRP-14 is a Ca^{2+} binding protein that forms a significant proportion of the cytoplasmic protein in neutrophils. It deactivates macrophages *in vitro* and suppresses inflammatory pain in mice (Giorgi et al., 1998). Under inflammatory conditions, neutrophils may secrete opioid peptides that bind to peripheral neuronal opioid receptors, mediating anti-nociception (Brack et al., 2004).

1.3.1.2.3. Peripheral immune cells: Macrophages

Macrophages are derived from bone marrow promonocytes. The main role of macrophages is to phagocytose foreign particles, such as microbes, and injured or dead tissue. There is a resident population of macrophages in the peripheral nerve and dorsal root ganglia. Activated macrophages in the injured nerve upregulate COX-2, and release PGE_2 and I_2 to directly sensitise primary afferents (Nathan, 1987; Ma and Eisenach, 2002; Ma and Eisenach, 2003b). Other algesic macrophage derived-mediators that most likely contribute to neuropathic pain include ROS and the cytokines tumour necrosis factor (TNF) α, IL-1β and IL-6 (Nathan, 1987; Sommer et al., 1998b; Sommer and Kress, 2004). Macrophage function has been explored in various models of neuropathic pain including chronic

constriction injury (CCI), partial nerve ligation (PNL) and spinal nerve ligation (SNL). Thermal hyperalgesia does not develop after CCI in the C57BL/Wld mouse, which has delayed recruitment of non-resident macrophages and retardation of Wallerian degeneration (Myers et al., 1996; Ramer et al., 1997; Sommer and Schafers, 1998; Araki et al., 2004). It has been demonstrated that depletion of macrophages in nerve-injured rats alleviated neuropathic hyperalgesia (Liu et al., 2000c; Barclay et al., 2007). However, another study found only a limited role of macrophages in the generation of mechanical allodynia following nerve injury (Rutkowski et al., 2000). It is noteworthy that macrophages also invade the DRG following peripheral nerve lesion and may contribute to neuropathic pain by release of excitatory agents that generate ectopic activity in sensory neurons (Hu and McLachlan, 2002).

1.3.1.2.4. Peripheral immune cells: Lymphocytes

Lymphocytes can be classed as B lymphocytes, responsible for antibody production, T lymphocytes, which are the mediators of cellular immunity, and cytotoxic natural killer cells. Both T cells and natural killer cells are found at the site of the nerve lesion in rat models of neuropathic pain (Cui et al., 2000). T cells are also found in increased numbers in the DRG and spinal cord after lesions of the peripheral nerve (Hu and McLachlan, 2002; Sweitzer et al., 2002a) and a role in neuropathic pain has been confirmed by the finding that congenitally athymic nude rats, which lack mature T cells, develop significantly less mechanical allodynia and thermal hyperalgesia following CCI surgery than their heterozygous littermates (Moalem et al., 2004). T cells are divided into $CD4^+$ (helper) and $CD8^+$ (cytotoxic) cells, the former further subdivided into type 1 (T_H1), T_H2 and T_H17 subsets according to their cytokine secretion profile (Sad et al., 1995; Mosmann et al., 2005; Korn et al., 2009). The functions of these subtypes correlate well with their distinctive cytokines, as T_H1 cells produce IL-2 and interferon (IFN) γ and are involved in cell-mediated inflammatory reactions, T_H2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13, are involved in antibody and allergic responses and inhibit synthesis of pro-

inflammatory cytokines by T_H1 cells (Mosmann and Sad, 1996) and T_H17 cells produce the proinflammatory cytokine IL-17 (Kleinschnitz et al., 2006). It has demonstrated that adoptive transfer of T_H1 cells into nude rats increased their sensitivity to noxious stimuli to a level comparable with that of heterozygous rats. By contrast, adoptive transfer of T_H2 cells into heterozygous rats reduced their pain sensitivity (Moalem et al., 2004). These findings indicate that T cells take part in the course of neuropathic pain and suggest that T_H1 and T_H2 cells have opposite effects on neuropathic pain as a result of the distinct sets of cytokines they release. T_H17 cells have a pronociceptive effect following infiltration of the site of injury following CCI (Kleinschnitz et al., 2006).

1.3.1.2.5. Peripheral immune cells: Endothelial cells

The vascular endothelial cells were originally considered to be passive lining cells, but are now known to play an active role in inflammation and are critically involved in the process of leukocyte recruitment and transendothelial migration of leukocytes from the vascular compartment into tissue at the site of injury or inflammation (Galley and Webster, 2004). The endothelial cells secrete nitric oxide (NO) causing relaxation of the underlying smooth muscle and thus regulates vasodilatation and delivery of plasma and blood cells to the area of inflammation (Galley and Webster, 2004). The cells of the post capillary venules have a regulatory role in the flow of exudate and thus in the delivery of plasma derived mediators such as kinins and complement (Galley and Webster, 2004).

Vascular endothelial cells express several adhesion molecules as well as a variety of receptors on the lumen, including those for histamine, cytokines, which can act to increase permeability allowing delivery of macrophages and neutrophils to the site of inflammation (Rang et al., 2003; Galley and Webster, 2004). They also synthesise and release PGI_2 and several cytokines (Galley and Webster, 2004), which act directly on nociceptors.

1.3.1.2.6. Peripheral immune cells: Schwann cells

The extracellular microenvironment of the peripheral nerve is highly regulated by Schwann cells, glial cells that provide myelin encapsulation of axons and paracrine trophic support to nerves (Campana, 2007). However, an immune function for Schwann cells in nerve injury has received increasing recognition since they undergo dramatic phenotypic modulation, regaining capacity to proliferate, migrate, and interact with the immune system in T-cell mediated immune responses by expressing major histocompatibility complex (MHC) class II molecules (Bergsteinsdottir et al., 1992; Campana, 2007). Schwann cells release several algesic mediators, including TNFα, IL-1β, IL-6, NGF, PGE_2 and ATP (Matsuoka et al., 1991; Bolin et al., 1995; Murwani et al., 1996; Shamash et al., 2002; Muja and DeVries, 2004; Liu and Salter, 2005). Schwann cells also express a number of ion channels and receptors for mediators including glutamate, ATP and IL-1β (Skundric et al., 1997; Liu and Bennett, 2003).

1.3.1.3. Peripheral immune mediators

The mediators released by inflammatory and immune cells may act directly to sensitise or activate neurons, or indirectly by acting on non-neuronal cells which may release mediators to activate neurons. These mediators include ATP and adenosine, neurotrophins, NO and ROS, histamine and opioid peptides and a further discussion can be found in Appendix A. Cytokines are key mediators that have particular importance in peripheral and central immune mediation of pain and are discussed below.

1.3.1.3.1. Peripheral immune mediators: Cytokines

Cytokines are low-molecular weight proteins that mediate actions between cells over relatively short distances, and in rare cases, are present in general circulation. They are mostly involved in responses to disease or infection. Many of them are referred to as interleukins, indicating a mediator released by one leukocyte and acting on another, and are synthesised by most cell types. IL-1β, IL-6 and TNFα are

proinflammatory, while IL-4 and IL-10 are antiinflammatory. IL-1β in particular appears to regulate basal pain sensitivity (Wolf et al., 2003) and exogenous administration of proinflammatory cytokines elicits pain and hyperalgesia (DeLeo et al., 1996; Tadano et al., 1999; Reeve et al., 2000; Falchi et al., 2001; Sommer and Kress, 2004). In the periphery, the algesic effects of proinflammatory cytokines are direct, via nociceptor cytokine receptors (Watkins et al., 1999; Zhang et al., 2008), as well as via indirect induction of agents, such as PGE_2. Chemokines are small chemotactic cytokines that are important for leukocyte migration and recruitment to damaged sites. They have been shown to contribute directly to nociception by producing excitatory effects on DRG neurons and inducing allodynia after intraplantar injection (Oh et al., 2001).

1.3.1.3.1.1. Peripheral immune mediators: Tumour necrosis factor α

TNFα is the prototypic proinflammatory cytokine due to its role in initiating a signalling cascade by activating IL-6 and IL-8, then IL-1β and nerve growth factor and finally PGs (Rittner and Brack, 2007). TNFα is constitutively expressed in cutaneous mast cells (Walsh et al., 1991), but following injury or inflammation, may be released by other cells including neutrophils and macrophages (Cunha and Ferreira, 2003; Cunha et al., 2005). TNF-α binds to the constitutively expressed TNF receptor, TNFR1 or inducible TNFR2 (Locksley et al., 2001) expressed on inflammatory cells and in the DRG. TNFR expression on peripheral nerve terminals has not been examined, but seems likely due to its expression in DRG and axons (Schafers et al., 2003b). Activation of either receptor results in p38 MAPK signalling (Schafers et al., 2003d), translocation of nuclear factor κB (NF-κB) to the nucleus and activation of COX-2 dependent prostanoid release (Dinarello, 1999). A direct action on nociceptors was postulated in electrophysiological studies, when TNFα was applied onto the sciatic nerve or injected subcutaneously as it led to lowered thresholds of the cutaneous C-fibres to mechanical stimulation and ongoing activity in some of these fibres (Junger and Sorkin, 2000). In rodents, the intraplantar, intramuscular or intraneural injection of TNFα into the paw produces a short-lived dose-dependent mechanical pain

through direct sensitisation or indirect recruitment of PGs or NGF (Cunha and Ferreira, 2003; Schafers et al., 2003c; Zelenka et al., 2005). Treatment with anti-TNFα antibodies, inhibition of TNF-α synthesis or knockout of TNFR1 significantly reduces signs of neuropathic pain (Sommer et al., 1998a; Sommer et al., 1998b; Schafers et al., 2001; Sommer et al., 2001; Cunha and Ferreira, 2003; Vogel et al., 2006). Sciatic nerve injury results in upregulation of TNFα mRNA in the nerve and DRG, and upregulation of TNFα protein and its receptors, mainly in Schwann cells and endothelial cells (Wagner and Myers, 1996; George et al., 2005; Uceyler et al., 2007b). *In vitro* TNFα perfusion of DRG cells elicits neuronal discharges in Aδ- and C- fibres, which are markedly higher and longer lasting after nerve injury (Schafers et al., 2003a). This increased sensitivity is mediated by TNFR1 (Schafers et al., 2003b).

1.3.1.3.1.2. Peripheral immune mediators: IL-1β

Binding of IL-1β to its receptor IL-1R on the cell surface initiates several signalling events, such as translocation of NF-κB into the nucleus (Dinarello, 1999) and upregulated transcription of several genes, including COX-2, inducible nitric oxide synthase (iNOS), IL-1β and IL-6 (Pahl, 1999; Tegeder et al., 2004). IL-1β may act directly on nociceptors, facilitating the release of calcitonin gene related peptide (CGRP) (Fukuoka et al., 1994) or indirectly by production of NO, bradykinin or PGs (Sommer and Kress, 2004). IL-1β elicits hyperalgesia when injected peripherally into the rat paw (Ferreira et al., 1988), intraneurally into rat sciatic nerve (Zelenka et al., 2005), intrathecally in the rat spinal cord (Sung et al., 2004) or centrally into various regions in the brain (Bianchi et al., 1998). Inflammatory pain induced by lipopolysaccharide or carrageenan can been reduced by IL-1R blockade (Cunha and Ferreira, 2003; Cunha et al., 2005). In neuropathic pain models, IL-1β is upregulated in the injured peripheral nerve (Gillen et al., 1998; Okamoto et al., 2001; Shamash et al., 2002) and the DRG (Uceyler et al., 2007b), where it may contribute to ectopic activity (Wolf et al., 2006). IL-1R knockdown or antagonism alleviates hypersensitivity in nerve-injured rodents (Sommer et al., 1999; Schafers et al., 2001; Sweitzer et al., 2001a).

1.3.1.3.1.3. Peripheral immune mediators: IL-6

IL-6 is synthesised by many cell types, including mast cells, monocytes, lymphocytes, glia and neurons. Once IL-6 has bound to its receptor, it initiates two major intracellular cascades- one involving Janus kinases, the other the Ras-dependent mitogen-activated protein kinase (MAPK) pathway (De Jongh et al., 2003). Crush injury of the sciatic nerve induces upregulation of IL-6 and its receptor in the region of the lesion, where it appears in macrophages and Schwann cells (Bolin et al., 1995). Upregulation of IL-6 in these macrophages appears to be induced by PGE_2 (Ma and Quirion, 2005). Sciatic nerve injury also increases IL-6 levels in the DRG and spinal cord, particularly in the superficial laminae of the dorsal horn (Murphy et al., 1995). IL-6 induces thermal hyperalgesia when injected into the lateral cerebral ventricles (Oka et al., 1995) and increases the heat-evoked release of CGRP from cutaneous nociceptors (Opree and Kress, 2000; Obreja et al., 2002). Intraplantar injection of IL-6 induces pain in normal rats via PGs or direct effects on nociceptors (Obreja et al., 2002; Cunha and Ferreira, 2003). Antisera neutralizing IL-6 or IL-6 knockout inhibits inflammatory pain (Xu et al., 1997; Cunha and Ferreira, 2003). In neuropathic mice, the IL-6 mRNA levels are upregulated in DRG and correlates well with the development of nerve injury-induced thermal pain and mechanical allodynia (Murphy et al., 1999).

1.3.1.3.1.4. Peripheral immune mediators: IL-10

IL-10 is generally regarded as anti-inflammatory. Its expression increases gradually over at least 6 weeks following nerve injury (Okamoto et al., 2001), and treatment with a single dose of IL-10 at the CCI site significantly reduces hyperalgesia, probably due in part to suppression of TNFα and macrophage recruitment (Wagner et al., 1998). These studies are consistent with findings that thalidomide, which inhibits TNFα synthesis, increases endoneurial IL-10 levels and alleviates hyperalgesia in rats with CCI nerve lesion (Sommer et al., 1998a; George et al., 2000). Studies confirm the efficacy of IL-10 in

alleviating neuropathic pain, using virally-driven spinal production of IL-10 in neuropathic pain models (Milligan et al., 2005a; Milligan et al., 2005b; Milligan et al., 2006).

1.3.1.3.1.5. Peripheral immune mediators: Chemokines

Chemokines are small proteins initially characterised as chemotactic peptides controlling the trafficking of leukocytes. The role of chemokines in neuropathic pain is has only been recently investigated and consequently, is not completely understood. The CC group has two adjacent cysteines, the CXC group has one amino acid that separates the two cysteine residues, and CX3C has three amino acids in between two cysteine residues. Chemokines are designated by L for ligand, while the receptors are designated with an R.

A key role for the receptor CCR2 and its ligand CCL2 (MCP-1) has been identified in neuropathic pain. The CCL2/CCR2 system is expressed in several cell types within damaged peripheral nerves, including Schwann cells, macrophages and neurons, involving a cascade of cytokines for non-neuronal upregulation (Tofaris et al., 2002), however the exact signal for neuronal expression of CCL2 remains to be elucidated. CCL2 mRNA and protein are upregulated in the DRG of nerve-injured animals (White et al., 2005; Morin et al., 2007). It is of interest that CCL2 is capable of activating DRG only from neuropathic rats (White et al., 2005). Disruption of the gene for CCR2, which is responsible for attracting monocytes, prevents mechanical allodynia and decreases the number of monocytes infiltrating the nerve (Abbadie et al., 2003). The role of the CCL2/CCR2 system in neuropathic pain has established *in vivo*, as neuropathic pain behaviours are significantly attenuated in null mutant CCR2 mice after PNL (Abbadie et al., 2003). It was concluded that there was likely to be more than one site at which CCL2 activates it receptor, including the nerve trunk, DRG and dorsal horn and it was proposed to involve macrophages and microglia (Abbadie et al., 2003). Recent work examining the expression of CCL2 in the DRG and spinal cord of the nerve-injured rat suggests that CCL2 may be involved in the induction rather than the maintenance of neuropathic pain (Jeon et al., 2009).

A range of other chemokines have also been implicated in neuropathic pain. CXCR4 and CCR4 are expressed on subpopulations of DRG neurons and their corresponding ligands stimulate Ca^{2+} influx in a small percentage of neurons *in vitro* (Oh et al., 2001). Direct intraplantar *in vivo* injection of chemokines such as CCL5, CXCL12 and CCL8 induces pain in healthy animals (Oh et al., 2001). Additionally, human CXCL8 or rat CXCL1 indirectly causes pain via catecholamine release when applied subcutaneously into the hindpaw (Cunha and Ferreira, 2003; Cunha et al., 2005). CCL3 (MIP-1α) appears capable of enhancing the sensitivity TRPV1 through a G-protein dependent signalling pathway, as activation of CCR1 by CCL3 results in increased TRPV1- mediated Ca^{2+} influx and increased capsaicin sensitivity (Zhang et al., 2005). Peripheral neuropathy following antiretroviral treatment is associated with increased CXCR4 and CXCL12 expression in the DRG and Schwann cells and this affliction was attenuated by administration of a CXCR4 antagonist (Bhangoo et al., 2007).

1.3.1.4. Dorsal root ganglion (DRG)

The DRG consists of cell bodies, or soma, of sensory neurons, satellite glia, dendritic cells, macrophages and endothelial cells (Olsson, 1990). Each neuronal cell body in the DRG is encapsulated by a layer of satellite cells with a basement lamina separating neighbouring glially encapsulated neuronal soma (Pannese, 1981; Olsson, 1990; Shinder et al., 1998). A capsule composed of connective tissue and a perineurium, similar to that of peripheral nerves, surrounds the entire DRG and keeps the microenvironment separate from surrounding extracellular fluid (Olsson, 1990). Almost every soma is bordered by a rich network of blood vessels, which is far denser than the peripheral nerve or dorsal root and are outside of the blood-brain barrier (Olsson, 1990). DRG neurons are 'pseudounipolar', which means that each neuron emits a single stem axon. A few tens or hundreds of microns from the soma this stem axon divides in two. One branch proceeds from the junction into the spinal nerve and from there into a sensory ending. The second branch enters the dorsal root and the spinal cord (Devor, 1999).

1.3.1.5. Central terminal

The central terminals of nociceptors are located in the superficial laminae dorsal horn of the spinal cord for somatic neurons and in the spinal nucleus of the trigeminal for those innervating the face. These terminals drive synaptic input to second order neurons, transferring information carried by action potentials about intensity and duration of peripheral noxious stimuli (Woolf and Ma, 2007). Once the action potential reaches the central terminal, neurotransmitter release is regulated by multiple factors that control or modulate Ca^{2+} influx such as L- and P/Q- type voltage dependent Ca^{2+} channels, however the major channels in nociceptors are N-type Ca^{2+} channels ($Ca_V2.2$), BK and PG receptors. These channels exert their functions in sensory transduction by increasing intracellular Ca^{2+} concentration in response to depolarisation, releasing neurotransmitters such as glutamate, neuropeptides like substance P and proteins, like BDNF (brain derived neurotrophic factor). Transmitter-modulated reductions in neurotransmitter release from nociceptors is a prominent control mechanism in nociception, increasing or decreasing access of nociceptor input to the CNS and includes endogenous opioids acting on μ- and δ- opioid receptors (Puehler et al., 2004; Guan et al., 2005; Kohno et al., 2005), γ-aminobutyric acid (GABA) on $GABA_B$ receptors and endogenous cannabinoids on cannabinoid $(CB)_1$ receptors (figure 1-4).

1.3.1.6. Peripheral characteristics of neuropathic pain

Neuropathy elicits a number of changes in nerves in terms of activity properties and transmitter content. The advent of a number of animal models of neuropathic pain states, including CCI, PNL and SNI (Bennett and Xie, 1988; Seltzer et al., 1990; Kim and Chung, 1992), has facilitated understanding of the peripheral mechanisms involved. Damaged nerves may start to generate ongoing ectopic activity due to the accumulation and clustering of Na^+ channels around the damaged axons and there is evidence that mechanoreceptors become highly sensitive to applied stimuli (Roza et al., 2003). This aberrant activity can then start to spread rapidly to cell bodies in the DRG. Nerve fibres can then start to cross-excite

each other (ephaptic transmission) and the same occurs in the cell bodies. In addition to the discussed action of immune cells at the site of injury, immune cells and their mediators may also contribute to ectopic activity in the DRG (Wolf et al., 2006; Hu et al., 2007; Morin et al., 2007; Shaw et al., 2008). Several lines of evidence stemming from the SNL model suggest that intact nociceptors sharing the nerve of injured fibres play a role in neuropathic pain. Li and colleagues (2000) demonstrated that dorsal rhizotomy of the lesioned L5 root did not prevent or reverse established hyperalgesia. Thus, interrupted inputs from the L5 spinal nerve fails to reverse the hyperalgesia in the foot, indicating that ectopic activity from the injured nerve is not essential for the development of neuropathic pain. Electrophysiological recordings from the uninjured L4 spinal nerve reveal abnormal spontaneous activity in C-fibre nociceptors, (Wu et al., 2001), possibly due to increased $Na_v1.8$ in peripheral nerves (Gold et al., 2003).

Under pathological pain conditions, like neuropathic pain, the sympathetic nervous system can be reorganised, leading to a separation between 'sympathetically maintained pain' and 'sympathetically independent pain' (Stanton-Hicks et al., 1995), the former being associated with noradrenergic sprouting in the periphery (Janig et al., 1996) and DRG (McLachlan et al., 1993). When noradrenaline (NA) is injected into healthy subjects, it evokes little or no pain (Torebjork et al., 1995; Fuchs et al., 2001), but when injected around stump neuromas in the skin of patients with post herpetic neuralgia, it induces an increase in spontaneous pain (Chabal et al., 1992; Choi and Rowbotham, 1997). Anaesthetic blockade of the sympathetic nervous system relieves pain and hyperalgesia (Raja et al., 1991). It has also been demonstrated that α_1-adrenoceptors are upregulated in the DRG and on peripheral blood mononuclear cells, via which catecholamine signalling results in ectopic discharges and stimulates proinflammatory cytokine production respectively (Heijnen et al., 1996; Rouppe van der Voort et al., 1999; Xie et al., 2001). Furthermore, glial secretion of proinflammatory cytokines in the CNS may increase sympathetic outflow (Niijima et al., 1991; Ichijo et al., 1994; Ando et al., 1995).

In conjunction with the evidence presented on the role of peripheral mediators, these macroscopic changes to peripheral nerve activity causally implicate, and demonstrate mechanisms for, the PNS in neuropathic pain.

1.3.2. The central nociceptive pathway

Primary afferents terminate in the spinal cord where the nociceptive signal is processed in the spinal dorsal horn and modulated by descending signals. The nociceptive stimulus is processed in various supraspinal sites where the nociceptive stimulus effectively initiates the pain experience in terms of the affective and cognitive components. Many neurotransmitters and neuromodulators act at these sites, including glutamate, GABA, 5-HT, acetylcholine, tachykinins, CGRP, opioid peptides, cannabinoids and cholecystokinin. A discussion of their mechanisms and sites of action can be found in Appendix A.

1.3.2.1. The dorsal horn

The nociceptors synapse in the dorsal horn of the spinal cord, which is organised into different laminae, extending from the superficial to the deep dorsal horn (figure 1-6). Most nociceptive Aδ- and C-fibres terminate superficially in laminae I-II, with a smaller number reaching deeper laminae, whereas Aβ-fibres predominantly innervate laminae III-VI (Todd, 2002).

Figure 1-6. The termination of primary afferent fibres in the six laminae of the dorsal horn of the spinal cord.

Adapted from Rang *et al.* (2003).

Synaptic transfer of information from the periphery to the dorsal horn is governed by the nature and amount of the transmitter released by primary afferents, the density and identity of postsynaptic receptors (ionotropic and metabotropic), the kinetics of receptor activation, ion channel opening and closing, and the uptake or breakdown of the transmitter. Each of these factors is subject to multiple modulatory influences (Millan, 1999). Further details on the innervation of the dorsal horn can be found in Appendix A.

1.3.2.2. Ascending pathways

Figure 1-7. The main ascending pain pathways.

Second order neurons project from the dorsal horn to supraspinal sites via the spinothalamic tract, the spinobulbar terminations and the spinohypothalamic pathways. PAG: periaqueductal grey; LC: locus coeruleus; PB: parabrachial nucleus; RVM: rostral ventromedial medulla. Adapted from DeLeo (2006).

The projection neurons transmit the nociceptive signal rostrally along the ascending pathways in the spinal cord to various supraspinal structures (Willis, 1985; Besson and Chaouch, 1987; Willis and Coggeshall, 1991; Berkley and Hubscher, 1995). The function of the ascending pathways is the transmission and integration of the nociceptive activity with homeostatic, arousal and autonomic processes. Within the supraspinal target structures of the ascending pathways, third order neurons further process the nociceptive signal and transmit it to cortical and limbic structures where the signal is interpreted (Millan, 1999; Tracey and Mantyh, 2007) (figure 1-7).

The organisation and neuroanatomy of the ascending pain pathways are quite complex (Willis, 1985; Besson and Chaouch, 1987; Willis and Coggeshall, 1991; Berkley and Hubscher, 1995; Willis and Westlund, 1997). There are a number of ascending pathways that are important for pain, including direct projections to the thalamus (the spinothalamic tract); direct projections to homeostatic control regions in the medulla and brain (the spinomedullary and spinobulbar projections); possible direct projections to the hypothalamus and forebrain (spinohypothalamic tract); as well as other indirect pathways. These pathways have been reviewed in Appendix A.

1.3.2.3. The pain matrix

The termination structures of the ascending tracts then project to a network of sites that process the nociceptive signal. Study of these structures has been aided by the advent of non-invasive brain-imaging techniques that provide novel opportunities to study the multiple distributed regions of the brain and the neural circuitry responsible for pain experience (Casey, 1999; Mackey and Maeda, 2004; Seminowicz et al., 2004; Apkarian et al., 2005; Mayer et al., 2006; Tracey and Mantyh, 2007; Zhuo, 2008). The functional anatomy of the ascending pathways indicates that pain is associated with multiple pathways; activity in multiple forebrain regions is integrated with past experience and present context to result in the complete, multidimensional pain experience (Millan, 1999) (figure 1-3 and figure 1-7). Although particular neurons and pathways may have a predominant contribution to one aspect of the

pain experience, it is the activity across the entire brain that constitutes the basis for the conscious experience of pain. The areas involved would predictably include the pathways and termination regions described above and potentially many other areas of the brain. This network of brain structures is referred to as the 'pain matrix' and includes the somatosensory primary (S1) and secondary (S2) areas, the cingulate, insula, the prefrontal cortex, thalamus and cerebellum (Casey et al., 1996; Craig et al., 1996; Jones and Derbyshire, 1996; Derbyshire et al., 1997; May et al., 1998)(figure 1-8). A discussion of the sensory-discriminative, the affective-motivational and the cognitive components of the pain matrix can be found in Appendix A.

Figure 1-8. Schematic representation of ascending pathway terminations and the subcortical and cerebral cortical structures involved in pain processing.

ACC: anterior cingulate cortex; Amyg: amygdala; BG: basal ganglia; HT: hypothalamus; M1: primary motor cortex; PAG: periaqueductal grey; PB: parabrachial nucleus; PCC: posterior cingulate cortex; PF: prefrontal cortex; PPC: posterior parietal complex; S1 and S2: primary and secondary somatosensory cortical areas; SMA supplementary motor area. Price (2000), reprinted, with permission, from Science, Volume 288 © 2000.

1.3.2.4. Descending modulatory controls of pain

Figure 1-9. The main descending pain pathways.

A major pain modulating pathway with critical links in the midbrain periaqueductal grey (PAG) and rostral ventromedial medulla (RVM). Regions of the frontal lobe and amygdala project directly and via the hypothalamus to the PAG. The PAG in turn controls spinal nociceptive neurons through relays in the RVM and Locus Coeruleus (LC). The RVM contains both serotonergic and non-serotonergic projection neurons; the LC provides noradrenergic innervation of the dorsal horn. The RVM exerts bidirectional control over nociceptive transmission in the dorsal horn. Adapted from DeLeo (2006).

The idea that pain undergoes modulatory effects from higher regions in the CNS was first introduced by Head and Holmes (1911) almost a century ago. Since then, a large body of knowledge has been gained regarding the mechanisms of pain perception and modulation (Reynolds, 1969; Fields and Basbaum, 1978; Millan, 2002). Several decades after Head and Holmes (1911) first theorized that pain is under the influence of higher areas in the CNS, studies confirmed their theory by providing evidence that a

number of supraspinal sites contribute to the control of ascending sensory input by exerting tonic inhibitory control of neurons in the dorsal horn (Hagbarth and Kerr, 1954; Carpenter et al., 1965; Wall, 1967). Further research into the contribution of supraspinal structures to nociceptive modulation showed that the mammalian CNS has several well-defined, supraspinally organised, descending pathways. These pathways form a network of neural systems that modulate the ascending transmission of nociceptive information, with the most well described being the circuitry mediating the brainstem control of nociceptive transmission at the level of the dorsal horn (Hagbarth and Kerr, 1954; Carpenter et al., 1965; Wall, 1967; Reynolds, 1969; Mayer et al., 1971; Oliveras et al., 1974; Fields and Basbaum, 1978; Willis, 1988; Fields et al., 1991; Millan, 2002).

Several supraspinal sites are known to contribute to the descending modulation of nociception, either by a direct projection of neurons to the spinal cord or indirectly by projections to the spinal cord via other brainstem regions. These include the periaqueductal grey (PAG), locus coeruleus (LC), and the rostral ventromedial medulla (RVM) (Figure 1-9), which subsequently modulate nociception in the dorsal horn. A discussion of these sites can be found in Appendix A.

1.3.2.4.1. Modulation in the dorsal horn and the gate control theory

Cells of lamina II of the dorsal horn, the substantia gelatinosa (SG), are mainly short inhibitory GABAergic or glycinergic interneurons projecting into lamina I and lamina V. They regulate transmission at the first synapse of the nociceptive pathway between the primary afferent fibres and the spinothalamic tract transmission neurons, hence the term ‘gate control’ theory, proposed by Melzack and Wall (1965). According to this view, the SG responds to the activity of primary afferents entering the cord and thus allows the arrival of impulses via primary afferent or descending fibres to regulate the transmission of impulses via the ascending pathway (Melzack and Wall, 1965). See Appendix A for more detail.

The effects of descending modulation from the PAG and RVM are exerted in the dorsal horn on the synapse between the primary afferent and projection neurons or on interneurons that synapse with projection neurons. This synapse in the dorsal horn is the point where nociceptive integration is first integrated before being transmitted to higher centres in the CNS (Melzack and Wall, 1965; Fields and Basbaum, 1978; Willis and Coggeshall, 1991; Millan, 1999). The descending modulatory effect is applied either by inhibiting the release of neurotransmitters from the primary afferent fibre or by inhibiting the function of neurotransmitter receptors on the post-synaptic neuron (Fields et al., 2006).

1.3.2.5. Central sensitisation

Many dorsal horn neurons are sensitised following brief bursts of activity in nociceptors and synaptic transfer is enhanced, potentiated or facilitated (Cook et al., 1987; Simone et al., 1991; Dubner and Ruda, 1992; Nichols et al., 1999). This is termed 'central sensitisation', and takes a number of different and distinct forms. One form is windup (Mendell and Wall, 1965), another is heterosynaptic activity-dependent plasticity that outlasts the initiating stimulus for tens of minutes (classic central sensitisation) (Thompson et al., 1990; Thompson et al., 1993) and a third is a largely homosynaptic potentiation elicited by brief high frequency inputs (long term potentiation) (Randic et al., 1993; Ikeda et al., 2003; Ji et al., 2003). Classic central sensitisation will be briefly discussed, however an expanded discussion, including windup and long-term potentiation, can be found in Appendix A.

Classic central sensitisation is 'an increased responsiveness of pain transmission to neurons in the spinal cord - usually caused by neurochemical changes in the spinal cord, brainstem or forebrain' (Mantyh et al., 2002) and manifests in three main ways (Woolf and Mannion, 1999; Woolf and Salter, 2000):

1. a reduction in threshold due to the recruitment of previously subliminal low-threshold Aβ-fibre inputs;

2. an increase in the responsiveness of the dorsal horn neurons, the number of action potentials elicited by a suprathreshold input is increased; and
3. an expansion of the extent of the receptive fields of dorsal horn neurons.

Several mechanisms involved in central sensitization have been described (Milligan and Watkins, 2009) including intracellular mechanisms (Ji and Woolf, 2001; Hucho and Levine, 2007), disinhibition of dorsal horn neurons by descending pathways (Woolf and Mannion, 1999; Gebhart, 2004), recruitment of Aβ-fibres (Woolf and Mannion, 1999; Woolf and Salter, 2000), cytokine modulation (Kawasaki et al., 2008). The most well characterised mechanism involves a change in the function of neuronal N-methyl-D-aspartate (NMDA) receptors in the spinal cord dorsal horn (Petrenko et al., 2003; South et al., 2003; Milligan and Watkins, 2009). Activation of sensory neurons by painful stimuli leads to the release of transmitters like substance P and excitatory amino acids that bind to and activate ascending projections in the spinal cord (Woolf and Mannion, 1999). During conditions that produce strong and/ or persistent nociceptive stimulation, including ectopic activity, sufficient amounts of substance P and glutamate are released to sustain the depolarisation of the spinal cord neurons. This results in removal of the Mg^{2+} plug that is normally present in the NMDA channel, allowing Ca^{2+} to activate signal transduction. The Ca^{2+} influx causes the production and release of nitric oxide by Ca^{2+}-activated neuronal NOS and PGs by COX enzymes (Ji and Woolf, 2001). These molecules both enhance the excitability of spinal cord neurons in response to incoming pain signals and cause an exaggerated release of neurotransmitters from sensory neuron presynaptic terminals to the spinal cord. Together, these downstream effects of NMDA activation result in the amplification of pain signals to the pain matrix (Petrenko et al., 2003).

1.3.2.6. Glia

Glial cells were originally described by Rudolf Virchow (1846) as non-neuronal cells constituting the 'glue' of the brain. Glial cells outnumber neurons somewhere between 10 and 50 to 1, collectively encompassing microglia (~10 %), astrocytes (~85 %) and oligodendrocytes (~5 %) in the CNS and

astrocytes and Schwann cells in the PNS (Chao et al., 1996; Moalem and Tracey, 2006; Savidge et al., 2007). Microglia are considered the resident macrophages of the CNS and, in a resting state, have a small soma with fine processes that sample the extracellular space for stimuli that threaten homeostasis (Tsuda et al., 2005). Astrocytes play critical roles in the development and physiology of the CNS, being involved in key aspects of neuronal function, such as trophic support. Furthermore, astrocytes contribute to CNS homeostasis, regulating the local concentrations of ions and neurotransmitters (Araque et al., 1999; Perea and Araque, 2002; Moalem and Tracey, 2006). Microglia and astrocytes are not likely to be the only glial cells involved in pain enhancement, but with greater accessibility than oligodendrocytes, most studies have focussed on these cells (Watkins et al., 2007). It is now thought that solely considering neuronal activity provides an incomplete understanding of the induction and maintenance of neuropathic pain (Scholz and Woolf, 2007).

1.3.2.6.1. Neuropathic pain and glia

In the early 1990's, glia were first associated with neuropathic pain when Garrison and colleagues (1991) reported that peripheral nerve damage that created allodynia and hyperalgesia also activated spinal cord glia. They also reported that the NMDA antagonist MK801, which blocks neuropathy-induced allodynia and hyperalgesia also blocked glial activation (Garrison et al., 1991). The question then arose as to whether glia are necessary for allodynia and hyperalgesia and it was answered by selectively removing glia from the picture. Fluorocitrate disrupts the Krebs cycle of glia by inhibiting the glia-specific enzyme aconitase (Hassel et al., 1992; Berg-Johnsen et al., 1993) and minocycline selectively disrupts the activation of microglia without directly affecting neurons or astrocytes. Both interventions have been shown to be effective in preventing allodynia and hyperalgesia in a wide range of pain models (Meller et al., 1994; Watkins et al., 1997; Milligan et al., 2000; Milligan et al., 2003; Raghavendra et al., 2003a; Ledeboer et al., 2005). Watkins and Maier (2003) note two findings from these studies. Firstly, fluorocitrate, by blocking astrocytes and microglia, seems to exert a more profound blockade of

exaggerated nociceptive states than minocycline and secondly that minocycline, blocking microglia alone, is far more effective in blocking rather than reversing exaggerated nociceptive effects. The individual roles of microglia and astrocytes in pain facilitation are further dissected in a study showing that surgical induction of neuropathic pain in rats results in upregulation of markers of spinal microglial activation, CD11b, TLR4 and CD14, at 4 h post surgery, increasing until postoperative day 14 and then declining to almost normal levels by postoperative day 28; however the spinal astrocyte marker, such as glial fibrillary acidic protein (GFAP), did not significantly increase until postoperative day 4, but then continued to increase until the conclusion of the study on postoperative day 28 (Tanga et al., 2004). Thus, on the basis of these animal studies, it seems evident that microglia are involved in the induction of initial exaggerated nociceptive response and this activation in turn leads to astrocyte activation that maintains persistent pain states (Raghavendra et al., 2003a).

1.3.2.6.2. Glial activation

Activation of glia is fundamentally different to neurons as neuronal activation is one dimensional, i.e. production of action potentials, whilst activation of glia is multidimensional (Watkins and Maier, 2003). Glia become activated in response to following trauma, ischaemia, tumours, neurodegeneration and the presence of viral and bacterial components (Pekny, 2001). Microglial activation is characterized by morphological changes from a resting, ramified shape to an active amoeboid shape, proliferation, increased expression in cell surface antigens including MHC classes I and II, CD11b, cellular adhesion molecules, CD4 and CD45, or receptors and changes in functional activities (Perry et al., 1987; Perry and Gordon, 1987; Hickey and Kimura, 1988; Flaris et al., 1993; Ford et al., 1995; Carson et al., 1998; Sweitzer et al., 2001b; Watkins and Maier, 2003). Astrocyte activation is morphologically characterised by hypertrophy and increased production of intermediate filaments, such as GFAP, vimentin and/or nestin (Pekny, 2001). Importantly, when glia are activated they begin producing and releasing a variety of neuroexcitatory substances such as ROS, NO, PGs, excitatory amino acids, proinflammatory

cytokines and growth factors (Watkins and Maier, 2000). This is not without controversy, however, as expression of microglial and astrocyte activation markers, CD68 (ED1) and GFAP respectively, have been reported in the absence in proinflammatory cytokines (Walsh et al., 2001).

1.3.2.6.3. Neuropathic mechanisms of glial activation

Glia isolated from various CNS regions have marked heterogeneity (Cholewinski et al., 1988; Beaujouan et al., 1990) in terms of activation phenotype (M1 vs. M2) (Mantovani et al., 2004), and so extrapolation of a mode of activation cannot be made of glia residing in one CNS region to the other. There is even heterogeneity between glia in nocisponsive layers of the superficial dorsal horn (laminae I-III) and other spinal regions (Ochalski et al., 1997; Li and Nagy, 2000; Sung et al., 2003). Given that dorsal horn glial activation occurs in response to peripheral injury and inflammation (Watkins et al., 2001), it would seem reasonable to predict that spinal neurotransmitters released in response to these stimuli will also activate glia. However study of the effects of these cells *in vivo* would be confounded by the fact that spinal neurons would also be activated. Despite this difficulty a number mechanisms of glial activation have been proposed and tested (Figure 1-1).

1.3.2.6.3.1. Toll-like receptors and neuropathic pain

The CNS has the ability to mount an innate response against potential pathogens. It is therefore critical that glia and neurons have the ability to discriminate between self-molecules and pathogen-associated non-self structures or endogenous danger signals (Guo and Schluesener, 2007). The presence of infection is recognized by pattern recognition receptors that bind to specific elements called the pathogen-associated molecular patterns (PAMPs). Recognition of these PAMPs by myeloid cells is the first step of a complex inflammatory reaction that characterises, and is crucial to, innate immune responses. Increasing evidence implicates membrane bound toll-like receptors (TLRs) 2 and 4 in neuropathic pain (Tanga et al., 2005; Kim et al., 2007). As most data to date has focussed on TLR4, the remainder of this discussion will focus on TLR4.

TLR4 is expressed by the immunocompentent cells of the CNS, microglia (Bsibsi et al., 2002; Guo and Schluesener, 2007) and perhaps astrocytes (Miyake, 2007), but not neurons. T lymphocytes (Kabelitz, 2007), B lymphocytes, macrophages (Banerjee and Gerondakis, 2007) and neutrophils (Parker et al., 2005) all express TLR4. Activation of TLR4 stimulates NF-κB and MAPK signalling pathways that result in synthesis of proinflammatory cytokines, chemokines, proteins of the complement system, enzymes such as COX2 and iNOS and adhesion molecules (DeLeo et al., 2004).

TLRs were implicated in earlier studies where the effect of injecting the immunogenic portions of bacteria and viruses over the spinal cord was examined. Glial activation and exaggerated pain responses occurred as a result and were subsequently blocked by disruption of glial function (Meller et al., 1994; Milligan et al., 2000; Milligan et al., 2001). By activating TLR4, these studies demonstrated that glial activation is causal to allodynia and hyperalgesia. Tanga and colleagues (2004) showed a correlation between increased spinal microglial TLR4 activation with the onset of behavioural hypersensitivity. In a later study, Tanga and colleagues (2005) showed that *TLR4* gene knockout (GKO) mice had significantly attenuated behavioural hypersensitivity, decreased expression of spinal microglial markers and proinflammatory cytokines following L5 nerve transection. In addition to detecting lipopolysaccharide (LPS), TLR4 detects host cell stress and damage that causes the release of host DNA, RNA, heat shock proteins and cell membrane components (Miyake, 2007; Hutchinson et al., 2009b). In the case of neuropathic pain, Tanga and colleagues (2005) demonstrated that sensory nerve damage in *TLR4* GKO and point mutation mice lead to the release of these substances and others to activate microglial TLR4 which then results in NF-κB activation and subsequent induction of proinflammatory cytokines (Vabulas et al., 2002; Tsan and Gao, 2004). TLR4 blockade not only prevents the initial development of neuropathic pain (Tanga et al., 2005), but also reverses established neuropathic pain (Hutchinson et al., 2008c). These studies demonstrate a role for TLR4 in the induction and maintenance of neuropathic pain (Figure 1-10).

1.3.2.6.3.2. Cytokines, chemokines and neuropathic pain

Glia not only secrete proinflammatory cytokines in response to activation, but express receptors for, and are hence activated by, these proinflammatory cytokines (Watkins et al., 1999; John et al., 2003). In addition, a range of chemokines activate glia and may constitute putative neuron to glia activation signals.

1.3.2.6.3.2.1. CCL2 and neuropathic pain

CCL2 (MCP-1) is upregulated in the DRG by CCI, transported to the dorsal horn and released in response to neuronal impulses (Tanaka et al., 2004; Zhang and De Koninck, 2006; Thacker et al., 2009). Microglial CCR2 is upregulated by peripheral nerve injury (Abbadie et al., 2003; Zhang and De Koninck, 2006; Thacker et al., 2009) and intrathecal CCL2 administration induces microglial activation, which is abolished in *CCR2* GKO mice (Zhang et al., 2007). Furthermore, behavioural nociceptive hypersensitivity and microglial activation from nerve injury is prevented in *CCR2* GKO mice or by intrathecal CCL2 neutralising antibodies (Zhang et al., 2007; Thacker et al., 2009). Given that CCL2 upregulation in the spinal cord closely precedes microglial activation (Zhang and De Koninck, 2006), CCL2 secretion by primary afferents appears to be an initiating neuron-glia activation signal via microglial CCR2 (Figure 1-10).

1.3.2.6.3.2.2. Fractalkine and neuropathic pain

Fractalkine (CX3CL1) is a protein tethered to the extracellular surface of neurons that can be released and diffuse away in response to strong neuronal activation, such as pathological pain conditions (Harrison et al., 1998; Chapman et al., 2000). In the spinal cord, neurons alone express fractalkine and microglia alone express the CX3CR1 receptors for fractalkine and so fractalkine is considered a putative neuron-to-glia signal (Harrison et al., 1998; Hughes et al., 2002b; Watkins et al., 2003; Verge et al., 2004; Milligan et al., 2008). The administration and blockade of exogenous fractalkine induces and

reverses exaggerated pain states respectively and the blockade of endogenous fractalkine attenuates these symptoms in animal models of neuropathic pain (Watkins et al., 2003; Milligan et al., 2004; Milligan et al., 2008), demonstrating that peripheral nerve injury leads to the release of fractalkine from neurons in the dorsal spinal cord. Administration of a CX3CR1 antagonist after establishment of a neuropathic pain model also reduces nociceptive responses, which suggests prolonged release of fractalkine and a role in the maintenance of neuropathic pain (Milligan et al., 2004). More recent evidence suggests that microglial activation is required as a precursor to cleavage of fractalkine from the neuronal membrane. Cathepsin S is released by activated microglia and is responsible for the proteolytic cleavage of fractalkine from the neuronal membrane via a bioinformatically identified cleavage site, as exogenous intrathecal administration resulted in increased spinal fractalkine (Clark et al., 2007). Cathepsin S expression was correlated with microglial activation and in the maintenance of pain hypersensitivity (Clark et al., 2007). Furthermore, inhibition reversed established exaggerated pain states and reduced microglial activation. As such fractalkine may not represent an initiating neuron-glia activation signal.

1.3.2.6.3.3. ATP and neuropathic pain

There is evidence that ATP is involved in pain transmission and hypersensitivity via P2 purinoceptors (Salter et al., 1993; Liu and Salter, 2005; Burnstock, 2006). Microglia express $P2X_4$ receptors (Tsuda et al., 2003) and $P2X_7$ (Ferrari et al., 1996; Collo et al., 1997; Moller et al., 2000; Chakfe et al., 2002; Ulmann et al., 2008). The involvement of $P2X_4$ was demonstrated by attenuation of established exaggerated pain responses induced by nerve injury following administration of a $P2X_4$ antagonist (Tsuda et al., 2003). Although it is accepted that microglial $P2X_7$ is involved in inflammatory pain, a study has reported that disruption of the $P2X_7$ receptor gene prevents both chronic inflammatory and neuropathic pain hypersensitivity (Chessell et al., 2005). It has also been demonstrated that antagonism of $P2X_7$ is anti-allodynic in three rat neuropathic pain models and dose dependently reduces IL-1β

release in peripheral macrophages (McGaraughty et al., 2007). Given that $P2X_7$ is also expressed on macrophages, astrocytes and the presynaptic terminals of neurons, the reported action of the receptor may not be solely mediated via the receptors located on microglia (Trang et al., 2006). P2 signalling results in IL-1 and BDNF secretion (Chessell et al., 2005; Ulmann et al., 2008). In addition, the source of ATP is unknown, but may potentially be actively released from injured primary afferents and dorsal horn neurons, or may increase as primary afferent neurons degenerate (Figure 1-10).

Figure 1-10. Pro-inflammatory roles for glia.

If a noxious input persists, such as during chronic inflammation or nerve damage, sustained central sensitization leads to transcriptional changes in dorsal horn neurons that alter these neurons' function for prolonged periods. Astrocytes respond to this ongoing synaptic activity by mobilizing internal Ca^{2+}, leading to the release of glutamate (Glu), ATP that binds to $P2X_4$, TNFα, IL-1β, IL-6, NO and PGE_2. Activated microglia are also a source of all of these pro-inflammatory factors. Astrocytes and microglia express the chemokine receptors CX3CR1 (not shown) and CCR2 and become activated when the respective chemokines bind. After nerve damage, heat shock proteins (HSPs) are released and can bind to Toll-like receptors (TLRs) expressed on both astrocytes and microglia, leading to the further activation of these cell types. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience (Milligan and Watkins, 2009), © 2009.

1.3.2.6.3.4. Other neuropathic mechanisms of glial activation

Subtance P: Microglia and astrocytes express the substance P receptors, as intrathecal substance P administration is associated with hyperalgesia and activation of glial, but not neuronal, p38 MAPK activation (Palma et al., 1997; Lai et al., 2000; Svensson et al., 2003). Glutamate: Microglia express mainly α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) receptors that act to inhibit proinflammatory cytokine release, whereas astrocytes express ionotropic non-NMDA and NMDA receptors as well as metabotropic glutamate receptors, that result in proinflammatory cytokine secretion via intracellular signalling pathways (Porter and McCarthy, 1997; Milligan and Watkins, 2009). PGs: Microglia and astrocytes both express PG receptors, facilitating glial activation by PGE_2 secreted from other glial cells, and neurons (Palma et al., 1997; Zhao et al., 2007; Cimino et al., 2008; Telleria-Diaz et al., 2010). Further glial activation occurs via reactive oxygen species including NO (Meller and Gebhart, 1993; Freeman et al., 2008) (Figure 1-10).

1.3.2.6.4. Glial enhancement of nociception

The mechanisms by which activated glia enhance neuronal transmission of nociception are only partially understood (Figure 1-10). Currently, the body of evidence supports a role of the glial proinflammatory cytokines TNFα, IL-1β and IL-6, which synergize, such that far more powerful effects are observed when more than one cytokine is present (Dinarello, 1999). Upon activation, glia secrete proinflammatory cytokines that act directly on cytokine receptors expressed by neurons and other glia (Watkins et al., 1999; Zhang et al., 2008). The injection of exogenous proinflammatory cytokines over the spinal cord enhances nociception (DeLeo et al., 1996; Tadano et al., 1999; Reeve et al., 2000; Falchi et al., 2001), and electrophysiological studies document rapid enhancement of neuronal excitability in response to noxious stimuli following injection of proinflammatory cytokines to the region (Reeve et al., 2000). Conversely, the blockade of proinflammatory cytokine function, using either IL-1 receptor antagonist (IL-1ra), soluble TNFRs or anti-IL-6-neutralizing antibodies, prevents their effects in brain (Maier and

Watkins, 1998) and prevents and reverses exaggerated nociception in virtually all animal models of inflammation and injury to peripheral tissues, peripheral nerves, spinal nerves and spinal cord. (Watkins et al., 1997; Milligan et al., 2001; Sweitzer et al., 2001a; Milligan et al., 2003). The fact that established hyperalgesia and allodynia can be reversed by proinflammatory cytokine antagonists supports the conclusion that these glial proteins are involved in the maintenance, as well as the initial induction of these enhanced nociceptive states.

Aside from directly activating neurons via cytokine receptors (Watkins et al., 1999; Zhang et al., 2008), proinflammatory cytokines alter synaptic transmission in dorsal horn neurons. TNFα and IL-1β increase neuronal excitability and synaptic strength by increasing the conductivity and number of AMPA and NMDA receptor on the neuronal surface (Beattie et al., 2002; Stellwagen and Malenka, 2006). Specifically, proinflammatory cytokines increase the frequency and amplitude of spontaneous excitatory postsynaptic currents, perhaps by enhancing glutamate release and by enhancing NMDA- and AMPA-induced currents respectively (Viviani et al., 2003; Ozaktay et al., 2006; Kawasaki et al., 2008). Proinflammatory cytokines also facilitate disinhibition, decreasing the frequency and amplitude of spontaneous inhibitory postsynaptic GABA- and glycine- induced currents (Kawasaki et al., 2008). Glial activation has been shown to increase phosphorylation of the NMDA receptor subunit, NR1, and attenuation of activation with fluorocitrate or IL-1ra reduced NR1 phosphorylation (Guo et al., 2007; Zhang et al., 2008). Direct stimulation with IL-1β was then shown to induce NR1 phosphorylation, which was blocked by IL-1ra, but not fluorocitrate, indicating that glial cells secrete IL-1β responsible for NR1 phosphorylation (Guo et al., 2007). Further studies have demonstrated that IL-1β phosphorylates the subunits, NR2A and B (Viviani et al., 2003). Activation of NMDA receptors leads to production of PGE_2 and NO, which are involved in amplifying the excitability of pain projections neurons (Besson, 1999).

Part of the synaptic homeostasis maintained by astrocytes includes a regulation of glutamate concentrations by Na^+ dependent GLT-1 and GLAST glutamate transporters in the astrocyte

cytoplasmic membrane (Rothstein et al., 1996; Gegelashvili and Schousboe, 1997; Lehre and Danbolt, 1998; Berger and Hediger, 2000). This transported glutamate is metabolised to glutamine via the enzyme glutamine synthetase and shuttled back to the neuron for reconversion to glutamate (Sonnewald et al., 1997). Dysfunction of these transporters can produce marked changes in spinal processing, as inhibition causes an elevation in extracellular glutamate concentrations and results in spontaneous nociceptive hypersensitivity (Liaw et al., 2005; Weng et al., 2006). Furthermore, the development of neuropathic pain is associated with deficiency and downregulation of these transporters (Sung et al., 2003; Binns et al., 2005; Weng et al., 2005; Xin et al., 2009). Nerve injury has been associated with upregulation of GLT-1 and GLAST on microglia, however the functional consequences are currently unknown (Nakajima et al., 2008; Xin et al., 2009). Some evidence demonstrates that proinflammatory cytokines downregulate glutamate transporters, however the intracellular mechanisms are unknown (Prow and Irani, 2008; Carmen et al., 2009). Depending on the temporal resolution, glutamate transporter downregulation may account for increased frequency of excitatory post synaptic potentials observed by Kawasaki and colleagues (2008).

1.3.2.7. CNS leukocyte infiltration in neuropathic pain

The CNS is no longer considered an entirely immune privileged organ (Hickey, 1999) and immune cell infiltration has been implicated in many diseases including AIDS-associated dementia, Alzheimer disease, Guillain-Barré syndrome, Parkinson's disease, stroke, traumatic brain and spinal cord injury and demyelinating diseases such as multiple sclerosis (Wilson et al., 2010). Research over the last decade has similarly implicated CNS leukocyte infiltration in the pathophysiology of neuropathic pain, and evidence suggests an adaptive immune component within the CNS by infiltrating T lymphocytes (Hu and McLachlan, 2002; Sweitzer et al., 2002a; Hu et al., 2007; Cao and DeLeo, 2008; Cao et al., 2009), which is further supported by attenuation of nociceptive hypersensitivity in nerve-injured nude rodents (Moalem et al., 2004; Cao and DeLeo, 2008; Costigan et al., 2009a) and *MHC class II* (Sweitzer

et al., 2002b), *CD4* (Cao and DeLeo, 2008), *CD40* (Cao et al., 2009), *Rag1* (Kleinschnitz et al., 2006; Costigan et al., 2009a) and *IFN-γR1* (Costigan et al., 2009a) GKO mice. Cao and DeLeo (2008) identified lumbar spinal cord infiltrating $CD3^+$ $CD4^+$ T lymphocytes in nerve injury and demonstrate a role for $CD4^+$ T lymphocytes, distinct from $CD4^+$ macrophages and microglia, in nerve injury. Further identification of lumbar spinal cord infiltrating T lymphocytes has been thoroughly explored by Costigan and colleagues (2009a), comparing the lumbar ipsilateral dorsal horn RNA expression profile of adult rats that develop allodynia to nerve injury with neonatal rats that do not. Pathway analysis of the RNA expression profile in the ipsilateral dorsal horn of the lumbar spinal cord revealed a significant microglial, as well as $CD2^+$, $CD3^+$ and TCR^+ T lymphocyte response and related extravasation and signalling pathways in the nerve-injured adult. This role for T lymphocytes was further confirmed by the demonstration that *Rag1* KO and nude mice do not develop nociceptive hypersensitivity, in contrast to B cell deficient mice. These data are supported by the detection of T_H1, T_H2 and T_H17 cytokine upregulation in the nerve-injured adult and by attenuation of allodynia in nerve-injured *IFN-γR1*, a T_H1 signalling pathway, GKO mice. A respective proinflammatory and protective role for T_H1 and T_H2 cells in neuropathic-like pain is also supported by previous reports (Moalem et al., 2004; Rutkowski et al., 2004). Nerve injury modifies vascular endothelial permeability by inducing endothelial expression of PECAM and ICAM (Rutkowski et al., 2002; Sweitzer et al., 2002b), which facilitates leukocyte transendothelial migration (Muller, 2009).

Once in the CNS, infiltrating T lymphocytes aggregate in regions of glial activation (Hu et al., 2007; 2009a). Microglial MHC II expression is upregulated in nerve injury, and nerve-injured *MHC II* KO mice develop attenuated allodynia compared to wild type controls (Sweitzer et al., 2002b) which suggests a T_H1 interaction and causal mechanism in neuropathic pain. Microglial CD40 expression is upregulated in nerve injured rats and the CD154-CD40 complex formed by T lymphocytes and microglia respectively leads to downstream proinflammatory signalling, however allodynia is initiated, but not maintained, in

nerve-injured *CD40* KO mice compared to wild type controls (Cao et al., 2009). Microglial expression of these surface antigen in nerve injury, in addition to expression of proinflammatory cytokines, such as IL-1, IL-6 (Milligan and Watkins, 2009) and IL-15 (Gomez-Nicola et al., 2008) and various chemokines (White et al., 2007), provides an immunocompetent microenvironment in the lumbar spinal cord for immune cell infiltration. These infiltrating T lymphocytes may be a key partner to glial activation mechanisms in neuropathic pain. The pattern of allodynia induction, but lack of maintenance, recurs in nerve-injured nude (Cao and DeLeo, 2008), *Rag1* (Costigan et al., 2009a) and *CD4* KO (Cao and DeLeo, 2008) mice. Furthermore, CD11b, but not GFAP, expression was reported at 7 days post nerve injury in *CD4* KO mice, compared to wild type controls (Cao and DeLeo, 2008) suggesting that infiltrating T lymphocytes may be critical mediators in the transition from microglial to astrocytic mediated pain and therefore the transition of acute to chronic pain.

1.3.3. Pain processing summary

Neuropathic pain processing is not linear, but rather receives input at multiple levels and encompasses many overlapping neural and immune mechanisms. Furthermore, the system is plastic, undergoing dynamic modifications at the peripheral terminus, in terms of increased ion channel density and reduced activation thresholds; at the DRG, in terms of increased Na^+ channel clustering leading to ectopic activity of damaged nerves and cross-excitation to undamaged fibres; at the dorsal horn of the spinal cord, in terms of disinhibition of GABAergic and glycinergic neurons, increased synaptic strength and subthreshold activation of ascending pain transmission neurons, and recruitment of Aβ-fibres. Descending pathways from midbrain and brainstem regions then modulate the integrated signal at the dorsal horn in either an inhibitory or facilitatory fashion. The immune system acts on this neural circuitry at every level, whether it be immune cells in the PNS, or glia and infiltrating immune cells in the CNS, amplifying the mechanisms underlying peripheral and central sensitisation. Key brain regions process

the transmitted nociceptive information, resulting in a pain sensation and an emotional response, which, in either cause or effect, positively or negatively modulates the gain of pain.

Basic strategies of pain control are based on this concept of integration at peripheral, spinal and supraspinal sites by attenuation or blockade of pain through intervention at the periphery, by activation of inhibitory processes that gate pain at the spinal cord and brain by interference with the perception of pain.

1.4. Treatment of pain and the problem of heterogeneity

To date, most treatment approaches have been based on management of symptoms, rather than modification of the underlying neuropathic pain disease mechanisms (Dray, 2008). Generally, pharmacological treatment of neuropathic pain is organised into different recommendations (Dworkin et al., 2007; Jain, 2008), but due to heterogeneity across the treatment of different pain conditions, any algorithm must be tailored to the specific patient. The treatment algorithm recommended by the Australian Pain Society (2008) is that devised by Finnerup and colleagues (2005): tricyclic antidepressants/ selective noradrenaline-serotonin inhibitors > opioids ≥ tramadol ≥ gabapentin/ pregabalin.

1.4.1. Treatment of pain: Antidepressants

1.4.1.1. Tricyclic antidepressants (TCAs)

TCAs, such as amitriptyline, nortriptyline, imipramine and desipramine, have been extensively used in the treatment of neuropathic pain due to their predominant ability to block reuptake of NA and 5-HT in the CNS descending antinociceptive pathways (Barkin and Barkin, 2001; Wallace, 2001; Namaka et al., 2004). TCAs can also suppress neuronal hyperexcitability by blocking Na^+ and Ca^{2+} channels as well as adenosine and NMDA receptors (Popik et al., 2000; Bielefeldt et al., 2002). Recent *in silico* docking evidence suggested that TCAs may be TLR2 and TLR4 antagonists, which was confirmed in a series of *in vitro* experiments (Hutchinson et al., 2010b). There is little comparative evidence to indicate that one TCA is more effective than another. Selection of a specific TCA may be based on the side effect profile of the agent (Barkin and Barkin, 2001; Finnerup et al., 2010).

1.4.1.2. Selective- serotonin reuptake inhibitors (SSRIs)

SSRIs sertraline, paroxetine, fluoxetine and citalopram all represent another subclass of antidepressants used to treat neuropathic pain. SSRIs differ from traditional TCAs in their ability to

selectively inhibit 5-HT reuptake without affecting noradrenaline. There have been conflicting reports regarding the effectiveness of SSRIs in managing neuropathic pain (Verma and Gallagher, 2002). One study found citalopram more effective than placebo in treating diabetic neuropathy (Sindrup et al., 1992) and another found paroxetine and sertraline more effective than placebo in managing burning pain (Maina et al., 2002), while another found fluoxetine no more effective than placebo in managing burning pain (Max et al., 1992). Despite the mechanistic differences between the two antidepressant classes, the literature has not been able to conclusively demonstrate a difference between them in terms of their ability to manage neuropathic pain (Namaka et al., 2004). More recent evidence questions to use of this class entirely (Finnerup et al., 2010).

1.4.2. Treatment of pain: Anticonvulsants

As with epilepsy, the hallmark characteristic of neuropathic pain is neuronal hyperexcitability. As a result, many anticonvulsants have been effectively used in the management of neuropathic pain due to their inherent ability to suppress neuronal hyperexcitability. Neuronal inhibition by agents of this therapeutic class is accomplished by several different mechanisms, which include reducing neuronal influx of Na^+ and Ca^{2+}, indirect or direct enhancement of the of the inhibitory effects of GABA and reducing activity of glutamate by depleting its stores and/or blocking the NMDA receptors (Namaka et al., 2004; Cheng and Chiou, 2006). Various antiepileptics suppress neuronal hyperexcitability through one or more of these mechanisms which ultimately results in the alleviation of chronic pain, including gabapentin and pregabalin, and carbamazepine and its analogue oxcarbazepine to a lesser extent (Namaka et al., 2004).

1.4.3. Treatment of pain: Opioids

The term opioid applies to any substance, whether natural or synthetic, that produces morphine-like effects, whereas opiate refers to compounds derived from *Papaver somniferum* and their synthetic

analogues (Somogyi et al., 2007). Opioids mimic the action of endogenous opioids on neurons by binding to opioid receptors that presynaptically inhibit high-voltage activated Ca^{2+} channels (Stein et al., 2003) that then decreases the release of neurotransmitters Substance P, NA etc. Although it is well known that opioids induce membrane hyperpolarization as a result of increased K^+ currents in central neurons, this has not yet been detected in DRG neurons and thus the modulation of Ca^{2+} channels seems to be the primary mechanism for the inhibitory effects of opioids on peripheral sensory neurons (Stein et al., 2003). Opioids also inhibit adenylate cyclase, suppress tetrodotoxin resistant Na^+-selective and non-selective cation currents stimulated by PGE_2 (Ingram and Williams, 1994; Gold and Levine, 1996). Tetrodotoxin resistant Na^+ channels are selectively expressed in nociceptors, where they have an important role in impulse initiation and action potential conductance, as well as mediating spontaneous activity in sensitised nociceptors (Laird et al., 2002). Consistent with their effects on ion channels, opioids attenuate the excitability of peripheral nociceptor terminals, the propagation of action potentials, the release of excitatory proinflammatory neuropeptides from peripheral sensory nerve endings and the vasodilation evoked by stimulation of C-fibres. All these mechanisms result in analgesia or antiinflammatory actions (Stein et al., 2003).

Opioid treatment is generally considered for neuropathic pain when other therapies fail, when pain relief from an opioid analgesic is significant and sustained, and the quality of life is improved enough to tolerate adverse effects and risk of long-term adverse effects of opioid therapy (Breivik, 2005). Traditionally, prescribers have been cautious of opioid treatment for chronic pain, based on fear of abuse and diversion. However, other problematic clinical issues include physical dependency, development of tolerance, cognitive disorders, abnormal pain sensitivity, dysfunction of the immune and reproductive systems and respiratory depression, nausea and vomiting, pruritus, miosis, constipation and sedation (Schug et al., 1992; Hojsted and Sjogren, 2007). Unfortunately, adverse effects associated with opioid treatment itself may limit the clinical benefit experienced by patients, the most common being

tolerance, hyperalgesia, nausea, constipation and sedation (Slatkin and Rhiner, 2003), which is supported by a recent Danish epidemiological study showing that long-term opioid treatment is generally not achieving the key goals of pain relief, improved functional capacity and quality of life, although a small proportion have reported an improvement in the key outcomes (Hojsted and Sjogren, 2007).

1.4.3.1. Adverse effects: Opioid tolerance

Opioid tolerance is a phenomenon in which repeated exposure to an opioid results in decreased therapeutic effect of the drug or need for a higher dose to maintain the same effect (Chang et al., 2007), resulting in rightward shift of the dose-response curve. Tolerance to adverse effects does not develop at the same rate as analgesic tolerance (Trescot et al., 2008) and is clinically problematic, as adverse effects limit the maximum analgesic dose. There are several aspects of tolerance: innate tolerance is a genetically determined sensitivity that is observed during the first administration (Chang et al., 2007); acquired tolerance can be divided into pharmacokinetic and pharmacodynamic tolerance; pharmacokinetic tolerance refers to changes in the distribution or metabolism of the opioid, resulting in reduced concentrations of the opioid in the blood or at the sites of drug action (Chang et al., 2007). Pharmacodynamic tolerance refers to adaptive changes in opioid receptor sensitisation and/or density. Christie (2008) has further characterises this pharmacodynamic tolerance at:

1. The μ opioid receptor: partial loss of capacity to signal to intracellular effectors over time due to decreased expression and/or reduced coupling efficacy.
2. The cell: homeostatic adaptations to the signalling system due to continued μ opioid receptor activation, such as cAMP hypertrophy.
3. The system: adaptations in networks linked to the μ opioid receptor, such as ORL1-receptor-nociceptin/OFq systems, NK_1 signalling, etc. that may function as anti-opioids.

Recent advances implicate glia in the development of analgesic tolerance and will be discussed in greater detail below.

1.4.3.2. Adverse effects: Opioid-induced hyperalgesia (OIH)

OIH is the paradoxical increased sensitivity to pain that develops following acute and chronic opioid exposure (Doverty et al., 2001; Angst and Clark, 2006; Pud et al., 2006; Hay et al., 2009; Hay et al., 2010). Suggested mechanisms include glutamate associated NMDA receptor activation, causing spinal neuron sensitisation, which is further supported by blockade of OIH following NMDA receptor antagonism (King et al., 2005; Ossipov et al., 2005; Mao, 2006). Other studies have documented that OIH results from increased excitatory neurotransmitters such as CCK, which are released from neurons in the RVM and activate spinal pathways that upregulate spinal dynorphin. Both of these substances act as pronociceptive agents (Dourish et al., 1988; Xu et al., 1992; Vanderah et al., 2000; Vanderah et al., 2001; Gardell et al., 2002). Crain and Shen (2000) have investigated mechanisms whereby the neuronally-bound GM1 ganglioside may induce a switch from the inhibitory $G_{i/o}$ to stimulatory G_s coupling of the μ opioid receptor. Their *in vitro* data also suggests that ultra-low doses of opioid antagonists may selectively block G_s-coupled μ opioid receptor due to lower activation thresholds (Crain and Shen, 2000). Recent advances implicate glia in the development of OIH and will be discussed in greater detail below.

1.4.3.3. Glia and opioid tolerance and OIH

In addition to direct neuronal effects, opioids have also been demonstrated to activate spinal glia and upregulate proinflammatory cytokines in the spinal cord (Song and Zhao, 2001; Raghavendra et al., 2002; Johnston et al., 2004) via NO-induced p38 MAPK activation (Cui et al., 2006; Liu et al., 2006; Hutchinson et al., 2008a). Key cytokines include TNFα, IL-1β, IL-6, IFNγ, CCL2 and fractalkine (Shavit et al., 2005; Hutchinson et al., 2008a; Hutchinson et al., 2010a) and their upregulation is correlated with analgesic tolerance and hyperalgesia (Raghavendra et al., 2004). Central administration of proinflammatory cytokines also inhibits opioid analgesia (Gul et al., 2000; Szabo et al., 2002). Further converging evidence for the role of glial-derived proinflammatory cytokines in opioid tolerance and OIH

is that glial activation by other phenomena, such as neuropathic pain (Raghavendra et al., 2003b; Mika et al., 2007) or LPS administration (Johnston and Westbrook, 2005; Wu et al., 2006b), also reduces the analgesic efficacy of morphine, which has been referred to as 'naïve tolerance' (Hutchinson et al., 2007). Whilst glia do possess classical opioid receptors (Chang et al., 1998), it seems unlikely that they are responsible for glial activation, as triple opioid receptor knockout animals do not attain opioid analgesia, but still develop OIH (Juni et al., 2007).

Administration of glial modulators or cytokine antagonists attenuates the development of analgesic tolerance and prevents the development of hyperalgesia and allodynia in response to systemic morphine (Song and Zhao, 2001; Raghavendra et al., 2002; Raghavendra et al., 2003b; Johnston et al., 2004; Raghavendra et al., 2004), as well as restoring analgesia (Raghavendra et al., 2003b; Hutchinson et al., 2008b; Hutchinson et al., 2009a). Pretreatment or co-administration of IL-10 or IL-1ra attenuates acute morphine tolerance (Fairbanks and Wilcox, 2000; Johnston et al., 2004), prevents the development of hyperalgesia and allodynia and potentiates analgesia (Johnston et al., 2004). Intrathecal administration after established tolerance reverses hyperalgesia, prevents the development of additional tolerance and allodynia (Johnston et al., 2004) and prolongs and potentiates opioid analgesia (Hutchinson et al., 2008a).

The sum of morphine's neuronal antinociceptive activity and its pronociceptive glial activation results in a net reduction in analgesia, manifested as analgesic tolerance, eventually resulting in hyperalgesia (Hutchinson et al., 2007). Recent data demonstrate higher IL-6 levels in the CSF of chronic pain patients treated with opioids long term compared to chronic pain patients treated with opioids over a shorter period of time (Zin et al., 2010). Recent evidence demonstrates that opioids activated glia via TLR4 (Hutchinson et al., 2007), as well as $P2X_4$ (Horvath and DeLeo, 2009).

1.4.3.3.1. Opioid action at TLR4

The opioid receptor antagonist naloxone has demonstrated antagonism of the effects on the TLR4 ligand, LPS (Chang et al., 2000; Liu et al., 2000a; Liu et al., 2000b; Lu et al., 2000). However both the opioid active (-)-isomer and the opioid inactive (+)- isomer of naloxone exerted identical inhibitory effects on LPS microglial activation, suggesting that the actions of naloxone on LPS-induced microglial activation are not mediated by classical opioid receptors (Liu et al., 2000a). Furthermore (+)- naloxone has demonstrated ability to potentiate acute and chronic morphine analgesia (Watkins et al., 2007). Morphine-induced anti-analgesia, in which a prior, glial priming, low dose of morphine attenuates subsequent analgesia from a higher dose of morphine (Wu et al., 2005), is prevented by administration of (+)- naloxone (Wu et al., 2005; Wu et al., 2006a; Wu et al., 2006b).

Hutchinson and colleagues (2007) confirmed *in vitro* that (+)- naloxone attenuates opioid withdrawal and OIH by antagonism of TLR4 and subsequently demonstrated a reversal of CCI-induced allodynia 90 mins following systemic administration of (+)- naloxone. Hutchinson and colleagues (2007) thus propose that opioid tolerance and OIH are mediated by glial TLR4 activation, which is further confirmed by a leftward shift of the morphine analgesic dose-response curve in the TLR4 KO compared to the wild type control (Hutchinson et al., 2010c). (+)- Naltrexone has also demonstrated non-stereoselectivity in antagonising TLR4 (Hutchinson et al., 2007)

Further characterisation has demonstrated that other TLR4 antagonists, including mutant LPS and LPS-RS, (+)- naltrexone, inhibition of the TLR4 intracellular signalling pathway, as well as inhibition of the TLR4 cofactor, heat shock protein-90, potentiated acute morphine analgesia and attenuated the development of analgesic tolerance and OIH (Hutchinson et al., 2007; Hutchinson et al., 2009b; Hutchinson et al., 2010c).

In vitro characterisation has subsequently demonstrated that many opioids and their metabolites are non-stereoselective TLR4 agonists (Hutchinson et al., 2010c) and that the (+)- isomers, which possess no classical opioid receptor activity, cause glial activation, hyperalgesia and upregulation of proinflammatory cytokine mRNA protein and release (Watkins et al., 2005; Hutchinson et al., 2010a).

1.4.3.4. Adverse effects: Opioid-induced sedation

It is well known that a common adverse effect of opioid therapy is sedation (Benyamin et al., 2008). Indeed the name morphine is derived from the Greek god of dreams, 'Morpheus'. It has been postulated that opioids cause sedation by decreasing sensory input and thereby increasing the probability of sleep. However, the work of Moruzzi and Magoun (1949) demonstrated that awake and sleep states are not determined by the amount of sensory input but rather are dependent on intact systems that are intrinsic to the CNS. Consciousness is a complex physiologic process accomplished through coordinated actions within the CNS (Young-McCaughan and Miaskowski, 2001). Plum and Posner (1980) proposed that consciousness is composed of 2 interrelated domains: arousal and content.

Arousal is the organism's state of responsiveness to sensory stimuli and is understood to be mediated by the reticular formation, which is distributed throughout the medulla, pons and midbrain (Moruzzi and Magoun, 1949; Role and Kelly, 1991; Amaral, 2000) and project to the thalamus, which also plays an integral role in arousal (Newman, 1995). The thalamic neurons are reciprocally linked with neurons in the cerebral cortex and have two distinct settings that correspond to awake and sleep states, under the control of aminergic and cholinergic neurotransmitters (Hobson, 1990). The depolarized, or awake, setting responds to sensory input, whereas the hyperpolarized, or sleep, setting is relatively unresponsive to sensory input (Steriade et al., 1993). The mechanism that triggers the switch from on setting to the other is not well understood (Hobson, 1990). Activation of the LC is also important for the maintenance of arousal states, information processing as well as regulating sleep cycles (Berridge and Waterhouse, 2003).

Content addresses how the organism interprets the environment by means of sensation, thought, speech, imagination and interpretation of somatic modalities (Crigger and Strickland, 1985). Content processing is primarily a function of the cerebral cortex, which integrates sensory and motor information from the thalamus and coordinates appropriate responses to various inputs (Amaral, 2000).

The expression of these domains as a consciousness is a time-sequenced process that fluctuates in a diurnal pattern of wakefulness and sleep. Various pathologic or drug-induced conditions can disrupt arousal and can be manifested in the extreme as either hyperactive delirium or unconsciousness (Young-McCaughan and Miaskowski, 2001). Opioid-induced sedation, sleep disruption and respiratory depression are at least in part mediated by depression of central cholinergic activity. A direct link to the effects of opioids comes from studies showing that morphine and other opioids inhibit cholinergic activity in multiple brain regions (Beleslin and Polak, 1965; Domino and Wilson, 1973; Jhamandas and Sutak, 1976; Yaksh and Yamamura, 1977; Rada et al., 1991; Sandor et al., 1992). It therefore seems reasonable to hypothesise that opioid-induced sedation and mental clouding arise from the inhibition of central cholinergic activity in multiple functional areas of the central nervous system. Young-McCaughan and Miaskowski (2001), conclude that 'opioid-induced sedation represents a unique, disordered level of consciousness in which both arousal mechanisms and content processing are functional but attenuated because of the action of opioids at receptors within the CNS. Attenuated arousal is manifested as decreased wakefulness, and attenuated content processing is manifested as a slowed interpretation of the environment'.

1.4.4. The problem of heterogeneity

Opioids are currently the most efficacious analgesics for moderate to severe pain; however they are only partially effective in treating the symptoms of neuropathic pain. Anticonvulsants and antidepressants work very well in selected patient populations, but not at all in others. Long term opioid treatment results in hyperalgesia and tolerance due to the narrow therapeutic index and thus opioids

may not be the ideal drug for the treatment of neuropathic pain. Thus, there is a problem of heterogeneity in pain treatment that creates further problems when novel therapeutic agents are trialled. Heterogeneity raises the NNT; the epidemiological measure that indicates how many patients would require treatment for a 50 % reduction in pain score. For example, the average NNT (95 % CI) across all neuropathic conditions is 3.3 (2.9, 3.8) for antidepressants, 2.5 (2.0, 3.2) for opioids and 4.2 (3.8, 4.8) for anticonvulsants (Finnerup et al., 2005). A higher NNT may result in a 'trial and error' selection of pain therapies, which may ultimately be futile. This exposes the patient to unnecessary adverse effects and increases the cost to the patient, without necessarily providing pain relief (Woolf and Decosterd, 1999). It is likely that heterogeneity in neuropathic pain mechanisms underlies this heterogeneity to treatment response. Whilst many interviews and questionnaires have been developed in an attempt to alleviate this problem by identifying the underlying mechanisms (Rowbotham and Fields, 1996; Galer and Jensen, 1997; Woolf and Decosterd, 1999), an objective, mechanism-based biomarker is desirable (Woolf and Decosterd, 1999; Finnerup and Jensen, 2006).

1.5. Biomarker discovery and development for pain and sedation

The discovery and development of biomarkers for pain and sedation has the heuristic goal of a better understanding of the underlying mechanisms, however a number of translational goals are described below.

1.5.1. Biomarkers of pain

Neuropathic pain remains poorly managed for many patients (Finnerup and Jensen, 2006; Dworkin et al., 2007), however this problem may be alleviated as outlined in the following translational goals for pain biomarker discovery and development:

1. Diagnostics: As described above, neuropathic pain is not a homogeneous condition, but a constellation of different sensitivities in normal and diseased states. The current aetiological or disease-based clusters, anatomical, or symptomatic classification of neuropathic pain is insufficient and should be replaced by mechanism-based classification in order to treat neuropathic pain effectively (Woolf and Decosterd, 1999; Woolf and Mannion, 1999; Woolf, 2004; Finnerup and Jensen, 2006). The reasons for this are that the primary disease only the initiates the cascade of changes that lead to and sustain neuropathic pain (Costigan et al., 2009b) and the resulting symptoms are not equivalent to the underlying mechanisms, although they do reflect them. The pain that manifests in these diverse diseases may operate through common mechanisms, and the same disease may activate different mechanisms. However, no pain mechanism is an inevitable consequence of a particular disease process; only a few patients are affected and there are no predictors to indicate which patient will develop neuropathic pain. One mechanism could be responsible for many different symptoms, but the same symptom in two patients may be caused by different mechanisms. Furthermore, more than one mechanism can operate in a single patient and these mechanisms may change with time. Thus, in patients with neuropathic pain it is impossible to predict the mechanisms responsible for their pain on

the basis of only aetiology of the neuropathy or on the distribution or nature of their symptoms (Woolf and Decosterd, 1999; Woolf and Mannion, 1999; Finnerup and Jensen, 2006). For these reasons, mechanism-based, objective biomarkers of pain have enormous utility due to their potential to predict the development of neuropathic pain in patient subsets; diagnose the existence of neuropathic pain; and to characterise the underlying mechanisms responsible for the pain. Elucidation of these factors may then be used to devise the optimum, targeted treatment strategy and hence reduce the NNT.

2. An objective measure of pain: in addition to sensory alterations, neuropathic pain is an emotional experience (Merskey and Bogduk, 1994). As such, interpretation of interindividual differences is hampered by questions about differences in the manner in which each individual reports experiences or uses rating scales (Algom and Marks, 1984), and there is currently no objective measure by which to compare between patients, to titrate analgesics, or to determine the efficacy of analgesic therapy. Subjective pain reports are influenced by environmental factors, as well as expectations of treatment effect (Colloca and Benedetti, 2005), which may mask treatment efficacy. Therefore a biomarker of pain may be a useful adjunct to the visual analogue scale and other subjective measures of pain reporting. There are additional cases where a subjective report is not sufficient. Subjective reports may not always be relied upon if the clinician suspects drug abuse or diversion. In other cases, it may not be possible to obtain a subjective pain report when the patient cannot communicate effectively, such as the elderly, the young or the comatose.

3. Drug discovery and development tool: as described in section 1.1.2., mechanistic biomarkers may expedite the process of neuropathic pain drug discovery and development, mainly for reasons 1 and 2 described above. The high intra- and inter- patient variability is a major problem when evaluating the efficacy of novel treatments (Moore et al., 1998). However, objective biomarkers of pain may reduce this problem by targeting the novel treatment to a particular mechanism, rather than on the basis of a particular aetiology (Woolf and Decosterd, 1999). This necessitates enriched trial designs, whereby the

patient population is identified on the basis of the neuropathic pain mechanism targeted by using these biomarkers (Chizh et al., 2008; Woodcock, 2009). Symptomatic therapies may be evaluated on the basis of reduction of a particular symptom, however disease-modifying treatments should be detectable by the biomarkers used.

While there are many potential objective pain biomarker candidates, none are currently well accepted due to incomplete validation, or failure to meet one or more of the biomarker criteria described in section 1.1.3. The potential pain biomarker candidates are described below are organised in the same fashion used for section 1.3 Pain transmission: biomarkers found in the basal system and peripheral to spinal to supraspinal signalling pathways.

1.5.1.1. Biomarkers of pain: The basal system

Genetic factors govern the broad spectrum of pain sensitivities to noxious stimuli and visual analogue scales (VAS) can range from 'almost no pain' right through to 'worst pain imaginable' (Nielsen et al., 2005). Low pain thresholds reduce the quality of life and increase susceptibility to postoperative pain (Granot et al., 2003) and chronic pain (Tegeder et al., 2006). High pain tolerance, whilst appearing desirable, limits protective behaviour to injury (Indo et al., 1996).

Quantitative sensory testing (QST) in monozygotic (shared genetic and environment) and dizygotic (different genetic, but shared environment) twins has reported 22 % - 55 % genetic components for the majority of noxious stimuli modalities (Norbury et al., 2007; Nielsen et al., 2008). However there is poor correlation between modalities, suggesting different genetic contributions to different modalities (Mogil et al., 1999).

Many single nucleotide polymorphisms have been demonstrated to influence the response of nociceptive neurons, pain perception and treatment response (Kim et al., 2004; Edwards, 2006; Foulkes

and Wood, 2008; Somogyi and Hardy, 2010; Tremblay and Hamet, 2010), and as such, the genotype is an important biomarker of basal pain thresholds and sensitivity (Wagner, 2009).

1.5.1.2. Biomarkers of pain: Primary afferents

1.5.1.2.1. Primary afferents: Nociceptor activation

Objective activation of peripheral nociceptor activation can be a useful biomarker for detecting interindividual differences in pain sensitivity and pharmacodynamic activity of analgesics that are targeted against peripheral and spinal mechanisms of pain. This can be achieved using imaging techniques such as infrared thermography and laser-Doppler imaging (LDI). These techniques detect minute changes in blood flow, particularly in the skin, which is related to the release of vasoactive neuropeptides, like CGRP. Since many nociceptive fibres contain CGRP, their activation causes axon reflex-mediated retrograde release of neuropeptides and vasodilation (Sauerstein et al., 2000; Schmelz et al., 2000; Weidner et al., 2000; Klede et al., 2003a). Importantly, pharmacological agents reducing nociceptor excitability, such as opioids and Na^+ channel blockers, reduce flare evoked by noxious stimuli (Koppert et al., 1999; Koppert et al., 2001; Klede et al., 2003b).

This technique may be a useful diagnostic to detect peripheral sensitisation by measuring CGRP activity following exposure to a noxious stimulus. LDI was used to detect changes in the blood flow of postherpetic neuralgia patients following stimulation of the allodynic areas with a cotton wool bud, however, no changes were detected (Brooks and Tracey, 2005). Potential changes may be too subtle for detection and the degree of activation of the affected area may not be standardised between patients. Further research using this technology should examine the effects of capsaicin on blood flow to the affected area in this population.

1.5.1.2.2. Primary afferents: Microdialysis

Microdialysis allows the chemical components of the extracellular fluid (ECF) to be quantified by inserting a semi permeable probe into the tissue. The dialysate is then slowly pumped through the tube allowing molecules in the tissue to diffuse into the dialysate as it is pumped through the probe. The dialysate is then collected and analysed to determine the identities and concentrations of ECF molecules. This technique can be combined with methods assessing superficial blood flow as described above (Geber et al., 2007; Paterson et al., 2009).

Microdialysis techniques have been used as pain models. Histamine, bradykinin, serotonin, SP and CGRP have been pumped through the probe to activate nociceptors, which is quantified by recording VAS, itch and flare, as well as analysis of the dialysate (Weidner et al., 2000; Lischetzki et al., 2001; Klede et al., 2003a). Other studies have used the dialysate as an endpoint in various pain models (Angst et al., 2008b), such as sensitised skin (Angst et al., 2008a; Paterson et al., 2009), noxious stimuli (Sauerstein et al., 2000; Eberle et al., 2010), surgical models (Gordon et al., 2008), and capsaicin (Geber et al., 2007). Treatment has been demonstrated to modify the dialysate (Angst et al., 2008a; Gordon et al., 2008).

Microdialysis probes have also been implanted at the dorsal root entry zone to successfully sample amino acids such as glutamate and GABA in the dorsal horn of neuropathic pain patients, showing a significant increase in the ratios aspartate/GABA and aspartate/glycine in neuropathic patients (Mertens et al., 2000; Mertens et al., 2001; Parrot et al., 2004).

1.5.1.2.3. Primary afferents: Nerve biopsy

The peripheral nerve or skin can be biopsied to quantify characteristics of the peripheral nerve and to quantify neurotransmitter content.

Lindenlaub and Sommer (2003) quantified IL-1β, IL-6 and TNF-α expression and T cell and macrophage infiltration in sural nerve biopsy specimens from healthy controls and patients with vasculitic neuropathy (VANP), chronic inflammatory demyelinating neuropathy (CIDP) and non-inflammatory chronic axonal neuropathy (CANP) using immunohistochemistry. Cytokine immunoreactivity was highest in VANP, less strong in CIDP and lowest in CANP and this was correlated with the degree of axonal degeneration and T cell and macrophage infiltration. The cytokine content was correlated with a 4 point neuropathic pain scale for VANP and CANP. Similar studies have taken skin biopsies to diagnose peripheral neuropathy (Quattrini et al., 2007; Hlubocky et al., 2010; Liguori et al., 2010) and correlated changes to peripheral nerves with quantitative sensory testing outcomes (Quattrini et al., 2007; Bachmann et al., 2010; Hlubocky et al., 2010). The obvious limitation to this technique as a biomarker is that collection and analysis of a biopsy is not simple and convenient. Additionally, the biopsy must be specific to the condition being investigated.

1.5.1.3. Biomarkers of pain: Ascending and descending pathways

Currently, the only method of measuring activity of ascending and descending pathways in a clinical population is to measure soluble factors in the CSF.

In general, CSF levels of neurotrophins (Capelle et al., 2009), CGRP and substance P (Tsigos et al., 1993) and endogenous opioids (Tsigos et al., 1995; Joseph et al., 2006) show no correlation with neuropathic pain, although endogenous opioid levels may be used to monitor opioid treatment efficacy and tolerance (Raffaeli et al., 2006). CSF cystatin C was proposed as a biomarker of pain following a correlation in a labour pain model (Mannes et al., 2003), however subsequent research showed an association with pregnancy and acute labour pain, rather than neuropathic pain (Eisenach et al., 2004).

There has been limited success in correlating CSF components with pain. Yuan and colleagues (2002) analysed the CSF of idiopathic low back pain patients using a proteomics approach to identify a number

of proteins as potential biomarkers. Of these proteins, pigment epithelium-derived factor has subsequently been identified as a potential biomarker for neuropathic pain (Conti et al., 2005). Furthermore, decreased CSF adenosine levels have been reported in neuropathic pain patients (Guieu et al., 1996). CSF proinflammatory cytokines are showing the most promise, showing strong correlation that with neuropathic pain (Empl et al., 2001; Alexander et al., 2005; Uceyler et al., 2007a; Ludwig et al., 2008). A recent study suggests CSF IL-6 may be a useful biomarker of opioid-induced glial activation (Zin et al., 2010). CSF IL-8 levels at full crusting of a herpetic rash has also been shown to predict post herpetic neuralgia (Kotani et al., 2004).

S100B is secreted by astrocytes and has recently been touted as the "C-reactive protein of the brain", as it is released in neuropathologic states (Sen and Belli, 2007). S100B may show a correlation with neuropathic pain, however has not been investigated.

Overall, analysis of CSF of neuropathic pain patients has not yielded any reliable biomarkers and is limited by the invasiveness of the collection procedure, however a number of leads have been identified and may yet translate.

1.5.1.4. Biomarkers of pain: Imaging the pain matrix

The functional anatomy of pain has mainly been studied with the various analogues of PET and functional magnetic resonance imaging (fMRI), which have been reviewed elsewhere (Stephenson and Arneric, 2008). The use of brain imaging as a biomarker, as highlighted by Borsook and Becerra (2006), could range from (a) diagnosis and evaluation of a mechanistic pain condition; (b) evaluation of drug efficacy in responders vs. non-responders; (c) evaluation of novel drug efficacy and (d) provision of new insights into the mechanisms of endogenous pain systems. There is a wealth of data on the brain regions involved in pain processing (Peyron et al., 2000; Borsook and Becerra, 2003; Moisset and Bouhassira, 2007; Tracey, 2008; Neugebauer et al., 2009) that have been discussed previously

(1.3.2.3.), and analysis of these regions may indeed yield a diagnostic biomarker of neuropathic pain (Borsook and Becerra, 2005). Chizh and Hobson (2007) highlight a number of caveats to the use of brain imaging as a biomarker for pain. Firstly, the cortical activation evoked by pain is not fully understood and shows considerable overlap with many other mechanisms of reward, cognition, anticipation and so on. Secondly, responses to analgesic therapies do not always align with other objective markers, such as electroencephalography, or with subjective scales. This review briefly will cover the imaging of brain regions that respond to analgesic interventions.

1.5.1.4.1. Imaging the pain matrix: Positron emission tomography (PET)

PET measures the concentrations of isotopes in a given body volume. The isotopes are carried by natural or almost-natural molecules (water, deoxyglucose, L-dopa), which are usually injected and enter the brain via the blood stream. The physical variable measured by PET cameras is the distribution of radioactivity, while the associated physiological variable depends on the molecule that carries the positron emitting isotope. In studies where relatively rapid changes in activity are to be measured, isotopes with a short half life are preferred, allowing repeated measurements in short amounts of time. The preferred isotope in this case is ^{15}O, which has a half-life of only 2 minutes and can be included in natural molecules such as water or butanol to provide information on regional cerebral blood flow (rCBF). Measurement of rCBF is a sensitive, simple and versatile approach to functional brain mapping (Fox and Mintun, 1989). Local CBF increases reflect increases in local synaptic activity (Sokoloff et al., 1991). Short scan interval (50-90 s) and interscan interval (10-15 min) permit multiple studies in rapid succession. Interpretation of results is usually based on a voxel by voxel subtraction of images, seeking areas where blood flow is significantly changed in one condition with respect to another. The major limitations of PET studies are:

1. low temporal resolution due to signal averaging over approximately 1 min;
2. the need for pooled group analysis data of five to six subjects to obtain meaningful results;

3. the need for a nearby cyclotron facility to prepare radioactive tracers;
4. the need to give intravenous injections;
5. radioactivity.

The use of PET as a biomarker of pain involves assessment of rCBF in a basal pain state, which is then compared with rCBF following analgesic administration. So whilst there are many PET studies imaging the brain under acute or chronic pain conditions and pharmacological PET studies of various analgesics, to my knowledge, only a few studies have compared rCBF of pain (acute experimental or chronic) as a baseline followed by rCBF analysis once analgesia has been administered. These studies highlight the motor cortex (Adler et al., 1997; Casey et al., 2000; Davis et al., 2000; Kupers et al., 2000), thalamus (Adler et al., 1997; Casey et al., 2000; Kupers et al., 2000; Buvanendran et al., 2007; Wagner et al., 2007), anterior cingulate cortex (Casey et al., 2000; Davis et al., 2000) and the cerebellum (Casey et al., 2000; Davis et al., 2000; Buvanendran et al., 2007) as brain regions involved in mediating analgesia. PET may have utility in identifying differences in physiology that may be linked with basal pain states. For example, Jones and colleagues (2004) demonstrated a reduction in neuronal opioid receptor binding capacity in central neuropathic pain patients as compared with age-matched healthy controls. This technique has potential as a biomarker of pain, but together with the paucity of data and contradictory findings, much further work needs to be done in this area, particularly in patients with established chronic pain.

Recently, a selective PET radiotracer for activated microglia (peripheral benzodiazepine receptor) has been developed, allowing temporal imaging of activated microglia (Stephenson and Arneric, 2008). Challenges to this application include poor signal-to-noise ratio, as well as difficulty in imaging the spinal cord.

1.5.1.4.2. Imaging the pain matrix: Functional magnetic resonance imaging (fMRI)

fMRI analysis is based on changes in the blood oxygenation level dependent (BOLD) signal, which reflects simultaneously rCBF changes and variations in deoxyhaemoglobin content (Turner, 1992; Rosen et al., 1998). fMRI has some advantages over PET, including the operation in a non-radioactive environment and thus the possibility to repeat recordings. Even though new-generation PET cameras have been applied to single subject analysis (Chmielowska et al., 1998), the possibility to take into account anatomy and other individual characteristics by fMRI is a clear advantage over PET. Furthermore, the access to single subject analysis will be an important gain in pain studies, since pain is dependent on individual factors. Finally the temporal resolution of fMRI of 1-3 s is another advantage and appears to be an intermediate solution between PET resolution (tens of seconds) and electrophysiology (tens of milliseconds).

The major limitations of fMRI are:

1. the requirement of MRI-compatible equipment (non-ferro-magnetic)
2. the need for strict timing between stimuli and acquisition in rapidly alternating conditions
3. pulsation artefacts, which impair analysis of brainstem and thalamic responses
4. limited to activation responses and is not able to provide information on resting state or on neurotransmitters or receptors;
5. limited to relative rather than absolute changes.

A number of studies have been conducted in healthy volunteers examining the effects of opioids, COX inhibitors and ketamine on acute noxious stimuli. Key regions modified include the insula, thalamus, anterior cingulate and to a lesser degree, S1 and S2 (Wise et al., 2002; Rogers et al., 2004; Hoffman et al., 2007; Maihofner et al., 2007). Other studies have more closely modelled peripheral (Maihofner et al., 2007) and central sensitisation (Iannetti et al., 2005) and quantified the effects of COX inhibitors and gabapentin respectively. Similar modulation of the brain regions described above was observed.

As with PET scanning, further validation of the treatment response is required in chronic pain patients for the development of fMRI as a biomarker of pain.

1.5.1.5. Biomarkers of pain: Other systems

Various proteins have been identified in the saliva as potential biomarkers of pain. Salivary α-amylase, an enzyme responsible for hydrolysis of polysaccharides to simple sugars, has been correlated with VAS of chronic pain patients (Shirasaki et al., 2007), however more recent research suggests it may be a biomarker of sympathetically-mediated stress (Nater and Rohleder, 2009). Other groups have suggested that neuropeptides in saliva can be accurately measured as biomarkers of pain (Parris et al., 1993; Fischer et al., 1998), but has not been investigated further.

Various cytokines, including TNFα, G-CSF and IL-6, have been identified in the serum of peripheral neuropathy patients (Ludwig et al., 2008; Doupis et al., 2009), and for long-term opioid treatment (Zin et al., 2010), showing promise and therefore requiring further investigation. β-endorphin is released into the plasma following an acute painful stimulus (Bruehl et al., 2007; Rasmussen and Farr, 2009) and may be a useful endpoint in pain models, such as QST, but has not been quantified in neuropathic conditions.

A crude assay showed significant proliferation of a cancer cell line treated with serum from nerve-injured rats compared with control (Peterson and Servinsky, 2007). This protocol may be further developed using serum from chronic pain patients.

Given the recently identified role of the immune system in neuropathic pain, identification of immune factors in the peripheral circulation, such as the cytokines already discussed, is an ideal target for the development of pain biomarkers (Achur et al., 2010). As such, serum markers of immune activation, such as tryptophan (Huang et al., 2002; Schrocksnadel et al., 2006) and neopterin (Murr et al., 2001) may prove successful biomarkers of pain.

In 2004, Hutchinson and colleagues reported that the enhancement of concanavalin A proliferation by 10^2 μM morphine in isolated human peripheral blood mononuclear cells (PBMCs) was correlated with cold pain tolerance (Figure 1-11). In the light of recent findings, the mechanism for this finding may be morphine activation of TLR4 following the concanavalin A priming signal. This assay may thus mirror glial activation within the CNS and requires further characterisation.

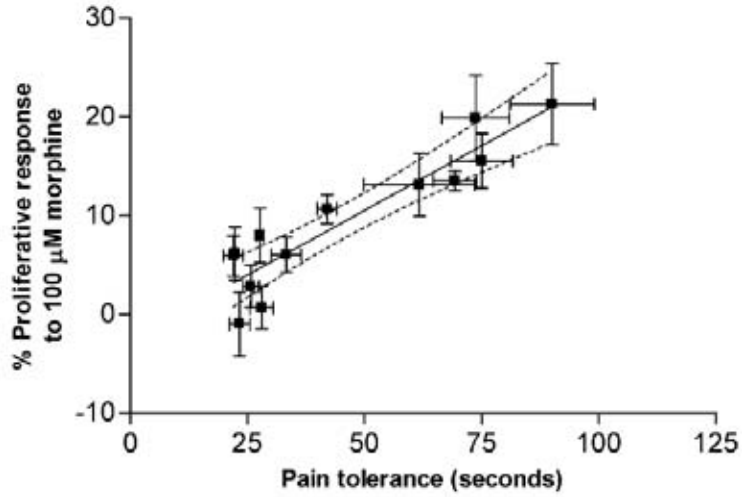

Figure 1-11. The correlation between proliferative response of peripheral blood mononuclear cells following *ex vivo* exposure to 100 μM morphine and cold pressor pain tolerances.

Hutchinson et al. (2004) reprinted, with permission, from Pain, Volume 110 © 2004.

These other systems have the advantage of easy access and a shared mechanism with neuropathic pain. Potential limitations in measuring single factors is that it is likely that they are influenced my multiple factors and may not be reliable. Cellular responses, such as the biomarker identified by Hutchinson and colleagues (2004) may have greater reliability due to an overlap of multiple mechanisms.

1.5.2. Biomarkers of sedation

There is particular interest in biomarkers of sedation from the perspective of industrial medicine in regard to shift work; psychiatry and psychology in the measurement of sleepiness; and from a clinical

pharmacological viewpoint in the assessment of the safety of sedative drugs. As an example of safety, opioids are used to treat many forms of chronic pain, however they are associated with many unwanted effects, such as sedation, which has the ability to affect daily activities, like driving. In this scenario, sedation has the ability to affect not only the patient's wellbeing and safety, but also other drivers.

Current subjective measures of sedation include visual analogue scales and self-reported questionnaires of which the Epworth sleepiness scale (Johns, 1991) and the Stanford Sleepiness Scale (Hoddes et al., 1973) are among the most well validated. However, subjective measures may not be the most appropriate measure for sedative drugs, as unpublished observations in our laboratory have shown: when additional buprenorphine was administered to opioid substitution patients, a marked degree of sedation was demonstrated objectively, whilst the patient was subjectively unaware. There have been similar reports previously, following alcohol administration (Holdstock and de Wit, 1999). As such, the patient's ability to self-report sedation may be affected by the drug itself or an inability to clearly articulate the feeling.

Current objective measures used to assess driving ability have included psychometric biomarkers such as Digit Symbol Substitution Test (DSST), test of variables of attention, ART 2020, which is a set of seven traffic psychology tests, as well as recorded driving exams and obstacle courses (Dellemijn et al., 1998; Galski et al., 2000; Meijler, 2000; Sohn, 2003; Schindler et al., 2004; Byas-Smith et al., 2005; Kress and Kraft, 2005; Baewert et al., 2007). Although psychometric testing is objective in nature, it is limited by the cooperation and motivation of the subject to complete the tests properly. Additionally, it may not be possible to extrapolate data relating to sedation from tests that only assess one aspect of cognitive function, such as DSST. Further, some of these tests take a significant amount of time to complete and are consequently not easily applied outside of the laboratory.

Another methodological factor that must be taken into account is the "learning effect". A relevant example is presented by Carrier and Monk (1999): A subject had to sort a pack of 96 playing cards into the four suits about six times a day, 7 days a week. The subject reached his best averaged speed in about 18 weeks (after more than 750 trials). Thus, even if hundreds of trials are given before the actual experiment, the practice effect must be taken into account.

Therefore there is a strong demand for convenient, objective measurement of sedation.

1.5.2.1. Ocular biomarkers of sedation

Oculomotor measures such as binocular convergence, eyelid closures, saccadic eye movements and pupil responses have been widely applied, showing sensitivity to drug use (Rowbotham et al., 1984; Rowbotham et al., 1987; Tennant, 1988; Jasinski et al., 1989; Pickworth et al., 1989; Cone, 1990; Pickworth et al., 1990; Pickworth et al., 1991; Pickworth et al., 1993; Jenkins et al., 1994; Paut et al., 1995; Fant et al., 1998); hypoxemia (Cymerman et al., 2005); and fatigue and sleep deprivation (Lowenstein and Loewenfeld, 1951; Yoss et al., 1970; Horne, 1975; De Gennaro et al., 2000; Crevits et al., 2003; Russo et al., 2003; Thomas et al., 2003; LeDuc et al., 2005; Rowland et al., 2005). Among the best validated and sensitive to a wide range of sedative states, independent of aetiology, are saccadic eye movements and pupillometry.

1.5.2.1.1. Ocular biomarkers of sedation: Saccadic eye movements

The line of sight is redirected to an area of interest by very rapid movements of the eyes, usually lasting less than 100 ms, called saccades. Saccades can target an object in the visual scene, a sound, a memory or a somatosensory stimulus, or they can occur spontaneously with no apparent target (Scudder et al., 2002). Saccadic eye movements (SEMs) can be elicited by instructing a subject to follow a target that jumps instantaneously from one point to another. A typical horizontal saccade will start approximately 180 ms after target movement (Baloh and Honrubia, 1976). During this time, the

CNS must identify the target, decide to move the eyes towards it and initiate the saccade. Once the saccade is triggered, the eyes accelerate rapidly to velocities approaching 1000 degrees per second (º/s), the peak velocity being a function of amplitude (Bahill et al., 1975). The eyes then decelerate to bring the foveae accurately on the target, usually with no overshoot or oscillations. When, on occasion, a normal subject misses the target, either by over or undershooting, a secondary automatic corrective saccade will be executed (Weber and Daroff, 1971). These properties make saccades the fastest and best controlled movements of which the body musculature is capable (Fuchs et al., 1985).

1.5.2.1.1.1. Neural control of saccadic eye movements

The programming and execution of a saccadic eye movement requires different operations that overlap in time, rather than following in a serial manner (Catz and Thier, 2007). Visual information is transmitted via both striate and extra striate visual cortex to the parietal cortex (eg, the lateral intraparietal area (LIP), the frontal eye fields (FEF), the supplementary eye fields (SEF), and the superficial layers of the superior colliculus (SC) (See Figure 1-12). LIP, FEF and SEF project heavily into the thalamus, the intermediate and deep layers of the SC ($SC_{i/d}$), the caudate nucleus of the basal ganglia and the basal pons. The saccade related output nucleus of the basal ganglia, the substantia nigra pars reticulata, provides inhibitory projections to the $SC_{i/d}$, which also receives a weak excitatory input from the superficial layers of the SC (SC_{sup}), in addition to the usual projections to the thalamus. Thus, the intermediate and deep layers of the SC appear to be a major point of convergence of saccade related signals (Scudder et al., 2002).

The SC, in turn, projects to the pre-motor saccade generation circuitry in the mesencephalic, pontine and medullary reticular formations, where neurons discharge preferentially for vertical and horizontal saccades. Further SC efferents include the reticulospinal neurons in the reticular formation and neurons in the cervical spinal cord for the simultaneous control of head movements and the cerebellum (via

nucleus reticularis tegmenti pontis), which exerts modulatory control over saccades and possible head movements (Scudder et al., 2002).

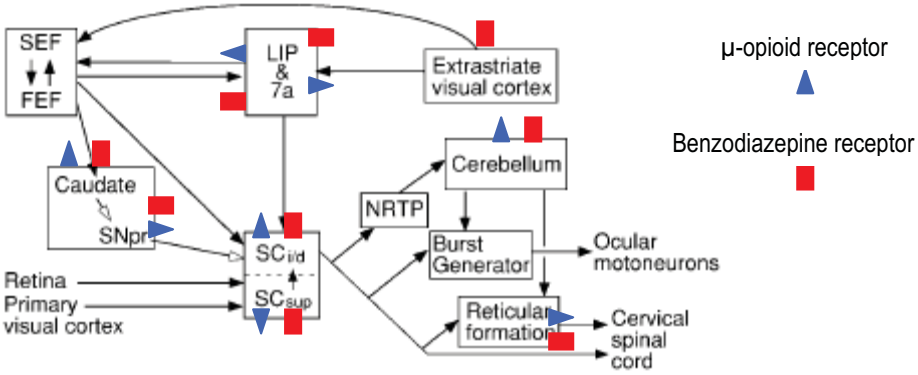

Figure 1-12. Schematic block diagram of areas of the brain believed to be involved in the generation of saccades. Excitatory connections shown by solid arrows, inhibitory connections by open arrows. ($SC_{i/d}$: intermediate and deep layers of the superior colliculus; SC_{sup}: superficial layers of the superior colliculus; NRTP: nucleus reticularis tegmenti pontis; 7a posterior parietal cortex; FEF frontal eye fields; LIP: lateral intraparietal cortex; SEF: supplementary eye fields; SNpr substantia nigra pars reticulata). Reprinted with kind permission from Springer Science: Experimental Brain Research, The brainstem burst generator for saccadic eye movements: a modern synthesis, vol. 142, 2002, p. 439, Scudder et al., figure 1, © 2002.

Saccades are generated by forces that consist of three components: a saccade force pulse that precedes and continues during the saccade, a force step to hold the eye at its new eccentricity against the elastic forces of the muscles and the globe (together, “the plant”) and a force slide between the pulse and step to counteract long time-constant viscoelastic elements of the plant (Collins et al., 1975; Miller and Robins, 1992). This pulse-slide-step profile, in turn is generated by six sets of oculomotor neurons that all exhibit a pulse-slide-step of discharge rate, known as a “burst-tonic” discharge pattern (Fuchs and Luschei, 1970; Fuchs et al., 1985; Goldstein and Robinson, 1986).

The “burst” component of the motoneuron discharge is generated by short-lead burst neurons located in the ipsilateral paramedian pontine reticular formation (PPRF) rostral to the abducens nucleus (Luschei

and Fuchs, 1972), the contralateral medullary reticular formation (medRF) caudal and ventral to the abducens nucleus, the bilateral nuclei prepositus hypoglossi (NPH) and medial vestibular nuclei (MVN). Short-lead burst neurons exhibit a burst of spikes in an "on-direction" but are silent during steady gaze. PPRF neurons, or excitatory burst neurons (EBNs), are responsible for activation of the agonist motoneurons of the abducens nucleus which activate the ipsilateral rectus muscle, and via internuclear neurons (INs) in the abducens nucleus neurons they are also responsible for the activation of the agonist motoneurons in the oculomotor nucleus controlling the contralateral medial rectus muscle (Highstein and Baker, 1978; Steiger and Buttner-Ennever, 1979). The lateral and medial rectus muscles direct the eye from side-to-side (Figure 1-13). These neurons exhibit sharp and vigorous bursts of action potentials during the saccade and the number of action potentials fired during the saccade increases with saccadic amplitude (Strassman et al., 1986). medRF neurons, or inhibitory burst neurons (IBNs), inhibit the motoneurons and IN of the contralateral abducens nucleus (Hikosaka et al., 1978; Hikosaka et al., 1980). The IBNs are involved in the process of relaxation of the antagonist muscles (Catz and Thier, 2007). The PPRF also contains omnidirectional pause neurons (OPNs), which are critical for the timing of saccades (Catz and Thier, 2007). They discharge at a high, constant rate when the eye is fixating and exhibit a pause in firing for saccades in all directions (Scudder et al., 2002).

The "tonic" signal is needed to stabilize the eyes in the new position acquired by the saccade, a signal related to eye position, counteracting position-dependent elastic forces trying to move the eyes back towards straight ahead. This signal is provided by tonic neurons, located in the PPRF, making excitatory connections with motoneurons (Catz and Thier, 2007). It has been suggested that eye position-related signal of TNs could be the result of a mathematical integration of the eye velocity-related signal provided by the EBNs. A copy of the eye velocity signal would be sent to a 'neural integrator' in order to generate a tonic command coding for eye position. This command would allow the motoneurons to offer the

constant position-dependent firing rate needed in order to stabilize the eyes at an eccentric position (Robinson, 1975).

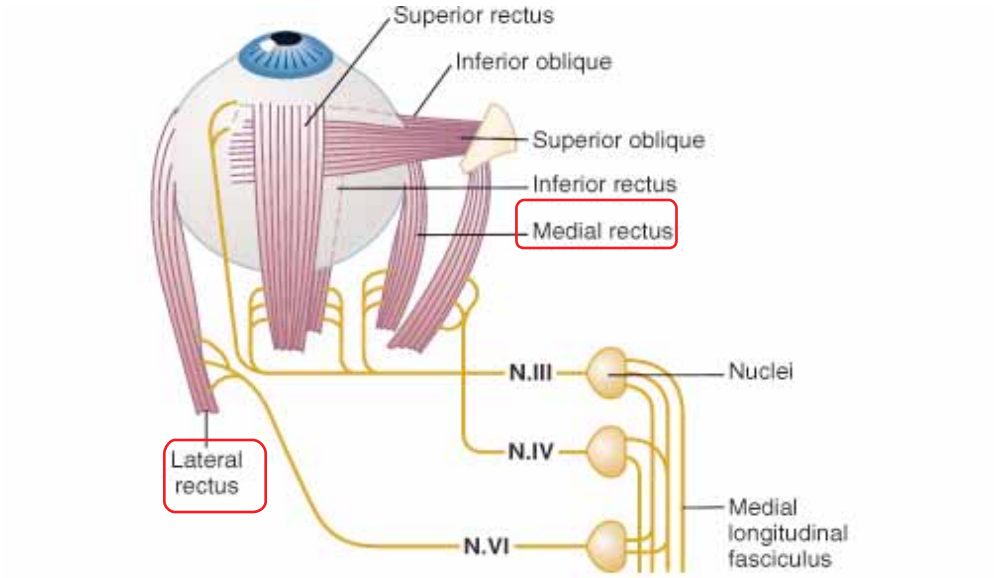

Figure 1-13. Extraocular muscles of the eyes and their innervation.

The lateral rectus and the medial rectus muscles are highlighted. Guyton (2006) reprinted, with permission, from Elsevier © 2006.

1.5.2.1.1.2. Measurement of saccadic eye movements

Aschoff (1968) first described the use of saccadic eye movements as a marker of drug-induced CNS depression, however it was not until the early 1980's when advances in microelectronics led to the development of quick and robust methods of generating and analysing saccadic eye movements using electro-oculography (EOG) (Smith et al., 1981). EOG is based on the ocular dipole concept (Taumer et al., 1974), where the eye is compared to a battery resting in a conducting medium, the cornea being positive in relation to the fundus by 10-30 mV. The electrical field created varies with eye position and can be recorded by electrodes in appropriate locations. EOG is only reliable for recording horizontal eye movements because lid movement artefacts prevent accurate recording of vertical eye movements (Barry and Jones, 1965). We have previously and reliably measured peak saccadic velocity (PSV) in our

laboratory using the Cardiff Saccades Generating and Analysis System (CSGAAS) (Marshall et al., 1985), which utilises EOG.

Many sedative drugs including benzodiazepines (Tedeschi et al., 1985; Griffiths et al., 1986; Tedeschi et al., 1986; Aantaa et al., 1990; Roy-Byrne et al., 1993; van Steveninck et al., 1993; Harron et al., 1995; Green et al., 1996; Roy-Byrne et al., 1996; van Steveninck et al., 1996; Bauer et al., 1997; Dingemanse et al., 1997; van Gerven et al., 1997; van Steveninck et al., 1997; van der Post et al., 2002; Blau et al., 2005; Wang et al., 2005a; Wezenberg et al., 2007; de Haas et al., 2008); ethanol (Lehtinen et al., 1979; Ali et al., 1985; Tedeschi et al., 1986; van Steveninck et al., 1996; Moser et al., 1998; Holdstock and de Wit, 1999; Blekher et al., 2002; King and Byars, 2004; Nyberg et al., 2004); zoplicone (Griffiths et al., 1986; Aantaa et al., 1990); triptans (van der Post et al., 2002); antihistamines (Hopfenbeck et al., 1995); thienobenzodiazepines (Wezenberg et al., 2007); clonidine (Glue et al., 1991; Harron et al., 1995); barbiturates (Tedeschi et al., 1986); antipsychotics such as chlorpromazine (Green et al., 1996); kava (Cairney et al., 2003a; Cairney et al., 2003b); hypnotics such as zolpidem (Richens et al., 1993); antidepressants such as mirtazapine (Wezenberg et al., 2007); and general anaesthetics (Gao et al., 1991; Yoshizumi et al., 1991) have been shown to reduce PSV. Opioids were first investigated in 1980, where Rothenberg and colleagues (1980) reported no reduction in PSV by methadone. Richens and colleagues (1983) found that meptazinol and papaveretum did cause a reduction in PSV, as did intravenous pethidine and fentanyl (Rottach et al., 2002). Hydromorphone challenge in methadone maintained subjects also elicited a reduction in PSV (Melichar et al., 2003). Amphetamines (Tedeschi et al., 1986) and SSRIs (Bell et al., 2003) have been shown to increase PSV.

1.5.2.1.1.3. Opioid modulation of the saccadic generator

Since the effects of opioids on saccadic eye movements have not been widely studied, there is not a strong evidence base as to why opioids alter the parameters of saccadic eye movements. Rottach and colleagues (2002) studied the effects of intravenous pethidine and fentanyl on the eye movements of

healthy volunteers and concluded that opioids may be acting in the cerebellum and the burst generator. Rottach and colleagues (2002) found that pethidine and fentanyl administration resulted in a mild cerebellar syndrome with some intention tremor, ataxia and dysarthria as the oculomotor findings of reduced smooth pursuit gain. Opioid receptors have been located on the cerebellum (Schadrack et al., 1999; Henderson and Wijdicks, 2000; Platzer et al., 2000) and so it is conceivable that opioids may inhibit neurons in the cerebellum, which has involvement in the generation of saccadic eye movements. Rottach and colleagues (2002) also hypothesise that opioids may act on OPNs as their malfunction has been shown to result in the slowing of saccades (Bronstein et al., 1990; Kaneko, 1996). Melichar and colleagues (2003) also highlighted the impact of opioid receptors in the cerebellum on saccadic eye movements.

1.5.2.1.1.4. Saccadic parameters

Mercer (1992) extensively studied the parameters of saccadic eye movements: saccade latency, peak saccadic velocity, saccade duration, acceleration, deceleration and found that peak saccadic velocity, peak deceleration and the acceleration to deceleration ratio were the most sensitive measures. Melichar and colleagues (2003) found peak deceleration to be the most sensitive measure of opioid-induced sedation following hydromorphone challenge in opioid dependent subjects.

1.5.2.1.1.5. Potential caveats to the use of SEMs as a biomarker of sedation

The effect of ambient light intensity on SEMs has been investigated and shown to have no impact on PSV (Yu et al., 2007). One study showed that saccadic velocity was unaffected by age (Shafiq-Antonacci et al., 1999), however another found a negative correlation between age and PSV (Wilson et al., 1993). There are no significant gender differences on any saccade parameters (Wilson et al., 1993). The effects of hormone replacement therapy on saccadic parameters, which is probably due to effects on $GABA_A$ receptors similar to benzodiazepines, has been well documented (Wihlback et al., 2001; Wihlback et al., 2005; Timby et al., 2006; van Broekhoven et al., 2006; van Broekhoven et al., 2007),

and unmedicated schizophrenia patients have a higher peak saccadic velocity than controls (Mahlberg et al., 2001).

1.5.2.1.2. Ocular biomarkers of sedation: Pupillometry

The pupil is formed by the iris and acts as a diaphragm to control the amount of light reaching the retina. The pupil is regulated by the autonomic nervous system with two antagonistic smooth muscle systems of continuously-varying activity resulting in a dynamic equilibrium that is expressed as pupil diameter. The pupillary sphincter, the constrictor responsible for miosis, is activated by the parasympathetic innervation of the eye and mediated by acetylcholine. The opponent of the pupillary sphincter, the dilator responsible for mydriasis, is the sympathetically innervated pupillary dilator muscle where NA is the neurotransmitter.

1.5.2.1.2.1. Neural control of pupil activity

The pupillary light reflex and near response are driven primarily by increased parasympathetic activity, while the resting size of the pupil diameter in darkness and in response to emotional changes are determined primarily by changes in sympathetic activity (Lowenstein et al., 1963). The pupillary dilator muscle exerts a much smaller force on pupillary size than does the pupillary sphincter. However, central modulation of sympathetic and parasympathetic activity results in a dynamic equilibrium of pupillary size. Increases in sympathetic activity are characteristically accompanied by central inhibition of parasympathetic activity. This central inhibition is mediated by two separate pathways. A noradrenergic pathway connects the LC to the midbrain area of the Edinger Westphal (EW) nucleus, while a second pathway connects the A1/A5 nuclei in the brainstem to the EW nucleus through the hypothalamus (Szabadi and Bradshaw, 1996). Parasympathetic pupilloconstrictor neurons of the EW nucleus fire continually and travel via parasympathetic reflex pathways to activate the sphincter muscle and constrict the pupil (Szabadi and Bradshaw, 1996).

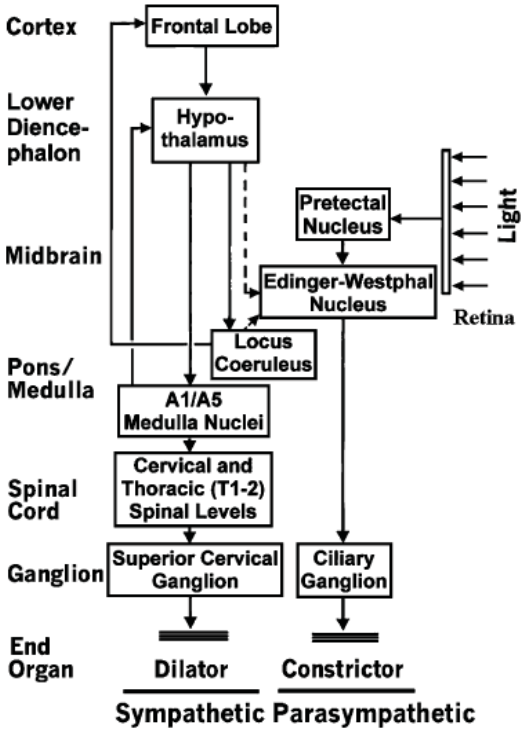

Figure 1-14. Autonomic neural control of pupil diameter.

Solid lines depict excitatory connections between brain areas, broken lines depict inhibitory ones. Szabadi et al. (1996) reprinted, with permission, from Journal of Psychopharmacology, Vol. 10, Suppl. 3 © 1996.

Central influences, such as alertness and emotion, and the peripheral sympathetic branch activate the dilator muscle. In alert individuals, excitatory impulses arise from the cerebral cortex and travel through the reticular activating system and hypothalamus to cause pupil dilation by inhibiting the EW nucleus and parasympathetic constrictor activity and activating the peripheral sympathetic pathway to the dilator muscle (Szabadi and Bradshaw, 1996) (Figure 1-14). Progressive loss of central sympathetic influences is the basis for pupillary oscillations and miosis. With ensuing sleepiness, central and hypothalamic centres cease to function in an orderly manner, central inhibition of the EW nucleus decreases, sympathetic tone is steadily lost and parasympathetic activity dominates, which is reflected in decreasing pupil size and large, slow pupillary oscillations (Lowenstein et al., 1963; Kardon, 1997). The second pathway is thought to be GABA-ergic. Activity in the central sympathetic inhibitory pathway

diminishes with age. Average pupillary size is maximal between the ages of 15 and 20 years and shows a continuous decrease of 0.4 mm per decade (Loewenfeld, 1993).

1.5.2.1.2.2. Pupillometric biomarkers of sedation

As the changes in pupil response and activity directly correlate with arousal (Loewenfeld, 1993), pupillometry provides the potential for a convenient, objective biomarker of sedation. The two parameters that have previously been examined are the pupil dark adaptation test and the pupil light reflex.

1.5.2.1.2.2.1. Pupil dark adaptation

Lowenstein and Loewenfeld (1962) used electronic video pupillometry to observe that alert individuals seated in a quiet, dark environment with eyes open can maintain a stable, dilated pupil size for 10 min or longer. However, in manifestly sleepy people, the pupils demonstrated slowly oscillating, large amplitude changes that were accompanied by a progressive decrease in size.

Pupil size in darkness is controlled by the nucleus LC in two ways: the noradrenergic neurons of the LC innervate sympathetic spinal column nuclei and exert an excitatory influence on the peripheral sympathetic nervous system, including the iris dilator muscle; an important central role is played by an inhibitory tone on the parasympathetic of the pupilloconstrictor EW nucleus directly from the ascending noradrenergic systems of the medulla and the LC (Koss et al., 1984; Szabadi and Bradshaw, 1996). Thus, the reduction of pupil size in darkness is believed to be the result of reduced inhibition of the EW nucleus as a result of reduced LC activity. Equally, the ensuing spontaneous pupillary fluctuations in darkness are believed to be the result of variable inhibition of the EW nucleus as a result of ensuing fluctuations in LC activity (Szabadi and Bradshaw, 1996). Clinically, drugs that “switch on” the LC increase arousal and the pupillary diameter, with a reduction in the ensuing pupillary oscillations and the inverse also holds true (Phillips et al., 2000). Therefore it appears that the regulation of arousal and

pupillary functions are linked via activity in the LC, a structure implicated in the regulation of attention, arousal (Berridge and Waterhouse, 2003), maintenance of wakefulness (Nelson et al., 2002; Nelson et al., 2003) and pupillary control (Koss et al., 1984; Koss, 1986).

Bitsios and colleagues (2006) developed a 5-min Pupillary Alertness Test (5-min PAT), where subject's pupil diameter was sampled for 15 consecutive 20 s periods in a dark, sound attenuated room. The outcome measures were the average resting pupil diameters (RPDs) for each 1 of the 15 twenty-second periods and the collapsed RPD for the entire 300 s recording. The 5-min PAT was tested in patients with obstructive sleep apnoea (OSA) who suffer from excessive daytime sleepiness, where it was found that the patients had lower tonic RPD levels compared to controls and that RPD correlated inversely with objective polysomnographic indices of OSA severity and subjective sleepiness within the patient group. The sensitivity of the 5-min PAT was tested with modafinil, an alertness promoting drug (Nikolaou et al., 2008), where the response was attenuated with treatment. Given the modulatory effects of modafinil on the pupil, the results should be treated with caution.

1.5.2.1.2.2.2. Pupil light reflex

The pupil light reflex (PLR) is not a well established means of assessing sleepiness. PLR is a constriction of the pupil when light is focussed on the eye. Pupil response latency, the time from onset of the stimulus the onset of pupil constriction, and pupil constriction amplitude, the maximal amount of constriction that occurs following the stimuli, are the two main parameters used previously to assess sleepiness. The theoretical rationale first advanced by Lowenstein and Loewenfeld (1958; 1961) proposed that sleepiness was associated with increased pupil constriction velocity, increased pupil constriction duration and slowed redilation time. There have been multiple attempts characterise circadian changes in the parameters of the PLR, with partially contradictory results (Doring and Schaefers, 1951; Tiedt, 1963; Borgmann, 1966; Lavie, 1979). Pressman and Fry (1989) showed the multiple sleep latency test to be significantly negatively correlated with pupil constriction velocity under

dark adapted conditions, such that increased sleepiness was related to increased constriction velocity in individuals with sleep disorders, an increase in number of apnoeas and hypoapnoeas was also significantly correlated with an increase in constriction velocity and a decrease in redilation velocity. Consistent with these findings, Russo and colleagues (2003) demonstrated significant increases in pupil constriction latency with increasing sleep debt, which provides some evidence for PLR sensitivity in sleepiness. Newman and Broughton (1991) compared 10 narcoleptic patients with 10 normal individuals and found no differences in any pupillary variables. Ranzijn and Lack (1997) found that partial sleep deprivation did not significantly alter the PLR and they consequently concluded that the PLR, under most circumstances, cannot be used to measure sleepiness. However, since Ranzijn and Lack's study (1997), constriction latency and constriction amplitude have successfully been used to identify sleepiness and mental fatigue (Fant et al., 1998; Perry, 1998; Russo et al., 2003; LeDuc et al., 2005). Thus, the literature is unclear as to the utility of the PLR as a biomarker of sedation. To my knowledge, PLR has not been used to measure the magnitude of opioid induced sedation.

1.5.2.1.2.3. Opioid modulation of pupillary activity

It is well known that opioids cause miosis and the current theory is primarily based on two canine studies. Lee and Wang (1975) anesthetized dogs with nitrous oxide and demonstrated that neurons in the EW nucleus increased their firing rate following administration of morphine. The miotic effect was not dependent upon interference with sympathetic control of pupil size, stimulation of the optic nerve or a local effect on the iris. It was concluded that opioids stimulate the preganglionic neurons in the EW nucleus. Sharpe and Pickworth (1985) noted that micro molar injections of morphine into the periaqueductal grey induced miosis in dogs and confirmed that opioid-induced pupillary effects appear to be brought about by effects on structures close to the EW nucleus.

Since morphine acts in a similar manner to the LC and increases activity in the EW leading to the reduction in pupil size, the 5-min PAT may not be an appropriate measure of sedation as the miotic

property of opioids may mask the decrease in arousal revealed by decreasing RPD. However, to my knowledge, this has not been investigated. It has previously been shown that a 25 ng/ml alfentanil plasma concentration did not diminish the PLR in volunteers under isoflurane anaesthesia (Larson et al., 1997). Conversely 60 mg of oral dihydrocodeine did depress the pupil light reflex (Freye et al., 2001). Further investigation is needed in this area as it is not clear whether an opioid will cause maximal constriction, inhibiting the pupillary response to light stimulus.

1.5.3. Biomarker discovery

Biomarker discovery is a 'Catch-22' situation, as described by Rolan (1997), as innovation is inversely related to validity, and the areas with the most poorly validated biomarkers are in fact the areas with the greatest clinical need. Biomarker discovery from extrapolation of disease mechanisms is not a straight-forward process, as many pathologies involve a complex integration of multiple processing systems. Potentially, modulation of any pathophysiological input will affect the clinical outcome. A biomarker that measures single factors may be insufficient, as it is unlikely that the clinical outcome solely relies on one gene or peptide. Therefore biomarkers are needed that encompass overlapping mechanisms. As reviewed in section 1.5. , there are many leads for pain biomarker discovery and development, based on a link to biological and clinical endpoints. However, returning to the criteria for a useful biomarker established in section 1.1.3. , it is possible to filter these leads even further.

Genetic factors may govern basal pain sensitivity and genetic mutations, of receptors targeted by analgesic therapies for example, may account for heterogeneity in treatment response. However, given the diverse variability and incidence of neuropathic pain that cannot be explained solely by genetic analysis, potential biomarkers should encompass both genetic (including epigenetic) and environmental factors. Laser Doppler imaging, thermography and microdialysis are limited by the requirement of skilled operators, specialised equipment and the necessity to provoke a response that can be measured, and as such, they do not hold the potential to be simple, routine tests. Similarly, a biopsy is limited to

particular neuropathic pain conditions and routine samples are impractical. Sampling the CSF is highly invasive and as such cannot be routinely employed unless the patient has been catheterised intrathecally, which only occurs in rare cases. PET imaging is highly specialised and expensive, whereas fMRI, is more readily available and may be a suitable marker of supraspinal activity. The immune system appears to be an ideal target for development of pain biomarkers, as it is involved in many of the mechanisms underlying neuropathic pain. Immune mediators and peripheral immune cells are present in general circulation and thus can be sampled via a simple venepuncture. Through careful investigation and validation, it may be possible to develop a routine test that can be run without the need for highly skilled personnel.

Given the discussed limitations to current subjective and objective measures of sedation, analysis of oculomotor function may be an appropriate alternative. Saccadic eye movements, in particular, appear to encompass the integrated brainstem processes of alertness and sedation, and thus may be a reliable tool to further examine the sedative effects of medications. Analyses of oculomotor measures are limited to the laboratory, with the need for a skilled operator and specialised equipment. Nonetheless, objective measurement of sedation is likely to predominantly occur in this setting.

1.6. Summary and Aims

The heterogeneity in neuropathic mechanisms, severity of symptoms and treatment response poses a clinical problem, resulting in unsatisfactory treatment outcomes for many patients. The development of novel biomarkers may alleviate these problems, however this has proved a difficult task for pain and pain therapy. Two approaches may be successful in the development of novel, mechanism-based biomarkers and will be explored in the context of neuropathic pain and opioid-induced sedation.

The first is a bottom-up approach, where the mechanisms are somewhat understood and are accessible to investigation. This may be applicable to the development of a biomarker for neuropathic pain. Current evidence suggests that the traditional neuronal model undergoes modulation by the immune system and is potentially a readily accessible biomarker via the blood, in contrast to many of the other pain mechanisms. Mechanisms can be investigated in concert with behaviour, with the goal of discovering novel biomarkers that may capture overlapping mechanisms. The bottom-up approach of elucidation of mechanisms may provide a more fertile hunting ground for a novel mechanistic biomarker, as well as beginning the process of construct validation.

The second is a top-down approach, which is applicable when the mechanisms are not completely understood and may be difficult to probe. This approach may be useful in the development of a biomarker of sedation, as many of the mechanisms are not properly understood and are even a mystery in some cases, such as sleepiness. The purpose of sleep and the reason that all animals get tired and consequently function poorly is really not understood. Even though some of the mechanisms by which sedative drugs induce sedation have been elucidated, such as opioid depression of cholinergic activity in pathways mediating cortical arousal, the reasons that these exist and how they mediate arousal is not understood. A ‘sedation’ neuron or neurotransmitter does not exist and so sedation may seem difficult to quantify objectively. The top-down approach to biomarker development is one solution to these

problems, in which the functional consequences of the complex neural integration resulting in sedation are examined.

Therefore, the aims of this PhD project were:

Aim 1: To develop a biomarker of pain through investigation of the peripheral immune system in the CCI model of neuropathic pain, using a bottom-up approach.

a): To develop a graded model of neuropathy, mimicking the heterogeneity in pain severity observed clinically. This model will increase the statistical power of subsequent investigations of the immune system in neuropathic pain.

b): To begin the construct validation process by confirming a causal role of the peripheral immune system in CCI mediated neuropathic pain.

c): To compare and correlate the graded transcriptional profile between the blood and spinal cord as potential biomarkers of pain.

Aim 2: Using a top-down approach, oculomotor measures will be investigated as biomarkers of sedation. The combination of opioids and sleep deprivation has not been investigated, and thus saccadic eye movements and dynamic pupillometry will be used to detect an interaction and to compare between opioid naïve and opioid tolerant populations.

Chapter 2. A novel animal model of graded neuropathy

Grace PM, Hutchinson MR, Manavis J, Somogyi AA and Rolan PE (2010) A novel animal model of graded neuropathic pain: Utility to investigate mechanisms of population heterogeneity. *J Neurosci Methods* **193**:47-53. Reprinted with permission from Elsevier, © 2010.

To begin the process of bottom-up development of a novel, mechanistic pain biomarker, I believe that animal models provide the optimal starting point. Animal models of neuropathic pain either reduce or control for many of the variable aspects that affect the clinical endpoint within the chronic pain population, such as depression, treatment response and disease aetiology. The homogeneity in pathophysiology is largely due to the fact that experiments often utilise one strain of animals, minimising genetic variability, as well as the use of the same precipitating nerve injury. The second advantage is that spinal and supraspinal mechanisms can be probed and correlated with the potential accessible biomarker. This approach facilitates the development of a mechanism-based biomarker, which is more difficult to achieve in man, where correlations can only been drawn with subjective VAS scores, or with accessible mechanisms of pain, such as those described in Chapter 1.

Existing models, however, are limited by their binomial (pain vs. control) design. This design reduces the statistical power to investigate mechanisms, as a statistically significant difference must be observed between the two groups, when in fact we are interested in a correlation between pain mechanisms and the behaviour. Therefore current models may overlook important, subtle changes that are associated with pain mechanisms.

For these reasons, I sought to develop a graded model of neuropathy, as a tool to more thoroughly investigate mechanisms of neuropathic pain and for pain biomarker development.

In this study I successfully advanced the CCI model of neuropathic pain by varying the chromic gut sutures subcutaneously and around the sciatic nerve. Allodynic behaviour was correlated with graded

glial activation in the ipsilateral dorsal horn of the lumbar spinal cord to demonstrate that the novel graded model was closely linked with established mechanisms of neuropathic pain.

Grace, P.M., Hutchinson, M.R., Manavis, J., Somogyi, A.A. and Rolan, P.E. (2010) A novel animal model of graded neuropathic pain: Utility to investigate mechanisms of population heterogeneity.
Journal of Neuroscience Methods, v.193 (1), pp. 47-53, October 2010

Chapter 3. Role of the peripheral immune system in neuropathic-like pain

Grace PM, Hutchinson MR, Bishop A, Somogyi AA, Mayrhofer G and Rolan PE (2010) Adoptive transfer of peripheral immune cells potentiates allodynia in a graded chronic constriction injury model of neuropathic pain. *Brain Behav Immun* (*in review*).

The immune system is receiving increasing recognition for its role in the induction and maintenance of neuropathic pain and, understandably, the majority of research has focussed on glia within the spinal cord. However, an appreciation for the role of peripheral immune cells in neuropathic pain mechanisms is now gaining momentum. If the peripheral immune system contributes to the generation of pain at the site of injury and to central sensitisation in the DRG and dorsal horn, then it presents a promising target for pain biomarker development. The peripheral immune system is easily accessible via a simple venepuncture in order to sample leukocytes and their mediators.

However, given the inconsistent reports of peripheral blood analysis as a pain biomarker, I believed it necessary take a step back and begin the process of construct validation. To achieve this, I aimed to build on the existing literature in order to demonstrate a role for peripheral immune cells in neuropathic pain.

In the novel graded model developed in Chapter 2, it was initially found that allodynia was inversely correlated with splenomegaly, which was initially interpreted as a substantial peripheral immune cell population shift. Subsequent experiments indicated that splenomegaly was probably due to subcutaneous chromic gut, and hence attributable to experimental design. However, this finding prompted a series of experiments, which showed that splenocytes and PBMCs contributed significantly to CCI-induced allodynia, as adoptive transfer of splenocytes from high pain donors to low pain recipients potentiated allodynia. Importantly, this was not simply a function of the cells alone, as adoptive transfer of allodynia was not achieved in a sham or splenectomised recipients, indicating that

peripheral immune cells were capable of potentiating allodynia rather than establishing allodynia and that the spleen was pivotal to the coordination of an allodynic response. Finally, intrathecal transfer of high pain donor $CD45^+$ spinally-derived immune cells to low pain recipients potentiated allodynia.

As such, this study confirmed that infiltrating immune cells are not passive bystanders, but actively contribute to nociceptive hypersensitivity in the lumbar spinal cord, thus confirming that the peripheral immune system is a valid target for biomarker development.

Title: Adoptive transfer of peripheral immune cells potentiates allodynia in a graded chronic constriction injury model of neuropathic pain.

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Conflict of Interest Statement

All authors declare that there are no financial or commercial conflicts of interest.

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Abstract

Recent evidence demonstrates that peripheral immune cells contribute to the nociceptive hypersensitivity associated with neuropathic pain by infiltrating the central nervous system (CNS). We have recently developed a rat model of graded chronic constriction injury (CCI) by varying the exposure of the sciatic nerve and control non-nerve tissue to surgical placement of chromic gut. We demonstrate that splenocytes can contribute significantly to CCI-induced allodynia, as adoptive transfer of these cells from high pain donors to low pain recipients potentiates allodynia ($P < 0.001$). The phenomenon was replicated with peripheral blood mononuclear cells ($P < 0.001$). Adoptive transfer of allodynia was not achieved in sham recipients, indicating that peripheral immune cells are only capable of potentiating existing allodynia, rather than establishing allodynia. As adoptively transferred cells were found by flow cytometry to migrate to the spleen ($P < 0.05$) and potentiation of allodynia was prevented in splenectomised low pain recipients, adoptive transfer of high pain splenocytes may induce the migration of host-derived immune cells from the spleen to the CNS as observed by flow cytometry ($P < 0.05$). Importantly, intrathecal transfer of $CD45^+$ cells prepared from spinal cords of high pain donors into low pain recipients led to potentiated allodynia ($P < 0.001$), confirming that infiltrating immune cells are not passive bystanders, but actively contribute to nociceptive hypersensitivity in the lumbar spinal cord.

Keywords: Adoptive transfer; Allodynia; Animals; Chronic constriction injury; Central nervous system; Neuropathic pain; Rat; T lymphocyte

Introduction

Neuropathic pain is initiated by nerve damage or disease and is associated with spontaneous pain and hypersensitivity to noxious (hyperalgesia) and non-noxious (allodynia) stimuli. This altered sensory processing may be persistent, even in the absence of ongoing injury. Pharmacotherapy is unsatisfactory for many patients and the dearth of effective novel therapies reflects an incomplete understanding of the mechanisms underlying neuropathic pain. A growing body of evidence supports a neuro-immune model of neuropathic pain, whereby glia are viewed as integral members of the tripartite synapse and are posited to make a causal contribution to exaggerated pain states (Araque et al., 1999; Milligan and Watkins, 2009).

The central nervous system (CNS) is no longer considered to be an entirely immunologically privileged organ, especially with respect to entry of activated T lymphocytes (Mason et al., 1986; Hickey et al., 1991; Furtado et al., 2008; Wilson et al., 2010) and research over the last decade has implicated immune cells in many animal models of pain hypersensitivity that resemble human neuropathic pain (Liu et al., 2000; Perkins and Tracey, 2000; Hu and McLachlan, 2002; Hu et al., 2007; Li et al., 2007; Morin et al., 2007; Shaw et al., 2008). The presence of infiltrating T lymphocytes in the lumbar spinal cord of nerve-injured rodents suggested that there might be an adaptive immune component in the pathogenesis of neuropathic pain (Hu and McLachlan, 2002; Sweitzer et al., 2002; Hu et al., 2007; Cao and DeLeo, 2008; Cao et al., 2009). These data were supported by the observation that nociceptive hypersensitivity was attenuated in nerve-injured T lymphocyte deficient rodents (Moalem et al., 2004; Kleinschnitz et al., 2006; Cao and DeLeo, 2008; Costigan et al., 2009). Using DNA microarray, Costigan et al. (2009) have recently advanced the understanding of peripheral immune cells in neuropathic pain by comparing the profiles of RNA transcripts from pooled ipsilateral lumbar dorsal horns of adult rats that develop allodynia following nerve injury and from neonatal rats that are not disposed to development of allodynia. Pathway analysis of the 148 genes found to be differentially regulated

revealed pronounced differences in the signalling pathways of microglia and T lymphocytes. A critical role for T lymphocyte signalling was supported by the observation that allodynia was attenuated in nerve-injured *Rag1* and *interferon-γ receptor 1* (*IFN-γR1*) gene knockout (GKO) mice compared to wild-type controls (Costigan et al., 2009).

The comparison of nerve-injured neonatal and adult rats by Costigan et al. (2009) introduced variables related to neural and immune system immaturity. In particular, neonatal rodents are effectively T lymphocyte-deficient, so that differences in expression of T lymphocyte-related genes in the spinal cords between neonatal and adult rats may be explained on this basis. We aimed to advance these findings, using a model in which test and control animals differ only with respect to specific experimental interventions. Our novel model of graded neuropathy (Grace et al., 2010) allows comparison of varying degrees of allodynia in intact adult animals, facilitating investigation of subtle neuro-immune mechanisms responsible for neuropathic-like pain. Here we demonstrate that graded chronic constriction injury (CCI) induced-allodynia is inversely correlated with spleen weight of animals exposed to chromic gut suture material, and that CNS infiltration by immune cells from the spleen may be causally involved in exaggerated pain states. Supportive evidence was obtained by adoptive transfer of spleen cells or peripheral blood mononuclear cells from 'high pain' donors to syngeneic 'low pain' recipients, which was found to potentiate allodynia. We conclude that immune cells are critical contributors to allodynia in our model of graded neuropathy, a behavioural correlate of neuropathic pain.

Methods

Animals

Inbred male Dark Agouti (DA CD45.1; 10 - 12 wk old) and outbred male Sprague Dawley (SD; 12 wk old) rats were purchased from Animal Resource Centre (Perth, WA, Australia). Male DA CD45.2 rats (10 - 12 wk old) were bred at the University of Adelaide as described (Spargo et al., 2006). These

congenic DA lines are histocompatible (Spargo et al., 2006), differing only in expression of functionally normal CD45 alleles that can be distinguished by a monoclonal antibody and thus can be used to differentiate between host and donor cells. CD45 is expressed by all nucleated cells of the hematopoietic lineage, including monocytes / macrophages, natural killer cells, and B and T lymphocytes (Hermiston et al., 2009). DA CD45.2 rats were used reciprocally as either donors or hosts in adoptive transfer experiments. All rats were housed in rooms that were temperature-controlled (18 - 21 °C) and light-controlled (12 h light/ dark cycle; lights on at 07:00 h) and provided with standard rodent food and water *ad libitum*. Rats were allowed to habituate to the animal facility for at least one week prior to experimentation. All procedures were approved by the Animal Ethics Committee of the University of Adelaide and were conducted in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Surgery

Graded chronic constriction injury (CCI): The CCI model of graded sciatic nerve injury was performed aseptically at the mid-thigh level of the left hindleg, as previously described (Grace et al., 2010). Briefly, animals were anaesthetised with isoflurane, the skin of the hindquarters was shaved and the sciatic nerve gently elevated. Either 0, 1, 2 or 4 sterile chromic gut sutures (cuticular 4-0 chromic gut; Ethicon, Somerville, NJ, USA) were loosely tied around the isolated sciatic nerve (N). Once the superficial muscle overlying the nerve was sutured with silk, and prior to surgical stapling of the skin incision, additional equal lengths of chromic gut were placed subcutaneously (S), such that each animal was exposed to 4 equal lengths of chromic gut in total. Thus the treatment groups, differing in neurogenic pain behaviour, were: sham (N0S0), no neurogenic pain (N0S4); low pain (N1S3); medium pain (N2S2); and high pain (N4S0). Surgery was performed at day -10 for all DA lines used in adoptive transfer experiments and at day 0 for SD rats used in the spleen and thymus weight experiment (experiment 6).

Splenectomy: Splenectomy was performed aseptically under isoflurane anaesthesia either prior to CCI surgery at day -10 for recipients used in experiment 6, or on day 0 for donors used in experiments 1, 3, 4, 6, 7) through a midline abdominal incision. The spleen was gently elevated from the abdominal cavity, the pedicle was ligated with a silk suture, and the organ was excised. The abdominal muscle layer was closed with silk suture and the skin incision closed with surgical staples.

Mechanical allodynia

Testing was conducted blind with respect to group assignment and thresholds were verified by an independent researcher. Rats received three 60 min habituations to the test environment prior to commencement of behavioural testing. The von Frey test (Chaplan et al., 1994) was performed within the region of the hindpaws innervated by the sciatic nerve, as previously described in detail (Milligan et al., 2000; Chacur et al., 2001; Milligan et al., 2001). Assessments were made prior to (baseline) and at specific times after experimental manipulations (Experiments 1-8 post surgery: days -3 and 0; Experiments 1-6, 8 post adoptive transfer: 3 h, 1, 3, 5, 7, 10, 14 and 21 days; Experiment 6 [Spleen and thymus weight]: days 3, 10 and 14; Experiment 6, 7 [flow cytometry] post adoptive transfer: 3 days; see Table 1). A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA) was applied in random sequence to the left and right hindpaws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs was determined by $\log_{10}$ (milligrams x 10) and ranged from manufacturer designated 3.61 (0.407 g) to 5.18 (15.136 g) filaments. An absolute threshold (the 50 % paw withdrawal threshold) was calculated by fitting a Gaussian integral psychometric function, using a maximum-likelihood fitting method (Harvey, 1986; Treutwein and Strasburger, 1999) as described (Milligan et al., 2000; Milligan et al., 2001). This fitting method allows parametric analyses that otherwise would not be appropriate (Milligan et al., 2000; Milligan et al., 2001). A statistically significant reduction in paw withdrawal threshold was interpreted as an increase in allodynia.

Cell and serum preparation

Splenocyte isolation: Following behavioural testing on day 0, donor DA CD45.1 or DA CD45.2 rats were splenectomised as described above. The excised spleen was diced and then homogenised with a loose fitting glass homogeniser to a single cell suspension. The suspensions were pooled within treatment groups, the cells were collected by centrifugation and washed in phosphate buffered saline pH 7.4 (PBS). The final cell pellet was resuspended in red blood cell lysis buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA) and incubated for 5 min at room temperature. Splenocytes were then washed twice and resuspended in PBS at a concentration of 1 x 10^8 cells·ml^{-1}.

PBMC isolation: Following final behavioural testing on day 0, donor DA CD45.2 rats were anaesthetised with sodium pentobarbital. Blood was obtained by cardiac puncture, using a syringe containing 1 ml citrate (3.8 % w/v) and pooled within treatment groups. Peripheral blood mononuclear cells (PBMCs) were isolated with Optiprep™ (Axis-Shield PoC AS, Oslo, Norway), using the mixer flotation method for rat PBMCs. An additional red blood cell lysis step was performed, as described above. Following washing, cells were resuspended in PBS at a concentration of 5 x 10^5 cells·ml^{-1}.

Preparation of serum: Following final behavioural testing on day 0, donor DA CD45.2 rats were anaesthetised with sodium pentobarbital. Blood was obtained by cardiac puncture, allowed to clot at room temperature for 5 h and after extraction of the clot, the serum was centrifuged at 4000 rpm for 10 min. The individual sera were then pooled within treatment groups for immediate transfer.

Isolation of lumbar spinal $CD45^+$ cells: Following final behavioural testing on day 0, donor DA CD45.2 rats were anaesthetised with sodium pentobarbital and perfused transcardially with isotonic saline. The L4-L6 segment of spinal cord (supplying the sciatic nerve) was carefully excised and following homogenisation (Beeton and Chandy, 2007), pooled within treatment groups. Peripheral immune cells were extracted from the lumbar spinal cord homogenate using a Percoll (GE Healthcare, Little Chalfont,

Bucks, UK) density gradient (Beeton and Chandy, 2007). To isolate $CD45.2^+$ cells from this extract, the immune cells were incubated on ice for 50 min in the dark with mouse primary anti-rat CD45.2 antibody (eBioscience, San Diego, CA, USA), containing 10 % normal rat serum (NRS). After washing the cells twice (wash buffer: PBS; 0.5 % bovine serum albumin; 2 mM EDTA), cells were incubated on ice in the dark for 15 min with microbeads coated with secondary monoclonal rat anti-mouse IgG1 (Miltenyi Biotec, Bergisch Gladbach, Germany). Labelled cells were positively selected through a MACS MS column (Miltenyi Biotec, Bergisch Gladbach, Germany) as directed by the manufacturer, with similar protocols obtaining a purity of ~95 % (Lucin et al., 2009). Cells were suspended in 100 μl of wash buffer and stored overnight at 4 °C. The following morning, cells were washed and resuspended in PBS. Cells from N0S0 rats were resuspended at 1.0×10^2 cells·μl^{-1} and those from N4S0 rats at 1.6×10^2 cells·μl^{-1}.

Transfer of cells and serum

A summary of all experiments is presented in Table 1.

Adoptive transfer of splenocytes: On day 0, DA CD45.1 or DA CD45.2 recipients received an intraperitoneal (i.p.) injection of 2×10^8 splenocytes from donors of the reciprocal strain in 2 ml of PBS, as detailed in Experiments 1, 3, 4, 6, 7. The donor to recipient ratio was 1:2. Behavioural testing was conducted following adoptive transfer as described above.

Adoptive transfer of PBMCs: On day 0, N1S3 DA CD45.1 recipients received an i.p. injection of 1×10^6 PBMCs from N0S0 or N4S0 DA CD45.2 donors in 2 ml of PBS. The donor to recipient ratio was 1:1. Behavioural testing was conducted following adoptive transfer as described above.

Intrathecal transfer of $CD45^+$ cells derived from the spinal cord: Intrathecal (i.t.) injections were performed as previously described (Ledeboer et al., 2005). Briefly, an 18-gauge sterile needle was inserted between L5 and L6 under isoflurane anaesthesia. The injection, over 1 minute, consisted 1 μl

of dead-space air followed by $CD45^+$ cells prepared from the spinal cords of N4S0 (1.6 x 10^3 cells) or N0S0 (1.0 x 10^3 cells) donors in 10 μl of PBS and then a flush with 14 μl of saline. Injections were performed with a 25 μl glass Hamilton syringe attached to sterile PE-10 tubing, threaded through the 18-gauge needle for delivery at the level of the lumbosacral enlargement (7.7 cm). The acute injection was completed in ~2 min and the rats were monitored until fully ambulatory, prior return to their home cage. No abnormal motor behaviour was observed after any injection. The donor to recipient ratio was 1:1. However, due to the greater numbers of $CD45^+$ cells recovered from N4S0 compared to N0S0 donors, the numbers of cells injected were intended to mimic those in host lumbar spinal cord. Behavioural testing was conducted following adoptive transfer as described above.

Transfer of serum: On day 0, N1S3 DA CD45.1 recipients received 2 ml of serum from N0S0 or N4S0 DA CD45.2 donors by i.p. injection. The donor to recipient ratio was 1:1. Behavioural testing was conducted following transfer as described above.

Flow cytometry

Following final behavioural testing (Experiment 6: 3 days post adoptive transfer; Experiment 7: day 0, 3 days post adoptive transfer; Table 1), DA rats were anaesthetised with sodium pentobarbital and the spleen was excised. The liver was removed after transcardial perfusion with isotonic saline. After dicing, the liver and spleen were homogenised separately to obtain a single cell suspension. The cells were washed by centrifugation in PBS, followed by removal of red blood cells by lysis, as described above. The cells were then suspended in PBS containing 2% foetal calf serum and 0.01 M azide (PBS-FCS-Az). The perfused L4-L6 segment of spinal cord was carefully removed, micro-dissected to isolate dorsal and ventral horns and homogenised to a single cell suspension. The cells were washed in PBS and resuspended in 2 ml of PBS-FCS-Az.

Approximately 1 x 10^6 liver and spleen cells, or 50 μl of the lumbar spinal cord homogenate, were incubated on ice and in the dark with either rat anti-rat CD45.1-FITC antibody (USBiological, Swampscott, MA, USA) or mouse anti-rat CD45.2-FITC antibody (eBioscience, San Diego, CA, USA) diluted in 10 % NRS. After 50 min, the cells were washed twice with PBS-FCS-Az. To compare the numbers of cells in samples, 10 ml of fixing buffer (1 % formalin (v/v), 2 % glucose (w/v) and 0.02 % azide (w/v) in PBS) was spiked with a drop of PE-polystyrene beads (CaliBRITE™; BD Pharmingen, San Diego, CA, USA), as directed by the manufacturer. Labelled cells were resuspended in 500 μl of the spiked fixing buffer for analysis. Control preparations, in which the primary antibody was replaced by washing buffer or by an isotype-matched antibody of irrelevant specificity, were included in each analysis.

Labelled cells were analysed using a Coulter Epics XL 4MCL flow cytometer and Beckman Coulter Expo32 ADC 1.1B software (Beckman Coulter, Brea, CA, USA). A $CD45^{hi}$ gate was established within the dot plot of forward and side scatter events, excluding dead cells and debris, thus defining the leukocyte population (hereafter referred to as $CD45^+$ cells), distinct from the $CD45^{lo}$ microglial population (Cao and DeLeo, 2008). Analysis was confined to events within this gate, from a total of 2 x 10^5 ungated events. Numbers of PE- polystyrene beads were estimated simultaneously. Number of cells of interest per tube, relative to 1 x 10^3 PE- polystyrene beads, was calculated as follows: number of cells per tube relative to 1 x 10^3 beads = number of cells (cytometer gate) x 10^3 / number of beads (cytometer gate).

Spleen and thymus weight

Following final behavioural testing on postoperative (PO) day 14, the groups of SD rats (N0S0, N0S4, N1S3, N2S2 and N4S0) were euthanised by overdose of sodium pentobarbital. The spleen and thymus were then excised and weighed using an Ohaus balance (Pine Brook, NJ, USA; 0.001-210 g ± 0.002 g).

Statistics

Data from the von Frey test were analysed as the interpolated 50 % thresholds (absolute threshold) in log base 10 of stimulus intensity (monofilament stiffness in milligrams x 10). Differences between adoptive transfer treatment groups were determined using repeated measures two-way ANOVA, followed by Bonferroni *post hoc* test. Lumbar spinal cord $CD45^+$ cell traffic at PO day 14 was analysed using Students t test. Lumbar spinal cord $CD45^+$ cell traffic at 3 days post adoptive transfer was analysed using a one-way ANOVA, followed by Tukey's *post hoc* test. The relationship between spleen weight and allodynia was determined using a Spearman rank correlation and one-way analysis of variance (ANOVA). Statistical comparisons are indicated on the figures for clarity and represent mean ± standard error of the mean. Statistical significance was set at $P < 0.05$.

Results

Exp. 1. Allodynia is potentiated by transfer of splenocytes from high pain donors to low pain recipients

A role for immune cells in the mechanisms responsible for nociceptive hypersensitivity was investigated in our graded CCI model. As this experimental design was the first of its kind, it was necessary to use a wide, non-specific population of immune cells, such as that in the spleen. Therefore, splenocytes (2×10^8) from high pain DA strain (N4S0) donors were transferred i.p. to low pain (N1S3) syngeneic recipients (representative pre adoptive transfer allodynia shown for N1S3 and N4S0 rats [Fig. 1]; $n = 6$; Fig. 2 A). Control recipients received vehicle alone. Two-way ANOVA revealed a statistically significant interaction in the ipsilateral paw between time and treatment ($P < 0.001$) as well as significant effects of time ($P < 0.05$) and treatment ($P < 0.001$). Similar results were obtained for the contralateral paw (two-way ANOVA analysis: $P_{\text{time x treatment}} < 0.001$; $P_{\text{time}} = 0.15$; $P_{\text{treatment}} < 0.001$; Fig. 2 A2). The Bonferroni *post hoc* test revealed that relative to controls ($n = 6$), rats receiving adoptive transfer of splenocytes

from N4S0 rats exhibited a striking potentiation of allodynia in both ipsilateral and contralateral paws that commenced as early as day 1 post transfer ($P < 0.05$) and persisted to the conclusion of behavioural testing at 21 days post transfer ($P < 0.01$). In a separate experiment, intravenous transfer of splenocytes from N4S0 donors produced a similar potentiation of allodynia (data not shown).

In order to examine whether transfer of cells alone is sufficient to induce potentiation of allodynia, splenocytes were prepared from sham N0S4 donors and transferred i.p. to syngeneic DA low pain N1S3 recipients (representative pre adoptive transfer allodynia shown for N0S4 rats [Fig. 1]; $n = 6$; ipsilateral two-way ANOVA analysis: $P_{time \times treatment} = 0.22$; $P_{time} = 0.14$; $P_{treatment} = 0.36$; contralateral two-way ANOVA analysis: $P_{time \times treatment} < 0.01$; $P_{time} < 0.01$; $P_{treatment} = 0.35$; Fig. 2 B). The Bonferroni *post hoc* test revealed that splenocytes from donors without direct nerve-injury had no significant effect on allodynia in either the ipsilateral or the contralateral paws of the recipients compared with those that received vehicle alone ($n = 6$).

Exp. 2. Allodynia is potentiated by transfer of PBMCs from high pain donors to low pain recipients

Since splenocytes from high pain donors potentiated allodynia, blood was examined for the presence of cells with similar activity. PBMCs were harvested from high pain N4S0 and control N0S0 donors, and 1 x 10^6 cells were transferred i.p. to syngeneic DA low pain N1S3 recipients (representative pre adoptive transfer allodynia shown for N0S0 rats [Fig. 1]; $n = 6$/ group; ipsilateral and contralateral two-way ANOVA analysis: $P_{time \times treatment} < 0.001$; $P_{time} < 0.001$; $P_{treatment} < 0.001$; Fig. 2 C). The Bonferroni *post hoc* analysis revealed a significant potentiation of allodynia in both ipsilateral and contralateral paws from day 1 compared to cell controls ($P < 0.01$), persisting until the conclusion of behavioural testing at 21 days post transfer ($P < 0.001$). Remarkably, the potentiation of allodynia achieved by this small inoculum of PBMCs was equivalent to that achieved with 2 x 10^8 splenocytes. The findings indicate that

cells capable of potentiating allodynia are present in the circulation of rats with CCI-induced allodynia in numbers relative to total mononuclear cells that are at least equivalent to those in the spleen.

Exp. 3. Nerve injury is a prerequisite for adoptive transfer of allodynia

To examine whether existence of CCI-induced allodynia in recipients is necessary for successful potentiation of allodynia by adoptive transfer, splenocytes were prepared from high pain N4S0 donors and transferred i.p. to syngeneic DA low pain N0S4 recipients. The results, compared with recipients that received vehicle alone, are presented in Fig. 2 D (n = 6/ group; ipsilateral two-way ANOVA analysis: $P_{\text{time x treatment}}$ = 0.55; P_{time} = 0.49; $P_{\text{treatment}}$ = 0.32; contralateral two-way ANOVA analysis: $P_{\text{time x treatment}}$ = 0.78; P_{time} = 0.38; $P_{\text{treatment}}$ = 0.28). The Bonferroni *post hoc* analysis demonstrated that low pain N0S4 recipients displayed no significant increase in allodynia in either ipsilateral or contralateral paws compared to vehicle controls (n = 6), indicating that nerve injury in the recipient is necessary for transfer of allodynia by splenocytes.

Exp. 4. Transfer of splenocytes from low pain donors to low pain recipients results in delayed potentiation of allodynia

To examine whether 'priming' of donor immune cells by induction of low-level CCI-induced allodynia is sufficient to facilitate adoptive transfer of allodynia, splenocytes were prepared from low pain N1S3 donors and transferred i.p. to syngeneic DA low pain N1S3 recipients (n = 6; ipsilateral and contralateral two-way ANOVA analysis: $P_{\text{time x treatment}} < 0.001$; $P_{\text{time}} < 0.001$; $P_{\text{treatment}} < 0.001$; Fig. 2 E). The Bonferroni *post hoc* analysis for ipsilateral and contralateral paws revealed that compared to vehicle controls (n = 6), there was a significant potentiation of allodynia, reaching maximum between day 7 and 10 post-transfer ($P < 0.001$) and persisting until conclusion of behavioural testing at 21 days post transfer ($P < 0.001$). In comparison with the results of adoptive transfer of splenocytes from high pain

N4S0 donors (Fig. 2 A), the potentiation of allodynia following transfer of cells from low pain donors was delayed significantly ($P < 0.001$), but was ultimately similar in magnitude.

Exp. 5. Transfer of serum from high pain donors induces transient potentiation of allodynia in low pain recipients

The experiments described above indicate that allodynia can be potentiated by mononuclear cells from high pain donors, implicating cellular mechanisms in the pathogenesis of neuropathic pain. However, the possible involvement of humoral factors remained unresolved. Serum was collected from high pain N4S0 and control N0S0 donors and transferred i.p. to low pain N1S3 recipients ($n = 6$/ group; ipsilateral two-way ANOVA analysis: $P_{time \times treatment} < 0.05$; $P_{time} < 0.001$; $P_{treatment} = 0.37$; contralateral two-way ANOVA analysis: $P_{time \times treatment} = 0.09$; $P_{time} < 0.001$; $P_{treatment} = 0.60$; Fig. 2 F). The Bonferroni *post hoc* analysis revealed a significant potentiation of allodynia in the ipsilateral paw of recipients of serum from N4S0 donors relative those that received control serum. This potentiation of allodynia was transient, peaking at 3 days post transfer ($P < 0.05$) and no longer observable by day 5. Although a similar profile was observed for the contralateral paw, the Bonferroni *post hoc* analysis revealed no significant potentiation of allodynia.

Exp. 6. Transferred donor cells migrate to recipient spleen and splenectomy of recipients abolishes adoptive transfer of allodynia

Transferred donor cells were expected to enter the recirculating pool of the recipient and thus enter secondary lymphoid tissues. Some, in particular activated T cells, might enter non-lymphoid organs such as the liver (Crispe, 2003) and the CNS (Hickey et al., 1991). Therefore, recruitment of donor cells to these sites was verified. Three days after adoptive transfer of 2×10^8 splenocytes from high pain N4S0 DA CD45.2 donors to low pain N1S3 DA CD45.1 recipients, $CD45.2^+$ donor-derived cells were not detected in either the dorsal or ventral horns of the lumbar spinal cord by flow cytometry (Fig. 3 A, B),

but small numbers were detected in the spleen and liver (Fig. 3 C, D). Although the presence of small numbers of donor-derived cells in the spinal cord cannot be excluded, at this stage of the disease their numbers must be very low. These observations raise the possibility that cells involved in the potentiation of allodynia either exert their effects at sites outside of the CNS, have transitory presence in the CNS, or are effective in very small numbers.

To investigate a site of action outside of the CNS and since T lymphocytes have been implicated in neuropathic pain-like hypersensitivity (Cao and DeLeo, 2008; Costigan et al., 2009) and contribute up to 45 % of the splenocyte population (Morris and Komocsar, 1997), changes in the spleen weight were investigated in SD rats with graded allodynia from our previous study (Grace et al., 2010). When the spleen weights of animals in groups receiving chromic gut suture material (N0S4, N1S3, N2S2 and N4S0) were compared at PO day 14, there was a direct relationship with allodynia (n = 23, r_S = 0.65, P < 0.001). That is, the weight of the spleen was inversely related to level of allodynia. However, one-way ANOVA followed by Tukey's *post hoc* analysis only revealed a significant difference in the spleen weights between sham N0S0 animals and N0S4 (P < 0.05) and N1S3 (P < 0.05) animals, but not N2S2 or N4S0 animals. The results suggest that rats in all groups mounted an immune response due to the presence of the chromic gut, that all had splenomegaly relative to controls, and that the degree of splenomegaly was inversely related to level of allodynia (N4S0 > N2S2 > N1S3 > N0S4). Lymphoid atrophy induced by the stress of neuropathic pain is an unlikely explanation, because there were no significant differences in thymus weights between any of the experimental groups at PO day 14 (data not shown) and it has been previously demonstrated that cortisol levels are not affected in the Bennett CCI model of neuropathic pain (Bomholt et al., 2005). Nonetheless, these data highlight that graded CCI is associated with immune system alterations, in particular the spleen.

To determine whether the spleen holds any significance for the potentiation of allodynia by adoptive transfer of immune cells, given the converging lines of evidence described above, DA rats were

splenectomised immediately prior to preparation as recipients by N1S3 surgery. These animals received transfer of 2 x 10^8 splenocytes from high pain N4S0 DA donors, or transfer of vehicle alone, by i.p. injection (n = 6/ group; ipsilateral two-way ANOVA analysis: $P_{\text{time x treatment}} < 0.05$; $P_{\text{time}} < 0.05$; $P_{\text{treatment}} = 0.70$; contralateral two-way ANOVA analysis: $P_{\text{time x treatment}} < 0.30$; $P_{\text{time}} < 0.05$; $P_{\text{treatment}} = 0.80$; Fig. 4). The Bonferroni *post hoc* analysis revealed no significant difference in allodynia in either the ipsilateral or contralateral paws between recipients of splenocytes and those that received vehicle control. Statistical comparison was also made between splenectomised N1S3 recipients and non-splenectomised recipients receiving N4S0 splenocytes (Exp. 1) and Bonferroni *post hoc* analysis revealed significantly greater allodynia in the ipsilateral and contralateral paws of the non-splenectomised N1S3 recipients at days 1, 3, 7, 10, 14, and 21 ($P < 0.001$), but not day 5. These data provide evidence that the spleen plays a pivotal role in potentiation of allodynia by adoptive transfer of immune cells.

Interestingly, development of allodynia appears unaffected in the splenectomised N1S3 recipients at day 0 (Fig. 4) compared with non-splenectomised N1S3 recipients (Fig. 1). Furthermore, removal of the spleen from the N4S0 donors when allodynia is already established at day 0 did not reverse allodynia during the remaining 21 days of behavioural testing (data not shown).

Exp. 7. Adoptive transfer accompanied by infiltration of the spinal cord by host-derived CD45+ cells

While the foregoing experiments showed that transfer of splenocytes from high pain donors potentiated allodynia, and splenectomy of the recipients prior to N1S3 surgery prevented such potentiation, donor-derived cells were not detected in the spinal cord. However, Hu et al. (2007) have previously reported leukocyte infiltration of the spinal cord in the CCI model of neuropathic pain. It was important, therefore, to investigate whether the action of donor cells was to facilitate recruitment of endogenous $CD45^+$ cells into affected areas of the spinal cord.

First, flow cytometry was used to confirm that there is an increase in numbers of $CD45^+$ cells in the lumbar spinal cords of DA CD45.2 rats during the course of neuropathic pain in N4S0 compared to N0S0 rats at day 0 (n = 3/ group). Significantly greater numbers of $CD45^+$ cells were observed in the N4S0 group compared to the N0S0 group ($P < 0.05$; Fig. 5 A).

Numbers and origin of $CD45^+$ cells in the spinal cords of DA CD45.2 recipients were then examined after adoptive transfer of DA CD45.1 splenocytes prepared from sham (N0S0), no pain (N0S4) and high pain (N4S0) donors into N1S3 congenic recipients of the CD45.2 strain. At three days following i.p. transfer (n = 3/ group), numbers of host-derived $CD45.2^+$ cells were measured in the lumbar spinal cord by flow cytometry. Tukey's *post hoc* analysis revealed significantly higher numbers of endogenous $CD45.2^+$ cells in spinal cords from rats that received N4S0 splenocytes compared to recipients of either control N0S0 or N0S4 splenocytes (Fig. 5 B). As described above, no donor-derived $CD45.1^+$ cells were detected in the spinal cord preparations (data not shown).

Exp. 8. Intrathecal transfer of CD45+ cells obtained from spinal cords of high pain donors potentiates allodynia in low pain recipients

To examine whether $CD45^+$ cells prepared from the spinal cords of rats with CCI-induced allodynia can potentiate allodynia, and are not simply bystanders, $CD45.2^+$ cells were isolated from the lumbar spinal cords of high pain (N4S0) and sham (N0S0) rats of the DA CD45.2 strain. These cells (1.6×10^3 and 1.0×10^3 respectively, in order to mimic the conditions of the donor lumbar spinal cord) were injected i.t. into low pain N1S3 DA CD45.1 recipients (n = 6/ group; ipsilateral and contralateral two-way ANOVA analysis: $P_{\text{time x treatment}} < 0.001$; $P_{\text{time}} < 0.001$; $P_{\text{treatment}} < 0.001$; Fig. 6). The Bonferroni *post hoc* analysis identified a potentiation of allodynia in both the ipsilateral and contralateral paws of recipients of cells from N4S0 donors, significant from 3 days post transfer ($P < 0.001$) and persisting to the conclusion of behavioural testing at 21 days post transfer ($P < 0.001$). The findings show that $CD45^+$ cells not only

infiltrate the spinal cord during the pathogenesis of neuropathic pain, but that the cells are potent potentiators of allodynia when applied directly to the spinal cords of low pain recipients.

Discussion

Our graded CCI model (Grace et al., 2010) has allowed us to select conditions that induce either mild or severe allodynia. Rats with mild allodynia are ideal experimental subjects in which to examine whether cells and/or soluble factors from syngeneic high pain donors can transfer allodynia, in that they represent an intact biological system that is not affected by differences between neonatal and adult animals or by GKO. Involvement of the immune system in the pathogenesis of CCI-induced neuropathic pain was demonstrated by the capacity of splenocytes and PBMCs, but not serum, from high pain donors to transfer allodynia to low pain recipients. Importantly, this was not simply a function of the pain status of the donor, as adoptive transfer of allodynia was not achieved following transfer of splenocytes from high pain donors to control non-neurogenic pain recipients. An additional finding was that transfer of cells from high pain donors did not potentiate allodynia in splenectomised low pain recipients. This suggests that donor cells may require either a period of residence in the spleen or an interaction with host spleen cells, in order to exert an effect on pain sensitivity. Nevertheless, direct i.t. transfer of $CD45^+$ cells isolated from the spinal cords of high pain donors to low pain recipients was sufficient to potentiate allodynia. This finding indicates that the spleen is unnecessary when the cells have proven ability to enter the CNS, and they are reintroduced by direct injection. The spleen may therefore have an accessory function, such as promoting the expression of molecules that are necessary for $CD45^+$ cell recruitment to the CNS. The ability of spinal cord-derived $CD45^+$ cells to potentiate allodynia provides direct evidence that these cells contribute to nociceptive hypersensitivity in the lumbar spinal cord.

Our findings indicate that the spleen has a role in the pathogenesis of the graded CCI model of neuropathic pain. The significance of the inverse relationship between spleen size and degree of

allodynia to neuropathic pain is unclear as the observed increases in spleen weight may be the result of immunisation against antigens in chromic gut which may be greater subcutaneously than in the compartment of the sciatic nerve; nevertheless the results of adoptive transfer show clearly that there are cells in the spleen of nerve-injured rats that can potentiate neuropathic-like pain in low pain recipients (Fig. 2). The spleen may function as a staging post for expansion of cell numbers, for inducing expression of receptors that are necessary for their entry into the CNS, or for recruiting host-derived cells into the disease process. It is unclear whether these cells are generated locally or are delivered via the blood from local lymph nodes draining the sciatic nerve ligation site. Some support for the latter comes from the observation that the spleen plays an essential role in facilitating the activities of adoptively transferred allodynia-potentiating effector cells (Fig. 4). Future studies should be directed towards examining the potential role and mechanisms of local lymph nodes as sources of allodynia-potentiating immune cells.

At this stage, the nature of the effector cells responsible for adoptive transfer of enhanced pain sensitivity have not been identified, but the results of others suggest that they are likely to be $CD45^+$ T lymphocytes (Sweitzer et al., 2002; Moalem et al., 2004; Kleinschnitz et al., 2006; Cao and DeLeo, 2008; Cao et al., 2009; Costigan et al., 2009). In particular, direct involvement of T lymphocytes in the pathogenesis of neuropathic pain was suggested by the findings of Cao and DeLeo (2008). They identified the presence of $CD3^+CD4^+$ T lymphocytes in the lumbar spinal cords of nerve-injured mice thus distinguishing the cells from $CD4^+$ macrophages and microglia. Furthermore, Costigan et al. (2009) used DNA microarray analysis of the ipsilateral lumbar dorsal horns to reveal significant contributions from T lymphocytes and molecules associated with migration and signalling in the adult nerve-injured rat compared with neonates (which are essentially T lymphocyte deficient). However, the functional importance of T lymphocytes in sciatic nerve injury was confirmed by the demonstration of nociceptive hypersensitivity in B cell deficient mice, but not in either *Rag1* GKO or nude mice. These data are

supported by the detection of increased T_H1, T_H2 and T_H17 cytokine expression in the spinal cords of nerve-injured adults, while allodynia is attenuated in nerve-injured *IFN-γR1* GKO mice, suggested particular importance of the T_H1 subset in neuropathic pain (Costigan et al., 2009). Indeed, previous reports (Moalem et al., 2004; Rutkowski et al., 2004) have supported respective proinflammatory and protective role for T_H1 and T_H2 cells in neuropathic-like pain. In the light of the above studies and others (Sweitzer et al., 2002; Sweitzer et al., 2002; Hu et al., 2007), future investigations of adoptive transfer of allodynia should focus on the subsets of T lymphocytes that are responsible.

An important discovery is that potentiation of allodynia by adoptively transferred cells is successful only in animals with a pre-existing nerve injury (Fig. 2 D). This suggests that nerve injury alters the CNS in a way that facilitates the potentiating effects of the transferred cells on pre-existing allodynia. Whilst we do not demonstrate a mechanism, current literature suggests that the most likely mechanism is facilitation of access by the cells into the CNS. Nerve injury has been shown to induce expression of adhesion molecules PECAM-1 and ICAM-1 by vascular endothelium in the spinal cord (Rutkowski et al., 2002; Sweitzer et al., 2002), and these molecules would be expected to facilitate transendothelial migration (Muller, 2009), aided by chemokines such as CCL2 (Stamatovic et al., 2003; Song and Pachter, 2004). Such changes would be expected to facilitate transmigration of activated T lymphocyte selectively (Hickey et al., 1991), as these cells express higher levels of adhesion molecules than resting T lymphocytes (Dustin and Springer, 1991) and nerve injury is not associated with neutrophil invasion into the CNS (Sweitzer et al., 2002).

Following nerve injury, T lymphocytes aggregate in regions of glial activation (Hu et al., 2007; Costigan et al., 2009). Glia play a pivotal role in nociceptive hypersensitivity associated with neuropathic pain (Milligan and Watkins, 2009) and nerve injury may induce expression of molecules such as MHC class II and CD40 by activated microglia, the primary competent antigen presenting cells within the CNS (Sweitzer et al., 2002; Cao et al., 2009). These molecules are important for both presentation of

antigens to T lymphocytes and for co-stimulation. Ligation of CD154 on activated T lymphocytes by CD40 on microglia would be expected to induce downstream proinflammatory signalling (Tan et al., 1999; Tan et al., 1999). It is interest, therefore, that allodynia is attenuated in nerve-injured *MHC II* and *CD40* GKO mice compared to that in wild-type controls (Sweitzer et al., 2002; Cao et al., 2009). Furthermore, the trait of neuropathic pain has significant linkage to certain MHC class II polymorphisms in rats (Dominguez et al., 2008). Given that microglia present antigens to $CD4^+$ lymphocytes via MHC class II molecules (Olson and Miller, 2004), the possible existence of a 'neuropathic pain antigen' remains an interesting but unanswered question.

In addition to increased expression of MHC class II molecules and CD40, production of proinflammatory cytokines, such as IL-1β, IL-6 (Milligan and Watkins, 2009), IL-15 (Gomez-Nicola et al., 2008) and a range of chemokines (White et al., 2007) by microglia could combine with T lymphocyte derived factors to create a microenvironment within the lumbar spinal cord that is conducive to recruitment and activation of immune cells. This process could have contributed to the delay in potentiation of allodynia that was observed when low pain splenocytes were transferred to low pain recipients (Fig. 2 D). While the cells from low pain donors might require additional time for activation and expansion at external sites (e.g. the spleen), it is also possible that upregulation of the antigen presenting capacity of local microglia is necessary for optimal local recruitment and activation of effector cells.

Tsuda et al. (2003) have demonstrated that allodynia can be induced in naïve recipients by i.t. injection of ATP-activated microglia. Because of the interactions between activated microglia and T lymphocytes, our demonstration of potentiated allodynia in low pain rats following i.t. injection of $CD45^+$ cells from the spinal cords of high pain rats (Fig. 6) complements these findings. Our results may also complement the pattern of transient allodynia described for nerve-injured nude mice (Cao and DeLeo, 2008) and *Rag1* (Costigan et al., 2009), *CD4* (Cao and DeLeo, 2008) and *CD40* (Cao et al., 2009) GKO mice. In the case of *CD4* GKO mice, CD11b (microglial activation marker), but not glial fibrillary acidic protein

(astrocyte activation marker), was detected 7 days post nerve injury (Cao and DeLeo, 2008). Previous findings (Tanga et al., 2004) suggest a model in which the initial nerve injury induces transient activation of microglia in the affected segment of the spinal cord, which is responsible for transient allodynia, however maintenance of allodynia depends on astrocyte activation. Our results and others (Sweitzer et al., 2002; Moalem et al., 2004; Kleinschnitz et al., 2006; Cao and DeLeo, 2008; Cao et al., 2009; Costigan et al., 2009) indicate that the maintenance and magnitude of allodynia may require infiltration of activated T lymphocytes into the affected region, possibly aided by proinflammatory factors produced by microglia, followed by engagement with this cell type.

As discussed previously (Grace et al., 2010), we have observed allodynia in the contralateral paw following graded CCI. In the current study, potentiation of allodynia was also detected in the contralateral paw following adoptive transfer of high pain N4S0 splenocytes and PBMCs to low pain N1S3 recipients. A potential mechanism for contralateral allodynia following unilateral nerve injury is a spread of glial activation from the ipsilateral to the contralateral dorsal horn (Milligan et al., 2003; Spataro et al., 2004). As discussed above, infiltrating immune cells may interact with CD40 and MHC class II expressed by microglia in the contralateral dorsal horn to potentiate contralateral allodynia, although expression of these molecules in the contralateral dorsal horn needs to be confirmed. The other explanation for this phenomenon is that adoptive transfer of high pain immune cells causes generalized nociceptive sensitivity. Therefore future adoptive transfer studies should quantify allodynia in other regions, such as the forepaws or face.

This study has focussed on CNS mechanisms by which adoptive transfer of high pain immune cells may potentiate allodynia, however action in the peripheral nervous system should also be considered. Neutrophils, macrophages and T lymphocytes have been demonstrated to invade the site of nerve injury, contributing to the generation of nociceptive hypersensitivity (Perkins and Tracey, 2000; Hu and McLachlan, 2002; Moalem et al., 2004; Hu et al., 2007; Morin et al., 2007). A similar role for these

infiltrating immune cells has been reported in the dorsal root ganglia (DRG) and may also contribute to ectopic activity (Hu et al., 2007; Morin et al., 2007; Shaw et al., 2008). Furthermore, recruitment or depletion of these specific immune cell populations results in respective enhancement or attenuation of nociceptive hypersensitivity in rodent nerve injury models (Liu et al., 2000; Perkins and Tracey, 2000; Li et al., 2007). Therefore, donor or host cells may be present at the site of injury and the DRG and further adoptive transfer studies should characterise the immune infiltration at these sites.

We have demonstrated that allodynia can be transferred by immune cells and that it is accompanied by leukocyte infiltration of the lumbar spinal cord. Nerve injury may induce a microenvironment in the CNS that facilitates leukocyte extravasation and promotes engagement of T lymphocytes with antigen presenting cells and other targets in the CNS. Further investigation of the peripheral immune system in neuropathic pain may uncover mechanisms contributing to population heterogeneity, and presents a potential range of novel, disease-modifying therapeutic targets.

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Table and Legends

Table 1. Experiment summary. CCI surgery was performed at day -10, unless indicated otherwise, and adoptive transfer was performed on day 0. DA: Dark Agouti; SD: Sprague Dawley; i.t.: intrathecal; i.p.: intraperitoneal; N: neuronal chromic gut suture; PBMC: peripheral blood mononuclear cell; S: subcutaneous chromic gut suture.

Exp.	Rat strain	Experimental manipulation	Experimental control	Timecourse to endpoint	Experimental endpoint
1	DA CD45.1	N4S0 splenocytes i.p. to N1S3 recipient	Vehicle	21 days post transfer	Behavior
	DA CD45.1	N0S4 splencoytes i.p. to N1S3 recipient	Vehicle	21 days post transfer	Behavior
2	DA CD45.1	N4S0 PBMCs i.p. to N1S3 recipient	N0S0 PBMCs	21 days post transfer	Behavior
3	DA CD45.1	N4S0 splenocytes i.p. to N0S4 recipient	Vehicle	21 days post transfer	Behavior
4	DA CD45.1	N1S3 splenocytes i.p. to N1S3 recipient	Vehicle	21 days post transfer	Behavior
5	DA CD45.1	N4S0 serum i.p. to N1S3 recipient	N0S0 serum	21 days post transfer	Behavior
6	DA CD45.1 & CD45.2	CD45.2 N4S0 splenocytes i.p. to CD45.1 N1S3 recipient	-	3 days post transfer	Flow cytometry
	SD	Graded allodynia (N0S0, N0S4, N1S3, N2S2, N4S0)	-	14 days post surgery	Spleen and thymus weight
	DA CD45.1	N4S0 splenocytes i.p. to splenectomized N1S3 recipient	Vehicle	21 days post transfer	Behavior
7	DA CD45.2	N4S0 surgery	N0S0 surgery	10 days post surgery	Flow cytometry
	DA CD45.1 & CD45.2	CD45.1 N4S0 splenocytes i.p. to CD45.2 N1S3 recipient	N0S0, N0S4 splenocytes	3 days post transfer	Flow cytometry
8	DA CD45.1 & CD45.2	CD45.2 N4S0 spinal immune cells i.t. to CD45.1 N1S3 recipient	N0S0 spinal immune cells	21 days post transfer	Behavior

Figures and Legends

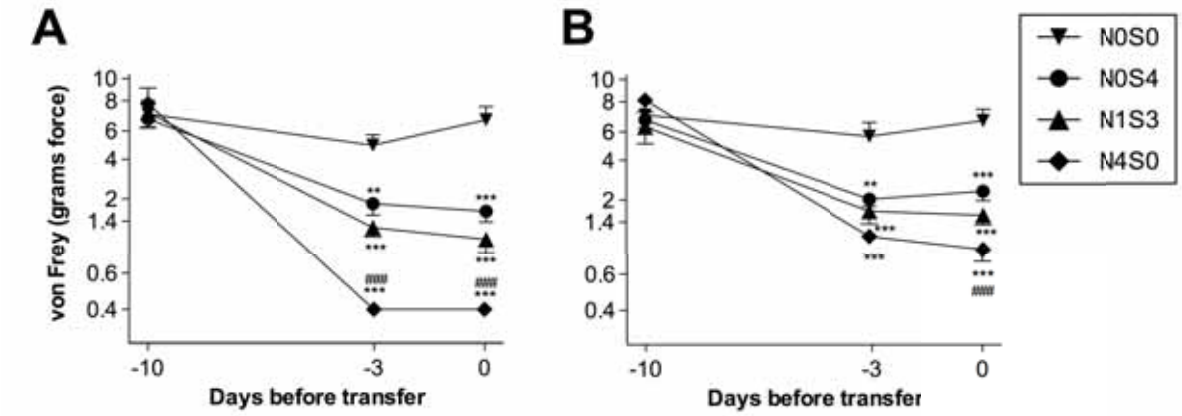

Fig. 1. Representative behavioural responses for sham, control non-neurogenic pain, low pain and high pain rats. Dark Agouti rats were placed in groups, in which chromic gut material was placed subcutaneously (S) following isolation of the sciatic nerve of the left hindpaw (N) to cause no neurogenic pain (N0S4), or a combination of subcutaneous and nerve sutures to induce low pain (N1S3) or high pain (N4S0) at day -10. The sciatic nerve was exposed in the sham group (N0S0), but no sutures were placed. Representative data is shown, quantifying the minimum force required to elicit paw withdrawal to

10 days post surgery for (A) the ipsilateral paw and (B) the contralateral paw. *n* =6/ group; Comparison to N0S0: ***P* < 0.01, ****P* < 0.001; Comparison to N0S4: ###*P* <0.001.

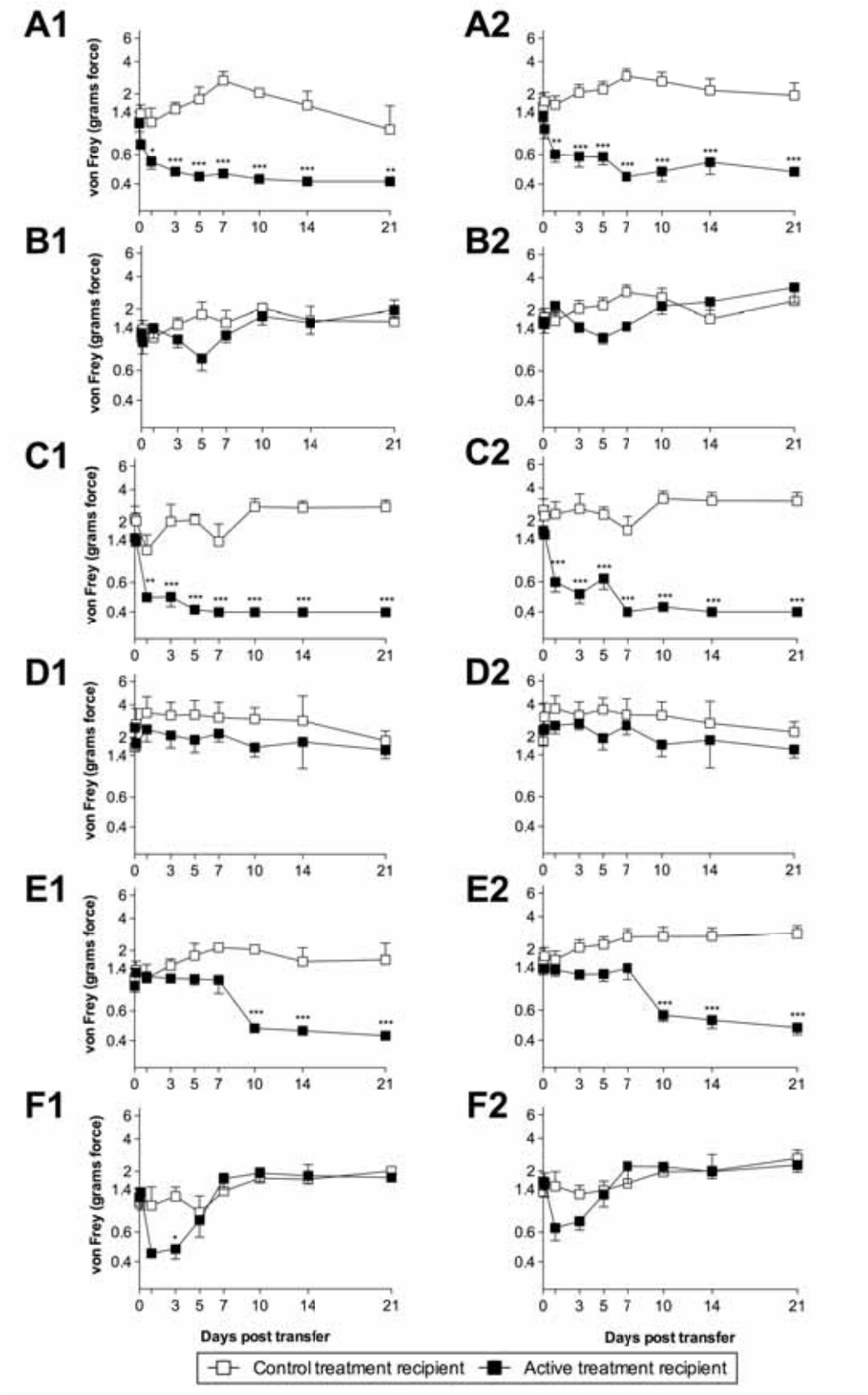
A1
A2
B1
B2
C1
C2
D1
D2
E1
E2
F1
F2
von Frey (grams force)
Days post transfer
Control treatment recipient
Active treatment recipient

Fig. 2. Transfer of splenocytes, peripheral blood mononuclear cells or serum potentiates allodynia. In all experiments, donor cells or serum prepared from Dark Agouti (DA) rats at day 0 (10 days post CCI surgery) were transferred to syngeneic recipients by intraperitoneal injection. Allodynia was quantified in both (1) ipsilateral and (2) contralateral paws at 3 h, 1, 3, 5, 7, 10, 14 and 21 days post adoptive transfer. (A) transfer of splenocytes from high pain N4S0 donors to low pain N1S3 recipients potentiates allodynia compared to vehicle control. (B) transfer of splenocytes from N0S4 donors to low pain N1S3 recipients does not potentiate allodynia compared to vehicle control. (C) transfer of peripheral blood mononuclear cells (PBMCs) isolated from high pain N4S0 donors to low pain N1S3 recipient potentiates allodynia compared to transfer of control N0S0 PBMCs. (D) transfer of splenocytes from high pain N4S0 donors to no neurogenic pain N0S4 recipients does not potentiate allodynia compared to vehicle control. (E) transfer of splenocytes from low pain N1S3 donor to low pain N1S3 recipients delays onset of allodynia potentiation compared to vehicle control and splenocytes from high pain N4S0 donors. (F) transfer of serum from high pain N4S0 donors to low pain N1S3 recipients results in a transient potentiation of allodynia compared to control N0S0 serum. n = 6/ group; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

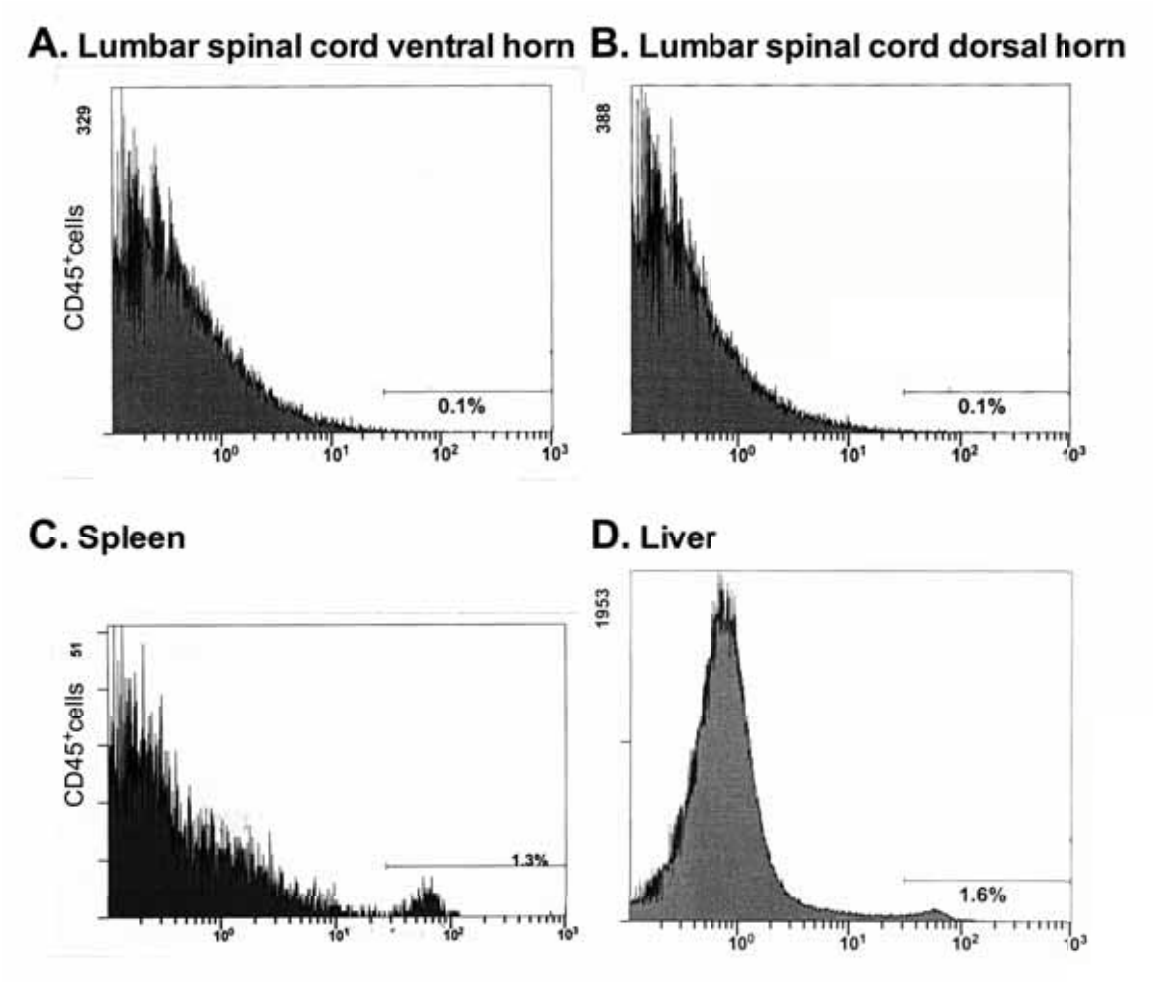

Fig. 3. Distribution of donor-derived splenocytes following adoptive transfer. Splenocytes from N4S0 high pain DA CD45.2 donors were transferred to DA CD45.1 low pain N1S3 recipients by intraperitoneal injection. Cell suspensions prepared 3 days after transfer of 2 x 10^8 cells were stained with antibody against CD45.2 (FITC) and analysed by flow cytometry. Percentages indicate the proportion of events within the respective analysis regions. No peak of donor-derived cells was found in either the ventral (A) or dorsal (B) horns of the lumbar spinal cord, However, donor-derived cells constituted ~1.3 % of splenocytes (C) and ~1.6 % of cells isolated from the liver (D).

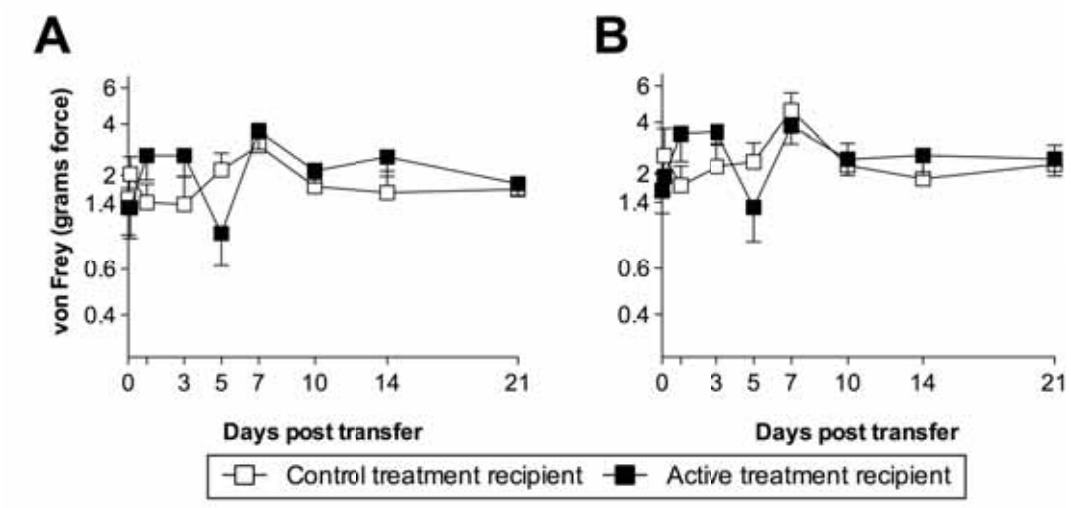

Fig. 4. Splenectomy prevents potentiation of allodynia by adoptive transfer. Splenocytes from DA high pain N4S0 donors were transferred to splenectomised syngeneic low pain N1S3 recipients by intraperitoneal injection. Controls were splenectomised low pain N1S3 rats receiving vehicle alone. Neither group exhibited potentiation of allodynia in either (A) ipsilateral or (B) contralateral paws at 3 h, 1, 3, 5, 7, 10, 14 and 21 days post adoptive transfer. *n* = 6/ group.

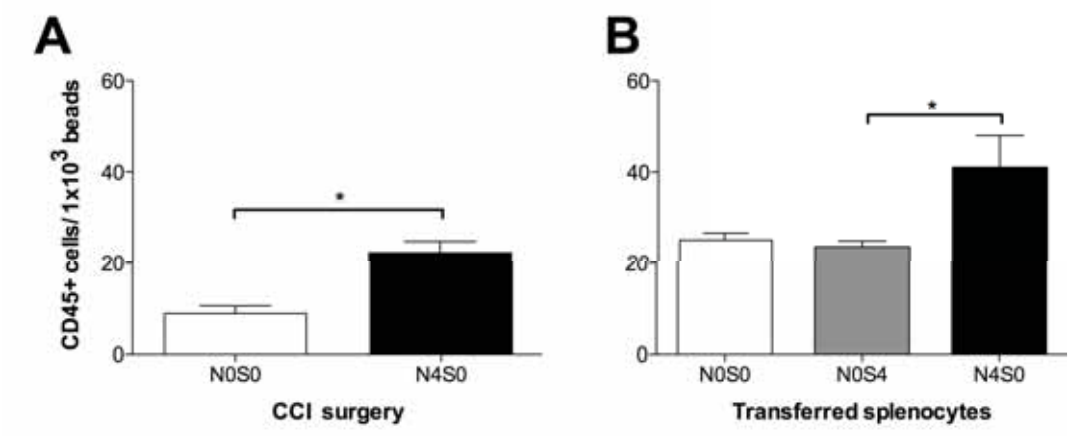

Fig. 5. CD45$^+$ cells in the lumbar spinal cord during chronic constriction injury-induced allodynia. Cells prepared from the lumbar spinal cords of DA rats were stained with antibody against CD45 (FITC) and analysed by flow cytometry. Cell numbers were estimated by addition of fixed numbers of FITC-conjugated polystyrene beads to the samples. (A) Numbers of CD45$^+$ cells in the lumbar spinal cords of DA CD45.2 high pain N4S0 and control N0S0 rats. (B) DA CD45.2 low pain N1S3 rats received intraperitoneal transfers of 2 x 10^8 splenocytes from DA CD45.1 donors that were either high pain (N4S0), or control no neurogenic pain (N0S4) or control sham (N0S0). At 3 days post transfer, cells were prepared from the lumbar spinal cord and host-derived CD45$^+$ cells were labelled with antibody against CD45.2 (FITC) and enumerated by flow

cytometry. Recipients of cells from high pain donors had significant accumulation of host-derived $CD45^+$ cells in the lumbar spinal cord relative to recipients of cells from N0S4 or N0S0 donors. *n* = 3/ group; **P* < 0.05.

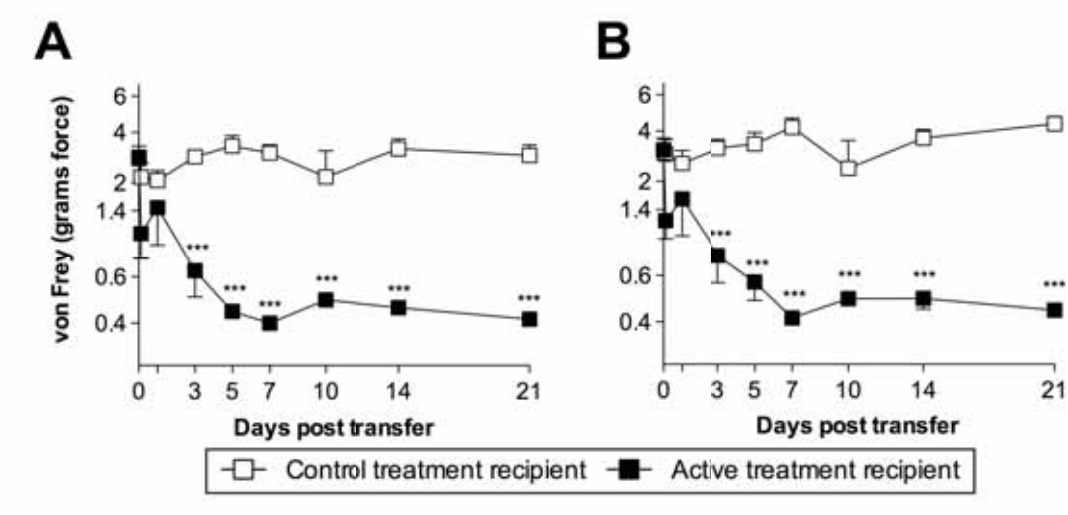

Fig. 6. Intrathecal transfer of $CD45^+$ cells obtained from allodynic lumbar spinal cord potentiates CCI-allodynia in recipients. $CD45^+$ cells were purified from the spinal cords of high pain N4S0 and sham control N0S0 DA rats at postoperative day 10. High pain N4S0 cells were transferred to syngeneic low pain N1S3 recipients by intrathecal injection (1.6×10^3). Sham control N0S0 cells were transferred to syngeneic low pain N1S3 recipients by intrathecal injection of (1.0×10^3). Compared with the recipients of control cells, rats receiving cells from N4S0 donors exhibited significant potentiation of allodynia in both (A) ipsilateral and (B) contralateral paws at 3 h, 1, 3, 5, 7, 10, 14 and 21 days post adoptive transfer. *n* = 6/ group; ****P* < 0.001.

Chapter 4. Identification of pain biomarkers by RNA microarray

Grace PM, Somogyi AA, Rolan PE and Hutchinson MR (2010) Potential biomarkers of pain identified by RNA microarray correlation of ipsilateral dorsal horn of the lumbar spinal cord with whole blood in a graded chronic constriction injury model of neuropathic pain.

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This study was the culmination of the series of experiments that aimed to develop a biomarker of pain using a bottom-up approach. Chapter 3 demonstrated a mechanistic link between the peripheral immune system and neuropathic pain, identifying immune cells present in general circulation as a suitable target for biomarker development. Therefore, using the graded model developed in Chapter 2, the graded transcriptome from the ipsilateral dorsal horn of the lumbar spinal cord was compared with that in the blood. The graded spinal cord transcriptome has major importance for understanding the pathophysiology of neuropathic pain. However, this study also aimed to correlate the two tissues in a heterogeneous pain population in order to identify a peripheral immune biomarker of pain and, to the best of my knowledge, this study is the first of its kind for neuropathic pain.

Using two different data analysis methods, this study identifies 10 genes as potential biomarkers of pain, including 4 transcription factors (zinc finger proteins) that may regulate inflammatory processes. In addition, blood CX3CL1 was found to correlate with spinal cord CX3CR1, which has a well established role in neuropathic pain processing and is considered a neuron- glia signal. The genes identified in this study require confirmation with RT-PCR and a prospective validation trial, as well as investigation of the corresponding proteins, but may represent the first generation of pain biomarkers.

Title: Potential biomarkers of pain identified by RNA microarray correlation of ipsilateral dorsal horn of the lumbar spinal cord with whole blood in a graded chronic constriction injury model of neuropathic pain.

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Abstract

Neuropathic pain is poorly treated for many patients largely due to the heterogeneity in underlying disease mechanisms and treatment response. These problems may be reduced by the development of biomarkers of pain as diagnostics. Therefore, we aimed to use DNA microarray to identify biomarkers of pain in the blood. We compared the transcriptomes of the whole blood and the ipsilateral dorsal horn of the lumbar spinal cord in rats that had undergone graded chronic constriction injury surgery (n = 2 sham, low pain; n = 3 medium pain, high pain). In the first analysis, genes from the spinal cord that were significantly correlated with von Frey threshold ($r < -0.73$ or > 0.5) were analysed using DAVID 6.7 to identify 17, primarily immune, functional pathways. The normalised expression of the genes from each pathway were summed for each animal and correlated with the respective blood transcriptome. The top 20 correlated genes for each pathway were consolidated and ranked according to the number of pathway correlations, revealing that 4 of the top 10 genes were transcription factors (zinc finger proteins) that may regulate inflammatory processes ($r > 0.7$ or < -0.8, $P < 0.001$). In the second analysis, genes correlated with von Frey threshold from the spinal cord ($r < -0.73$ or > 0.5) and blood ($r < -0.7$ or > 0.7) were analysed together using DAVID 6.7 to identify functional pathways. Each pathway was examined for blood/ spinal cord gene pairing. Blood CX3CL1 was found to correlate with spinal cord CX3CR1 ($r > 0.8$, $P < 0.001$). The genes identified in this study require confirmation with RT-PCR and a prospective validation trial, but may represent the first generation of pain biomarkers.

Introduction

Neuropathic pain remains poorly managed for many patients (Dworkin et al., 2007), due to population heterogeneity in the underlying mechanisms and treatment response. Pain treatment is further hindered by the current availability of symptomatic, rather than disease-modifying therapies (Dray, 2008). These problems may be alleviated by the development of pain biomarkers that serve as diagnostics, enable targeted therapy, and tools to expedite drug discovery and development by enriching trial designs and rapidly generating data on dose-concentration-response relationships (Rolan, 1997; Chizh et al., 2008; Lathia et al., 2009; Woodcock, 2009).

Despite inroads made in the genetics of pain (Foulkes and Wood, 2008), quantification of primary afferent activity (Chizh and Hobson, 2007), sampling of the cerebrospinal fluid (Uceyler et al., 2007; Zin et al., 2010), and imaging of the pain matrix (Chizh and Hobson, 2007; Tracey and Mantyh, 2007), to date there are no well accepted objective biomarkers for pain. This is primarily due to the fact that they fail to meet one or more of the biomarker criteria: a) evidence of a shared mechanism with biological and clinical endpoints; b) sensitive to treatment effect; c) reliable, practical and non-invasive, and; d) simple for routine utilisation without the need for expensive equipment, skilled operators or extensive time commitment (Lesko and Atkinson, 2001; Lee et al., 2006). The peripheral immune system is a potential target that may satisfy these criteria, as it: is involved in many of the mechanisms underlying neuropathic pain (Cao and DeLeo, 2008; Cao et al., 2009; Costigan et al., 2009; Grace et al., 2010a); is sensitive to disease-modifying treatments (Orhan et al., 2010); easily accessible via a simple venepuncture, and; validation and refinement of a peripheral biomarker may be simple for routine testing, without the need for highly skilled personnel.

The development of microarray technology has enabled researchers to measure genome-wide transcription in the central nervous system (Griffin et al., 2003; Mirnics and Pevsner, 2004; Reilly et al.,

2004). This technology has since been widely applied in animal models to understand the mechanisms underlying neuropathic pain (Ko et al., 2002; Yang et al., 2004; LaCroix-Fralish et al., 2006; Lacroix-Fralish et al., 2006; Griffin et al., 2007; Costigan et al., 2009; Persson et al., 2009; Vega-Avelaira et al., 2009). However, to our knowledge, gene microarray has not been applied to identify pain biomarkers. Our novel model of graded chronic constriction injury (CCI) (Grace et al., 2010b) presents an opportunity to investigate transcriptional changes in a heterogeneous population that is not confounded by genetic differences between rodent strains (Persson et al., 2009). Therefore, the aim of this study was to correlate the transcriptome of the ipsilateral dorsal horn of the lumbar spinal cord with that in the blood, in order to identify potential blood-borne biomarkers of pain.

Methods

Subjects

Pathogen-free adult male Sprague-Dawley rats (300-350 g; Animal Resource Centre, Perth, Australia; *n* = 10) were used in all experiments. Rats were housed in temperature- (18 - 21 °C) and light- controlled (12 h light/ dark cycle; lights on at 07:00 h) rooms with standard rodent food and water available *ad libitum* and allowed to habituate to the holding facility for 1 week prior to experimentation. All procedures were approved by the Animal Ethics Committee of the University of Adelaide and were conducted in accordance with the NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Surgery

The CCI model of graded sciatic nerve injury was performed aseptically at the mid-thigh level of the left hindleg, as previously described (Grace et al., 2010b). Briefly, animals were anaesthetised with isofluorane, the skin of the hindquarters was shaved and the sciatic nerve gently elevated. 0, 1 or 4

sterile chromic gut sutures (cuticular 4-0 chromic gut; Ethicon, Somerville, NJ, USA) were loosely tied around the isolated sciatic nerve (N). Once the superficial muscle overlying the nerve was sutured with silk, and prior to surgical stapling of the skin incision, additional equal lengths of chromic gut were placed subcutaneously (S), such that each animal was exposed to 4 equal lengths of chromic gut in total. Thus the treatment groups, differing in pain behaviour, were: sham (N0S0; *n* = 2), low pain (N0S4; *n* = 2); medium pain (N1S3; *n* = 3); and high pain (N4S0; *n* = 3).

Pain behaviour

Testing was conducted blind with respect to group assignment. Rats received at least three 60 min habituations to the test environment prior to behavioural testing. The von Frey test (Chaplan et al., 1994) was performed within the sciatic innervation region of the hindpaws as previously described in detail (Chacur et al., 2001; Milligan et al., 2001). Assessments were made prior to (baseline) and at PO days 3, 10 and 21. A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA) was applied randomly to the left and right hindpaws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs was determined by $\log_{10}$ (milligrams x 10) and ranged from manufacturer designated 3.61 (0.407 g) to 5.18 (15.136 g) filaments. The behavioural responses were used to calculate absolute threshold (the 50 % paw withdrawal threshold) by fitting a Gaussian integral psychometric function using a maximum-likelihood fitting method (Harvey, 1986; Treutwein and Strasburger, 1999), as described previously (Milligan et al., 2000; Milligan et al., 2001). This fitting method allows parametric analyses that otherwise would not be appropriate (Milligan et al., 2000; Milligan et al., 2001).

Tissue preparation, RNA extraction, chip hybridisation

Following final behavioural testing on day 21, the rats were anaesthetised with sodium pentobarbital and circulating blood collected via cardiac puncture. Blood was collected in RNAprotect Animal Blood Tubes

(QIAGEN, Doncaster, Vic, Australia) and stored at -80 °C. Following transcardiac perfusion with isotonic saline, the L4-L6 segment of spinal cord (innervating the sciatic nerve) was carefully excised and the ipsilateral dorsal horn was dissected, rapidly frozen, and stored at -80 °C. Total RNA (including microRNA) was purified from blood using a RNeasy Protect Animal Blood Kit (QIAGEN, Doncaster, Vic, Australia), as directed by the manufacturer. Spinal cord tissue was disrupted over dry ice in an RNase free loose fitting glass homogeniser. Total RNA (including microRNA) was purified using a miRNeasy kit (QIAGEN, Doncaster, Vic, Australia), according to the manufacturer's instructions. The A260/A280 Absorbance Ratios were calculated on an Implen NanoPhotometer (Munich, Germany) and was > 2 for all samples. The RNA integrity number was determined using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) and was > 7 for all samples. cRNA was produced from the total RNA and hybridised to the Affymetrix Rat Gene 1.0 ST Array (Santa Clara, CA, USA), according to the manufacturer's instructions. Array intensity was quantified using the Affymetrix GeneChip 3000 7G scanner (Santa Clara, CA, USA).

Data analyses

Array CEL files (Affymetrix, Santa Clara, CA, USA) were normalised, differential gene expression in the blood and ipsilateral dorsal horn of the lumbar spinal cord for each animal was paired with the respective von Frey withdrawal threshold at day 21, and a Pearson correlation was performed. Two approaches were used to identify biomarkers. The first was to correlate the blood transcriptome with significant spinal cord functional pathways that were correlated with von Frey threshold, whereas the second was to correlate the combined spinal cord and blood transcriptomes directly with von Frey threshold.

Correlation of whole blood transcriptome with spinal cord functional pathways

It was necessary to define a threshold at which correlations between mRNA expression in the spinal cord and von Frey thresholds were no longer due to chance. Given that whole blood has virtually no mechanistic link with von Frey threshold, the blood transcriptome can be used as a control to determine random correlations. Therefore the number of correlations with the von Frey threshold for the blood and spinal cord were plotted (Figure 1), as described previously (Mansson et al., 2004). The threshold at which correlations were deemed statistically significant was defined as the point at which the spinal cord curve crossed the random correlation curve for the blood. This threshold is conservative, as we hypothesise that a biomarker can be identified in the blood and that some correlations will therefore be statistically significant. This preprocessing of the data replaces that used previously, in which significant fold change between control and treatment is a requirement for further analysis (Ko et al., 2002; Yang et al., 2004; LaCroix-Fralish et al., 2006; Lacroix-Fralish et al., 2006; Griffin et al., 2007; Costigan et al., 2009; Persson et al., 2009; Vega-Avelaira et al., 2009). Our method includes subtle changes for analysis that may otherwise be overlooked. The statistically significant threshold for negative correlations (positive correlation with allodynia) is $r < -0.73$, whereas for positive correlations the threshold is $r > 0$ and as such, an arbitrary data reduction threshold was set at $r > 0.5$.

The $r < -0.73$ or > 0.5 spinal cord gene list was analysed with DAVID 6.7 software (http://david.abcc.ncifcrf.gov) (Dennis et al., 2003; Huang et al., 2009) to identify functional pathways represented within the dataset. The pathways identified were further refined by excluding those that contained less than 1 % of the gene list and those that did not contain unique genes, in order to reduce the likelihood of spurious associations. The differential expression of each significantly expressed gene was normalised to account for fold changes so that the relative expression of each gene would not artificially weight the pathway. Normalised expression was then summed within the spinal cord

functional pathways for each animal to obtain a single value for each pathway. A Pearson correlation was then performed between the sum of each pathway and the entire blood transcriptome.

Correlation of combined whole blood and lumbar spinal ipsilateral dorsal horn transcriptomes with von Frey threshold

The threshold of statistically significant correlations for the spinal cord transcriptome was determined as described above. The threshold of statistically significant correlations for the blood was defined as the point at which the number of correlations between the von Frey threshold and gene expression crossed the curve for randomly generated correlations (Figure 2), as described previously (Mansson et al., 2004). This blood curve did not cross the random curve, and therefore an arbitrary threshold was set at $r < -0.7$ or > 0.7. The blood and spinal cord gene list was combined and analysed with DAVID 6.7 software (Dennis et al., 2003; Huang et al., 2009). The pathways identified were further refined by excluding those that contained less than 1 % of the gene list, in order to reduce the likelihood of spurious associations. Each pathway was examined for genes present in the blood that were functionally paired with those in the spinal cord.

Results

Pain behaviour

Behaviour was quantified to day 21 post graded CCI surgery (Figure 3). The degree of allodynia corresponded to treatment group as found previously (Grace et al., 2010b) and the general aim to achieve heterogeneous allodynia in the total population (n = 10) was achieved. Von Frey allodynia scores dropped from 8.96 ± 0.97 g (mean ± standard error of the mean) at baseline to 1.87 ± 0.37 g (N0S0), 1.82 ± 1.25 g (N0S4), 0.88 ± 0.20 g (N1S3) and 0.44 ± 0.00 g (N4S0) at 21 days post surgery.

Correlation of whole blood transcriptome with spinal cord functional pathways

We used oligonucleotide microarrays to detect a correlation in mRNA expression in the ipsilateral dorsal horn of the lumbar spinal cord with graded von Frey threshold. A statistically significant gene list (513 genes) was generated from those with r < -0.73 or > 0.5 and was further refined by excluding pathways that contained less than 1 % of the gene list and those that did not contain unique genes (Table 1; 87 genes). The refined list of genes was classified into functional pathways (Figure 4) and the blood transcriptome was correlated with the sum of the normalised differential expression from each of the 17 pathways. The top 20 genes from each pathway correlation were consolidated and ranked according to the number of pathways that each gene was correlated with. Following exclusion of genes that were correlated with only one pathway, a list of 56 genes was generated (Table 2). The mean *P*-value and Pearson r were calculated for each gene.

Correlation of combined whole blood and ipsilateral dorsal horn of lumbar spinal transcriptomes with von Frey threshold

The spinal cord r < -0.73 or > 0.5 and blood r < -0.7 or > 0.7 gene lists were classified into functional pathways (Figure 5). Each of the 12 pathways were examined for functional pairing of spinal cord and blood genes, of which only the “cytokine-cytokine receptor interaction pathway” contained receptor-ligand pairing. The ligands granulocyte colony stimulating factor (G-CSF; CSF3) (r = 0.80, *P* = 0.0052) and CX3CL1 (fractalkine) (r = -0.90, *P* = 0.0003) were significantly correlated with von Frey threshold in the blood and their respective receptors G-CSF-R (CD114; CSF3R) (r = -0.90, *P* = 0.0004) and CX3CR1 (r = -0.83, *P* = 0.0026) were significantly correlated with von Frey threshold in the spinal cord. An inverse relationship was found between G-CSF and G-CSF-R, however no significant correlation was found (r = -0.47, *P* = 0.17; Figure 6 A). A significant correlation was found between CX3CL1 and CX3CR1 (r = 0.84, *P* = 0.0025; Figure 6 B).

Discussion

The aim of this study was to compare the transcriptome of the ipsilateral dorsal horn of the lumbar spinal cord with the transcriptome of the whole blood in the Grace model of graded neuropathic pain (Grace et al., 2010b), in order to identify potential biomarkers of pain. This approach necessarily required a correlational approach to the analysis of the data set. The first approach classified the r < -0.73 or > 0.5 spinal cord gene list into functional pathways and each pathway was then correlated with the blood transcriptome. This gene list was collapsed and ranked according to the genes in the blood that were correlated with more than one spinal cord pathway, resulting in a list of 56 genes (Table 2). The second approach combined the spinal cord r < -0.73 or > 0.5 and blood r < -0.7 or > 0.7 gene lists and classified the genes into functional pathways. Each pathway was examined for receptor-ligand pairing between the blood and spinal cord, resulting in the identification of ligands G-CSF and CX3CL1 in the blood and their respective receptors G-CSF-R and CX3CR1 in the spinal cord. Of these pairs, only the CX3CL1 CX3CR1 combination was significantly correlated (r = 0.84, *P* = 0.0025; Figure 6 B).

The top 10 genes from the list identified from the first approach do not appear to be mechanistically linked to pain pathophysiology and consist of mainly of transcription factors (zinc fingers). Gene I.D. 10733477 “zinc finger CCHC domain containing 10” (*P* = 0.0002, r = -0.93; 15 pathways) and gene I.D. 10721145 “zinc finger protein” (*P* = 0.0008, r = 0.88; 10 pathways) are both highly correlated with the significant spinal cord functional pathways (Figure 4). Gene I.D. 10870259 (*P* = 0.0006, r = -0.90; 16 pathways) is currently unknown, but 14 nucleotides of the probe sequence (representing 60 % of the 25 nucleotide probe) share complete homology with the human “zinc finger and BTB domain containing 8A” transcript (NM_001040441.1). On the rat genome, this gene is located between the genes encoding “Janus kinase 1” and “adenylate kinase 3 like 1”. Similarly, gene I.D. 10772167 (*P* = 0.000375, r = 0.774728; 12 pathways), is also currently unknown, but 17 nucleotides of the probe sequence, (representing 68 % of the 25 nucleotide probe) share complete homology with the human “zinc finger

protein 131" transcript (NM_003432.1). On the rat genome, this gene is located between the genes encoding "serine/threonine protein kinase NIM1" and "hypothetical protein LOCI00132356". Very little is currently known about these particular factors, however the domain and protein designations define the structure-activity binding relationship with DNA and/or RNA (Klug, 2010). Gene I.D. 10734853 aurora kinase B (P = 0.0009, r = -0.88; 10 pathways) is a mitotic enzyme that functions in the attachment of the mitotic spindle to the centromere (Meyer et al., 2010). Given that these genes are highly correlated with what are predominantly immune pathways (Figure 4), these proteins may be upstream regulators of the immune response (immune cell proliferation, cytokine secretion etc). Indeed, other zinc finger proteins have been shown to regulate cytokine-mediated inflammatory process at a transcriptional level (Sharif-Askari et al., 2010; Shifera, 2010; Shin et al., 2010). Gene I.D. 10903725 "tumor necrosis factor receptor superfamily, member 11b" (P = 0.0006, r = -0.89; 12 pathways), is also known as the soluble cytokine receptor osteoprotegerin, which has been linked to cardiovascular disease (Lieb et al., 2010), osteoporosis (Wagner and Fahrleitner-Pammer, 2010), painful diabetic neuropathy (Doupis et al., 2009) and has utility as a biomarker for inflammation (Caidahl et al., 2010). Osteoprotegerin regulates vascular permeability, cytokine release and monocyte transmigration (Caidahl et al., 2010), which may have profound implications, given the role of peripheral immune cells in neuropathic pain (Grace et al., 2010a). The relationship of the other top 10 genes to neuropathic pain is unclear: Gene I.D. 10914209 "xylulokinase homolog (H. influenzae)" (P = 0.0009, r = -0.88; 11 pathways) has been investigated in *e. coli* and is a catalysis enzyme involved in the degradation of alcohols (Buhler et al., 2000); gene I.D. 10849267 "Src homology 2 domain containing F" (P = 0.0009, r = -0.88; 10 pathways) is involved in the signal transduction pathways and has been investigated in cancer (Daino et al., 2009); gene I.D. 10920195 "RNA binding motif protein 6" (P = 0.0011, r = -0.87; 10 pathways) has also been investigated in cancer and, based on the structural similarities with other RNA binding motif proteins, may be an apoptosis signal (Sutherland et al., 2005); gene I.D. 10937496 "similar to ubiquitin specific protease 27, X" (P = 0.0011, r = -0.87; 10 pathways) currently has an unknown function. Not only do these genes

require validation with RT-PCR and in prospective trials in order to identify them as biomarkers of pain, but further investigation may reveal a role in the mechanisms underlying neuropathic pain.

The two genes, CX3CL1 and G-CSF, identified by the second approach, encode for chemokines that are posited to play a role in neuropathic pain (Abbadie et al., 2009; Milligan and Watkins, 2009; Ro et al., 2009). A significant correlation was found between CX3CL1 in the blood and its receptor CX3CR1 in the spinal cord ($r = 0.84$, $P = 0.0025$; Figure 6 B). Therefore circulating CX3CL1 is a potential biomarker of pain and requires a prospective validation study. Whole blood never crossed the random correlation curve for statistical significance (Figure 2) and so it is possible that the CX3CL1 correlation occurred by chance. However, given the strong mechanistic link to neuropathic pain (Abbadie et al., 2009; Milligan and Watkins, 2009), this seems unlikely. G-CSF in the blood is positively correlated with von Frey threshold (negatively correlated with allodynia) ($r = 0.80$, $P = 0.0052$), whilst spinal cord G-CSF-R is negatively correlated with von Frey threshold, resulting in a non-significant, inverse ligand-receptor relationship ($r = -0.47$, $P = 0.17$; Figure 6 A). The overall significance of G-CSF in the graded CCI model is unclear. G-CSF is considered an anti-inflammatory chemokine (Ro et al., 2009) and in the blood, may be down-regulated by proinflammatory factors. In the spinal cord G-CSF-R is upregulated, in a similar fashion to another anti-inflammatory cytokine, IL-10 (Milligan and Watkins, 2009), although no relationship was observed between blood IL-10 and von Frey threshold in the current study ($r = 0.15$). G-CSF has also been indentified in the serum of diabetic neuropathy patients (Doupis et al., 2009). Irrespective of this unusual and somewhat contradictory relationship, G-CSF and G-CSF-R protein should be quantified before G-CSF is ruled out as a biomarker of pain due to the insignificant ligand/ receptor RNA transcript relationship.

A potential limitation of these putative biomarkers is that they may lack specificity. The 10 genes in the blood identified by correlation with spinal cord functional pathways, are involved in general cellular function, whilst CX3CL1 is generally involved in inflammatory processes (Zhang and Patel, 2010) and

cardiovascular complications (Ikejima et al., 2010). However, the particular combination of any or all of these genes as a panel may be unique to CCI-induced neuropathic pain. Therefore a prospective validation study is a required.

Statistical analysis of the data set generated by microarray presents a problem, as there is no real consensus on the appropriate methodology to identify statistically significant gene expression. Previous studies (Ko et al., 2002; Yang et al., 2004; LaCroix-Fralish et al., 2006; Lacroix-Fralish et al., 2006; Griffin et al., 2007; Costigan et al., 2009; Vega-Avelaira et al., 2009), including those that used a correlational approach to analysis (Cameron et al., 2007; Persson et al., 2009), preprocess the data by excluding non-significant expression of normalised fold-changes compared to a baseline. We had no baseline expression, whereas we correlated all transcripts with the von Frey threshold and identified a statistically significant threshold whereby correlations were no longer due to chance. This method potentially allows detection of subtle changes rather basing selection for further analysis on overabundance. Furthermore, to our knowledge, there are no studies that correlate graded transcriptomes between tissues. Therefore we used two approaches, one of which yielded a chemokine that has a known role in neuropathic pain processing. Prospective validation studies will not only validate the putative biomarkers identified by this study, but also the data analyses used. Another feature of this study was the use of the blood transcriptome as a control for non-specific expression, being a mechanistically unrelated tissue. The principle of using the transcriptome of an unrelated tissue from the same animal as a control may be useful in all microarray experiments, in that it may facilitate detection of more subtle changes rather than relying on the control tissues of interest from sham/control-treated animals. Furthermore, this approach has the potential to reduce the number of animals required for microarray experimentation, in accordance with the animal welfare principle of the “Three Rs” (reduction, refinement and replacement).

The graded transcriptome of the spinal cord was correlated with the graded transcriptome of the blood using two different approaches that identified different gene sets as potential biomarkers of pain. The genes identified in this study require a prospective validation trial in combination with assessment of treatment, but may represent the first generation of pain biomarkers.

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Figures, Tables and Legends

Table 1. Refined spinal cord gene list. The ipsilateral dorsal horn of the lumbar spinal cord transcriptome was correlated with von Frey threshold and the r < -0.73 or > 0.5 gene list was classified into functional pathways using DAVID 6.7 software. The gene list was refined by excluding pathways containing less than 1 % of the gene list and those that did not contain unique genes in order to reduce the likelihood of spurious associations.

ID	r	*p*	Gene name
10823363	-0.9800	0.0001	purinergic receptor P2Y, G-protein coupled, 13
10769788	-0.9566	0.0001	Fc fragment of IgG, low affinity IIb, receptor (CD32); Fc fragment of IgG, low affinity IIa, receptor (CD32)
10769825	-0.9370	0.0001	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide
10939293	-0.9337	0.0001	Bruton agammaglobulinemia tyrosine kinase
10821689	-0.9245	0.0001	prostaglandin E receptor 4 (subtype EP4)
10719394	-0.9242	0.0001	gastric inhibitory polypeptide receptor
10897419	-0.9170	0.0002	neutrophil cytosolic factor 4
10920981	-0.9047	0.0003	chemokine (C-X3-C motif) receptor 1
10825153	-0.9022	0.0004	Fc fragment of IgG, high affinity Ia, receptor (CD64)
10871957	-0.9011	0.0004	colony stimulating factor 3 receptor (granulocyte)
10760946	0.8967	0.0004	erythropoietin
10865463	-0.8841	0.0007	protein tyrosine phosphatase, non-receptor type 6
10720797	-0.8749	0.0009	CD22 molecule
10711268	-0.8731	0.0010	integrin alpha M
10820908	-0.8716	0.0010	similar to neuronal apoptosis inhibitory protein; NLR family, apoptosis inhibitory protein 2
10730131	-0.8687	0.0011	B-cell linker
10895705	-0.8684	0.0011	glucosamine (N-acetyl)-6-sulfatase
10754176	-0.8678	0.0011	CD80 molecule
10872489	-0.8648	0.0012	lysosomal multispanning membrane protein 5
10858315	-0.8612	0.0014	interleukin 17 receptor A
10914618	-0.8610	0.0014	chemokine (C-C motif) receptor 5
10817429	-0.8608	0.0014	cathepsin S
10732725	-0.8596	0.0014	lymphocyte cytosolic protein 2
10768376	-0.8585	0.0015	phospholipase A2, group IVA (cytosolic, calcium-dependent)
10777677	-0.8580	0.0015	SH3-domain binding protein 2
10832306	-0.8564	0.0016	integrin beta 2
10880731	-0.8537	0.0017	complement component 1, q subcomponent, C chain
10867461	-0.8497	0.0019	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
10769771	-0.8480	0.0019	Fc fragment of IgG, low affinity IIb, receptor (CD32); Fc fragment of IgG, low affinity IIa, receptor (CD32)
10810549	-0.8438	0.0021	similar to H3 histone, family 3B; similar to Zgc:56193; similar to H3 histone, family 3A; H3 histone, family 3B; similar to Histone H3.3; similar to histone 1, H2ai
10701924	-0.8416	0.0023	interferon gamma receptor 1
10757898	-0.8387	0.0024	neutrophil cytosolic factor 1
10922871	-0.8351	0.0026	interleukin 18 receptor 1
10905316	-0.8306	0.0029	ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)
10775731	-0.8300	0.0030	chemokine (C-X-C motif) ligand 13
10880727	-0.8293	0.0030	complement component 1, q subcomponent, beta polypeptide
10880734	-0.8280	0.0031	complement component 1, q subcomponent, alpha polypeptide
10907815	-0.8247	0.0033	caspase 1
10754384	-0.8246	0.0033	CD86 molecule
10768138	-0.8224	0.0035	protein tyrosine phosphatase, receptor type, C
10828778	-0.8174	0.0039	mitogen activated protein kinase 13
10860831	-0.8149	0.0041	calcitonin receptor
10811663	-0.8109	0.0044	galactosamine (N-acetyl)-6-sulfate sulfatase
10733982	-0.8100	0.0045	NLR family, pyrin domain containing 3
10865585	-0.8097	0.0045	CD4 molecule
10898862	-0.8093	0.0046	interleukin-1 receptor-associated kinase 4
10830951	0.8073	0.0047	histocompatibility 2, M region locus 11
10721728	-0.8039	0.0051	CD37 molecule
10840975	-0.8023	0.0052	hemopoietic cell kinase
10717967	0.8004	0.0054	plasminogen
10933345	-0.7927	0.0062	toll-like receptor 7
10837424	-0.7884	0.0067	apelin receptor
10802065	-0.7865	0.0070	colony stimulating factor 1 receptor
10757986	-0.7862	0.0070	glucuronidase, beta
10765497	-0.7827	0.0074	Fc fragment of IgG, low affinity IIIa, receptor
10750193	-0.7811	0.0076	interleukin 10 receptor, beta
10706810	-0.7798	0.0078	luteinizing hormone beta
10818167	-0.7792	0.0079	adenosine A3 receptor
10819281	0.7788	0.0079	protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform
10820693	-0.7785	0.0080	hexosaminidase B
10769797	-0.7767	0.0082	Fc fragment of IgG, low affinity IIb, receptor (CD32); Fc fragment of IgG, low affinity IIa, receptor (CD32)
10915910	-0.7755	0.0084	junctional adhesion molecule 3
10811347	-0.7642	0.0101	phospholipase C, gamma 2
10823353	-0.7636	0.0102	purinergic receptor P2Y, G-protein coupled, 14
10880343	-0.7635	0.0102	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene homolog
10931717	-0.7605	0.0106	complement component 3
10897428	-0.7575	0.0112	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)
10931196	-0.7551	0.0116	vav 1 guanine nucleotide exchange factor
10780396	-0.7545	0.0117	proteasome (prosome, macropain) activator subunit 1
10756877	-0.7540	0.0118	caspase recruitment domain family, member 11
10831620	-0.7485	0.0128	major histocompatibility complex, class II, DM beta
10784641	0.7463	0.0132	PTK2B protein tyrosine kinase 2 beta
10839232	-0.7451	0.0134	beta-2 microglobulin
10827820	0.7445	0.0135	histocompatibility 2, T region locus 23; histocompatibility 2, T region locus 24
10940549	-0.7439	0.0136	complement component 5a receptor 1
10912161	-0.7438	0.0137	cathepsin H
10861923	0.7419	0.0140	ATPase, H+ transporting, lysosomal V0 subunit A4
10787491	-0.7413	0.0141	interferon gamma inducible protein 30
10809912	-0.7401	0.0144	mannosidase, alpha, class 2B, member 1
10731922	-0.7389	0.0146	Mediterranean fever
10797509	-0.7388	0.0147	spleen tyrosine kinase
10840565	0.7359	0.0153	somatostatin receptor 4
10813628	-0.7344	0.0156	prolactin receptor
10770197	0.7332	0.0158	v-akt murine thymoma viral oncogene homolog 3 (protein kinase B, gamma)
10809665	0.7328	0.0159	nucleotide-binding oligomerization domain containing 2
10759524	-0.7322	0.0160	chemokine (C-C motif) ligand 25
10877069	-0.7321	0.0161	lysophosphatidic acid receptor 1

Table 2. Refined list of genes in the blood significantly correlated with functional pathways in the spinal cord. The - r < -0.73 or > 0.5 spinal cord gene list was classified into functional pathways using DAVID 6.7 software. The gene list was refined by excluding pathways containing less than 1 % of the gene list and those that did not contain unique genes. The differential gene expression was normalised and summed for each animal within pathways. The summed gene expression was correlated with the blood transcriptome, the top 20 genes from each pathway were consolidated and ranked according to the number of pathways that the genes were correlated with (of a possible 17). Genes that were correlated with only one pathway were excluded. The mean *P*-value and Pearson r are presented.

No. of pathways	Average r	Average *p*	Gene I.D.	Gene name
16	-0.8964	0.0006	10870259	---
15	-0.9259	0.0002	10733477	ENSRNOT00000009098 // Zcchc10 // zinc finger, CCHC domain containing 10 // 10q22
12	-0.8936	0.0006	10903725	NM_012870 // Tnfrsf11b // tumor necrosis factor receptor superfamily, member 11b
12	0.7747	0.0004	10772167	---
11	-0.8788	0.0009	10914209	NM_001033704 // Xylb // xylulokinase homolog (H. influenzae) // 8q32 // 316067 /
10	-0.8794	0.0009	10734853	NM_053749 // Aurkb // aurora kinase B // 10q24 // 114592 /// ENSRNOT00000008492
10	0.8833	0.0008	10721145	NM_001106248 // Znf507 // zinc finger protein 507 // 1q21 // 292816
10	-0.8892	0.0006	10849267	ENSRNOT00000037023 // Shf // Src homology 2 domain containing F // 3q35 // 36220
10	-0.8729	0.0011	10937496	ENSRNOT00000043867 // LOC683561 // similar to ubiquitin specific protease 27, X
10	0.8842	0.0008	10920195	NM_001108186 // Rbm6 // RNA binding motif protein 6 // 8q32 // 315997 /// ENSRNO
10	-0.8975	0.0005	10723876	NM_001109436 // Mogat2 // monoacylglycerol O-acyltransferase 2 // 1q32 // 681211
10	-0.8856	0.0009	10711234	NM_019244 // Bckdk // branched chain ketoacid dehydrogenase kinase // 1q36 // 29
9	-0.8885	0.0007	10876157	NM_001109616 // LOC691024 // similar to Protein C9orf25 homolog // 5q22 // 69102
9	-0.8868	0.0007	10916709	NM_001106816 // Abcg4 // ATP-binding cassette, sub-family G (WHITE), member 4 //
8	-0.8835	0.0009	10904223	---
6	0.8757	0.0010	10706308	NM_001106250 // Siglecg // sialic acid binding Ig-like lectin G // 1q22 // 29284
6	0.8643	0.0013	10933651	NM_001033899 // Sms // spermine synthase // Xq21 // 363469 /// BC101870 // Sms /
6	-0.8717	0.0010	10931034	NM_001009717 // Lrg1 // leucine-rich alpha-2-glycoprotein 1 // --- // 367455 ///
6	0.8895	0.0006	10738056	NM_031134 // Thra // thyroid hormone receptor alpha // 10q31 // 81812 /// NM_001
6	0.8882	0.0007	10818314	NM_206881 // Amigo1 // adhesion molecule with Ig like domain 1 // 2q34 // 295365
6	0.8900	0.0007	10834014	XM_227820 // H28 // histocompatibility 28 // 2q45 // 310968 /// XM_001079914 //
5	-0.8706	0.0011	10845441	---
5	-0.6006	0.0006	10886244	---
5	-0.8702	0.0011	10876747	NM_021701 // Ppp3r2 // protein phosphatase 3, regulatory subunit B, alpha isofor
5	-0.8902	0.0008	10704895	NM_144757 // Zfp180 // zinc finger protein 180 // 1q21 // 246279 /// ENSRNOT0000
4	-0.8494	0.0019	10750421	NM_001105892 // Dscr6 // Down syndrome critical region homolog 6 (human) // 11q1
4	-0.8635	0.0013	10778379	NM_053751 // Wap // whey acidic protein // 14q21 // 114596 /// ENSRNOT0000001074
4	-0.8645	0.0013	10781745	NM_001107279 // Pcdh17 // protocadherin 17 // 15q12 // 306055 /// ENSRNOT0000004
4	0.8732	0.0010	10765462	NM_001033899 // Sms // spermine synthase // Xq21 // 363469 /// BC101870 // Sms /
4	0.8694	0.0011	10855187	NM_001109230 // Zfp398 // zinc finger protein 398 // 4q24 // 500108 /// ENSRNOT0
4	-0.8781	0.0008	10823303	NM_030873 // Pfn2 // profilin 2 // 2q31 // 81531 /// ENSRNOT00000023469 // Pfn2
4	-0.8941	0.0007	10722213	NM_172158 // Mrgprx1 // MAS-related GPR, member X1 // 1q22 // 282547 /// ENSRNOT
3	-0.8718	0.0010	10917149	---
3	-0.8731	0.0010	10791966	NM_001004212 // Tusc3 // tumor suppressor candidate 3 // 16q12.1 // 290783 /// E
3	-0.8955	0.0005	10934683	XR_006082 // LOC302346 // similar to Glyceraldehyde-3-phosphate dehydrogenase (G
3	-0.8808	0.0009	10892307	---
3	-0.8987	0.0005	10893343	NM_001000074 // Olr952 // olfactory receptor 952 // 7q11 // 288880 /// ENSRNOT00
2	-0.8593	0.0015	10922245	---
2	-0.8490	0.0019	10855836	NM_001009528 // V1rc43 // vomeronasal 1 receptor, C43 // 4q24 // 494248 /// ENSR
2	-0.8541	0.0017	10756987	ENSRNOT00000001716 // RGD1305593 // similar to RIKEN cDNA 1810042K04 // 12q11 //
2	-0.8703	0.0011	10819390	NM_001106475 // Adh6a // alcohol dehydrogenase 6A (class V) // 2q44 // 295498 //
2	-0.8636	0.0013	10895747	NM_053019 // Avpr1a // arginine vasopressin receptor 1A // 7q21 // 25107 /// ENS
2	-0.8783	0.0008	10847221	NM_001000623 // Olr715 // olfactory receptor 715 // 3q24 // 404815
2	0.8959	0.0005	10875698	NM_001024757 // Wwp1 // WW domain containing E3 ubiquitin protein ligase 1 // 5q
2	-0.8696	0.0011	10768346	NM_019336 // Rgs1 // regulator of G-protein signaling 1 // 13q21 // 54289 /// EN
2	-0.8833	0.0007	10827000	NM_001107727 // Mttp // microsomal triglyceride transfer protein // 2q44 // 3109
2	-0.8883	0.0006	10802040	NM_031525 // Pdgfrb // platelet derived growth factor receptor, beta polypeptide
2	-0.8823	0.0007	10824798	NM_001100996 // RGD1559714 // similar to TDPOZ3 // 2q34 // 502570 /// NM_0011009
2	-0.9020	0.0004	10730539	NM_134370 // Psd // pleckstrin and Sec7 domain containing // 1q54 // 171381 ///
2	-0.8850	0.0007	10723501	NM_001000116 // Olr13 // olfactory receptor 13 // 1q32 // 293075 /// ENSRNOT0000
2	0.8942	0.0005	10740895	NM_001105767 // Kremen2 // kringle containing transmembrane protein 2 // 10q12 /
2	0.8904	0.0006	10933755	NM_001106964 // Tbl1x // transducin (beta)-like 1 X-linked // Xq22 // 302711 ///
2	-0.9053	0.0003	10833673	---
2	-0.9131	0.0002	10898809	NM_057118 // Cntn1 // contactin 1 // 7q35 // 117258 /// ENSRNOT00000006219 // Cn
2	-0.9151	0.0002	10890522	NM_053865 // Rtn1 // reticulon 1 // 6q24 // 116644 /// U17604 // Rtn1 // reticul
2	0.9003	0.0009	10738657	NM_001012013 // Acbd4 // acyl-Coenzyme A binding domain containing 4 // 10q32.1

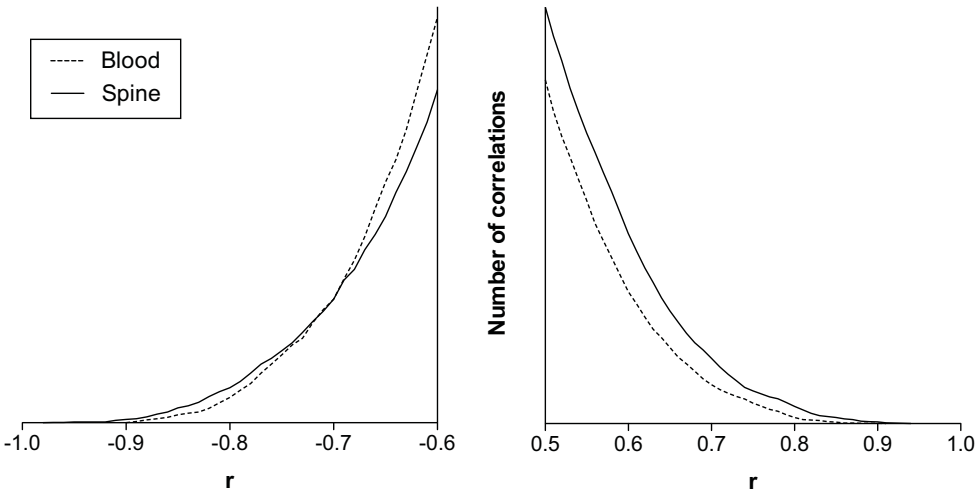

Figure 1. Relative number of correlations for blood and ipsilateral dorsal horn of the lumbar spinal cord with von Frey threshold. Whole blood is not considered mechanistically related to von Frey threshold and therefore the number of correlations occurs are random. As the ipsilateral dorsal horn of the lumbar spinal cord is mechanistically related to von Frey threshold, the point at which this curve crosses that of the blood determines the threshold of statistically significant correlations as described previously (Mansson et al., 2004). For negative correlations (positively correlated with allodynia), this threshold is $r < -0.73$. Whereas for positive correlations this threshold is $r > 0$ and as such, the threshold was set at $r > 0.5$ in order to reduce the data.

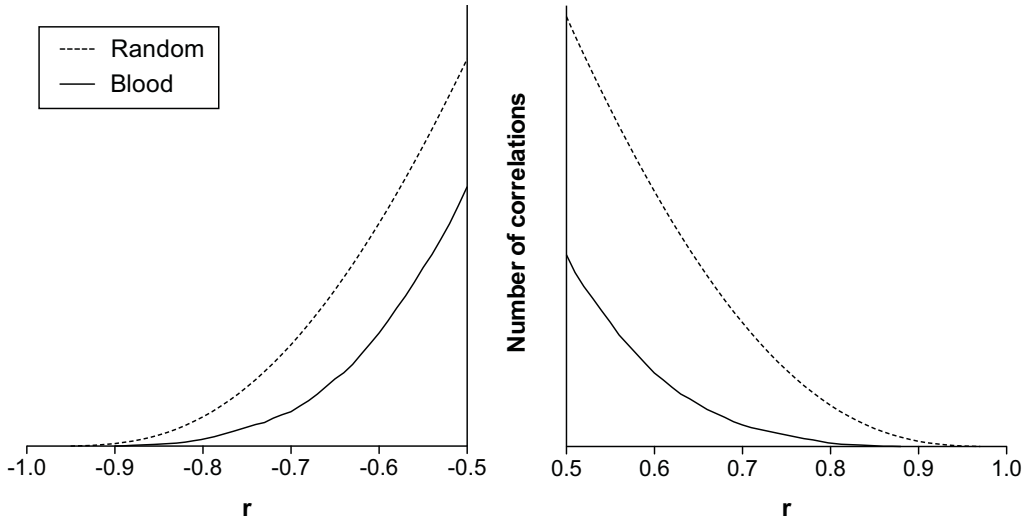

Figure 2. Relative number of correlations for blood and randomly generated correlations with von Frey threshold. The point at which this blood curve crosses the random curve determines the threshold of statistically significant correlations, as described previously (Mansson et al., 2004), however this threshold did not exist for this experiment. Therefore an arbitrary threshold was set at r < -0.7 or > 0.7.

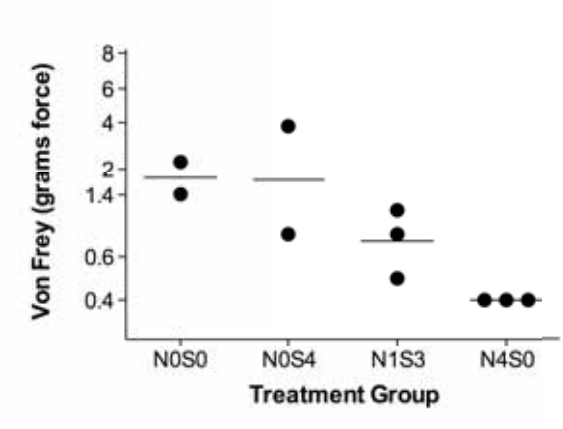

Figure 3. Graded chronic constriction injury of the sciatic nerve produces graded allodynia at day 21 post surgery. Graded neuropathy was induced by varying the distribution of 4 equivalent chromic gut pieces across the nerve (N) or subcutaneous (S) compartments. As such, treatment groups were N0S0 (*n* = 2), N0S4 (*n* = 2), N1S4 (*n* = 3) and N4S0 (*n* = 3).

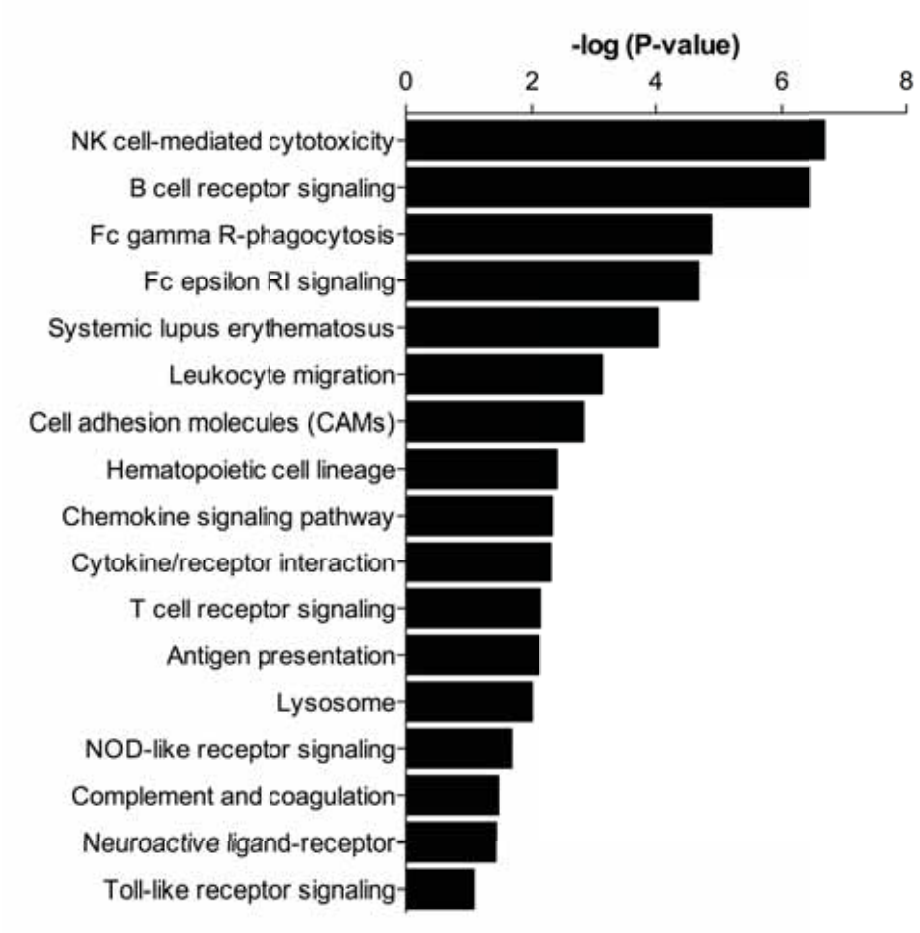

Figure 4. Pathways significantly associated with graded neuropathy in the ipsilateral lumbar dorsal horn of the lumbar spinal cord. The r < -0.73 or > 0.5 gene list was classified into functional pathways using DAVID 6.7 software. The gene list was refined by excluding pathways containing less than 1 % of the gene list and those that did not contain unique genes, in order to reduce the likelihood of spurious associations. Bars represent the -log(p value) for that pathway within the gene list (two-tailed Fisher's exact test).

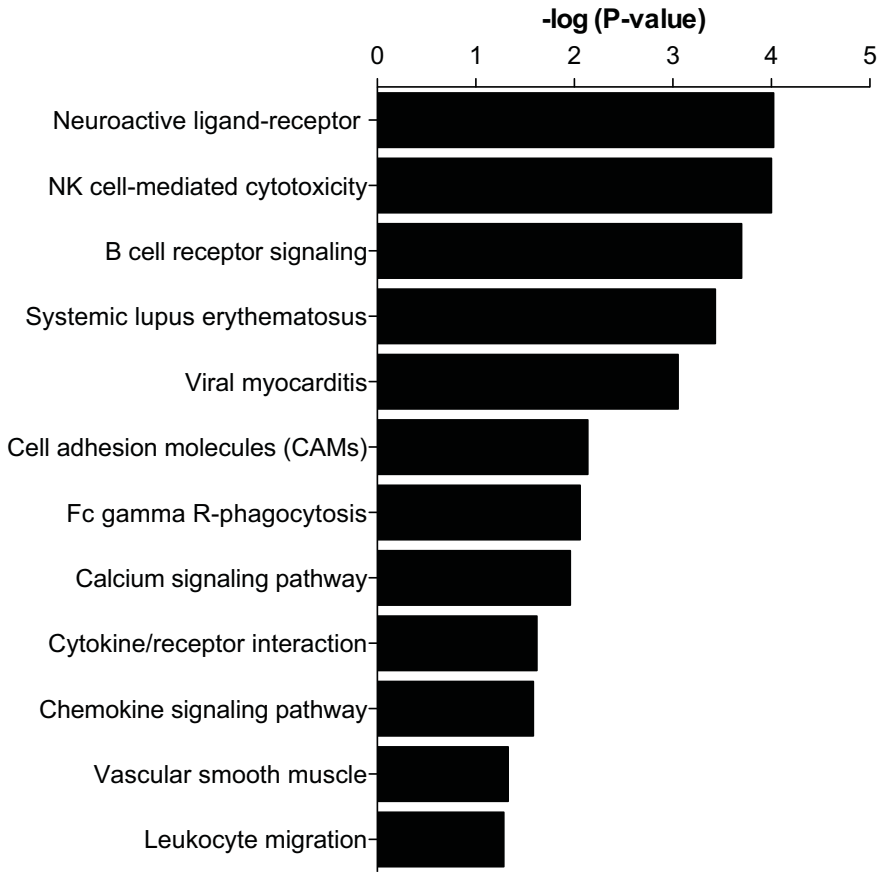

Figure 5. Pathways significantly associated with graded neuropathy in the ipsilateral lumbar dorsal horn of the lumbar spinal cord and whole blood. The combined spinal r < 0.73 or > 0.5 and blood r < -0.7 or > 0.7 gene list was classified into functional pathways using DAVID 6.7 software. The gene list was refined by excluding pathways containing less than 1 % of the gene list, in order to reduce the likelihood of spurious associations. Bars represent -log(p value) for that pathway within the gene list (two-tailed Fisher's exact test).

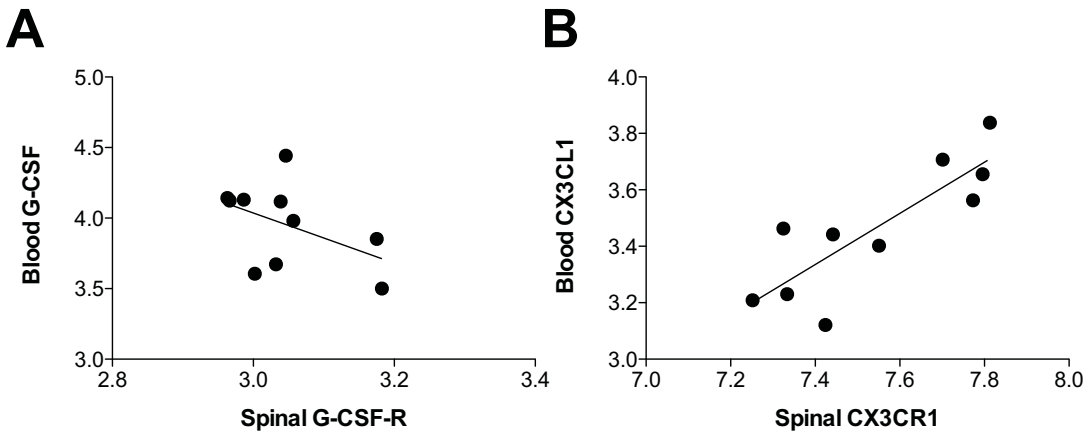

Figure 6. Relationship between blood ligand and spinal cord receptor in the graded CCI model. A significant correlation was found between the von Frey threshold and the ligands granulocyte colony stimulating factor (r = 0.80, *P* = 0.0052) and CX3CL1 (fractalkine) (r = -0.90, *P* = 0.0003) in the blood. A significant correlation was also found between the von Frey threshold and the respective receptors G-CSF-R (r = -0.90, *P* = 0.0004) and CX3CR1 (r = -0.83, *P* = 0.0027) in the spinal cord. A correlation between the ligands and their respective receptors found (A) no significant relationship between G-CSF and G-CSF-R (r = -0.47, *P* = 0.17); but (B) a significant relationship between CX3CL1 and CX3CR1 (r = 0.84, *P* = 0.0025).

Chapter 5. Oculomotor measures as objective biomarkers of sedation

Grace PM, Stanford T, Gentgall M and Rolan PE (2010) Utility of saccadic eye movement analysis as an objective biomarker to detect the sedative interaction between opioids and sleep deprivation in opioid-naive and opioid-tolerant populations. *J Psychopharmacol*. Epub Feb 8. Reprinted with permission from the Journal of Psychopharmacology, © 2010.

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- Royal Adelaide Hospital Medical Staff Society Research Prize, Best oral communication (2010)
- Oral Communication Best Free Paper Award, Australian Pain Society/ New Zealand Pain Society Combined Scientific Meeting (2010)

In contrast to mechanisms of pain investigated by this thesis, there are some situations, such as sedation, where the mechanisms are not well understood and cannot be directly characterised. In these scenarios, a 'top-down approach to develop a biomarker may be appropriate, by quantifying functional consequences. It has previously been demonstrated that saccadic eye movement analysis and dynamic pupillometry are sensitive to detect sedation induced by drug administration or sleep deprivation independently. As such, these oculomotor measures may be ideal candidates for top-down biomarker development.

Therefore, this study aimed to examine the utility of saccadic eye movements and dynamic pupillometry to detect an interaction between sleep deprivation and opioids in opioid-naïve and opioid-tolerant populations. Each factor has only been examined in isolation, and the effect of a combination has not previously been addressed. However, the question is extremely relevant, as many people experience sleep deprivation at one point or another, for example nightshift jobs. Interestingly, many nightshift jobs involve manual handling, where the risk of injury, potentially resulting in chronic pain, is higher. For this

reason, there could be a large number of chronic pain patients, on long-term opioid treatment, who experience sleep deprivation.

An additive sedative interaction between the opioid and sleep deprivation was detected with analysis of saccadic eye movements in the opioid-naïve populations, however the nature of the interaction between sleep deprivation and chronic opioid administration in the opioid-tolerant population could not be determined. This study also showed that dynamic pupillometry was unable to detect sleep deprivation or opioid administration. Both populations reported a subjective impairment by sleep deprivation, however only the opioid-tolerant population reported a subjective impairment by the opioid. The opioid-tolerant population revealed greater objective baseline impairment compared to the opioid-naïve population. Strikingly, this was not reflected on the subjective scale, suggesting that this population may be tolerant to the subjective effects and are consequently unaware of their objective level of impairment.

This study shows that objective measures should be used in combination with subjective measures, as they provide additional information. It also demonstrates the utility of a top-down approach, as conclusions in this study are based on quantification of complex brainstem processing, which does not require an understanding of largely unknown, complex neural mechanisms, but rather measures a functional consequence.

Grace, P.M., Stanford, T., Gentgall, M. and Rolan, P.E. (2010) Utility of saccadic eye movement analysis as an objective biomarker to detect the sedative interaction between opioids and sleep deprivation in opioid-naive and opioid-tolerant populations *Journal of Psychopharmacology, v.24 (11), pp. 1631-1640, November 2010*

Chapter 6. Conclusion

Interest in the clinical development and use of biomarkers for a wide range of diseases has been on the rise in recent years. Even during the course of this thesis, the number of publications on the development and utility of biomarkers has recently risen sharply, with special issues devoted to the subject (See *Clin. Pharmacol. Ther.* (2009) 86(1); *Nat. Biotechnol.* (2010) 28(5)). Despite the considerable interest surrounding biomarkers in general, a pain biomarker has remained elusive. In my view, this is largely due to an incomplete understanding of the mechanisms underlying neuropathic pain as well as the complex relationship that exists between the multiple processing pathways and systems that underlie neuropathic pain. Consequently, it is difficult to isolate single factors or pathways that encompass overlapping mechanisms. Furthermore, the CNS location of many of the mechanisms that have been elucidated presents a further obstacle, in that they are difficult to probe. To overcome these hurdles, the major aims of this thesis were to develop biomarkers of pain and pain pharmacotherapy using bottom-up and top-down methods.

I thought it most appropriate to begin the bottom-up method by using an animal model of pain, as CNS mechanisms can be more readily dissected than in a clinical scenario. However, it was my view that current animal models of neuropathic pain were somewhat lacking, in that they did not adequately model the heterogeneity in pain sensitivity that is present within this patient population. Therefore, the CCI model of neuropathic pain (Bennett and Xie, 1988) was modified by varying subcutaneous and sciatic nerve chromic gut sutures in order to generate graded nociceptive hypersensitivity in the rat. The graded model was also correlated with graded glial activation, providing an example of the application of the model and, as well as insight in the mechanisms underpinning the graded allodynia. The dynamic range of this novel graded model greatly improves the potential for pain biomarker discovery and development by allowing sensitive detection of the subtle interactions and influences on pain processing. Since biomarkers seek to quantify heterogeneity, the dynamic range of this model is

essential for thorough preclinical biomarker discovery and validation, as it provides the means through which to characterise the nociceptive hypersensitivity and associated pathophysiological changes that occur between the 'no pain' 'severe pain' extremes of traditional models. These characteristics may also provide greater scope to assessment of novel therapeutics by assessing analgesia on the 'pain dose-response' curve of the graded model.

The graded model was then applied to investigate the current evidence implicating the peripheral immune system in the induction and maintenance of neuropathic pain. As discussed in Chapter 1, the peripheral immune system was identified as the target for pain biomarker development in this thesis, as it satisfies some of the biomarker criteria identified by Lesko and Atkinson (2001) and Lee and colleagues (2006), in that it is linked to biological endpoints and can be readily accessed via a simple venepuncture. In order to identify peripheral immune factors critical to neuropathic pain pathophysiology, that would therefore be sensitive to treatment effect, further characterisation was required. As such, a series of experiments demonstrated that rat peripheral immune cells derived from high pain donors potentiated allodynia when adoptively transferred to low pain recipients. Strikingly, adoptive transfer of allodynia was not achieved in sham-operated recipients, suggesting that nerve injury may facilitate immune cell migration into the CNS. As adoptively transferred cells were found to migrate to the spleen, and potentiation of allodynia was prevented in splenectomised low pain recipients, it was hypothesised that adoptive transfer of high pain splenocytes may induce the migration of host-derived immune cells from the spleen to the CNS, which was indeed observed. Direct intrathecal transfer of peripheral immune cells derived from spinal cords of high pain donors into low pain recipients potentiated allodynia. These findings confirm that peripheral immune cells actively contribute to nociceptive hypersensitivity in the lumbar spinal cord and further investigation may demonstrate a contribution to population heterogeneity. As such, the aim of this study, to begin the process of construct validation, was successful, in that a pivotal role for the peripheral immune system and

identification of targets for further investigation was demonstrated in this model of neuropathic pain. This validation lays the groundwork for what could potentially be the first pain biomarker to become the criterion for further biomarker discovery and validation (Rolan, 1997).

The final step in developing a pain biomarker from the bottom-up approach was to take the adoptive transfer findings from Chapter 3 and apply them in order to identify pain biomarker leads in the circulating blood. The novel model from Chapter 2 was applied to generate a heterogeneous population with respect to nociceptive hypersensitivity. The transcriptomes from the spinal cords were then compared with those from the circulating blood in order to identify candidate genes as potential pain biomarkers. Two analytical approaches were used and identified transcription factors (zinc finger proteins) as well as blood CX3CL1, which was found to correlate with spinal cord CX3CR1. The genes identified in this study require confirmation with RT-PCR. A prospective validation trial will confirm firstly whether the genes identified are in fact biomarkers of pain, and secondly whether the hypothesis that the peripheral immune system could be used as a biomarker for pain was in fact correct. Furthermore this study provides enough evidence to proceed with a prospective clinical trial.

Biomarkers not only have utility as diagnostics, as described in Chapter 1, but also to report on drug safety (Rolan et al., 2007). One such safety concern associated with chronic pain pharmacotherapy is opioid-induced sedation. As the mechanisms of sedation are not well understood, a top-down method of biomarker development was also demonstrated by measuring the functional consequences of the complex brainstem processing of sedation. Review of the literature revealed that analysis of saccadic eye movements and dynamic pupillometry may have utility as biomarkers of sedation and thus were applied to answer a previously unaddressed question: is there an interaction between sleep deprivation and opioids in opioid-naïve and opioid-tolerant populations? These clinical trials found that saccadic eye movements had the greatest utility and that dynamic pupillometry was not appropriate in this setting. Saccadic eye movement analysis detected an additive sedative interaction between the opioid and

sleep in the opioid-naïve population. Subjectively, only the opioid-tolerant population reported impairment by the opioid, which could be attributed to the open label design of the study. The most striking finding was that the opioid-tolerant population showed greater baseline objective impairment compared to the opioid-naïve population, which was not reflected on the subjective scale. This discrepancy suggests that the opioid-tolerant population may be tolerant to the subjective effects and consequently unaware of their actual, objective level of impairment. This finding challenges the consensus that the opioid-tolerant population becomes tolerant to the adverse effect of sedation, which carries a public health concern and requires reconsideration. This study achieved the aim set out, in that a top-down approach was successful to develop a robust biomarker of sedation. This study further demonstrates that additional information can be gained by applying objective measures in combination with subjective measures.

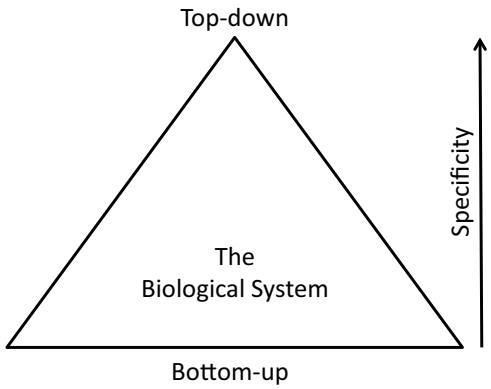

Figure 6-1. The relationship between bottom-up and top-down biomarker development is defined by specificity.

Bottom-up biomarker development will be associated with a number of non-specific factors that are involved in many disease mechanisms and validation will therefore increase the specificity by exclusion. Top-down biomarker development is specific to the application under investigation and so validation is required to increase these applications. The arrow indicates the degree of specificity associated with each method.

Each approach may be useful in different scenarios, depending on how well understood and accessible the mechanisms are. However, the main difference that distinguishes the two approaches is specificity, or proximity to the endpoint (Rolan, 1997), depicted as a pyramid in figure 6-1. The bottom-up method is

not specific, as it deals with a wide range of mechanisms: some associated purely with the disease in question, others that will be common to many diseases. Therefore, the goal of validation is to increase the specificity by excluding non-specific biomarkers, or tightening the net. This validation moves the potential biomarker towards the pinnacle of the pyramid. By contrast, the top-down method is associated with a high degree of specificity and it must not be assumed that the entire endpoint is completely captured. Therefore validation of a top-down biomarker will seek to expand the applications in which the biomarker has utility, by exploring the pinnacle of the pyramid. This level of validation was achieved in Chapter 5, by exploring the utility of an existing biomarker in a novel scenario.

The relatively different degrees of innovation associated with each approach reflect the particular applications rather than the method. This thesis took the ambitious step towards developing a pain biomarker by using a bottom-up method and was innovative simply due to the biological system studied and the absence of pain biomarkers. A well-established measure of sedation, saccadic eye movement analysis, was used to investigate a previously unanswered question. This is not to say that the top-down approach is not novel, as the idea of saccadic eye movements as a biomarker of sedation was highly innovative when proposed by Aschoff in 1968. A difficulty associated with searching for novel biomarkers is that a logical starting point is not always readily apparent. This was certainly the case during the course of this thesis, for whilst the immune system was identified as a target for investigation, it nevertheless led to several false starts and blind alleys (see Appendices B and C). The currently unavoidable 'fishing expedition' that is innovative biomarker discovery represents a barrier at present, and the absence of accepted biomarkers is therefore not surprising. However, as research is continued into the mechanisms underlying the pathophysiology of neuropathic pain and into biomarker development, momentum will build, with increased identification of new leads and refinement of these leads over time.

This thesis may well contribute to a building momentum. As described above, use of the graded model has many advantages for identifying leads, and it is recommended that this approach be continued. The graded model led to the chance finding that spleen weight was correlated with increased allodynia in this model. Whilst it subsequently became apparent that the spleen weight was a 'red herring' and probably related to subcutaneous chromic gut placement, it serendipitously led to the series of adoptive transfer experiments. The results of these experiments have laid the groundwork for further biomarker discovery, as they demonstrate a critical role for peripheral immune cells in neuropathic pain and can be used to inform further research in this field. The results confirm the initial hypothesis that the immune system is, at present, the most appropriate avenue for biomarker discovery and development. General directions that may be pursued from this thesis include characterisation of the effector cells responsible for adoptive transfer of allodynia as differences in these cells may reflect heterogeneity in pain sensitivity. The transcriptome or *in vitro* and *ex vivo* assays may be able to quantify differences in expression of surface molecules, reactivity and immune mediator secretion, as well as factors that facilitate CNS migration. A current barrier to validation of novel biomarkers is evidence of treatment response. As discussed in Chapter 1, current pharmacotherapies are symptomatic rather than disease modifying, and will likely have no effect on the biomarker. This may be overcome if there is translation of disease modifying therapies, such as glial inhibitors/attenuators.

Returning to the multidimensional understanding of pain that was described at the outset of Chapter 1, it seems likely that the application of biomarkers will be most successful in combination, as was found for the combination of objective and subjective measures of sedation in Chapter 5. Immune mechanisms constitute an important aspect of the nociceptive pain pathophysiology, but cannot explain the affective-motivational and cognitive aspects that may be detected by other technologies, such as fMRI. Nor are they likely to constitute the sole mechanisms responsible for neuropathic pain (Woolf and Decosterd,

1999; Woolf and Mannion, 1999; Finnerup and Jensen, 2006). To improve treatment outcomes for sufferers of chronic pain, a multidisciplinary approach is a necessity.

Chapter 7. References

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Appendix A. Pain transmission

A.1. Peripheral Chemical mediators

In most cases, stimulation of nociceptive endings in the periphery is chemical in origin. Excessive mechanical or thermal stimuli cause acute pain, but the persistence of such pain after the stimulus has been removed, or the pain resulting from inflammatory or ischaemic changes in tissues, generally reflects an altered chemical environment in pain afferents. The main groups of substances that stimulate nociceptors are kinins, PGs and substances released from damaged cells or tissues, such as 5-HT.

A.1.1. Kinins

Kinins belong to a group of 9-11 amino acid peptides including bradykinin, kallidin, T-kinin and their active metabolites, des-Arg^9-kinins (Couture et al., 2001). Evidence implicating bradykinin in the facilitation of nociception includes hyperalgesia after intradermal bradykinin administration (Manning et al., 1991). Damage to cells causes the release of plasma and tissue serine proteolytic enzymes, kallikreins, into the extracellular space. High molecular weight kininogen and low molecular weight kininogen, which are widespread throughout the body, are cleaved to generate bradykinin and kallidin respectively (Bhoola et al., 1992; Cesare and McNaughton, 1997). The major effects of kinins are mediated by the activation of at least two distinct receptors, B_1 and B_2. B_2 receptors are most well characterised, mediating most of the physiological effects of kinins and are constitutively and widely expressed throughout the central and peripheral nervous system (Calixto et al., 2000). With some exceptions, B_1 receptors are not expressed in significant levels in normal tissues, but their expression occurs rapidly under certain pathological conditions or by the action of proinflammatory agents such as lipopolysaccharide or cytokines (Marceau and Bachvarov, 1998). The induction of the B_1 receptor is controlled by mitogen activated protein kinase (MAPK) and by the transcription factor κB (NF- κB) (Larrivee et al., 1998; Ni et al., 1998). B_1 receptors are preferentially and selectively activated by the

metabolites des-Arg^{9}-bradykinin and des-Arg^{10}-kallidin. B_2 receptors are rapidly desensitised, however B_1 receptors are resistant to sensitisation, which may explain the long-term effects of bradykinin (Marceau et al., 1998).

Kinin receptors are coupled to $C\alpha_{q/11}$ proteins leading to an activation of phospholipase C-β (PLCβ) and phospholipase A_2 (PLA_2), with a subsequent generation of second messengers inositol-1,4,5-triphosphate, diacylglycerol. This leads to a release of internal Ca^{2+}, which in turn activates protein kinase Cε (PKCε) (Cesare and McNaughton, 1997; Blaukat, 2003). PKCε directly phosphorylates and facilitates the opening of the TRPV1 channel (Premkumar and Ahern, 2000). The TRPV1 channel is permeable to Na^+, Ca^{2+} and other cations causing depolarisation and initiation of action potentials (Tominaga and Tominaga, 2005) and subsequent release of neurotransmitters from primary afferent neurons in the dorsal horn. Activation of PLA_2 causes production of arachidonic acid (AA) from the cell membrane, which is the initial substrate for enzymatic cascades that generate other pro-inflammatory mediators that act to amplify nociception (McHugh and McHugh, 2000). Bradykinin also acts directly on some immune cells, including mast cells (Cohan et al., 1991), causing release of proinflammatory mediators.

A.1.2. 5-hydroxytryptamine (5-HT)

5-HT is released from platelets and neurons and acts on no less than 14 distinct receptor types that are heterogeneously distributed throughout tissues. Of these receptors, only the 5-HT_3 receptor is not linked to a G-protein mediated second messenger pathway. Rather, it forms a ligand-gated ion channel and causes Na^+ influx and neuronal excitation in response to ligand binding (Faerber et al., 2007) and is located on peripheral nociceptive neurons (Bedford et al., 1998). 5-HT has been demonstrated to mediate local hyperalgesia in the rat partial ligation model of neuropathic pain (Theodosiou et al., 1999).

A.2. Peripheral immune mediators

The mediators released by inflammatory and immune cells may act directly to sensitise or activate neurons, or indirectly by acting on non-neuronal cells which may release mediators to activate neurons. Key mediators will be discussed below.

A.2.1. Immune mediators: ATP and adenosine

Purines such as adenosine and ATP not only have a metabolic effect, but also affect immune cells and neurons (Moalem and Tracey, 2006). ATP is a classical neurotransmitter and is also released by non-neuronal cells and from disrupted cells in injured tissue (Cook and McCleskey, 2002). Adenosine is a neuromodulator that is produced in the extracellular space by degradation of extracellular ATP and is also transported from the cytoplasm into the interstitial space by transport proteins (Linden, 1994).

Bleehan and Keele reported that ATP, ADP, AMP and adenosine all produced pain when applied to a blister base in man (Bleehen and Keele, 1977). Since then, it has been found that adenyl compounds and other purines activate nociceptors and elicit pain by their actions on purinergic receptors (Sawynok, 1998; Hamilton and McMahon, 2000). Receptors for purines have been classified into adenosine (P1) receptors and nucleotide (P2) receptors (Ralevic and Burnstock, 1998). P2 receptors can be subdivided into P2X receptors, which are ATP-gated ion channels (Chizh and Illes, 2001) and P2Y receptors which are G-protein coupled receptors (GPCR) activated by purine or pyrimidine nucleotides such as ATP or UDP (von Kugelgen and Wetter, 2000).

$P2X_3$ receptors have been implicated in neuropathic pain as the level of DRG expression was reduced by antisense oligonucleotides to the receptor, resulting in inhibited initiation of hyperalgesia and reversal of established hyperalgesia in the rat PNL model (Barclay et al., 2002). This was confirmed by $P2X_3$ antagonism in the CCI model, attenuating thermal and mechanical allodynia (Jarvis et al., 2002). $P2X_7$ receptors are expressed by immune cells including mast cells, macrophages and T-cells, having a key

role in secretion of IL-1β. It was shown that $P2X_7$ is upregulated in the DRG and injured nerves of neuropathic pain patients and that disruption of the $P2X_7$ purinoceptor gene abolished neuropathic pain (Chessell et al., 2005).

$P2Y_1$ and $P2Y_2$ receptors are expressed by nociceptors (Molliver et al., 2002; Xiao et al., 2002; Stucky et al., 2004) and Schwann cells (Mayer et al., 1998). $P2Y_1$ neuronal mRNA expression increases threefold after sciatic axotomy, suggesting a role in neuropathic pain (Xiao et al., 2002).

A.2.2. Immune mediators: Eicosanoids

AA is a polyunsaturated fatty acid that is found in the cell membrane. It is the major precursor of the eicosanoids and prostanoids and once freed from the cell membrane by PLA_2, it is converted by constitutively expressed COX enzymes to prostanoids, and by the lipoxygenase pathway to leukotrienes (Wolfe and Horrocks, 1994; Smith et al., 2000; Moalem and Tracey, 2006; Okuse, 2007).

Prostanoids comprise the various bioactive PG isomers such as PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A2 (TXA_2) and exert their effects through various GPCRs, EP1, EP2, EP3, EP4 and IP, which differ in their agonist selectivity, tissue distribution and signal transduction pathways (Lin et al., 2006). Receptor knockout studies confirm the role of prostanoids in nociception and pain (Zeilhofer, 2007) and it has been demonstrated that PGE_2 and PGI_2 induce hyperalgesia in the periphery (Taiwo and Levine, 1990) where they act directly on the terminals of nociceptors (Taiwo and Levine, 1989). Inhibition of COX relieves hyperalgesia in nerve injured rats, thus implicating prostaglandins in neuropathic pain (Syriatowicz et al., 1999). PGE_2 exerts its effects through all 4 EP isoforms that target ion channels (Zeilhofer, 2007) via the PKA and PKC second messengers, including TRPV1 in a similar way to bradykinin, and $Na_V1.8$ and 1.9 (Zeilhofer, 2007). PKA directly phosphorylates $Na_V1.8$ and increases the magnitude of peak currents and causes a hyperpolarising shift in the voltage dependence of activation (England et al., 1996; Gold et al., 1996; Fitzgerald et al., 1999), increasing sensory neuron

excitability. Facilitation of TRPV1 by PGE_2 may also link increases in cyclic adenosine monophosphate (cAMP) to stimulation of CGRP and substance P (SP) release (Rathee et al., 2002).

There are three mammalian lipoxygenases, which catalyse the insertion of oxygen at positions 5, 12 and 15 of AA (5-, 12- and 15- lipoxygenases). The 5-lipoxygenases form leukotrienes, including leukotrienes B_4 (LTB_4) which produce hyperalgesia by eliciting the release of the 15-lipoxygenase product (8R, 15S)-dihydroxyeicosa-(5E,9,11,13Z)-tetraenoic acid (8R,15S-diHETE) from neutrophils (Levine et al., 1984; Levine et al., 1985; Levine et al., 1986). 8R,15S-diHETE was shown to sensitize nociceptors in the rat and may contribute to the role of neutrophils in neuropathic pain. It has been demonstrated that LTB_4, 12- and 15- HPETE and 5- and 12- HETE directly activate TRPV1 (Hwang et al., 2000) and facilitate TRPV1 activation by bradykinin (Shin et al., 2002).

A.2.3. Immune mediators: Neurotrophins

The neurotrophins are dimeric proteins that are essential for the normal development of the vertebrate nervous system. This family includes NGF, brain-derived neurotrophic factor (BDNF), neurotrophin (NT)-3 and NT-4/5 (Lewin and Barde, 1996). Glial-cell-line-derived neurotrophic factor (GDNF) also has neurotrophic properties, but is structurally unrelated to the dimeric neurotrophin family (Lewin and Barde, 1996). Neurotrophins are synthesised and released by a several cell types of immune cells, including mast cells and lymphocytes (Moalem et al., 2000; Bonini et al., 2003).

BDNF is constitutively expressed by peptidergic nociceptors (Thompson et al., 1999). Nerve injury produces marked changes in expression of neurotrophins and their receptors, primarily in Schwann cells, where the synthesis of NGF, BDNF and GDNF is dramatically upregulated (Lindholm et al., 1987; Meyer et al., 1992; Hammarberg et al., 1996; Frostick et al., 1998). BDNF is also upregulated in sensory neurons following axotomy (Tonra et al., 1998). Neurotrophins act on a trio of trk receptors, trkA, trkB

and trkC which primarily bind NGF, BDNF and NT-4/5 and NT-3 respectively (Moalem and Tracey, 2006).

A.2.3.1. NGF

The trk A mutation resulting in congenital pain hyposensitivity (Indo et al., 1996) demonstrates the importance of NGF for normal nociceptive functioning. Intraplantar or systemic injection of NGF in rodents induces thermal and mechanical hyperalgesia (Lewin et al., 1993; Theodosiou et al., 1999). In humans, intravenous injections of very small doses of NGF produce hyperalgesia at the injection site and widespread aching pains in deep tissues (Petty et al., 1994). The rapid onset and location of these effects suggests a role of peripheral terminals on nociceptors (Rueff and Mendell, 1996; Koltzenburg et al., 1999). NGF contributes to the development and maintenance of neuropathic pain in animal models. Hyperalgesia resulting from CCI is delayed by application of NGF anti-sera at the site of injury (Herzberg et al., 1997), and is reduced or even reversed by injection of anti-sera into the medial dorsal surface of the nerve-injured hind paw (Ro et al., 1999). DRG neurons cultured with NGF show hyperexcitability (Kitamura et al., 2005) by fluctuations in intracellular Ca^{2+}, which can be inhibited with lignocaine (Ozaki et al., 2009). Thus it appears that NGF may contribute to the maintenance of neuropathic pain by generating spontaneous action potentials.

The direct mechanisms involve both altered gene expression and post-translational regulation of receptors and ion channels including TRPV1 (Bonnington and McNaughton, 2003) and Na^{+} channels (Zhang et al., 2002). It is also possible that increased levels of NGF increase the sensitivity of dorsal horn neurons by releasing larger amounts of neuromodulators such as SP and CGRP from the central terminals of primary afferents (Ma et al., 1995). NGF indirectly modulates neuronal sensitisation via a number of peripheral immune cell types and sympathetic neurons (Lewin et al., 1994; Woolf et al., 1996; Bennett et al., 1998). Mast cells express trkA (Horigome et al., 1994; Leon et al., 1994) and NGF can result in degranulation and increased proliferation (Horigome et al., 1994). In peritoneal mast cell

cultures, NGF induced expression of a number of cytokines (Bullock and Johnson, 1996). Inhibition of 5-lipoxygenase inhibits release of LTB_4, accumulation of neutrophils and NGF associated hyperalgesia (Amann et al., 1996; Bennett et al., 1998), indicating that NGF produces hyperalgesia by inducing the release of LTB_4 from mast cells, leading in turn to recruitment of neutrophils (Bennett et al., 1998).

A.2.3.2. BDNF

BDNF also contributes to neuropathic pain by increasing the responsiveness of dorsal horn neurons in the spinal cord (Pezet et al., 2002). It is coexpressed with SP in 20–30% of sensory neurons in the dorsal root ganglia (Luo et al., 2001). BDNF is anterogradely transported from the cell bodies of these neurons in the DRG (Zhou and Rush, 1996) and increased by nerve injury (Tonra et al., 1998). Intrathecal administration of BDNF induces thermal hyperalgesia and tactile allodynia in mice (Groth and Aanonsen, 2002; Yajima et al., 2005), while intrathecal injection of antisense oligonucleotides for BDNF or its receptor, trkB, attenuates inflammatory hyperalgesia (Groth and Aanonsen, 2002). In animal models of neuropathic pain, nerve injury increases levels of BDNF in the dorsal horn (Yajima et al., 2005), and these levels are related to the extent of thermal hyperalgesia (Miletic and Miletic, 2002). The thermal hyperalgesia and tactile allodynia induced by nerve injury are completely suppressed by repeated intrathecal injection of an antibody to BDNF or a trkB/Fc chimera protein, which sequesters endogenous BDNF (Yajima et al., 2002; Yajima et al., 2005).

Other neurotrophins appear to inhibit neuropathic pain. NT-3 suppresses thermal, but not mechanical hyperalgesia following CCI, which is associated with decreased expression of TRPV1 (Wilson-Gerwing et al., 2005). Intrathecal GDNF prevents and reverses hyperalgesia following nerve injury (Boucher et al., 2000; Nagano et al., 2003; Wang et al., 2003). It was suggested that this inhibition of hyperalgesia is due to suppression of $Na_{V1.3}$ expression by injured fibres (Boucher et al., 2000), but other factors such as downregulation of $P2X_3$ receptors may also be involved (Wang et al., 2003).

A.2.4. Immune mediators: Nitric oxide and reactive oxygen species

ROS such as nitric oxide (NO) and superoxide play important roles in inflammatory and immune responses, including defence mechanisms against invading microbes (Guzik et al., 2003; Roos et al., 2003). ROS are released by a number of cell types, including macrophages (Bosca et al., 2005) astrocytes (Buskila et al., 2007) and microglia (Vilhardt, 2005). NO is a diffusible free radical that is synthesised by three distinct nitric oxide synthases (NOS), of which the neuronal and endothelial forms (nNOS and eNOS) are constitutive, while the inducible form (iNOS) is upregulated in immune cells. Once released, NO can react with superoxide radicals to form peroxynitrite, which is toxic and may cause tissue damage (Moalem and Tracey, 2006).

NO elicits pain when injected into the skin of human subjects (Holthusen and Arndt, 1994; Holthusen and Arndt, 1995) and contributes to peripheral hyperalgesia in the skin probably by contributing to PGE_2-induced sensitisation of primary afferents (Aley et al., 1998). NO is also implicated in central mechanisms of hyperalgesia (Meller et al., 1992) where nNOS and NO form part of a second messenger cascade involving cyclic guanosine monophosphate (cGMP) and partly responsible for central sensitisation (Meller and Gebhart, 1993; Riedel and Neeck, 2001; Sung et al., 2004). NO may also contribute to sensitisation of central neurons by disinhibition (Lin et al., 1999). However, NO can contribute to central mechanisms of analgesia as well as nociception since it enhances human opioid analgesia (Lauretti et al., 2002).

NO is implicated in neuropathic pain (Levy and Zochodne, 2004). In rats with a CCI, iNOS is induced in macrophages and Schwann cells at the injury site and distal to it (Levy and Zochodne, 1998; Levy et al., 1999). In the same model, eNOS could be demonstrated in endbulb-like structures of injured peripheral axons, while treatment with a non-specific NOS inhibitor alleviated hyperalgesia and blocked ectopic mechanosensitivity of injured A (Levy et al., 2000). NO also plays a role in central mechanisms of

neuropathic pain so that, in nerve injured rats, intrathecal delivery of a non-specific NOS inhibitor produced a dose-dependent reduction of thermal hyperalgesia (Lui and Lee, 2004).

Other ROS are implicated in neuropathic pain. While NOS catalyses the production of NO, xanthine oxidase catalyses the production of another free radical, superoxide (SO). Under physiological conditions, SO and NO react to form perioxynitrite, which is a powerful oxidant and potent cytotoxin. Xanthine oxidase levels are increased in the injured sciatic nerve (Khalil et al., 1999), and treatment with superoxide dismutase, which degrades the superoxide radical, alleviates thermal hyperalgesia resulting from CCI (Khalil et al., 1999). Reactive oxygen species also contribute to mechanical allodynia, which is abolished by superoxide dismutase treatment in an inflammatory model (Twining et al., 2004) and ROS scavengers in the SNL model (Kim et al., 2004). Perioxynitrite also contributes to neuropathic hyperalgesia as PNL results in increased levels of macrophages and Schwann cells in the injured nerve (Liu et al., 2000). Treatment with uric acid, a scavenger for perioxynitrite, alleviates thermal hyperalgesia (Liu et al., 2000).

A.2.5. Immune mediators: Histamine

Histamine is produced by neuronal and non-neuronal cells in the periphery (Panula et al., 1985). Histamine receptors are divided into three types, H_1, H_2 and H_3 (Arrang, 1994). Activation of H_1 and H_2 receptors induces a mobilisation of Ca^{2+} and an accumulation of cAMP respectively. These receptors are expressed in target cells. Histamine has sensitising effects on nociceptors (Mizumura et al., 2000; Baron et al., 2001; Herbert et al., 2001; Koda and Mizumura, 2002), and receptors are upregulated after crush injury to the sciatic nerve (Kashiba et al., 1999), but histamine is probably most important as a mast cell mediator, facilitating the recruitment of immune cells to the injured site (Metcalfe et al., 1997).

A.2.6. Immune mediators: Opioid peptides

In peripheral inflamed tissue, opioid peptides such as β-endorphin, met-enkephalin, dynorphin and endomorphins are produced by lymphocytes, monocytes, macrophages granulocytes and glia (Stein et al., 1993; Stein et al., 1996; Peterson et al., 1998; Mousa et al., 2001; Rittner et al., 2001; Mousa et al., 2002) and released upon stimulation by viruses, endotoxins, cytokines, corticotropin releasing hormone and adrenergic agonists (Smith, 2003; Stein et al., 2003; Labuz et al., 2006). Opioid peptides can bind to opioid receptors on sensory neurons. These receptors, the μ-, δ- and κ- opioid receptors, are synthesised in the DRG and are transported intra-axonally to peripheral nerve terminals (Puehler et al., 2004; Puehler et al., 2006; Mousa et al., 2007). Exogenous or endogenous agonist binding elicits potent analgesia in inflamed tissue (Stein et al., 2003). Opioid peptides will be covered in greater detail in the context of the central nociceptive pathway, as their roles are more clearly understood.

A.3. The central nociceptive pathway

Primary afferents terminate in the spinal cord where the nociceptive signal is processed in the spinal dorsal horn and modulated by descending signals. The nociceptive stimulus is processed in various supraspinal sites where the nociceptive stimulus effectively initiates the pain experience in terms of the affective and cognitive components.

A.4. The dorsal horn

The nociceptors synapse in the dorsal horn of the spinal cord, which is organised into different laminae, extending from the superficial to the deep dorsal horn. Most nociceptive Aδ- and C-fibres terminate superficially in laminae I-II, with a smaller number reaching deeper laminae, whereas Aβ-fibres predominantly innervate laminae III-VI (Todd, 2002).

Synaptic transfer of information from the periphery to the dorsal horn is governed by the nature and amount of the transmitter released by primary afferents, the density and identity of postsynaptic

receptors (ionotropic and metabotropic), the kinetics of receptor activation, ion channel opening and closing, and the uptake or breakdown of the transmitter. Each of these factors is subject to multiple modulatory influences (Millan, 1999).

Based on projections of their axons, dorsal horn neurons can be divided into three general classes: projecting neurons, propriospinal neurons and local interneurons (Willis and Coggeshall, 1991). Although projecting neurons transfer sensory information from the spinal cord to the brain, and therefore are the primary output from the spinal cord, they represent only a tiny minority of the total number of cells in the dorsal horn. Apart from transferring information to the higher brain centres, projection neurons are also involved in the activation of descending control systems, which in turn control the gain of dorsal horn neurons through excitatory and inhibitory mechanisms (DeLeo, 2006).

Propriospinal neurons transfer inputs from one segment of the spinal cord to another; their role in nociception is poorly understood, but they appear able to act as a multisynaptic pathway that transfers information to the brain. They have a major role in controlling locomotion and in organising coordinated reflex responses (Woolf and Salter, 2006).

The vast majority of intrinsic dorsal horn neurons are local interneurons, which send their axons for only a short distance within the same segment of the spinal cord and comprise both excitatory and inhibitory interneurons. Excitatory, glutamaterigic, neurons provide a source of polysynaptic excitatory input that is numerically greater than the monosynaptic input driven from primary afferents. For example, nociceptive withdrawal reflexes are mediated by a chain of excitatory interneurons linking the superficial dorsal horn with motor neurons.

Most inhibitory interneurons contain GABA and/or glycine as neurotransmitters and synapse both pre-synaptically on primary afferents and postsynaptically on dorsal horn neurons (Todd, 1990; Powell and Todd, 1992). Presynaptic inhibition decreases transmitter release from primary afferent terminals, while

postsynaptic inhibition hyperpolarizes or clamps the post synaptic membrane. Fast inhibitory postsynaptic potentials (IPSPs) produced by Cl^- currents are mediated by glycine and GABAA ionotropic receptors, and hyperpolarise the cell. The GABAB receptor is a GPCR and produces slower-onset and longer-lasting inhibition, predominantly presynaptic (Yoshimura and Nishi, 1995; Towers et al., 2000).

Like the vast majority of fast excitatory synapses in the CNS, most presynaptic excitatory terminals in the dorsal horn release glutamate, which activates postsynaptic glutamate receptors. These excitatory postsynaptic potentials (EPSPs) resulting from single presynaptic action potentials is caused primarily by activation of α-amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA) and kainite subtypes of the ionotropic glutamate receptor and typically last for only a few milliseconds (Yoshimura and Jessell, 1990; Li et al., 1999). The N-methyl-D-aspartate (NMDA) subtype of the ionotropic receptor which is localised at excitatory synapses, contributes little to the response to single presynaptic action potentials, because these receptors are tonically suppressed by extracellular Mg^{2+}, which block NMDA channels (Mayer et al., 1984). This type of fast excitatory synaptic transmission occurs even at synapses of slow nociceptor C-fibre primary afferents (Moore et al., 2000). With low frequency activation of nociceptors produced by mild noxious stimuli, these EPSPs signal to dorsal horn neurons on the onset, duration, intensity and location of noxious stimuli in the periphery (Woolf and Salter, 2006).

A.5. Ascending pathways

The ascending pathways that are important for pain include direct projections to the thalamus (the spinothalamic tract, STT); direct projections to homeostatic control regions in the medulla and brain (the spinomedullary and spinobulbar projections); and possible direct projections to the hypothalamus and forebrain (spinohypothalamic tract, SHT). There are other indirect pathways, but these will not be discussed.

A.5.1. Spinothalamic tract

The most prominent and well-described of the ascending pathways is the STT, which is thought to transmit sensations of pain, temperature and touch (Willis, 1985; Willis and Coggeshall, 1991; Millan, 1999). The majority of the projection neurons that travel in the STT originate in dorsal horn laminae I, II and V (Willis et al., 1979; Apkarian and Hodge, 1989). Before ascending, the STT neurons decussate to the opposite ventrolateral quadrant of the spinal cord white matter, where they ascend in the ventrolateral funiculus (VLF), a bundle of nerve fibres, to 6 distinct regions of the thalamus, depending on their origin in the dorsal horn lamina. These thalamic regions are the posterior portion of the ventral medial nucleus (VMPo); the ventral posterior nuclei (VPL, VPM and VPI); the ventral lateral nucleus (VL); the central lateral nucleus (CL); the parafascicular nucleus (Pf); and the ventral caudal portion of the medial dorsal nucleus (MDvc) (Willis et al., 1979; Apkarian and Hodge, 1989; Millan, 1999; Craig, 2003). The thalamus is thought of as the major supraspinal relay structure for the integration and transfer of ascending nociceptive information to the cerebral cortex (Willis and Coggeshall, 1991; Bushnell, 1995). Within the thalamus, nociceptive information regarding the type, temporal pattern intensity and topographic localisation of the pain is encoded prior to sending the information onward to limbic structures and cortical sites (Willis and Coggeshall, 1991; Bushnell, 1995; Millan, 1999).

The VMPo projects to the dorsal margin of the posterior insula cortex, constituting an interoceptive sensory representation of the physiological condition of the body (Craig, 2003). This view is consistent with the general view of the insula as limbic sensory cortex associated with autonomic activity. Embedded within this projection are distinct, highly resolved representations of several ‘feelings’ from the body, including pain, temperature, itch, muscle and visceral sensations and C-fibre touch (Craig, 2003). The VPL, VPM and VPI project to the motor cortex and thus associated with sensorimotor activity (Craig, 2003). The CL projects to the basal ganglia and to the superficial and deep layers of the motor and parietal cortices (Jones and Derbyshire, 1996). The Pf projects to the basal ganglia and motor

cortex (Sadikot et al., 1992). The MD projects to the cingulate, orbito-frontal and prefrontal cortices (Craig, 2003) and is important for the affective component of pain.

A.5.2. Spinobulbar tract

Spinal projections to the brainstem are important for the integration of nociceptive activity with processes that regulate homeostasis. There are also pathways that indirectly convey nociceptive activity to the forebrain following integration in the brainstem. In addition, spinal input to the brainstem influences the modulation of both spinal and forebrain activity, which can affect the experience of pain (Dostrovsky and Craig, 2006).

The spinobulbar projection originates in laminae I, II and V and ascend in the VLF to the mesencephalon (Willis et al., 1979; Mantyh, 1982; Wiberg et al., 1987; Millan, 1999). Within the mesencephalon, the neurons terminate in four main areas: the regions of the catecholamine cell groups, the parabrachial nucleus (PB), the periaqueductal grey (PAG) and the reticular formation (Kerr, 1975; Wiberg et al., 1987; Willis and Coggeshall, 1991; Keay et al., 1997).

The catecholamine cell groups include the ventrolateral medulla, the nucleus of the solitary tract, the locus coeruleus (LC) and the subcoerulear and Kölliker-Füse regions in the dorsolateral pons. These are integration sites for cardiorespiratory and homeostatic function which contain pre-autonomic bulbospinal neurons that drive sympathetic outflow (Loewy and Spyer, 1990). Spinal projections to this region activate somato-autonomic reflex arcs (Sato and Schmidt, 1973) as well as descending modulatory systems (Craig, 2003).

The PB has numerous interconnections with the reticular formation which is appropriate for its role in homeostasis and autonomic integration (Chamberlin and Saper, 1992). The PB projects to the hypothalamus, amygdala, and thalamus, which serves as a relay to the insular cortex for general and special visceral sensory activity (Bernard et al., 1993). Input to the PB provides a substrate for

integration of nociceptive activity with general visceral afferent activity, as well as an indirect relay to forebrain autonomic, neuroendocrine and emotional control regions (Craig, 2003).

Moderately dense spinal input occurs in the lateral and caudal portions of the PAG and adjacent tegmental area. The PAG is a major mesencephalic site for homeostatic control and limbic motor output that has both ascending and descending projections. Spinal input to the PAG may be integrated with descending antinociceptive modulation of the spinal cord by way of projections to the rostral ventromedial medulla, dorsolateral pons and the ventrolateral medulla (Basbaum and Fields, 1978). Notably, the portions of the PAG that receive spinal input also have ascending projections to the hypothalamus and the medial thalamus (Mantyh, 1983a).

Nociceptive neurons have been recorded in the reticular formation. Many neurons in the rostral brain stem project to the thalamus, so it has been suggested that some indirectly convey nociceptive spinal activity to the forebrain. A 'spinoreticulothalamic' pathway was hypothesised as a multisynaptic, alternative pathway for pain related activity that could serve the motivational and arousal aspects of pain (Melzack and Casey, 1968). However brain stem reticular neurons that project to the forebrain do not seem to receive spinal input (Blomqvist and Berkley, 1992); rather the spinal projections to the PB and PAG provide the major indirect routes for spinal input to reach the forebrain via the brain stem (Craig, 2003).

A.5.3. Spinohypothalamic tract

Retrograde labelling evidence in the rat indicates the presence of the SHT (Dado et al., 1994) that could have potentially important implications for autonomic, neuroendocrine and emotional aspects of pain.

A.6. The pain matrix

The termination structures of the ascending tracts then project to a network of sites that process the nociceptive signal. Study of these structures has been aided by the advent of non-invasive brain-

imaging techniques that provide novel opportunities to study the multiple distributed regions of the brain and the neural circuitry responsible for pain experience (Casey, 1999; Mackey and Maeda, 2004; Seminowicz et al., 2004; Apkarian et al., 2005; Mayer et al., 2006; Tracey and Mantyh, 2007; Zhuo, 2008). The functional anatomy of the ascending pathways indicates that pain is associated with multiple pathways; activity in multiple forebrain regions is integrated with past experience and present context to result in the complete, multidimensional pain experience (Millan, 1999). Although particular neurons and pathways may have a predominant contribution to one aspect of the pain experience, it is the activity across the entire brain that constitutes the basis for the conscious experience of pain. The areas involved would predictably include the pathways and termination regions described above and potentially many other areas of the brain. This network of brain structures is referred to as the 'pain matrix' and includes the somatosensory primary (S1) and secondary (S2) areas, the cingulate, insula, the prefrontal cortex, thalamus and cerebellum (Casey et al., 1996; Craig et al., 1996; Jones and Derbyshire, 1996; Derbyshire et al., 1997; May et al., 1998). The separate components of the pain matrix will be briefly discussed.

A.6.1. The sensory-discriminative (nociceptive) component of pain

Within the cortical regions described above, there is a complex network of interconnections that include the thalamus and limbic structures (Sherman and Guillery, 1996). This network of cortical structures is responsible for the sensory-discriminative component, that is, the perception or mapping of the intensity, location, duration, temporal pattern and quality of noxious stimuli (Kenshalo and Willis, 1991; Millan, 1999). The sensory-discriminative component of nociception is likely an adaptive measure in triggering evasive, or nocifensive, action to a threatening noxious stimulus.

A.6.2. The affective-motivational component of pain

The affective-motivational component of the pain experience is the relationship between pain and mood, attention, coping tolerance and rationalisation (Kenshalo and Willis, 1991; Millan, 1999). Components of

the affect dimension include the moment-by-moment unpleasantness of pain, composed of emotional feelings that pertain to the short-term future, such as distress or fear. Pain unpleasantness is often, but not always, closely linked to the intensity of the painful sensation. Another component, the 'secondary pain affect' includes emotional feelings directed towards the long-term implications of having pain such as 'suffering' (Price, 2000).

Numerous anatomical, physiological and behavioural studies have demonstrated the important role of the limbic system in the affective-motivational component of pain. Injection of a local anaesthetic into limbic structures such as the hypothalamus (Tasker et al., 1987), the cingulate (Vaccarino and Melzack, 1989) and the dentate nucleus of the hippocampus (McKenna and Melzack, 1992) temporarily blocks neural activity and may induce significant analgesia during late tonic perception. Vaccarino and Melzack (1989) reported that, relative to rats suffering brief pain, rats subjected to enduring pain displayed attenuated responses when lignocaine was injected into the cingulate, a major link between limbic structures and the cortex.

Clinical evidence and animal studies have demonstrated the importance of the anterior cingulate cortex in affective-motivational aspects of pain (Kenshalo and Douglass, 1995). Surgical lesions of the cingulate cortex are described as able to alleviate pain and it has been reported that cingulotomy reduced the emotional, but not the sensory component of chronic pain (Corkin and Hebben, 1981). The effects of cingulotomies are more substantial among anxious and depressed patients (Vaccarino and Melzack, 1989; Kenshalo and Douglass, 1995). As well, the lack of somatotopic organisation and the convergence of cutaneous, visceral and joint related information suggest that the ventrolateral orbital cortex plays a role in the affective motivational components of pain. Kenshalo and Douglass (1995) suggested that activation of this area may be responsible for the unpleasant experience that causes organisms to attempt escape from prolonged painful stimulation. Rainville et al. (1997) observed that pain affect is encoded in the anterior cingulate, but not in the somatosensory cortex. Casey (2000)

concluded from PET images that limbic and autonomic structures, such as the insula and anterior cingulate cortex, may reflect the affective aspect of the pain experience.

Various studies have demonstrated interactions between affect and cognitive states, showing that distraction modulates pain-evoked activity in the thalamus and in cortical regions associated with S1 and S2 and centres of the brain processing affect (Price, 2000; Villemure and Bushnell, 2002). Villemure et al. (2002) used pleasant and unpleasant odours to alter mood and anxiety to demonstrate that this had a clear impact on pain unpleasantness and had a clear impact on pain unpleasantness in direct association with mood state. Thus the emotional changes selectively modulated pain affect. Rainville et al. (1997; 1999) modulated pain related activity in the anterior cingulate cortex using hypnotic suggestion to change pain unpleasantness without altering the sensory input. It is noteworthy that both positive and negative mood states are capable of altering pain. De Weid and Verbaten (2001) demonstrated that exposure to positive visual imagery increased pain tolerance and negative visual imagery reduced tolerance. Religious imagery has also been demonstrated to reduce pain to a noxious stimulus in religious people (Wiech et al., 2008).

Descending neuromodulatory controls involving the amygdala, periaqueductal grey, rostral ventromedial medulla and dorsal horn may have a role in the emotional modulation of pain (Villemure and Bushnell, 2002). The amygdala plays a well known role in emotions and affective disorders (Pare et al., 2004; Maren, 2005; Phelps and LeDoux, 2005) and an increasing body of evidence supports an important role of the amygdala in the emotional-affective dimension of pain (Heinricher and McGaraughty, 1999; Rhudy and Meagher, 2001; Gauriau and Bernard, 2002; Neugebauer et al., 2004; Neugebauer, 2006; Carrasquillo and Gereau, 2007; Ikeda et al., 2007; Ji and Neugebauer, 2007; Pedersen et al., 2007). Fields (2000) asserts that descending pathways are capable of generating or enhancing perceived pain intensity. This would provide a physiological mechanism for the pain-enhancing impact of mood, attention or expectation.

A.6.3. The cognitive component of pain

The biobehavioural model presented by Flor and Turk (2006) asserts that people learn to predict events based on previous learning experiences and information processing. Information is filtered through pre-existing knowledge and beliefs to react accordingly. Patients' behaviours can elicit responses from those around the patient that can reinforce both adaptive and maladaptive modes of thinking, feeling and behaving. Because interaction with the environment is a dynamic process, healthcare support is given to ongoing reciprocal relationships among physical, cognitive, affective, social and behavioural factors (Flor and Turk, 2006).

Meichenbaum and Turk (1976) were the first to systematically propose a role for cognitive variables in the pain experience, suggesting that the way people appraise pain might have significant effects on their pain perception and tolerance. From this perspective, chronic pain patients are viewed as contributing to their chronic pain syndrome through negative expectations about their own ability to control certain motor skills, such as performing specific physical activities. Additionally, chronic pain patients tend to believe that they have a limited ability to exert any control over their pain. These negative, maladaptive appraisals about the situation and personal efficacy may reinforce the experience of demoralisation, inactivity and overreaction to nociceptive stimulation (Flor and Turk, 2006).

A.7. Descending modulatory controls of pain

The idea that pain undergoes modulatory effects from higher regions in the CNS was first introduced by Head and Holmes almost a century ago (1911). Since then, a large body of knowledge has been gained regarding the mechanisms of pain perception and modulation (Reynolds, 1969; Fields and Basbaum, 1978; Millan, 2002). Several decades after Head and Holmes (1911) first theorized that pain is under the influence of higher areas in the CNS, studies confirmed their theory by providing evidence that a number of supraspinal sites contribute to the control of ascending sensory input by exerting tonic

inhibitory control of neurons in the dorsal horn (Hagbarth and Kerr, 1954; Carpenter et al., 1965; Wall, 1967). Further research into the contribution of supraspinal structures to nociceptive modulation showed that the mammalian CNS has several well-defined, supraspinally organised, descending pathways. These pathways form a network of neural systems that modulate the ascending transmission of nociceptive information, with the most well described being the circuitry mediating the brainstem control of nociceptive transmission at the level of the dorsal horn (Hagbarth and Kerr, 1954; Carpenter et al., 1965; Wall, 1967; Reynolds, 1969; Mayer et al., 1971; Oliveras et al., 1974; Fields and Basbaum, 1978; Willis, 1988; Fields et al., 1991; Millan, 2002).

Several supraspinal sites are known to contribute to the descending modulation of nociception, either by a direct projection of neurons to the spinal cord or indirectly by projections to the spinal cord via other brainstem regions. These include the PAG, LC, and the rostral ventromedial medulla (RVM), which subsequently modulate nociception in the dorsal horn.

A.7.1. The periaqueductal grey (PAG)

The PAG area of the midbrain is a small area of grey matter surrounding the central canal of the brainstem and densely packed with heterogenous neurons (Mayer and Price, 1976; Bandler and Shipley, 1994). In 1969, Reynolds found that electrical stimulation of this brain area in the rat caused analgesia of such intensity, that abdominal surgery could be performed without anaesthesia and without eliciting any marked response . Non-painful sensations were unaffected by this stimulation (Reynolds, 1969). The PAG integrates input from the limbic forebrain and diencephalon and it is thought to represent the mechanism whereby cortical and other inputs act to control the nociceptive gate in the dorsal horn (Bandler and Keay, 1996). There are direct projections to the PAG from a number of medial prefrontal areas including the anterior cingulate, insula and amygdala (Aggleton, 1992). Analgesia resulting from microinjection of opioid agonists into the amygdala is blocked by lignocaine inactivation of, or opioid antagonist injection into, the PAG (Pavlovic et al., 1996; Helmstetter et al., 1998). The

nucleus accumbens, located in the ventromedial striatum, has also been implicated in pain modulation, as it receives projections from the amygdala and itself projects to the PAG via the hypothalamus (Zahm et al., 1999).

Given that few PAG efferents project directly to the dorsal horn (Kuypers and Maisky, 1975; Castiglioni et al., 1978; Mantyh and Peschanski, 1982), research has focussed on discovering other pathways that mediate the spinal effects of PAG stimulation. It was found that the modulatory effect of the PAG is exerted indirectly through efferent connections with a variety of brainstem structures, such as the RVM, PB nucleus, LC and the A5 and A7 noradrenergic cell groups (Gallager and Pert, 1978; Abols and Basbaum, 1981; Mantyh, 1983a; Mantyh, 1983b; Cameron et al., 1995a; Cameron et al., 1995b).

A.7.2. The locus coeruleus (LC)

The LC, a bilateral structure composed of noradrenergic neurons and is located in the pons on the border of the fourth cerebral ventricle (Westlund and Coulter, 1980). Bilateral projections from the LC and nearby A7 cell group descend primarily to the contralateral dorsal horn laminae I, II and V where they exert an antinociceptive effect (Westlund and Coulter, 1980; Kwiat and Basbaum, 1992; Yeomans and Proudfit, 1992). In addition to the intrinsic antinociceptive effects of the pontine noradrenergic cell groups, they also receive neuronal projections from the RVM and PAG, thus serving as relays for the modulatory effects from the RVM and PAG to the dorsal horn (Yaksh, 1979; Hammond et al., 1985).

It has been suggested that there is increased noradrenergic innervation and thus increased descending adrenergic inhibition to the dorsal horn after nerve injury (Ma and Eisenach, 2003a). Studies have shown nerve injury induced changes in noradrenergic pathways including upregulation of spinal α2-adrenoceptor, which is negatively coupled to adenylate cyclase (AC), (Cho et al., 1997; Birder and Perl, 1999; Stone et al., 1999) and increased spinal NA content (Satoh and Omote, 1996). The enhanced analgesic potency of α_2-adrenergic agonists after nerve injury (Obata et al., 2005) points to an

enhancement of descending inhibition, or at least to an increased noradrenergic innervation and sensitivity of the dorsal horn. Clonidine, which has been clinically successful in the alleviation of neuropathic pain (Eisenach et al., 1996) and which has been licensed for the treatment of cancer pain in the United States (Eisenach et al., 1995), acts by partial agonism at the spinal α_2-adrenoceptors.

Contrary to this inhibitory role of the LC, Brightwell and Taylor (Brightwell and Taylor, 2009) have demonstrated that neuronal activity markers were increased in the nerve injured LC compared to sham and correlated to the intensity of tactile allodynia and that destruction or anaesthetisation of the LC resulted in reversal of behavioural allodynia and hyperalgesia, suggesting a nociceptive facilitatory role for the LC in neuropathic pain.

A.7.3. The rostral ventromedial medulla (RVM)

The RVM has been studied at length and is recognised as a major component of the pain modulatory circuitry, exerting its own modulatory effects in addition to relaying the modulatory effects from higher brainstem sites (Fields and Basbaum, 1978; Behbehani and Fields, 1979; Pomeroy and Behbehani, 1979). The RVM is a large region of the medulla that includes the midline nucleus raphe magnus and portions of the adjacent reticular formation; the nucleus reticularis gigantocellularis pars alpha and the nucleus paragigantocellularis lateralis.

There are three distinct populations of neurons in the RVM: those that discharge just prior to the occurrence of withdrawal from noxious heat (on cells); those that stop firing just prior to a withdrawal reflex (off cells); and those that show no consistent changes in activity when withdrawal reflexes occur (neutral cells) (Fields and Heinricher, 1985; Fields et al., 1991). Off cells exert a net inhibitory effect on nociception, as activation of off cells is sufficient to produce behavioural antinociception (Heinricher et al., 1994). Conversely on cells exert a net facilitatory effect on nociception. Periods of ongoing on-cell discharge are associated with enhanced nociception (Heinricher et al., 1989; Ramirez and Vanegas,

1989; Bederson et al., 1990; Foo and Mason, 2003) and direct, selective action of on cells produces hyperalgesia in lightly anaesthetised rats (Neubert et al., 2004). The role of neutral cells remains to be understood, although a portion are serotonergic (Potrebic et al., 1994; Mason, 1997) suggesting some involvement of this cell class in pain modulation.

Efferent projections from the RVM extend bilaterally, have been identified in all levels of the spinal cord, and comprise a major portion of the neurons projecting to the dorsal horn (Fields et al., 1977; Basbaum et al., 1978; Basbaum and Fields, 1979; Watkins et al., 1980; Jones and Gebhart, 1988; Willis, 1988; Fields et al., 1991). These neurons have widely collateralised yet lamina-specific projections (Huisman et al., 1981), with dense bilateral terminations in laminae I, II and V (Ruda et al., 1981; Mason, 1999). While all of the components of the descending modulatory network are important, the PAG and RVM have been shown to play key roles in the underlying mechanisms of pain modulation (Fields and Basbaum, 1978; Pomeroy and Behbehani, 1979). Additionally, the projection from the PAG into the RVM is critical for the PAG to exert its descending modulatory effect on dorsal horn nociceptive neurons (Behbehani and Fields, 1979; Urban and Smith, 1994; Cameron et al., 1995b; Mason, 1999), as electrical stimulation or microinjection of excitatory amino acids into the RVM abolishes analgesia produced by stimulation of the PAG (Fields et al., 1991).

The RVM exerts its modulatory effect on nociceptive transmission at the spinal level, resulting in antinociception to painful stimuli (Oliveras et al., 1975; Gallager and Pert, 1978; Abols and Basbaum, 1981). During persistent noxious stimulation, such as during a prolonged inflammation, there is continued activation of the descending pain modulatory circuitry and increased neuronal activity in the RVM that results in a progressive enhancement of descending modulation of spinal nociceptive transmission (Montagne and Oliveras, 1994; Ren and Dubner, 1996; Ren and Ruda, 1996; Urban and Gebhart, 1999; Terayama et al., 2000; Renn et al., 2003; Renn et al., 2005).

The descending modulation of nociception is not only inhibitory. Several lines of evidence demonstrate time-dependent biphasic properties of the pain modulatory system that can both inhibit and facilitate nociceptive transmission (Fields, 1988; Schaible et al., 1991; Fields, 1992; Zhuo and Gebhart, 1992; Ren and Dubner, 1996; Zhuo and Gebhart, 1997; Hurley and Hammond, 2000; Terayama et al., 2000). Descending facilitatory pathways from the RVM are involved in the maintenance, but not the initiation of neuropathic pain in animal models (Kovelowski et al., 2000; Burgess et al., 2002; Vera-Portocarrero et al., 2006). Injection of the local anaesthetic lignocaine into the RVM reverses established behavioural hypersensitivity in nerve injured animals, but does not prevent the expression of this hypersensitivity (Burgess et al., 2002). In an electrophysiological study, injection of lignocaine into the RVM reduced dorsal horn neuronal responses to noxious stimuli in healthy animals. This effect was greater in nerve-injured animals and it was observed that descending facilitation from the RVM now influenced neuronal responses to non-noxious stimulation, suggesting a possible mechanism for mechanical allodynia (Bee and Dickenson, 2007). These opposing modulatory effects result in the RVM being a crucial site for balancing descending modulation.

A.7.4. Modulation in the dorsal horn and the gate control theory

Cells of lamina II of the dorsal horn, the substantia gelatinosa (SG), are mainly short inhibitory GABAergic or glycinergic interneurons projecting into lamina I and lamina V. They regulate transmission at the first synapse of the nociceptive pathway between the primary afferent fibres and the spinothalamic tract transmission neurons, hence the term ‘gate control’ theory, proposed by Melzack and Wall (1965). According to this view, the SG responds to the activity of primary afferents entering the cord and thus allows the arrival of impulses via primary afferent or descending fibres to regulate the transmission of impulses via the ascending pathway (Melzack and Wall, 1965) (Figure A-1).

The effects of descending modulation from the PAG and RVM are exerted in the dorsal horn on the synapse between the primary afferent and projection neurons or on interneurons that synapse with

projection neurons. This synapse in the dorsal horn is the point where nociceptive integration is first integrated before being transmitted to higher centres in the CNS (Melzack and Wall, 1965; Fields and Basbaum, 1978; Willis and Coggeshall, 1991; Millan, 1999). The descending modulatory effect is applied either by inhibiting the release of neurotransmitters from the primary afferent fibre or by inhibiting the function of neurotransmitter receptors on the post-synaptic neuron (Fields et al., 2006).

Figure A-1. Schematic diagram of the gate control system.

This system regulates the passage of impulses from the peripheral afferent fibres to the thalamus via transmission neurons originating in the dorsal horn. Neurons act in the substantia gelatinosa (SG) of the dorsal horn act to inhibit the transmission pathway. Inhibitory interneurons are activated by descending inhibitory neurons or by non-nociceptive afferent input. They are inhibited by nociceptive C-fibre input, so the persistent C-fibre activity facilitation excitation of the transmission cells by either nociceptive or non-nociceptive inputs. This autofacilitation causes successive bursts of activity in the nociceptive afferents to become increasingly effective in activating transmission neurons. Adapted from Rang et al. (Rang et al., 2003).

A.8. Central neurotransmitters and modulators

Many neurotransmitters and neuromodulators are present in the central nervous system and this review is not exhaustive, but will cover the main factors involved.

A.8.1. Glutamate

Glutamate is the major excitatory amino acid neurotransmitter found throughout the nervous system and therefore critical for pain signalling at every anatomical level. Thus the vast majority of primary afferents synapsing in the dorsal horn utilize this transmitter, regardless of category. Glutamate exerts an excitatory effect on a number of receptors found on post synaptic spinal neurons (Tolle et al., 1993; Bleakman et al., 2006) leading to membrane depolarisation via three distinct receptor subclasses, ionotropic AMPA (Kerr et al., 1998; Tracey and Mantyh, 2007; Polgar et al., 2008) and NMDA receptors (Tolle et al., 1993; Nagy et al., 2004) and the G-protein coupled metabotropic (mGluR) family of receptors (Young et al., 1994; Valerio et al., 1997; Young et al., 1998). In addition, pre-synaptic kainate receptors for glutamate have been described in the spinal cord (Hwang et al., 2001; Kerchner et al., 2001b; Kerchner et al., 2002; Lucifora et al., 2006). AMPA and NMDA receptors have been most well characterised in pain.

Glutamate is released from sensory afferents in response to acute and more persistent noxious stimuli and it is fast AMPA receptor activation that is responsible for setting the initial baseline response of dorsal horn neurons to both noxious and tactile stimuli.

NMDA activation has been clearly shown to play a key role in the hyperalgesia and enhancement of pain signalling seen in persistent pain states including inflammation and neuropathic conditions (Price et al., 1994; Sindrup and Jensen, 1999). The major mechanism by which the NMDA receptor acts is through the Ca^{2+} influx occurring when the channel is activated. Once inside the cell, Ca^{2+} can activate various effectors, such as nNOS (Kitto et al., 1992; Budai et al., 1995), calcium/calmodulin-dependent

kinases (Malinow et al., 1988; Malinow et al., 1989) and extracellular signal-regulated kinase (ERK) (Xia et al., 1996; Ji et al., 1999), promoting downstream changes.

NMDA receptors have been identified as pharmacological targets due to their role in windup and central sensitisation. Antagonists such as ketamine, dextromethorphan and memantine are antinociceptive in animal models of inflammation and nerve-injury, as well as healthy volunteer and patient studies (Kristensen et al., 1992; Price et al., 1994; Park et al., 1995; Qian et al., 1996; Sang, 2000). Overall, these studies indicate that aberrant peripheral activity is amplified and enhanced by NMDA receptor-mediated spinal mechanisms in tissue damage and neuropathic pain and that the receptor is critical for both the induction and maintenance of persistent pain.

A.8.2. GABA

GABA is found in almost every region of the brain and spinal cord as an inhibitory neurotransmitter (Elliott and Van Gelder, 1958). GABA is produced by decarboxylation of glutamate and like other classical neurotransmitters, is packaged into synaptic vesicles and released from axon terminals via Ca^{2+} dependent exocytosis. Following its release, GABA is removed by either glial or presynaptic reuptake. In the cytoplasm, GABA transaminase converts GABA to succinic semialdehyde (Iversen and Kelly, 1975), which enters the Krebs cycle, leading to glutamate production. In glial cells there is an additional step in which glutamate is converted to glutamine before being returned to neurons where it is converted back to glutamate and then GABA.

GABA acts through two receptor subtypes. Ionotropic $GABA_A$ receptors are ligand gated, inward Cl^- channels composed of a pentameric combination of subunits (Jasmin et al., 2004). Metabotropic $GABA_B$ receptors are positively coupled to AC, allowing K^+ to move out of the cells through inwardly rectifying K^+ channels, inhibiting neurotransmitter release (Jasmin et al., 2004). In contrast to $GABA_A$ receptors, $GABA_B$ receptors produce long lasting inhibition (Jasmin et al., 2004).

GABA is highly concentrated in the superficial dorsal horn of the spinal cord (Miyata and Otsuka, 1975) where primary nociceptive afferents terminate and is a major component of the gate control system. There, GABA is found in local interneurons (Kaduri et al., 1987; Magoul et al., 1987; Todd and McKenzie, 1989; Waldvogel et al., 1990), but not in projection neurons (Haring et al., 1990). GABA is responsible for balancing excitatory systems and is thus released upon glutamate signalling (Iyadomi et al., 2000; Kerchner et al., 2001a) and inhibits the further release of glutamate and SP pre-synaptically (Matthews et al., 1988; Aanonsen and Wilcox, 1989; Hwang and Wilcox, 1989; Malcangio and Bowery, 1993; Rossi and Scarpini, 1993; Teoh et al., 1996; Li et al., 2002). In addition to interneurons, a significant number of bulbospinal afferents also release GABA in all parts of the spinal grey matter (Holstege, 1991; Antal et al., 1996). An imbalance in GABAergic transmission results in aberrant and exaggerated nociceptive signalling (Jasmin et al., 2004). GABAergic neurons and terminals are found in large numbers at all supraspinal levels and in these regions, GABA release can have either pro or antinociceptive effects (Jasmin et al., 2004).

A.8.3. 5-HT

Pharmacological block of spinal 5-HT_3 receptors reveals a role for a serotonergic descending facilitatory influence in the modulation of spinal nociceptive transmission. 5-HT_3 receptors exert pronociceptive effects at the spinal level (Ali et al., 1996; Oyama et al., 1996; Green et al., 2000). The contribution of such descending serotonergic facilitation to neuropathic pain was further confirmed by the fact that the efficacy of the 5-HT_3 receptor antagonist, ondansetron, in suppressing spinal responses to mechanical punctuate stimuli following SNL, suggesting an increase in descending serotonergic facilitatory drive to the spinal cord (Suzuki et al., 2004). In support of this theory, depletion of spinal 5-HT attenuates signs of behavioural hypersensitivity after nerve ligation (Rahman et al., 2006).

Serotonergic neurons comprise about 20% of the total RVM population (Moore, 1981) and all serotonergic neurons recorded in vivo are neutral cells (Potrebic et al., 1994; Gao and Mason, 2000).

A.8.4. Acetylcholine

Nicotinic acetylcholine (ACh) receptors in the RVM contribute to pain modulation. Nicotinic agonists microinjected into the RVM inhibit hypersensitivity to noxious heat (Iwamoto, 1991; Bannon et al., 1998). In addition, iontophoretic (Willcockson et al., 1983) or systemic (Bitner et al., 1998) administration of a nicotinic agonist produces a dose related activation of RVM neurons. The pain modulating actions of nicotinic agonists in the RVM depend on serotonergic neurons as nicotinic receptors are located primarily on serotonergic neurons of the RVM (Bitner et al., 1998). Paradoxically, lignocaine blockade of the RVM prevents the antinociceptive effects of systemic nicotine administration, however it does not block the antinociceptive effects produced by a nicotinic cholinergic channel modulator, ABT-564 (Decker et al., 1998).

A.8.5. Tachykinins

There are 3 endogenous tachykinins widely distributed throughout the nervous system: SP, neurokinin A (NK_A) and NK_B, which bind the GPCRs NK_1, NK_2 and NK_3, respectively. NK_1 and NK_2- receptors elicit very slow excitatory synaptic potentials in dorsal horn neurons, which alone are insufficient to excite post synaptic neurons, but may build up during repetitive activity to produce a burst of action potentials lasting for a few seconds in response to each stimulus. It is estimated that 80 % of projection neurons express NK_1 receptors (Todd, 2002). NK1-positive cells in lamina I have been shown to project to areas in the brain such as the thalamus, the PAG and in particular the PB nucleus (Todd, 2002) and brainstem areas such as the RVM (Mantyh et al., 1997; Suzuki et al., 2002).

SP and NK_A are found especially in primary afferents and the dorsal horn, as they are expressed by nociceptive sensory neurons (Lawson et al., 1997), playing significant roles in spinal pain mechanisms (Hunt and Mantyh, 2001). Using antibody-coated microelectrodes, SP and NK_A are released in the superficial dorsal horn in response to noxious stimuli (Diez Guerra et al., 1988; Duggan et al., 1988; Duggan et al., 1990).

A.8.6. CGRP

Animals studies show that CGRP, a pronociceptive peptide existing as two (α and β) isoforms, is the most abundant peptide in primary afferent fibres and has the widest distribution across subtypes of primary afferents terminals in the dorsal horn (Tie-Jun et al., 2001). Various animal models of pain result in increased DRG expression of CGRP (Hanesch et al., 1993; Ma et al., 1999; Nohr et al., 1999; Fernihough et al., 2005). CGRP exerts its actions via at least two GPCR types, CGRP1 and CGRP2, both of which are positively coupled to AC.

CGRP is present in virtually all SP containing fibres and many others (Hokfelt et al., 1992), and cooperatively and synergistically elicit EPSPs in dorsal horn neurons. The initial trigger of their release is probably provided by activation of AMPA receptors (Millan, 1999).

A.8.7. Opioid peptides

Morphine produces an analgesic effect when microinjected into the PAG, RVM, amygdala or insula (Yaksh and Rudy, 1978). Furthermore microinjection of opioid antagonists into or inactivation of these sites reduces the analgesic effect of systematically administered opioids. With the cloning of the three classical opioid receptors (μ, δ and κ) and a fourth related receptor (ORL1) and the generation of selective antibodies for each, it became possible to map their CNS distributions. Each is present in the insula, amygdala, hypothalamus, hippocampus, RVM and dorsal horn (Mansour et al., 1995; Darland et al., 1998). At spinal sites, ligands for the classical opioid receptors can produce an analgesic effect, in part by reducing excitatory amino acid and neuropeptide release from primary afferents (Suarez-Roca et al., 1992; Glaum et al., 1994; Grudt and Williams, 1994), and in part by a direct postsynaptic inhibition of central neurons that are activated by noxious stimulation.

The contribution of endogenous opioid peptides to pain modulation was first suggested by reports that stimulation-produced analgesia in animals and humans is reduced by the opioid antagonist naloxone

(Oliveras et al., 1975; Adams, 1976; Akil et al., 1976), thus establishing the relevance of endogenous opioids to common clinical situations (Fields and Levine, 1984). Endogenous opioids are low molecular weight proteins that are widely distributed throughout the nervous system, operating as neurotransmitters or as modulators of neuronal function. Endogenous opioids (methionine- (met-) and leucine- (leu-) enkephalin, β-endorphin, dynorphins, nociceptin, nocistatin and endomorphins) are derived by the cleavage of larger, usually inactive, precursors.

Although anatomical studies indicate that the enkephalins are released in proximity to both μ- and δ-opioid receptors, it is unclear which of the endogenous opioids act at which receptors (Akil et al., 1997). Both leu- and met-enkephalin have somewhat higher affinity for δ- rather than μ-opioid receptors and very low affinity for κ- opioid receptors. Dynorphins have a relatively high affinity for the κ-opioid receptor and there is general agreement that dynorphins are the endogenous ligand for this receptor. Like met-enkephalin, β-endorphin has approximately equal affinity for δ- and μ- opioid receptors and much lower affinity for κ-opioid receptors. Nociceptin has negligible affinity for μ- δ- κ- opioid receptors, but binds selectively to ORL1. Endomorphins have over 1000-fold selectivity for μ-opioid receptors versus κ- and δ-opioid receptors, inhibit C-fibre evoked responses of dorsal horn neurons (Chapman et al., 1997) and have analgesic sites of action and potency similar to those of morphine (Stone et al., 1997; Goldberg et al., 1998).

A.8.8. Cannabinoids

The endogenous cannabinoid system is comprised of cannabinoid receptors (CBRs), their endogenous ligands (endocannabinoids) and enzymes for their biosynthesis and degradation (Salzet, 2000). The neuroanatomy of the endocannabinoid system is organised to facilitate its role in retrograde signalling, the process by which endocannabinoids released postsynaptically modulate neurotransmission via action at CBRs located pre-synaptically (Rea et al., 2007).

Two GPCR CBRs have been described, CB_1 and CB_2, and are negatively coupled to AC and positively to MAPK (Howlett et al., 2002). CB_1 receptors are expressed presynaptically on neurons in both the peripheral and central nervous systems as well as on a wide range of peripheral tissues. CB_2 receptors are expressed largely in non-neural tissues including immune cells (Rea et al., 2007). CB_1 activation modulates Ca^{2+} (Sugiura et al., 2002) and K^+ channels (Mackie et al., 1995) to inhibit neurotransmitter release, including glutamate (Levenes et al., 1998), GABA (Szabo et al., 1998), glycine (Jennings et al., 2001), ACh (Gifford and Ashby, 1996), NA (Ishac et al., 1996), dopamine (Cadogan et al., 1997), serotonin (Nakazi et al., 2000) and cholecystokinin (CCK) (Beinfeld and Connolly, 2001).

The brain produces at least five compounds that possess submicromolar affinity for cannabinoid receptors: anandamide, 2-arachidonylglycerol (2-AG), noladin ether, virodhamine and N-arachidonyldopamine (NADA) (Svizenska et al., 2008). It is believed that endocannabinoids are biosynthesised as required and immediately released from cells to exert their physiological effects. Anandamide was the first identified and is the best studied endocannabinoid (Devane et al., 1992). Anandamide is synthesised by postsynaptic neurons, acting as a retrograde messenger molecule to modulate neurotransmitter release from CB_1 expressing terminals (Egertova and Elphick, 2000). In addition to CBRs, anandamide also acts as a full agonist at TRPV1 (Zygmunt et al., 1999), however any pronociceptive action appears to be negated by the antinociceptive action at CBRs (Guindon and Hohmann, 2009). 2-AG is a natural ligand for both CBrs, but has less affinity for CB_1 than anandamide. Despite this, 2-AG is present in the brain at higher levels than anandamide and is therefore considered the primary endocannabinoid in the brain (Childers and Breivogel, 1998).

The metabolism of endocannabinoids occurs intracellularly, however the mechanism by which endocannabinoids are taken up into the cell is currently unknown. Endocannabinoids can be degraded through two different pathways, hydrolysis and oxidation (Vandevoorde and Lambert, 2007). The former includes fatty acid hydrolase for anandamide and monoacylglycerol for 2-AG. The latter is performed by

COX and lipoxygenase which induce oxidation of the arachidonic moiety of the endocannabinoids (Kano et al., 2009).

Cannabinoids are inhibitory at the central terminal of peripheral nociceptors, outweighing any pronociceptive action at peripheral terminals (Guindon and Hohmann, 2009). Supraspinally, microinjected cannabinoid agonists have antinociceptive effects when injected into the PAG, RVM and amygdala (Hohmann and Suplita, 2006).

A.8.9. Cholecystokinin

There is a dense concentration of CCK- immunoreactive nerve terminals in the ventrocaudal PAG and the RVM, colocalised with enkephalin-immunoreactive terminal fields (Skinner et al., 1997). Behavioural studies indicate that CCK peptides act as functional antagonists to opioid analgesia via the CCK_2 receptor (Crawley and Corwin, 1994), which decrease K^+ conductance (Cox et al., 1995) and increase GABA release (Miller et al., 1997). An anti-opioid action of CCK_2 agonists has been demonstrated in the spinal cord, at supraspinal sites including the RVM (Mitchell et al., 1998; Heinricher et al., 2001) and the basolateral amygdala (Mitchell et al., 2000). A consequence of this functional antagonism is that selective CCK_2 antagonists can potentiate the analgesic action of morphine and of endogenous opioids acting at the μ-opioid receptor (Crawley and Corwin, 1994) and reduce some forms of opioid tolerance (Zarrindast et al., 1999; Tortorici et al., 2003). Consistent with animal research, human studies have demonstrated that CCK antagonism enhances morphine and placebo analgesia (Price et al., 1985; Benedetti, 1996; Benedetti, 2008; Price et al., 2008).

In addition to these anti-opioid actions, there is evidence that CCK exerts a pronociceptive effect through the PAG-RVM system. CCK, acting spinally and supraspinally, contributes to the development of enhanced spinal cord nociceptive transmission that occurs with tonic noxious stimuli (Urban et al., 1996) and peripheral nerve injury (Kovelowski et al., 2000).

A.8.10. Other modulators

Neurotensin is codistributed with endogenous opioids in pain modulating networks. Initial studies confirmed a neurotensin antinociceptive action in the amygdala, PAG and RVM, however more detailed dose-response studies revealed that neurotensin microinjections in the RVM elicit a facilitatory effect on nociceptive transmission at low doses and the endogenous neurotensin exerts an anti-opioid action (Smith et al., 1997; Urban and Gebhart, 1997). The physiological significance of pronociceptive effects of neurotensin is confirmed by the finding that inflammatory hyperalgesia is blocked by microinjection of a neurotensin antagonist into the RVM (Urban et al., 1996). Focal application of neurotensin in the RVM activates putative pronociceptive neurons in the RVM (Neubert et al., 2004).

Neuropeptide tyrosine (neuropeptide Y; NPY) is widely expressed in the nervous system and has been shown to play a role in pain processing (Brumovsky et al., 2007; Hokfelt et al., 2007; Smith et al., 2007). NPY is expressed in dorsal horn interneurons, with its receptors Y_1 and Y_2, widely distributed in the dorsal horn and DRG (Brumovsky et al., 2007; Hokfelt et al., 2007; Smith et al., 2007). Studies in transgenic mice indicate that NPY acting at Y_1 receptors primarily has an antinociceptive action. Activation of postsynaptic Y_1 receptors inhibits both the excitatory glutamatergic interneurons in the superficial dorsal horn and projection spinothalamic neurons. In addition, activation of presynaptic Y_1 and Y_2 receptors reduces glutamate and SP release from primary afferents in the superficial dorsal horn (Smith et al., 2007).

A.9. Central sensitisation

Many dorsal horn neurons are sensitised following brief bursts of activity in nociceptors; synaptic transfer is enhanced, potentiated or facilitated (Cook et al., 1987; Simone et al., 1991; Dubner and Ruda, 1992; Nichols et al., 1999). This central sensitisation takes a number of different and distinct forms. One form is windup (Mendell and Wall, 1965), another is heterosynaptic activity-dependent

plasticity that outlasts the initiating stimulus for tens of minutes (classic central sensitisation) (Thompson et al., 1990; Thompson et al., 1993) and a third is a largely homosynaptic potentiation elicited by brief high frequency inputs (long term potentiation) (Randic et al., 1993; Ikeda et al., 2003).

A.9.1. Windup

Windup, or temporal summation, is a form of homosynaptic activity-dependent plasticity (only the synapses activated show a change) that is characterised by a progressive increase in action potential output from dorsal horn neurons elicited during the course of a train of repeated low-frequency C-fibre or nociceptor stimuli (Mendell, 1984; Dickenson and Sullivan, 1987). A behavioural correlate of windup can be induced in humans by repeated peripheral noxious heat or mechanical stimuli, where the pain increases with each successive stimulus even though the stimulus intensity does not change (Price et al., 1977). After peripheral nerve injury, repeated light touch can produce a progressively increasing pain (hyperpathia) (Jensen, 1998).

The enhanced activity results from the activation of the NMDA receptor. If there are only acute or low frequency noxious or tactile inputs to the spinal cord, then activation of NMDA receptors is not possible, since under normal physiological conditions, the ion channel of this receptor is blocked by normal Mg^{2+} ion levels found in nervous tissues. This unique Mg^{2+} plug of the channel requires a sustained depolarisation of the membrane in order to be removed and allow the NMDA receptor-channel to be activated and opened. Here it is likely that the co-release of peptidergic transmitters, such as SP and CGRP, is responsible for a prolonged slow depolarisation of the neuron and subsequent removal of the NMDA block, thus permitting windup to occur (Budai and Larson, 1996; Khasabov et al., 2002; Suzuki et al., 2003). The sustained depolarisation furthermore recruits voltage-gated Ca^{2+} currents, triggering plateau potentials mediated by Ca^{2+}-activated non-selective cation channels. AMPA receptor antagonists have little effect on windup (Stanfa and Dickenson, 1999; Seagrove et al., 2004) and the brief depolarisation induced by this receptor would not be expected to produce any prolonged removal

of the NMDA block, unlike the long lasting, slow (seconds) activations caused by peptides. The lack of peptides in large Aβ afferents explains the lack of windup after low threshold stimuli.

A.9.2. Classic central sensitisation

Classical central sensitisation refers to immediate-onset, activity- or use- dependent increase in the excitability of dorsal horn nociceptive neurons as a result of, and outlasting a short barrage or nociceptor afferent input for tens of minutes (Woolf, 1983; McMahon and Wall, 1984; Woolf and Wall, 1986; Cook et al., 1987).

Activity in nociceptors evokes a period of facilitated transmission in dorsal horn neurons, augmenting responses in the conditioning nociceptor pathway, which is referred to as homosynaptic potentiation. The activity-dependent form of central sensitisation is the consequence of activation of multiple intracellular signalling pathways in dorsal horn neurons by the neurotransmitter glutamate and the neuromodulators SP and BDNF. Thus this central sensitisation involves activation of ligand-gated ion channels (NMDA, AMPA and/or kainate receptors) and GPCRs (NK_1, mGluRs and trk B).

The NMDA receptor plays a key role in activity-dependent central sensitisation. This has been revealed both pharmacologically and by a conditional knockout of the NR1 NMDA receptor subunit, which eliminates both NMDA-sensitive synaptic currents and a behavioural manifestation of central sensitisation (South et al., 2003). Two major mechanisms appear to contribute to the resulting increased synaptic strength: alterations in ion channel and/or receptor activity owing to post translational processing and trafficking of receptors to the membrane (Woolf and Salter, 2000; Ji et al., 2003). Activation of several protein kinases leads to the NMDA phosphorylation, increasing synaptic strength by altering channel open time, increasing bursting, removing Mg^{2+} channel blockade and promoting trafficking of NMDA receptors to the synaptic membrane (Woolf and Salter, 2000; Ji and Woolf, 2001). A major mediator of NMDA receptor tyrosine phosphorylation appears to be the non-receptor tyrosine

kinase Src, which is activated by trkB and EphB receptor and subsequently enhances NMDA receptor currents (Yu et al., 1997). It is not yet clear exactly how the different tyrosine kinases that phosphorylate NMDA and AMPA receptors are activated and controlled in dorsal horn neurons in response to different nociceptor inputs. One major modulator may be ERK, which is phosphorylated in dorsal horn neurons following nociceptive afferent input. Inhibition of its activation suppresses behavioural manifestations of central sensitisation (Ji et al., 1999). Docking of Ca^{2+}/calmodulin-dependent protein kinase to the NMDA receptor and phosphorylation of the GluR1 subunit of the AMPA receptor might also play a role (Fang et al., 2002; Garry et al., 2003).

The balance between mGlu and $GABA_B$ receptor activation can switch intrinsic firing properties of deep dorsal horn neurons from a tonic pattern to a plateau or even to an endogenous bursting pattern through modulation of the inwardly rectifying K^+ channel (Derjean et al., 2003) and this contributes to afferent-induced altered excitability. Dorsal horn neuronal excitability might also be controlled directly through ERK regulation of $K_{v4.2}$ channels, major contributors to A-type K^+ currents (Hu et al., 2003). Finally PGE_2 produced by COX in the spinal cord acts on EPs expressed by the dorsal horn and primary sensory neurons (Tegeder et al., 2001; Yaksh et al., 2001) to 1) to facilitate transmitter release from nociceptor central terminals (Vasko, 1995), 2) produce a direct depolarisation of dorsal horn neurons (Baba et al., 2001), and 3) reduce glycine receptor activity (Ahmadi et al., 2002).

After the induction of this form of activity-dependent central sensitisation by a brief (as short as 10-20 s) intense nociceptor-conditioning stimulus, normally subthreshold inputs begin to activate dorsal horn neurons as a result of an increase in synaptic strength. A striking feature of the increase in synaptic strength in dorsal horn neurons is that while it includes those nociceptor central terminal synapses activated by the conditioning or initiating stimulus (a homosynaptic facilitation), it is also found for synapses not activated by the initiating stimulus (Simone et al., 1989). Allodynia results from low-threshold sensory fibre induction by innocuous stimuli that activate normally high threshold nociceptive

neurons after the induction of heterosynaptic central sensitisation. This facilitation manifests within seconds of an appropriate noxious stimulus and can outlast the stimulus for several hours, even longer if the stimulus is maintained.

Heterosynaptic central sensitisation manifests in three main ways:

1. a reduction in threshold due to the recruitment of previously subliminal low-threshold Aβ-fibre inputs;
2. an increase in the responsiveness of the dorsal horn neurons, the number of action potentials elicited by a suprathreshold input is increased; and
3. an expansion of the extent of the receptive fields of dorsal horn neurons.

The unmasking of subthreshold inputs causes the neurons to become sensitive to stimuli in surrounding regions of the periphery, producing a spread of sensitivity beyond the site of tissue damage (secondary hyperalgesia). Furthermore, not only are the responses of individual neurons amplified, but the number of pain pathway neurons activated by any given stimulus is also increased (Woolf and Salter, 2000).

A.9.3. Long term potentiation

Although long term potentiation (LTP) has been studied most extensively in the hippocampus and other cortical areas, a similar phenomenon can be elicited in the spinal cord, comprising an activity-dependent, long lasting homosynaptic facilitation of excitatory postsynaptic potentials in response to brief, high frequency repeated trains of nociceptor input (Randic et al., 1993; Liu and Sandkuhler, 1995; Sandkuhler and Liu, 1998; Sandkuhler, 2000). The biological significance of this is difficult to evaluate fully because C fibres do not normally fire beyond a few spikes at the frequencies required experimentally to produce the LTP (~100 Hz). However, dorsal horn AMPA-receptor-mediated EPSPs remain potentiated for tens of minutes following brief repeated trains of high-frequency afferent stimulation. However, the full duration of this spinal cord LTP is not known. Similar to classical central

sensitisation, LTP in the dorsal horn seems to occur particularly in NK_1-expressing spinobulbar lamina I neurons. Indeed, induction of LTP in these neurons requires a synergistic interaction between NMDA receptor and NK_1 activation, as well as activation of low-threshold T-type Ca^{2+} currents beyond a critical threshold (Ikeda et al., 2003).

Appendix B. Mononuclear cell proliferation as a potential biomarker of pain

B.1. Introduction

A novel, putative biomarker has been reported previously by Hutchinson *et al*. (Hutchinson et al., 2004). They found that the proliferative response of isolated human peripheral blood mononuclear cells (PBMCs) to morphine was correlated with cold pain tolerance. Briefly, 1 x 10^5 PBMCs isolated from 13 healthy volunteers were cultured for 24 h with 2.5 $\mu g \cdot ml^{-1}$ of concanavalin A and 10^2 μM of morphine. It was observed that morphine significantly enhanced the concanavalin A-induced cell proliferation and that this enhancement was highly correlated with pain tolerance. The recent work by Hutchinson and colleagues (2007; 2010c) implicating morphine as a TLR4 agonist may provide some insight into possible mechanisms. Concanavalin A may 'prime' the PBMCs, so that morphine induces further proliferation via the TLR4-MAPK pathway. Thus, the magnitude of the enhanced proliferation may be directly proportional to the sensitivity of TLR4 and with our current understanding of TLR4 mediated mechanisms of glial activation, the work of Hutchinson and colleagues (2004) suggests that TLR4 activity may be a critical driver of pain tolerance. TLR4 is also expressed by peripheral immune cells (Methe et al., 2005; Lian et al., 2009) and this assay may therefore quantify the reactivity of TLR4 by measuring the degree of morphine-induced proliferation on peripheral immune cells.

Other studies have observed the mitogenic effects of morphine *in vitro*. Chuang and colleagues (1997) measured MAPK induction and activation of human lymphocytes that were cultured with an unspecified concentration of morphine. They concluded that morphine's effects were mediated by μ-opioid receptors; however in light of recent evidence showing that morphine's activity at non-classical opioid receptors, it is possible the observed effect was mediated by TLR4. Another study reported the stimulatory effects of opioids on MAPK in Chinese Hamster Ovary (CHO) cells that were transfected to express μ-opioid receptors (Li and Chang, 1996). Although there are CHO cell lines that express

endogenous TLR4 (Oliveira et al., 2004), this study found that CHO cells not transfected with μ-opioid receptor cDNA did not show a mitogenic response to opioids. In addition, etorphine showed the greatest potency, which does not activate glia (Hutchinson et al., 2007) and so it seems that this observation is indeed mediated by the μ-opioid receptor. The rank order potency of MAPK induction by the opioids tested in this paper is consistent with opioid pharmacology in regards to analgesic potency (etorphine > DAMGO > morphine)(Lester and Traynor, 2006). Another recent study showed that astrocyte proliferation was mediated by the μ-opioid receptor activation of p38 MAPK (Xu et al., 2007). Increase in the astrocyte activation marker, GFAP, was observed in the lumbar spinal cord of partial sciatic nerve ligation mice, but not in mice pre-treated with a κ antagonist, κ knockout mice or knockout mice lacking the dynorphin gene. It was shown that proliferation was mediated by p38 MAPK by blocking proliferation with co-treatment of a p38 inhibitor. These studies highlight opioid-induced proliferation via classical opioid receptors, however Hutchinson and colleagues (2004) found no induction of proliferation by morphine alone. As there are active μ-opioid receptors on lymphocytes (Chuang et al., 1995), the TLR4 hypothesis requires confirmation.

B.1.1. Aims

Hutchinson and colleagues (2004) have reported a 'snapshot' of basal pain tolerance in humans and as such, there remains a great deal to discover in regard to mechanisms and translation to chronic pain populations.

Therefore, this study aimed to first replicate the findings of Hutchinson and colleagues (2004), before investigation of mechanisms or in patient populations.

B.2. Materials and methods

All experiments were based on the method of Hutchinson and colleagues (2004). 10 ml of venous blood from healthy volunteers was drawn into EDTA tubes. PBMCs were isolated using Optiprep™ (Axis-Sheild PoC AS, Oslo, Norway) as directed by the manufacturer, using the mixer flotation method. Isolated cells were diluted to 1 x 10^6 cells·ml^{-1} in enriched RPMI 1640 (10% foetal calf serum [FCS]) and plated into 96 well plates (100 μl per well) (Nunc, Roskilde, Denmark), followed by the addition of morphine concentration range (McFarlane Smith, Edinburgh, UK), with a media control and concanavalin A 2.5 μg·ml^{-1} (final well volume 200 μl, *n* = 3 for each concentration) (Sigma, St Louis, Missouri, USA). Plates were incubated for 24 hours at 37 ºC, 5% CO_2, in an humidified environment, followed by the addition of 25 μl of a 1 in 5 AlamarBlue solution (Invitrogen, Mulgrave, VIC, Australia) and further 3.5 hours incubation. Supernatant was then transferred (175 μl) to white 96 well plates (BMG Labtechnologies, Offenburg, Germany) and fluorescence quantified on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) (excitation 545, emission 590).

B.2.1. Optimisation in human PBMCs

Unless otherwise indicated, the protocol was optimised in triplicate, using PBMCs isolated from a single healthy volunteer.

B.2.1.1. Replication

In the first instance, it was necessary to generate a response to concanavalin A, and so 1 x 10^5 cells per well were stimulated with 2.5 μg·ml^{-1} concanavalin A in supplemented RPMI 1640 (5% FCS), resulting in a robust response, with ceiling proliferation being reached (Figure B-1).

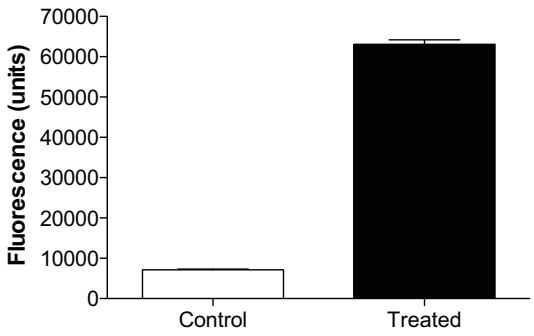

Figure B-1. 1 x 10^5 PBMC stimulation with 2.5 μg·ml^{-1} concanavalin A.

Variables	
Cell number per well	1 x 10^5
FCS concentration	5 %
Concanavalin A concentration	2.5 μg·ml^{-1}
TLR agonist	none
Reagent diluent:	Water

As such, a 10-fold lower concanavalin A concentration was used in order to enable detection of an enhancement of proliferation by morphine. Data are represented as % of concanavalin A proliferation, and a modest proliferative response to concanavalin A was observed. However, this was sufficient to elicit enhancement of proliferation by morphine at all concentrations (1, 10, 100 μM; Figure B-2). In contrast to these data, Hutchinson and colleagues (2004) had previously observed the greatest enhancement by morphine at 100 μM. In addition, 100 μM morphine alone had a proliferative effect, which had not been previously reported.

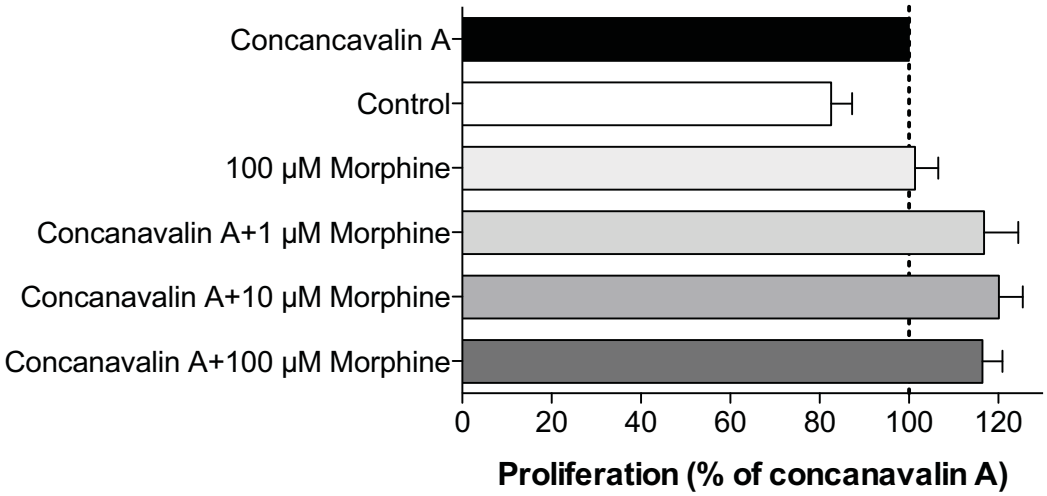

Figure B-2. PBMC stimulation with 0.25 μg.ml^{-1} concanavalin A and enhancement with morphine.

Variables	
Cell number per well	1 x 10^5
FCS concentration	5 %
Concanavalin A concentration	0.25 μg·ml^{-1}
TLR agonist	Morphine (1, 10, 100 μM)
Reagent diluent:	Water

The experiment was repeated in an attempt to account for previously unobserved results, however the cells did not respond to 0.25 μg·ml^{-1} concanavalin A. As such, the concanavalin A concentration was modified.

B.2.1.2. Concanavalin A concentration

To determine the optimum concanavalin A concentration, PBMCs were isolated from peripheral blood samples obtained from three healthy volunteers. 1 x 10^5 cells per well were incubated with a concanavalin A concentration range in supplemented RPMI 1640 (10% FCS). PBMCs did not respond to the concanavalin A at any concentration (Figure B-3).

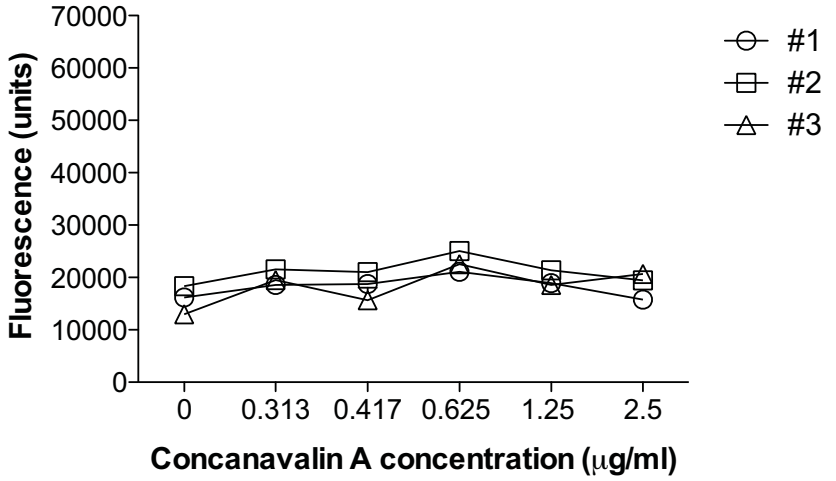

Figure B-3. Concanavalin A dose-response for PBMCs isolated from 3 healthy volunteers.

Variables	
Cell number per well	1 x 10^5
FCS concentration	5 %
Concanavalin A concentration	0.313, 0.417, 0.625, 1.25 and 2.5 μg·ml^{-1}
TLR agonist	None
Reagents diluent	PBS

The effect of a higher concanavalin A concentration range was then assayed across a range of cell concentrations with varied RPMI 1640 supplementation (10% and 20% FCS). Increasing the cell number had the expected effect of increasing background fluorescence, however no concentration of concanavalin A had an effect on PBMCs (Figure B-4). Furthermore, increasing the FCS supplementation simply increased background fluorescence.

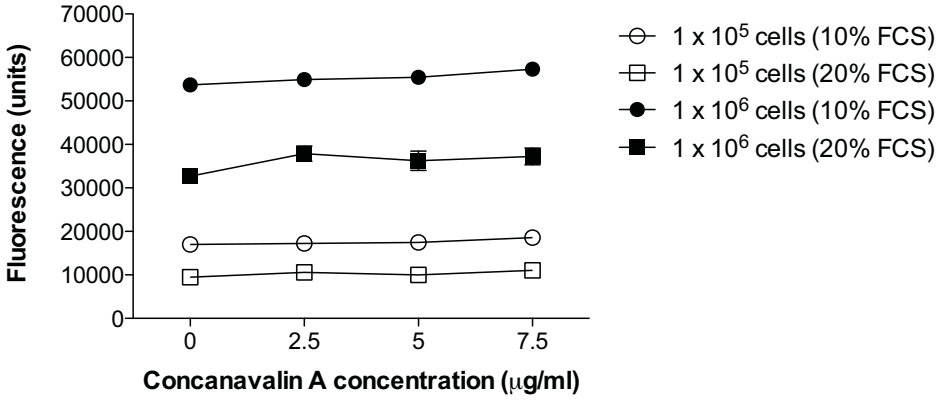

Figure B-4. Effect of concanavalin A concentration across varied cell numbers and concentration of media supplementation.

Variables	
Cell number per well	1 x 10^5, 1 x 10^6
FCS concentration	10 %, 20 %
Concanavalin A concentration	2.5, 5, 7.5 μg·ml^{-1}
TLR agonist	None
Reagent diluent:	Unsupplemented RPMI 1640

The reason for the sudden failure of concanavalin A to induce PBMC proliferation could not be explained and other factors were therefore investigated.

B.2.1.3. AlamarBlue concentration

AlamarBlue is a deep blue colour when unreduced. The dye is reduced through the cell's metabolic process to a fluorescent pink colour in the medium. To confirm that the peak fluorescence signal of AlamarBlue was 1.25 % (v/v) in supplemented RPMI 1640 (10 % FCS), AlamarBlue was fully reduced with ascorbic acid. Ascorbic acid was selected due to its stability, safety and miscibility in water. Ascorbic acid concentrations ranging from 10^{-5} to 10^{-2} M were made up in milliQ water and investigated with 10 % and 20 % AlamarBlue enriched RPMI 1640 (10% FCS) solutions (final well concentration). All

concentrations except 1 x 10^{-5} M of ascorbic acid bleached the AlamarBlue colourless and so a concentration range of AlamarBlue (0.01 - 10 % [v/v]) in 10^{-5} M ascorbic acid was assayed (Figure B-5).

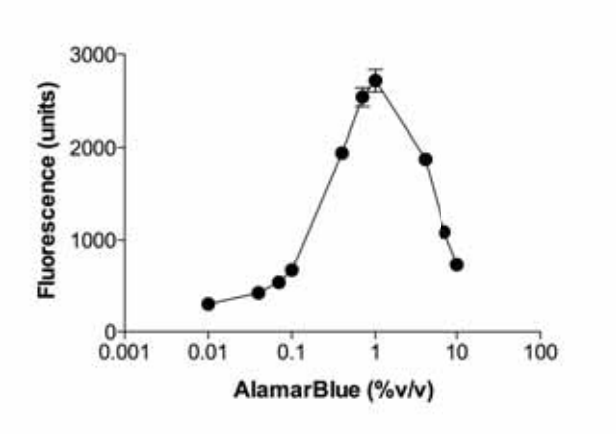

Figure B-5. Quantification of AlamarBlue fluorescence in 1 x 10^{-5} M ascorbic acid.

The fluorescence profile in figure 1-1 is similar to that previously observed (peak fluorescence at ~1.25 % v/v), however the maximum fluorescence is markedly lower than previously observed by Hutchinson (~60 000 units). The first of two possible reasons is that the AlamarBlue is not working correctly or the signal is being quenched. The second possibility is that while the ascorbic acid is reducing the AlamarBlue, it is also interfering and perhaps degrading the AlamarBlue. The latter appears most likely given the bleaching seen at higher concentrations of ascorbic acid. However, as a similar AlamarBlue concentration-response profile was observed, 1.25 % v/v was confirmed as the appropriate AlamarBlue concentration.

B.2.1.4. Cell and foetal calf serum concentration

The number of cells per well was investigated to ensure that the greatest dynamic range of cell proliferation with concanavalin A was obtained, allowing more robust detection of morphine enhancement of proliferation.

FCS autofluoresces, so FCS concentration was optimised to ensure that adequate nutrition for the cells while minimising interference with detection of fluorescence.

1 x 10^3 – 1 x 10^6 cells per well were stimulated with 2.5 μg·ml^{-1} concanavalin A in supplemented RPMI 1640 (5% and 10% FCS [Figure B-5]).

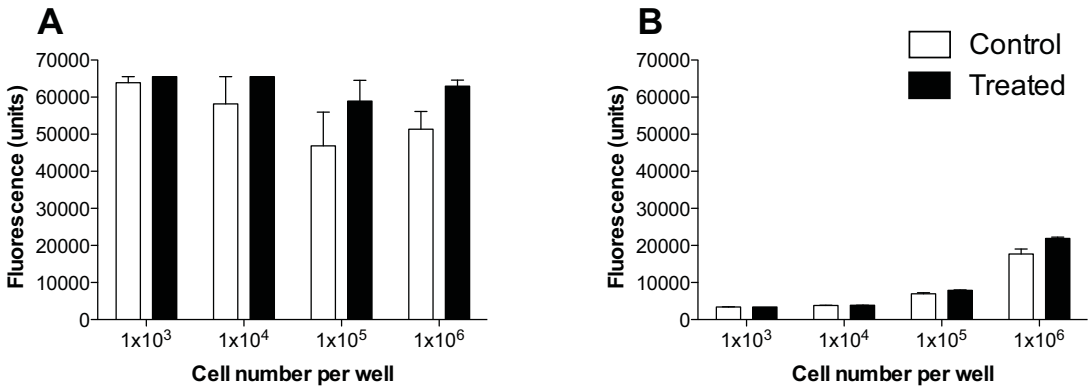

Figure B-6. Optimisation of cell number in (A) 5 % and (B) 10 % FCS.

Variables	
Cell number per well	1 x 10^3 – 1 x 10^6
FCS concentration	5 %, 10 %
Concanavalin A concentration	2.5 μg·ml^{-1}
TLR agonist	none
Reagent diluent:	Unsupplemented RPMI 1640

The large discrepancy between Figure B-6 A and B in can likely be attributed to different batches of FCS. While concanavalin A had a slight effect with 5% FCS, there is no clear cell concentration-response relationship.

The experiment was repeated using a wider range of the new FCS batch. A cell range of 1 x 10^3 – 1 x 10^6 cells per well were stimulated with 2.5 μg·ml^{-1} concanavalin A in supplemented RPMI 1640 (Figure

B-7). Each graph demonstrates a cell concentration-response relationship, however the concanavalin A caused minimal cell proliferation. These data indicate that the greatest dynamic range between control and stimulated cells occurred with 1 x 10^6 in 2.5 % FCS.

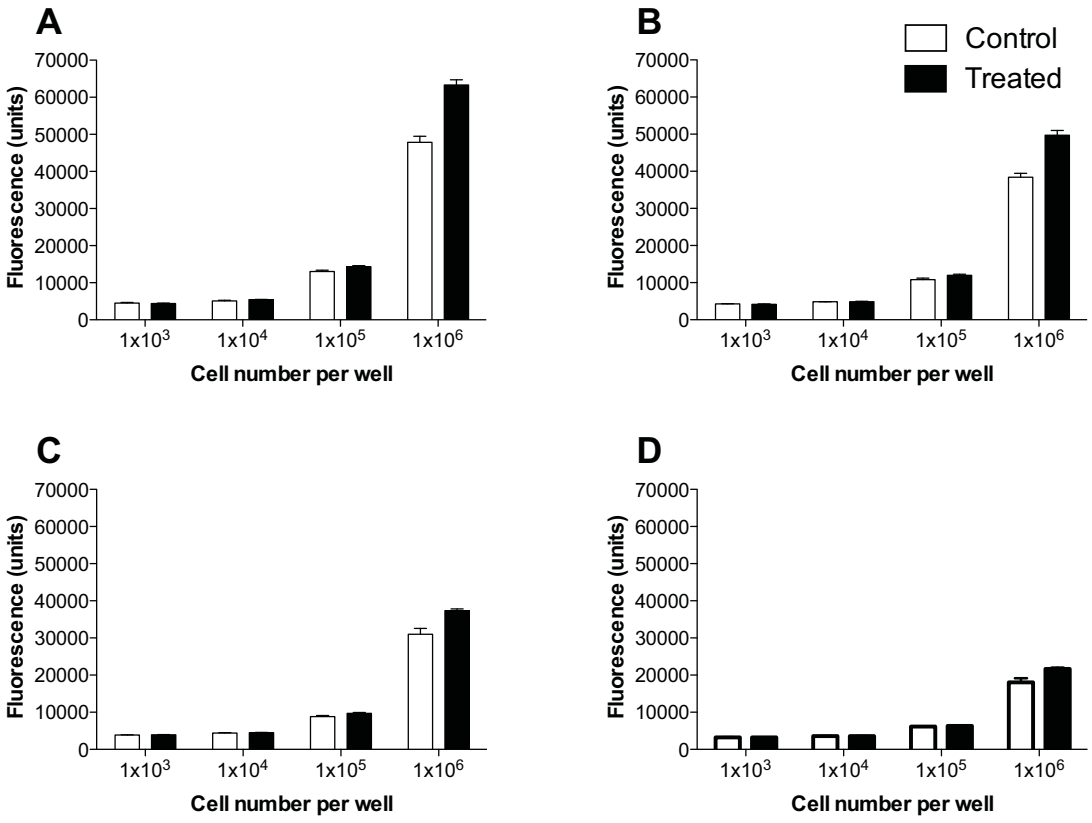

Figure B-7. Optimisation of cell numbers and FCS concentration at (A) 2.5 %, (B) 5 %, (C) 10 %, (D) 20 % in 2.5 μg·ml^{-1} concanavalin A.

Variables	
Cell number per well	1 x 10^3 – 1 x 10^6
FCS concentration	2.5%, 5%, 10% & 20%
Concanavalin A concentration	2.5 μg·ml^{-1}
TLR agonist	none
Reagent diluent:	Unsupplemented RPMI 1640

B.2.1.5. LPS concentration

Given the proposed mechanism of morphine-induced proliferation via TLR4 (Hutchinson et al., 2007; Hutchinson et al., 2010c), it seemed likely that the known TLR4 agonist, lipopolysaccharide (LPS), would similarly correlate with cold pain tolerance. Therefore LPS from *salmonella typhimurium* was investigated in the $\mu g \cdot ml^{-1}$ range at various cell concentrations from two healthy volunteers (Figure B-8 and Figure B-9).

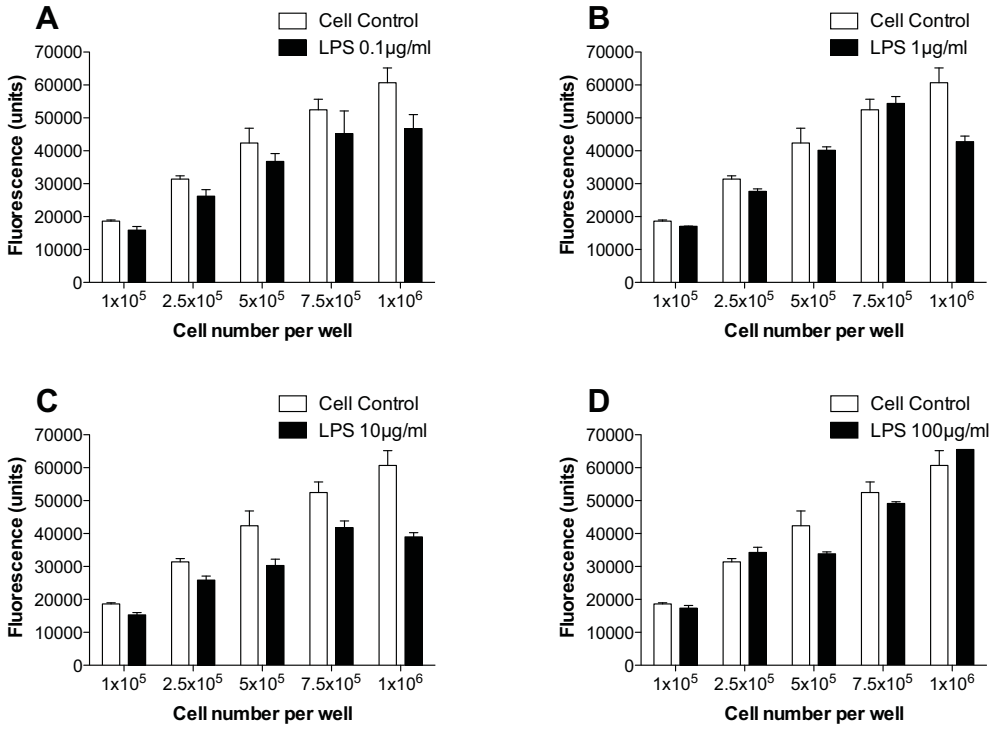

Figure B-8. Subject 1 optimisation of $\mu g \cdot ml^{-1}$ range of LPS and cell concentration in supplemented RPMI 1640 (2.5% FCS).

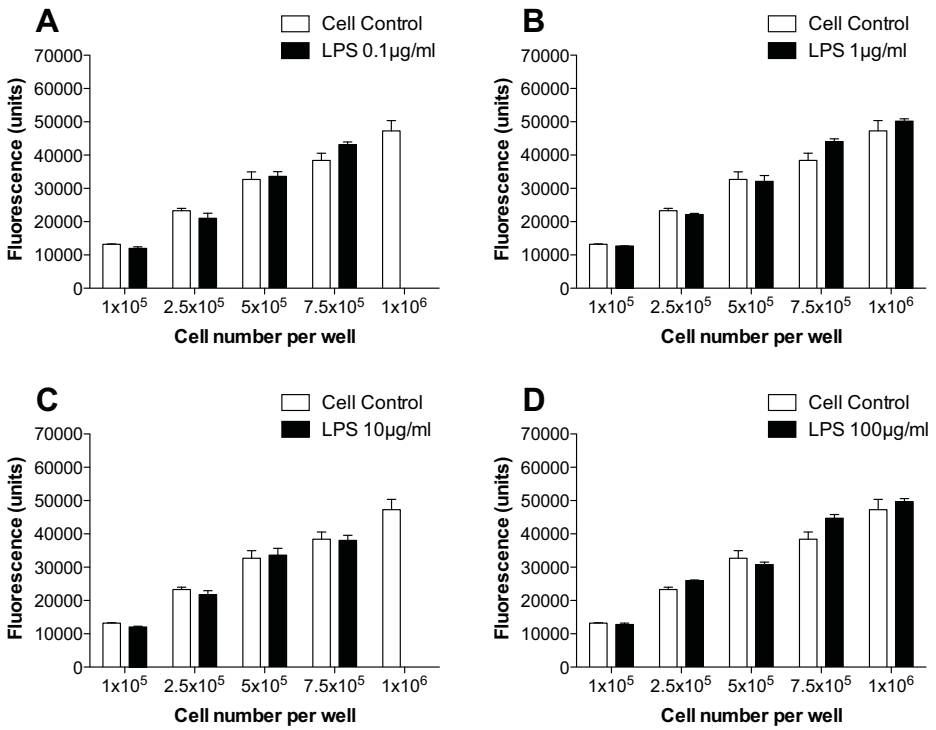

Figure B-9. Subject 2 optimisation of μg·ml^{-1} range of LPS and cell concentration in supplemented RPMI 1640 (2.5% FCS).

Variables	
Cell number per well	1 x 10^5, 2.5 x 10^5, 5 x 10^5, 7.5 x 10^5, 1 x 10^6
FCS concentration	2.5%
Concanavalin A concentration	None
TLR agonist	LPS (0.1-100 μg·ml^{-1})
Reagent diluent:	Unsupplemented RPMI 1640

Both Figure B-8 and Figure B-9 show toxicity to LPS, so the concentration range was lowered to the ng·ml^{-1} range (Figure B-10). However there was virtually no effect of LPS at any concentration.

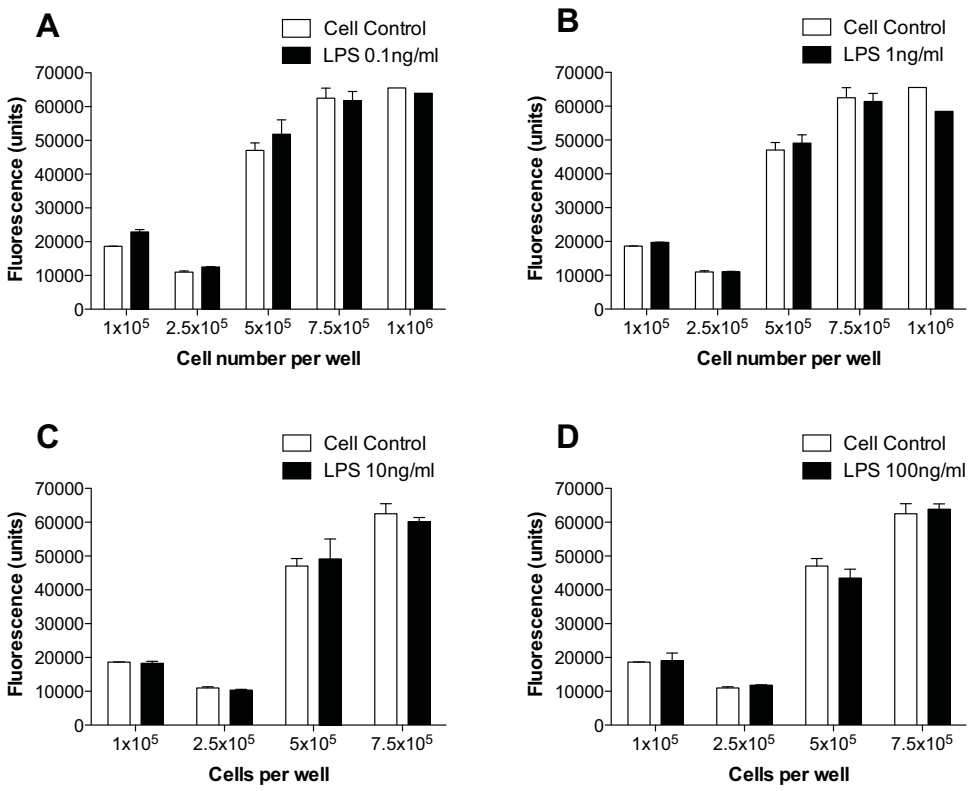

Figure B-10. Optimisation of ng·ml^{-1} range of LPS and cell concentration in supplemented RPMI 1640 (2.5% FCS).

Variables	
Cell number per well	1 x 10^5, 2.5 x 10^5, 5 x 10^5, 7.5 x 10^5, 1 x 10^6
FCS concentration	2.5%
Concanavalin A concentration	None
TLR agonist	LPS (0.1-100 ng.ml^{-1})
Reagent diluent:	Unsupplemented RPMI 1640

LPS from *e. coli* was used rather than *salmonella typhimurium* in an attempt to induce a proliferative response from the PBMCs isolated from 3 healthy volunteers (Figure B-11).

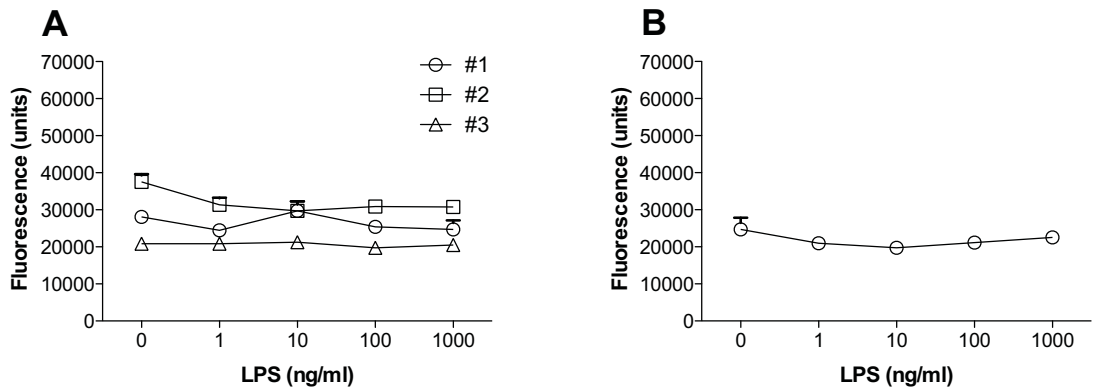

Figure B-11. Stimulation of 1 x 10^5 PBMCs with LPS.

Concentration range of LPS from *e. coli* in (A) PBMCs isolated from 3 healthy volunteers in unsupplemented medium, and (B) PBMCs isolated from a single healthy volunteer in supplemented medium (2.5 %).

Variables	
Cell number per well	1 x 10^5
FCS concentration	0, 2.5 %
Concanavalin A concentration	None
TLR agonist	LPS (0.1-1000 ng.ml^{-1})
Reagents diluted in:	Unsupplemented RPMI 1640

In summary, all attempts to induce PBMC proliferation using LPS were unsuccessful, in contrast to all other evidence.

B.2.1.6. Additional TLR4 agonists

Reserpine and morphine-3-glucuronide (M3G) may also be other TLR agonists (Hutchinson et al., 2010c)(unpublished observations) and thus were examined in combination with concanavalin A and LPS to observe any potential enhancement (Figure B-12). No significant effect of any treatments were observed.

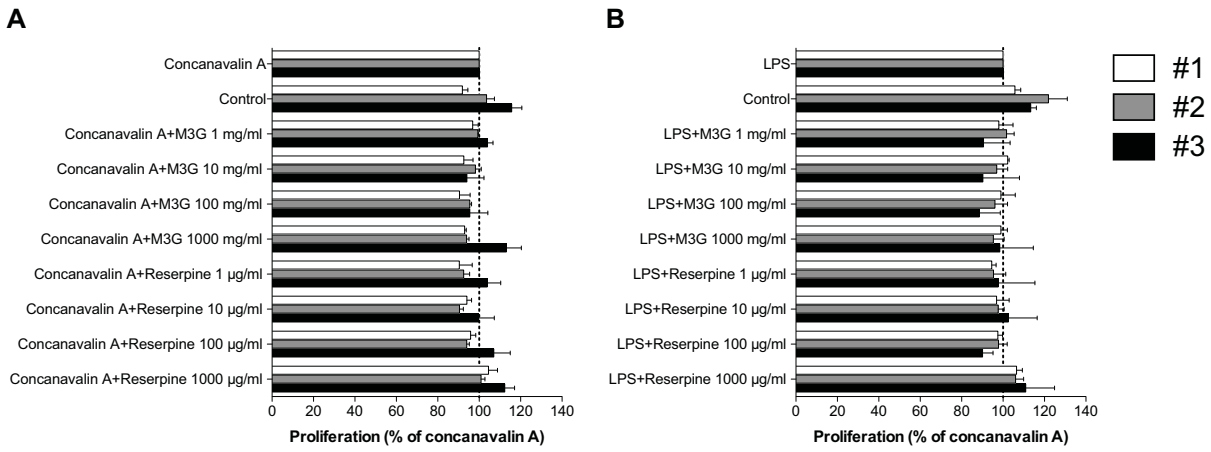

Figure B-12. Stimulation of 1 x 10^5 PBMCs with M3G, LPS and reserpine.

Dose response to M3G and reserpine in combination with (A) 2.5 μg·ml^{-1} concanavalin A, and (B) 100 ng·ml^{-1} LPS, in 1 x 10^5 PBMCs isolated from 3 healthy volunteers in unsupplemented medium.

Variables	
Cell number per well	1 x 10^5
FCS concentration	0%
Concanavalin A concentration	0, 2.5 μg·ml^{-1}
TLR agonist	LPS (100 ng·ml^{-1}), M3G (1-1000 ng·ml^{-1}), Reserpine (1-1000 μg·ml^{-1})
Reagent diluent:	Unsupplemented RPMI 1640

B.2.1.7. Medium

All experiments had been conducted in RPMI 1640 that did not contain phenol red or HEPES buffer. Therefore an additional experiment was conducted in supplemented RPMI 1640 (5% FCS), containing these additives (Figure B-13). No proliferation was observed following any treatments.

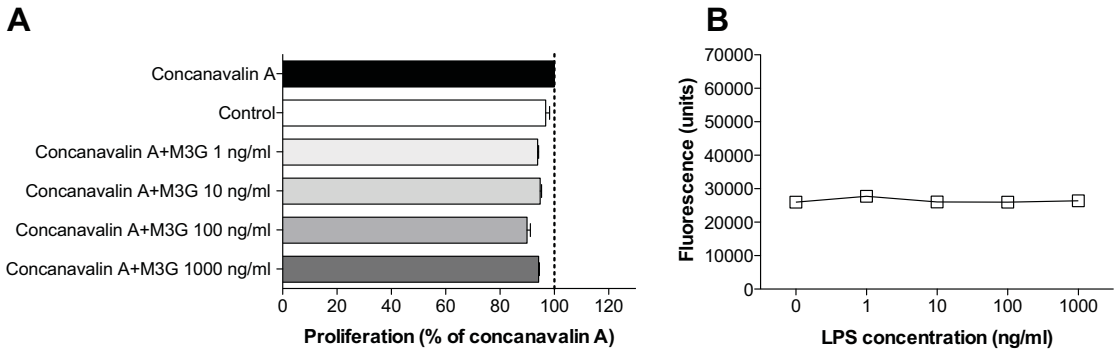

Figure B-13. Stimulation of 1 x 10^5 PBMCs with M3G and LPS.

(A) concanavalin A and M3G, and (B) LPS, in supplemented RPMI 1640 (5 % FCS) containing 25 nM HEPES and phenol red.

Variables	
Cell number per well	1 x 10^5
FCS concentration	5 %
Concanavalin A concentration	0, 2.5 μg.ml^{-1}
TLR agonist	LPS (1-1000 ng·ml^{-1}), M3G (1-1000 ng·ml^{-1})
Reagent diluent:	Unsupplemented RPMI 1640

Artificial cerebrospinal fluid (aCSF) was also experimented in place of RPMI 1640, with the advantage of an environment closer to physiological conditions. aCSF was prepared as previously described (Oka et al., 1996), supplemented with 5 % FCS, and 25 mM of HEPES was added to buffer the solution. 1 x 10^5 cells per well were investigated using a concentration range of LPS and 2.5 μg.ml^{-1} concanavalin A with the addition of a concentration range of M3G (Figure B-14). A slightly different profile was observed with aCSF, but no proliferation was detected.

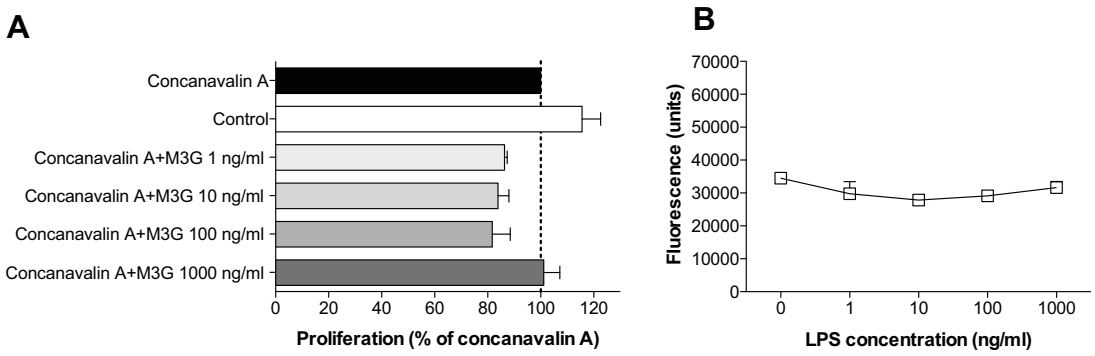

Figure B-14. Stimulation of 1 x 10^5 PBMCs with M3G and LPS.

(A) concanavalin A and M3G, and (B) LPS, in supplemented aCSF 1640 (5 % FCS) containing 25 nM HEPES.

Variables	
Cell number per well	1 x 10^5
FCS concentration	5%
Concanavalin A concentration	0, 2.5 μg.ml^{-1}
TLR agonist	LPS (1-1000 ng·ml^{-1}), M3G (1-1000 ng·ml^{-1})
Reagent diluent:	Unsupplemented aCSF

B.2.2. Optimisation in graded rat splenocytes

With the lack of proliferative response to either concanavalin A and LPS despite the attempted optimisation of many of the variables associated with the assay, human PBMCs were replaced by rat splenocytes. It was believed that the greater homogeneity between rats rather than human samples might lead to an improved response.

Experiment 1 graded neuropathic rats from Chapter 2 were euthanised by overdose with sodium pentobarbital and the spleen promptly excised. The spleen was finely minced in 2 ml of RPMI 1640 and massaged through a nylon mesh into a 50 ml tube with up to 15 ml RPMI 1640 (25 nM HEPES, L-glutamine, 5 ml penicillin- streptomycin solution per l). The cell suspension was centrifuged at room

temperature for 10 min at 500 x g, the supernatant was discarded and the cells resuspended in 1 ml of PBS followed by the addition of 7 ml of red blood cell lysis buffer (155 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA). The suspension was incubated at room temperature, centrifuged (10 min at 200 x g) and the supernatant discarded. Cells were washed in 10 ml PBS and then resuspended in 2 ml of RPMI 1640. Following quantification with the Trypan Blue exclusion method, suspensions were diluted to 1 x 10^6 cells·ml^{-1} in enriched RPMI 1640 and plated into 96 well plates (100 ml per well).

FCS concentration (0 %, 5 % and 10 %), incubation time (20 – 48 h), AlamarBlue incubation time (2 – 8 h) and mitogen concentrations were modified, however no proliferation was observed and no pattern emerged according the degree of nerve injury. Representative data is shown for a concentration range of concanavalin A and LPS, assayed across splenocytes in enriched RPMI 1640 (5 % FCS), isolated from N0S4, N1S3, N2S2 and N4S0 treated rats (Figure B-15 A,B).

A morphine concentration range across a concanavalin A concentration range was also assayed in splenocytes isolated from N0S4 and N4S0 rats (Figure B-15 C,D). Despite the lack of response to concanavalin A alone, a striking dose-response to morphine was observed. However, there appeared to be no pattern according to the degree of nerve-injury or concentration of 'priming' concanavalin A and further attempts to replicate this data were unsuccessful.

It was also observed that splenocyte counts were inversely related to the degree of nerve-injury (splenocyte count decreased as allodynia increased) (See Chapter 3). This observation raised the possibility that splenocytes from different treatment groups could contain different proportions of leukocyte subpopulation, such that cellular responses could not be compared between groups.

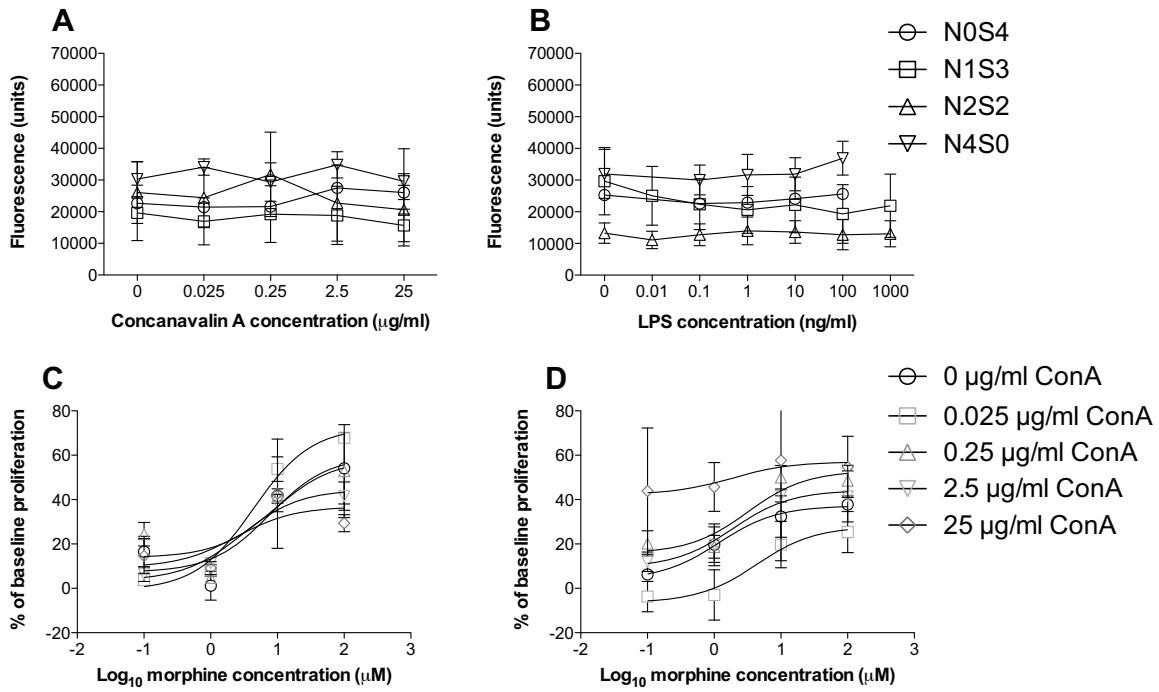

Figure B-15. Proliferation response of 1 x 10^5 rat splenocytes.

Graded splenocyte dose response to (A) concanavalin A, and (B) LPS. Dose response of (C) N0S4, and (D) N4S0 splenocytes to morphine concentration range at various concentrations of concanavalin A.

B.3. Conclusion

Insanity: doing the same thing over and over again and expecting different results.

- Albert Einstein

This study was ultimately unsuccessful in replicating the findings by Hutchinson and colleagues (2004) in either human PBMCs or rat splenocytes harvested from graded CCI donors.

Results in the human PBMCs were initially promising, with cells showing a strong response to concanavalin A and, although the response profile was substantially altered to that reported previously (Hutchinson et al., 2004), morphine enhanced cell proliferation at all concentrations, as well as independently showing an effect at least equal to 0.25 $\mu g.ml^{-1}$ concanavalin A at a concentration of 100 μM. Subsequent attempts at replication showed that the PBMCs failed to respond to concanavalin A or

LPS, despite the purchase of new reagents, fresh stock solutions for each experiment and manipulation of concanavalin A, LPS, FCS concentrations, as well as the use of other putative TLR4 agonists. The proliferative action of concanavalin A and LPS have been well documented (Stewart et al., 2008), so the lack of effect in the current study cannot be explained from my data or from any previous data. Some subtle, but currently undetectable, differences must exist between these data and those published previously. One example may be co-factors required for mitogen binding and proliferation, for example heat shock protein-90, which is required for LPS-induced proliferation (Hutchinson et al., 2009b).

The lack of concanavalin A- and LPS- induced proliferation in human PBMCs lead me to investigate the activity of these mitogens in rat splenocytes. Despite greater homogeneity between samples, results were extremely variable and no reliable response to either mitogen was observed. Recent results in our laboratory have shown that splenocytes robustly respond to LPS when an alternate, harsher red blood cell lysis buffer was used, perhaps priming a response to the mitogen. However, the potential variability in leukocyte subpopulations across the graded CCI model suggested that comparisons between groups might be limited.

As such, the lack of response by human PBMCs and the potentially limited applicability of the graded model to this assay meant that this work was abandoned to investigate the biological significance of the altered spleen weights in the search for a biomarker of pain (Chapter 3).

Appendix C. *Ex vivo* Transient Receptor Potential Vanilloid Type 1 (TRPV1) activation of leukocytes as a correlate of pain tolerance

C.1. Introduction

Volunteer models of pain are useful in the study of pain mechanisms and are used in clinical drug development to demonstrate the analgesic potential of new compounds. The selection of a model should be based on the mechanism of pain targeted by the compound under investigation. One putative model of neuropathic pain is the intradermal (i.d.) capsaicin model (Hughes et al., 2002a).

Capsaicin is the irritant compound in the capsicum plant (red pepper, chilli pepper etc.) and produces a profound alteration in nociception following i.d. injection:

1. Spontaneous pain (intense stinging sensation at the injection site), which is maximal at the time of injection, but short lived (10-30 mins).
2. Allodynia, occurring at the injection site (primary allodynia) and in the surrounding skin area (secondary allodynia), and usually short lived (< 20 min).
3. Hyperalgesia occurring at the injection site (primary hyperalgesia) and the surrounding skin area (secondary hyperalgesia), lasting between 6 and 24 h.
4. Neurogenic inflammation (the area of redness extending beyond the site of injection) is maximal between 10 and 20 mins following capsaicin administration and lasts between 30 and 90 mins.

The capsaicin model was first used by Simone and colleagues (1987) and has since been used extensively in the study of human pain (Koltzenburg et al., 1992; Torebjork et al., 1992; Park et al., 1995; Liu et al., 1996; Sang et al., 1996; Kinnman et al., 1997a; Kinnman et al., 1997b; Serra et al., 1998; Hughes et al., 2002a; Sumikura et al., 2003; Scanlon et al., 2006; Geber et al., 2007) due to its reliable induction of pain (Hughes et al., 2002a) and central sensitisation (Valeriani et al., 2003). The

pain induced by capsaicin has been attributed to its specific action on sensory nerve fibres (Fitzgerald, 1983) and both animal and human studies suggest that the spontaneous pain caused by capsaicin administration is mediated by C-fibre polymodal nociceptors, whereas the allodynia is mediated by Aβ-fibres and hyperalgesia by Aδ- and C-fibres (Dougherty and Willis, 1992; Torebjork et al., 1992; Park et al., 1995). In this way, capsaicin may effectively model central sensitisation.

Capsaicin acts directly on transient receptor potential vanilloid type 1 (TRPV1) (Lee et al., 2005), which belongs to a larger family of TRP channels whose core transmembrane structure resembles that of voltage-gated channel proteins with six transmembrane domain segments, a pore region between the 5th and 6th transmembrane domains and a voltage sensor (Lee et al., 2005). TRPV1 is a non-selective cation channel with high calcium permeability, first cloned in 1997 by Caterina and colleagues, being highly expressed in small sensory neurons .

TRPV1 mRNA and protein has recently been detected in human peripheral blood mononuclear cells (PBMCs) (Saunders et al., 2007; Spinsanti et al., 2008). Functionally, leukocyte TRPV1 expression may be linked to reduced lymphocytes in end-stage kidney disease (Saunders et al., 2009) and increased mRNA expression has been linked to pain insensitivity (Spinsanti et al., 2008), however the consequences for pain have not been well characterised.

If TRPV1 is expressed by leukocytes, characterisation of inter-individual *ex vivo* function may correlate with *in vivo* alterations in nociception following i.d. capsaicin administration. Such a correlation may represent a biomarker of central sensitisation and would further suggest a role for peripheral immune cells in the mediation of capsaicin-induced pain.

C.1.1. Aims

The aim of this study was to compare the dose-response and dose-duration curves for pain, flare, hyperalgesia and allodynia with the *ex vivo* TRPV1 mediated cytosolic calcium increase by capsaicin.

C.2. Methods

C.2.1. Preparation of capsaicin

A capsaicin solution was prepared as previously described (Gustafsson et al., 2009). Briefly, capsaicin (Fluka, Switzerland, Batch 21741) was added stepwise to 2 ml of a 38 % (w/v) hydroxypropyl-β-cyclodextrin (HP-β-CD) solution and suspended by vortexing. The solution was then spun on a rotor for 24 h to equilibrate, followed by centrifugation at 10 000 rpm for 3 min. The supernatant was collected (2 ml), further diluted with 3 ml of Hank's Balanced Salt Solution (HBSS, 20 nM HEPES; Sigma, St Louis, Missouri, USA) and stored at 4 °C for no longer than 30 days.

C.2.2. A23187 preparation

The calcium ionophore, A23187 (Sigma, St Louis, Missouri, USA), was selected as a positive control as previously described (Martina et al., 1994) and suspended in DMSO.

C.2.3. Isolation of PBMCs

Two subjects had 10 ml of venous blood drawn into EDTA tubes. PBMCs were isolated using Optiprep (Axis-Shield PoC AS, Oslo, Norway) as directed by the manufacturer using the mixer flotation method. Isolated cells were diluted to 2.5 x 10^6 cells·ml^{-1} in assay media (HBSS, 20 nM HEPES) and plated into white 96 well plates (Nunc, Roskilde, Denmark) (50 ml per well). Plates were then incubated for 1 h at 37 °C, 5% CO_2 in a humidified environment, allowing the cells to settle.

C.2.4. *Ex vivo* TRPV1 functional assay

A Fluo-4 NW Calcium Assay Kit (Invitrogen, Mulgrave, Vic, Australia) was used and reagents were prepared according to manufacturer's instructions. 96 well plates were removed from the incubator and, following a 10 min cooling period, 50 μl of the 2X probenecid dye loading solution was added to each well. The solution was mixed on a plate shaker (300 rpm) for 2 min at room temperature, followed by a further 30 min incubation at 37 °C, 5% CO_2, in a humidified environment and a 30 min incubation at room temperature. 10 min prior to the conclusion of room temperature incubation, 2 μM of A23187 was added to control wells (final volume 110 μl). Previous results from a similar experiment demonstrated that capsaicin caused a cytosolic Ca^{2+} elevation in rat neutrophils at concentrations ≥ 100 μM (Wang et al., 2005b). As such, 3 concentrations of capsaicin (100 - 300 μM) were used in the first experiment and 8 concentrations were used in a second experiment (1 - 13 mM) (final volume 110 μl). Fluorescence (a marker of intracellular calcium) was quantified on a BMG Polarstar microplate reader (BMG Labtechnologies, Offenburg, Germany) (Ex 485 Em 520).

C.3. Results

Experiment 1 examined the effect of 3 capsaicin concentrations, ranging from 100 - 300 μM (Figure C-1). The assay was successful, given that a strong signal was obtained from the positive control, however capsaicin did not induce a calcium influx at any concentration.

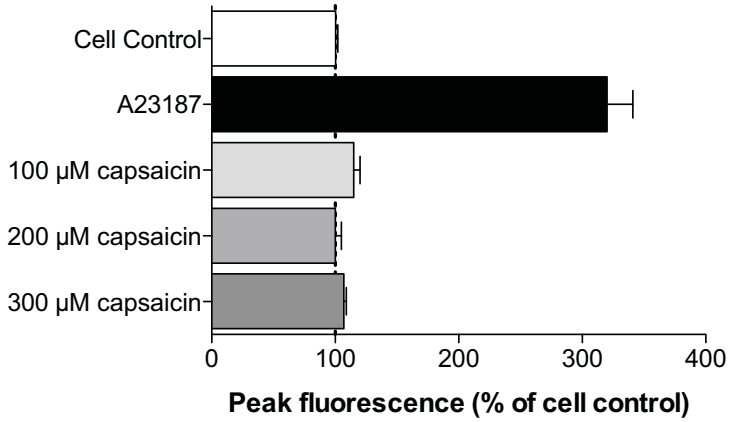

Figure C-1. Quantification of peak Fluo-4 fluorescence following addition of a capsaicin concentration range

Experiment 2 examined the effect of 8 capsaicin concentrations (1 - 13 mM) and found similar responses as experiment 1, in which capsaicin did not induce a calcium influx at any concentration (data not shown).

C.4. Conclusion

This study aimed to establish whether human mononuclear cells would respond to capsaicin *ex vivo* via TRPV1 and whether this response could be correlated with *in vivo* alterations in nociception following i.d. capsaicin administration. Whilst the assay was effective in detecting relative cytosolic calcium concentrations, capsaicin did not modulate cytosolic calcium at the concentrations tested (100 µM - 13 mM).

It should be noted that the current study deviates from previous work in a number of areas that may explain the disparity in findings (Wang et al., 2005b). This study examined human PBMCs in place of rat cells and it cannot be assumed that TRPV1 expression or function is identical across species. Furthermore, this study used a HP-β-CD capsaicin formulation in contrast to a DMSO formulation used previously. As such, any further work in this area should investigate lower capsaicin concentrations and alternate capsaicin formulations.

As this was to be the first functional study of TRPV1 in human PBMCs, it was not clear whether capsaicin would have any effect at all. Furthermore, the lack of expertise available for consultation on these assay methods may have resulted in lengthy optimisation process. Due to time constraints resulting from other unsuccessful *in vitro* experiments (see Appendix B), optimisation of the assay was abandoned in favour of the study conducted in Chapter 5.

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