The Translational Studies of Pain:

From Spinal Neurones to Human Perception

By

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I, Jessica O'Neill, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The discovery of new treatments for chronic pain relies on the detection of pre-clinical targets and the progression to successful clinical trials. In order to improve this transition reliable translational models must be identified, based on mechanisms that underlie the symptoms of chronic pain. This thesis aimed to validate the use of 3 potential translational models: topical capsaicin, ultraviolet irradiation (UVB) and UVB rekindling. Furthermore, using a mechanism based approach to treatment, the modulation of capsaicin induced sensitisation was explored in animals and humans.

In order to characterise the models in rats, in vivo electrophysiological recordings were made from single unit dorsal horn wide dynamic range neurones. Evoked responses to thermal, mechanical and electrical stimulation were quantified. To complement the animal studies, full QST profiling was undertaken on healthy human volunteers. Assessments of the pain thresholds were made, as well as numerical ratings to sub and supra threshold stimuli, in order to best compare these results with rodent data.

All of the models tested evoked similar sensory changes across species, and the symptoms induced in each of the models were used to infer the peripheral and central components. Sensory changes evoked by capsaicin included mechanical hypersensitivity accompanied by a facilitation of responses in the Aδ fibre range. These are reflective of both a peripheral and central sensitisation. Furthermore, these changes were prevented by pre-treatment with the adenosine receptor 1 (A1R) agonist, CPA. UVB appeared to be a strictly peripheral model, resulting in no secondary changes or receptive field expansion. On the other hand, the UVB rekindling model showed clear signs of engaging both peripheral and central mechanisms, including thermal allodynia, secondary brush hypersensitivity and a facilitation of Aβ fibre responses.

Overall, we confirmed that similar short-term sensory consequences, that may mimic certain pathophysiologies, could be engaged and quantified in rats and human volunteers in response to topical capsaicin, UVB irradiation and UVB rekindling. The UVB rekindling model induced signs of the engagement of a number of clinically relevant phenomena, such as peripheral inflammation/ sensitisation driving central modifications. As such this model will be useful in investigating mechanisms of inflammatory pain and assessing analgesic efficacy of novel medications.

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Abbreviations

5HT – 5-hydroxytryptamine (serotonin)

AC - adenylate cyclase

ADO - adenosine

ADP – adenosine diphosphate

AMPA - alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA – analysis of variance

ASIC – acid sensitive ion channel

ATP – adenosine triphosphate

BDNF - brain-derived neurotrophic factor

BK – bradykinin

CAMKII - Ca2+/calmodulin-dependent protein kinase II

Cavx – voltage gated calcium channel subtype x

CB- cannabinoid

CCI - chronic constriction injury

CCLx - chemokine (C-C motif) ligand x

CDT – cool detection threshold

CFA - complete Freund's adjuvant

CGRP - calcitonin gene related peptide

CNS - central nervous system

CPA - N6-cyclopentyladenosine

CPM – conditioned pain modulation

CPP - conditioned place preference

CPT – cold pain threshold

CXCLx - chemokine (C-X-C motif) ligand x

DAG- diacylglycerol

DH – dorsal horn

DDH – deep dorsal horn

DFNS - German research network on neuropathic pain

DMA - dynamic mechanical allodynia

DNIC – diffuse noxious inhibitory controls

DRG – dorsal root ganglia

δOR - delta opioid receptor

DPN - diabetic painful neuropathy

EEG – electroencephalography

EPSP - excitatory postsynaptic potential

ERK - extracellular signal-regulated kinases

fMRI - functional magnetic resonance imaging

GABA - γ amino butyric acid

GPCR - G-protein coupled receptor HIV - human immunodeficiency virus

HPT – heat pain threshold

IASP - international association for the study of pain

IB4 – isolectin B4

IBS - irritable bowel syndrome

IL – interleukin

IT - intrathecal

JAK – Janus kinase

KO – knockout

LC - locus coeruleus

LTP - long term potentiation

Lx – Lamina X of the DH

MAPK - mitogen-activated protein kinases

MDT - mechanical detection threshold

MED - minimal erthymal dose

MIA - monosodium iodoacetate

μOR - mu opioid receptor

MPS – mechanical pain sensitivity

MPT - mechanical pain threshold

NA - noradrenaline

Navx – voltage gated sodium channel subtype x

NGF – nerve growth factor

NK - neurokinin

NMDA - N-methyl-D-aspartate

NS - nociceptive specific

NSAID - non-steroidal anti-inflammatory drugs

OA - Osteoarthritis PAF- peripheral afferent fibres

PAG - periaqueductal grey

PG - prostaglandin

PHN - post herpetic neuralgia

PHS – paradoxical heat sensation

PI3K - phosphoinositide 3-kinase PK – protein kinase

PLC - phospholipase C

PPT – pressure pain threshold

QST - quantitative sensory testing

RA rheumatoid arthritis RTK – receptor tyrosine kinase

RVM - rostral ventromedial medulla

SNL - spinal nerve ligation

SNP - single nucleotide polymorphism

S/R – stimulus/ response

TMD - transmembrane domain

TNF-α - tumour necrosis factor

TrkA - tyrosine kinase A receptor

TRPxy - transient receptor potential cation channel subfamily x member y

TSL - thermal sensory limen

UVB - ultraviolet B

UVBR – UVB rekindling

VDT – vibration detection threshold

WDR - wide dynamic range

WDT – warm detection threshold

WUR – wind up ratio

Table of Contents

Table of Figures

Table of Tables

1. Introduction

1.1. Overview of Pain

Around 1664 Descartes proposed his alarm bell type model of pain. He postulated that the afferent nerve functioned like a tiny rope; the end triggered by damage in the skin and a sensor pulling on the cord located in a 'pain centre' in the brain:

"...ainsi que tirant l'un des bouts d'une corde on fait sonner en même temps la cloche qui pend à l'autre bout."

Although rather simplistic, this biomedical model conjures a wonderful image and captures the essence of the protective nature of pain. The somatosensory phenomenon can indeed result from the transmission of signals from peripheral nociceptors, initiated by tissue damage. A signal that is relayed through the spinal cord and reaches the somatosensory cortex in the brain, where it may be interpreted as painful. However, it is now well recognised that pain also incorporates psychological, social, and behavioural aspects. Furthermore, whilst this kind of alarm bell warning signal is necessary for survival, when the pain outlasts the initiating stimuli and becomes chronic it leads to great suffering.

Figure 1-1 'Traité de l'Homme'. Descartes' original proposition of the pain pathway.

1.1.1. Pain defined: A complex aetiology with multiple co-morbidities

Aristotle described pain as *"the passion of the soul"*, an early description which begins to hint at the subjective, conscious nature of the experience. Pain usually results from a nociceptive input, which is subsequently modified by a number of affective emotional and cognitive evaluative factors. The initial intensity of a given sensory stimulus is therefore not necessarily proportional to what is perceived by the subject. Whilst nociception is generally accepted to describe the neuronal activity, pain refers to the additional integration of complex emotional factors. This is reflected in the International Association for the Study of Pain (IASP) definition, which describes 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). In recent years it has become apparent that pain is also influenced by genetic factors, which contribute to the variation in perception between individuals (Mogil et al. 1999; Binder et al. 2011; Sikandar et al. 2013). This variable relationship between stimulus and percept often creates difficulties not only with regards to measurement, but also more importantly to the management and treatment of pain, particularly for those affected by chronic conditions.

Pain can be classified in a number of ways. Whilst clinicians sometimes use a disease-based classification, scientists more often try to classify pain on the basis of underlying mechanisms (Jensen and Baron 2003). Thus, several broad categories are recognised. These are: nociceptive pain, inflammatory pain, neuropathic pain (and syndromes that combine these), and a less well understood category referred to as 'dysfunctional' or 'generalised' pain. Nociceptive pain is that which transiently activates the nociceptive system, for instance stubbing one's toe. Inflammatory pain is associated with the release into tissues of inflammatory mediators, which not only activate but also sensitise the pain signalling system. It includes conditions such as rheumatoid arthritis (RA) and osteoarthritis (OA), as well as post-operative pain. Neuropathic pain is usually the result of a lesion or disease located at any point of the neuraxis – from the peripheral to the central nervous system (CNS). This category commonly includes disorders of the peripheral nervous system such as post herpetic neuralgia (PHN), diabetic neuropathy (DPN), or human immunodeficiency virus (HIV) related neuropathies. In all these cases, the pain appears to be related to damage of peripheral nerves. Neuropathic pain can however also arise following damage to the CNS; such as spinal cord injury, multiple sclerosis, and stroke. Cancer pain often includes a mixture of mechanisms with a contribution of space-occupying tumours, a release of inflammatory mediators and compression or invasion of nerves (Sikandar et al. 2013). Finally, generalised

pain refers to conditions that have no obvious tissue or nerve damage, and includes fibromyalgia, irritable bowel syndrome (IBS), and tension headaches.

All classifications of pain may be acute or chronic, the latter being defined as pain persisting for 3 (or 6) months, in duration (McMahon et al. 2013). Although it may be considered pathological when it outlasts a nociceptive stimulation, or is present in the absence of external stimuli, it may equally arise from a defined stimulus such as in OA, RA or nerve injury. Acute pain is necessary for survival, in order to drive protective reflexes and help avoid harm. This function provides considerable benefit, as evidenced for instance by the rare cases of congenital insensitivity to pain (resulting from mutations in genes such as SCN9A and those encoding NK1 receptors), which are potentially fatal since sufferers are particularly prone to burns, fractures and other such physical injuries (Cox et al. 2006; Sikandar et al. 2013). While pain may therefore be considered an adaptive behaviour, the development into a chronic state on the other hand may certainly be deemed maladaptive. When the acute responses outlast the initiating stimuli, and persevere long beyond the time that such sensations serve any useful purpose, unrelenting suffering can occur with a consequential decrease in quality of life. Chronic pain involves the emergence of changes in normal pain processing and thus involves different mechanisms to those in normal pain sensing. As a result it may be more difficult to manage and treatments are often associated with greater side effects.

In healthy volunteers, stimuli applied to peripheral tissues such as skin or muscle produce pain typically when the stimulus intensity rises to a level that might damage tissues, such as skin temperatures above 45°C. With even stronger stimuli, the perceived pain rises monotonically with stimulus strength. However, many chronic pain patients show stimulus response relationships that are shifted leftwards and upwards. In other words, for a given degree of painful stimulus, the chronic pain patient feels more intense pain than they would if healthy. The term for this phenomenon is hyperalgesia. Allodynia is a term that refers to the condition where a normally non-painful stimulus begins to evoke the sensations of pain. For example, the simple process of brushing ones hair becomes exquisitely painful. Allodynia is not simply an extension of hyperalgesia since it may reflect activity in innocuous sensory receptors being abnormally interpreted as pain.

Whilst chronic pain serves as an umbrella term, encompassing a wide variety of clinical features such as hyperalgesia and allodynia, these features can be broken down further into the modality affected. It is clear that different symptoms are the manifestation of distinct underlying mechanisms. For example, hyperalgesia to thermal stimuli may have different

mechanisms to mechanical hyperalgesia, and cold allodynia most certainly differs in its origins from dynamic mechanical allodynia (DMA) (Dworkin et al. 2003; Baron 2006; Baron et al. 2010). Furthermore, although there may be an association between mechanisms and symptoms, there is not necessarily any one specific mechanism which can be assigned to a single symptom and there are likely to be a number of mechanisms which can lead to the same symptom (Jensen and Baron 2003). Different symptoms therefore have different pharmacological sensitivities and as such it is unrealistic to attempt to find one treatment that will suffice for all chronic pain sufferers. Moreover, it seems likely that a mechanism-based approach to treatment will bring more success into helping patients improve their quality of life.

These mechanisms will be discussed in further detail throughout this thesis, however as it stands we still do not have a full understanding of the mechanisms underpinning chronic pain and as such many patients find their treatment inadequate. It is estimated up to one in five of the adult population in Europe suffer (Breivik et al. 2006). Similar figures were found in a more recent evaluation of non-cancer related moderate-severe pain in Europe, where it was established that 19% of the population were affected (Reid et al. 2011). In Britain it is believed that up to 10 million suffer from pain each day (BPS 2005). Furthermore, it was shown that up to 40% of the patients studied receive inadequate treatment, highlighting the large gap in pharmacological and non-pharmacological interventions for such conditions (Breivik et al. 2006; Attal et al. 2011).

This treatment shortfall may have a direct impact on patient's everyday lives, inhibiting normal daily activities such as going to work and socialising, eventually leaving some completely disabled. In addition to this suffering, many clinical studies have shown there to be a correlation with affective disorders in patients suffering from chronic conditions. Common co-morbidities include anxiety, depression and sleep disturbances (Gormsen et al. 2010; Rehm et al. 2010), with up to 36% of patients reporting that chronic pain has a further negative impact on their family and friends (Baker 2010). Furthermore, the direct and indirect cost to the economy as a result of chronic pain is estimated to be up to €200 billion in Europe alone (Baker 2010). Thus, there are a number of pressing reasons for better management and treatment of chronic pain. Knowledge of the underlying mechanisms is imperative for the development of such pharmaceuticals and as such this thesis will focus on the exploring translational models with mechanisms that are likely to contribute to chronic pain.

1.1.2. Pain is detected by a specific set of primary sensory neurones

Pain is most commonly initiated by excitation of specialised peripheral afferent fibres (PAFs), named nociceptors (Sherrington 1900). These sensory neurones detect noxious stimuli of chemical, mechanical and thermal nature. They include unmyelinated C fibres and myelinated Aδ fibres (Burgess and Perl 1967; Bessou and Perl 1969; Meyer et al. 2006) and under certain pathological conditions it is believed that myelinated Aβ fibres may also contribute to the sensation of pain (Treede and Cole 1993; Latremoliere and Woolf 2009). The thick myelinated Aβ fibres are the fastest conductors, with velocities (in humans) of around 30-80 m/s, whereas the thinly myelinated Aδ fibres conduct around 6-30 m/s, and finally C fibres are the slowest with velocities around 0.5-2 m/s. These somatosensory afferents innervate virtually all tissues of the body. Their cell bodies are found mainly within the dorsal root ganglia (DRG), or the trigeminal ganglion. They have bifuricating axons, allowing branching into peripheral tissues and into the dorsal horn (DH) of spinal cord (or brainstem) where they synapse with second order projection neurones. The action potentials arising from nociceptor terminals are relayed to the CNS where they are integrated and interpreted in a manner that motivates behaviour aimed at limiting the damaging influence of the noxious stimulus.

Fibres may be classified by their distinct functions or expression patterns. C fibres are the smallest of the primary afferents and unmyelinated, which renders them the slowest in conducting. Activation of C fibres results in a burning, pricking, tingling or warm sensation (Mouraux et al. 2010). Studies of rodent cutaneous nociceptors suggest they can be broadly split into two classes depending on the genes they express and therefore their peripheral sensitivity, and their central termination patterns (Snider and McMahon 1998). These two groups, roughly equally numerous, are commonly referred to as peptidergic and non peptidergic, as the former but not the latter normally express neuropeptides such as substance P, calcitonin gene related peptide (CGRP) and somatostatin (Snider and McMahon 1998). Non-peptidergic neurones contain fluoride-resistant acid phosphotase and stain positively for the plant binding lectin isolectin B4 (IB4) (Silverman and Kruger 1988). Peptidergic neurones are also characterised by their expression of the high affinity receptor for nerve growth factor (NGF), tyrosine kinase A receptor (TrkA), whereas non peptidergic are generally found to express glial cell line-derived neurotrophic factor receptors such as GFRα1-4 and receptor tyrosine kinase Ret (Averill et al. 1995; Bennett et al. 1998; Orozco et al. 2001). More recently it has become clear that there is a significant population of low-

threshold C fibres that are more common in proximal body sites that may mediate pleasant touch sensations (Löken et al. 2009).

Aδ fibre stimulation can lead to tingling and pricking sensations, as well as that of light touch (Mouraux et al. 2010). All of these afferents are believed to express neurofilament-200 and the brain-derived neurotrophic factor (BDNF) receptor (Priestley et al. 2002). These fibres can also be split into two subgroups, which are both thinly myelinated; type I are generally found to respond to heat greater than 50°C, whereas type II have a lower threshold around 42-45°C (Treede et al. 1995). The high threshold Aδ and C fibres are collectively thought of as nociceptors. The differences in neurotransmitters/neuromodulators, receptors and sensitivities of nociceptors suggests that they all contribute to pain signalling in slightly different ways and each have their own specific subtle functions.

1.1.3. Stimuli are transduced via specific receptors on nociceptor peripheral terminals

The ability to detect and transduce potentially damaging external stimuli is required for survival. This process is carried out by a myriad of receptors (detailed in table 1-1), which are found on the unencapsulated nerve endings of peripheral nociceptors. Transduction converts stimuli into an electrical signal known as a generator potential, which can drive action potentials, and allow the message to be relayed to the spinal cord and higher centres. Although there are a number of ways in which nociceptors can be classified, one way to do so is by the range of stimuli that the peripheral afferent responds to. This classification system includes: polymodal nociceptors (responding to mechanical, thermal and chemical stimuli); C-heat, C-mechano-heat, C-mechano-cold fibres; mechanically insensitive afferents (do not, at least in normal conditions, respond to mechanical stimuli) (Meyer and Campbell 1988; Davis et al. 1993; Schmidt et al. 1995; Weidner et al. 1999).

Polymodal afferents express a number of receptors detailed in the table 1-1 below. They may be ionotropic or metabotropic and activation generates either inward or outward currents, resulting in a depolarisation or hyperpolarisation of the afferent, respectively. Ionotropic receptors allow either an influx or efflux of certain ions, whereas metabotropic G protein coupled receptors activate specific signalling cascades and second messengers. It is important to note that with regards to mechanotransduction, the receptors remain poorly defined (Coste et al. 2012).

Stimulus Type	Stimuli	Candidate Receptor	Receptor Type
Thermal	>42C, Capsaicin	TRPV1	Ionotropic
	Heat	TRPV2-4	Ionotropic
	Cold, Menthol	TRPM8	Ionotropic
Mechanical		Unknown ASIC? TRP? Piezo 1-2?	
Chemical	H^+	TRPV1/ASIC	Ionotropic
	Ci;8nnamaldehyde, Mustard Oil, Formalin	TRPA1	Ionotropic
	ATP/ADP	P2X	Ionotropic
	Adenosine	A1-4	Metabotropic
	Bradykinin	BK1-2	Metabotropic
	Prostaglandins	EP1-4	Metabotropic
	Histamine	H1	Metabotropic
	Serotonin	5HT3	Ionotropic

Table 1-1 The major mammalian receptor types located on peripheral afferent nociceptors. Thermosensation is governed almost exclusively by TRP channels, whilst chemosensitivity is dictated by the expression of a number of different channels. Suggested candidate mechanotransducer molecules includes TRPs, potassium channels, Piezos, and ASICs, although this process is not yet fully understood (Basbaum et al. 2009).

1.1.4. TRP Channels

Transient receptor potential (TRP) channels are one of the largest families of ion channels and have a wide variety of functional roles. In 1969 Cosens and Manning isolated a mutant photoreceptor from the drosophila, which caused the specimen to become blind upon exposure to bright light (Cosens and Manning 1969). This was the first TRP channel to be discovered and since then 28 mammalian isoforms have been identified, which are split into 7 different subfamilies (Clapham 2003; Venkatachalam and Montell 2007). They are made up of 6 transmembrane domain (TMD) polypeptide subunits, which assemble as tetramers that can form pores in the cell membrane (Ramsey et al. 2006).

Currently, they are one of the most extensively studied families of ion channels present in sensory neurones. The seven sub-families include: TRPV, TRPC, TRPM, TRP TRPML, TRPA and TRPP (Venkatachalam and Montell 2007). TRPM8 and A1 are thought to be involved in cold sensing (McKemy et al. 2002; Story et al. 2003), whereas seven others are activated by heat, over a distinct range of temperatures: TRPV1-4, TRPM2, TRPM4 and TRPM5 (Venkatachalam and Montell 2007). Collectively, these nine channels are expressed on Aδ and C fibres and are known as thermoTRPs. They are activated at different ranges of temperature, both noxious and non-noxious (Venkatachalam and Montell 2007). It has been suggested that TRPV1 and TRPM8/TRPA1 are the first to detect noxious hot and cold stimuli, respectively, with activation thresholds of 42°C for TRPV1 and 14°C for TRPA1 (McKemy et al. 2002; Dhaka et al. 2006), thus activation of one of these receptors is proposed to lead to the perception of hot or cold thermal pain. TRPV2 on the other hand appears to be expressed on Aδ rather than C fibres, and has the highest threshold for activation (over 52°C) and contributes to noxious heat perception (Caterina et al. 1999).

1.1.5. The TRPV1 Receptor

The transient receptor potential vanilloid type 1 (TRPV1) receptor is a non-selective ligand gated ion channel. It is an integrator of many physical and chemical stimuli, including capsaicin and noxious heat (>43 °C), as well as being activated by protons (pH <5.2), endogenous lipids and certain inflammatory mediators (Szallasi and Blumberg 1999). All compounds are lipophilic and therefore act at intracellular binding sites. Stimuli are detected and transduced through opening of the ion channel, which results in entry of cations such as Na^+ and Ca^{2+} to the neurone; it has been shown that although the channel is non-selective, it has a high permeability to Ca^{2+} (Caterina et al. 1997).

TRPV1 is believed to exist as a homo or heteromeric complex consisting of 4 subunits, with a pore-forming hydrophobic stretch between TMDs 5 -6 (Caterina et al. 1997; Moiseenkova-Bell et al. 2008). The presence of specific amino acid residues are required for sensitivity to different stimuli; it is believed that Y511 and S512 located between TMDs 2-3 dictate vanilloid/capsaicin sensitivity since mutations to tyrosine or alanine render the channel capsaicin insensitive (Jordt and Julius 2002).

The receptors are known to be expressed on primary sensory neurones. They have been detected on terminals of small- to medium- diameter nociceptors, such as peptidergic and

non-peptidergic C fibres, as well as some Aδ fibres (Caterina et al. 1997). Caterina and colleagues demonstrated that capsaicin sensitivity is eliminated in TRPV1$^{-/-}$ mice, though interestingly they only display impaired heat detection and reduced thermal hypersensitivity in response to inflammatory agents (Caterina et al. 2000). This highlights the importance of TRPV1 for heat sensing and the induction of thermal hyperalgesia in inflammatory states, whilst suggesting there may be other receptors also involved.

Recently, the function of TRPV1 expressing afferents has been explored with the profiling of TRPV1-DTA mice, generated by Mishra and colleagues. As noted, it was previously shown that TRPV1 knockout (KO) mice maintained some thermosensation, which was only impaired over 50°C (Caterina et al. 2000). However, these mice, whose TRPV1 afferents are completely ablated, have no response to capsaicin and are also totally insensitive to both hot and cold (Mishra and Hoon 2011). The lack of cold sensitivity is believed to be due to co-expression of the channels early on in development, which is normally lost during adulthood (McKemy 2011). The mice also exhibited no hypothermia in response to intraplantar capsaicin, which is seen in normal animals. This suggests that the group of TRPV1 positive afferents are imperative in the detection of noxious heat and thermoregulation.

1.1.6. The TRPM8 Receptor

Another member of the TRP family, TRPM8, may be more important for the detection of cooling (30-15°C) and noxious cold (<15°C) (McKemy et al. 2002). TRPM8 is activated by menthol, which has been shown to cause cooling and eventually irritation and pain when applied to the skin of human volunteers (Wasner et al. 2004). TRPM8 is a non-selective cation channel and activation generates currents required for cold sensing. TRPM8 KO mice show deficiencies in cold detection, as well as impaired development of cold hypersensitivity, suggesting that the receptor is indeed involved in cold sensing and the pathological sensations (Bautista et al. 2007; Colburn et al. 2007; Dhaka et al. 2007).

A second channel suggested to take part in detection of cold stimuli is TRPA1, which does not appear to have any co-localisation with TRPM8 and thus may contribute to cold sensing in a separate set of neurones (Story et al. 2003; Kwan et al. 2006). However, the data is conflicting as although some studies have shown cold induced Ca^{2+} influx through TRPA1, others were unable to reproduce this and thus the full contribution of TRPA1 in cold sensing remains controversial (Story et al. 2003; Jordt et al. 2004). Since there are also a number of neurones

which do not express either TRPM8 or TRPA1, but are cold sensitive, this suggests that other channels must also be involved in cold sensing (Munns et al. 2007).

Together TRPV1 and TRPM8 can sense temperatures from 42°C+ and <15-30°C (Caterina et al. 1997; McKemy et al. 2002); a range which may be expanded under certain pathological conditions. Despite the indicated function in the detection of heat and cold pain, with possible involvements in chronic pain, it has also been suggested that when simultaneously activated their actions may oppose one another. Premkumar and colleagues found that intraplantar injection of both capsaicin and menthol reduced nocifensive behaviour in mice, in comparison to capsaicin alone (Premkumar et al. 2005). They suggest that TRPM8 activation may indeed be able to counteract that of TRPV1.

1.1.7. Subsets of peripheral sensory neurones respond to distinct stimuli

Although it is believed that many nociceptors are polymodal and respond to a number of different stimuli, it has recently been suggested that in mice there are a specific set of peptidergic fibres which are important in the detection of heat pain, and a set of non-peptidergic which contribute to mechanical pain (Abrahamsen et al. 2008; Cavanaugh et al. 2009; Cavanaugh et al. 2011; Minett et al. 2012). These data suggest that modality specific pain behaviours are generated by different subsets of nociceptors. In addition they showed that each set was carried into a different section of the DH. It was suggested that nociceptors that respond to mechanical stimuli are mainly fed into lamina I (LI), whereas those responding to heat were terminating in deeper sections of the DH (Cavanaugh et al. 2011). This indicates that perhaps there are segregated pathways for the different stimuli at least at the peripheral level (this is not to say that integration will not take place further up the pain pathway).

In addition to this specificity in transmission, it was further suggested that the sets of nociceptors have separate regulatory controls (Cavanaugh et al. 2011). In addition to modulation by γ amino butyric acid (GABA)ergic interneurones, peripheral afferents are also under the control of endogenous opioids (Basbaum and Fields 1984). Bausbaum and colleagues proposed that in mice, peptidergic neurones express mainly the mu opioid receptor (μOR), whereas the non-peptidergic express the delta opioid receptor (δOR) (Cavanaugh et al. 2011). The implication of this is that it would be possible to control for different pain modalities by targeting these different receptors. For example, a patient

suffering from a mechanical hypersensitivity could be treated with a δOR agonist, whereas a heat hypersensitivity may respond better to a μOR agonist. However, as this study was conducted only in mice, it is not certain that such specificities would translate into humans. It may be interesting to investigate this hypothesis further in human models of heat and mechanical sensitivity to identify any translational hurdles.

1.1.8. Primary afferent action potentials require voltage gated sodium channels

Activation of a sensory nerve terminal as a consequence of a stimulus activating receptors at the terminal membrane leads to membrane depolarization. There has been considerable speculation about the functional role of different voltage gated ion channels in the propagation of noxious stimuli. This has been fuelled in part by the selective expression of three sodium channel subunits on nociceptors, Nav 1.7, 1.8 and 1.9 (Dib-Hajj et al. 2010). Nav 1.7 is expressed in peripheral sensory neurones and has fast activation and inactivation kinetics (Toledo-Aral et al. 1997); without this inactivation the channel is predicted to produce prolonged bursts of activity, leading to increased nociceptive signalling. Whilst Nav 1.9 is believed to be responsible for the resting potential, Nav 1.7 and is believed to amplify slow subthreshold generator potentials towards the threshold for an action potential (Dib-Hajj et al. 2010; Dib-Hajj et al. 2013). This signal recruits Nav 1.8, a channel almost selectively expressed in nociceptors, which is crucial for the upstroke of these action potential and thus transmission (Stirling et al. 2005; Momin and Wood 2008). Humans carrying null mutations in Nav 1.7 exhibit a congenital insensitivity to pain, whilst other mutations affecting the fast inactivation can lead to conditions such as paroxysmal extreme pain disorder (Cox et al. 2006; Fertleman et al. 2006; Sikandar et al. 2013). These findings have of course generated considerable evidence in the role of this channel. Although out of scope of this thesis, further discussion on the roles of these channels can be found in Dib-Hajj et al. (Dib-Hajj et al. 2013).

1.1.9. Chemical mediators in the periphery may lead to nociceptor sensitisation

Evidence supports the general hypothesis that persistent or chronic pain is, in part, due to an increase in the excitability of the peripheral terminals of fine calibre afferents to a given stimulus intensity (Baron 2006; Basbaum et al. 2009; Gold and Gebhart 2010; Baron et al. 2013). This process, dubbed peripheral sensitisation, has been much studied (Basbaum et al. 2009; Gold and Gebhart 2010). There are a myriad of chemical stimuli that can activate

nociceptor endings, including both endogenous and exogenous substances. The equilibrium of chemicals in the environment surrounding the nerve terminals determines the baseline sensitivity and thus the normal phenotype (Basbaum et al. 2009; Gold and Gebhart 2010).

Inflammation is a complex biological response and classical signs include - dolor (pain), calor (heat), rubor (redness), tumor (swelling), in addition to a possible functio laesa (loss of function). Tissue damage is often accompanied by a release of endogenous mediators from activated nociceptors, as well surrounding non-neural cells such as mast cells, basophils, platelets, macrophages, neutrophils, endothelial cells, keratinocytes, and fibroblasts (Basbaum et al. 2009; Gold and Gebhart 2010; Sikandar et al. 2013). Numerous activating and sensitising substances can be released in an inflammatory immune response often referred to as a cocktail or 'inflammatory soup'. Recognised components of this 'soup' include adenosine triphosphate (ATP), bradykinin (BK), NGF, prostaglandins (PGs), serotonin (5HT), protons and other pro-inflammatory cytokines and chemokines such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), chemokine (C-C motif) ligand 3 (CCL3) and chemokine (C-X-C motif) ligand 5 (CXCL5) (Zhang and Oppenheim 2005; Jin and Gereau IV 2006; Dawes et al. 2011; Sikandar et al. 2013). The hallmarks of such sensitisation include decreased activation thresholds and an increased frequency of action potential discharge to suprathreshold stimuli.

That an increase in peripheral excitability is at the root of certain chronic pain syndromes raises the possibility that drugs acting on these chemical messages may be developed that remain outside CNS, thereby avoiding some of the complications of present therapies. A large number of non-redundant mechanisms have been causally associated with increasing the excitability of sensory nerve terminals (Pethő and Reeh 2012). Activation of certain metabotropic receptors, discussed below, can lead to signalling events that converge on a few downstream second messengers, which ultimately interact with ion channels.

Key players include G-protein coupled receptors (GPCRs), TrkA activation and other tyrosine kinase receptors (RTKs)(Basbaum et al. 2009; Gold and Gebhart 2010). The second messenger systems involved post-activation of G protein coupled receptors are largely regulated by adenylate cyclase (AC)/protein kinase A (PKA) and phospholipase C (PLC)/ protein kinase C (PKC). Activation of TrkA receptors engages mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K) and PLC signalling pathways, whilst RTKs are linked to p38 MAPK, Janus kinase (JAK) and Stat transcription factors. The downstream effect of these messengers involves transcriptional, translational and post-translational

modifications to a number of ion channels (Huang et al. 2006; Basbaum et al. 2009). This may include modulation of ion channels through phosphorylation or methylation, Ca^{2+} mobilisation, altered expression of channels, receptors or enzymes. Whilst mediators such as BK, PG's, 5HT and numerous chemokines act at GPCRs (their respective actions depend on the type of G protein activated), many cytokines and neurotrophic factors such as NGF, BDNF, IL-1 and TNF-α act at RTKs (Basbaum et al. 2009).

K^+ channels are vital for normal neuronal function; setting the resting potential of the cells and regulating action potentials. Downstream messengers that interact with these channels may inhibit currents that are at least partially open in the resting state and depolarize the membrane, driving it closer to activation threshold. In addition, by inhibiting the channel activity, the membrane resistance would be increased. This in turn may increase the amplitude of the membrane depolarization for any stimulus that would open depolarizing ion channels. Mediators that block K^+ channels would therefore not necessarily activate the nerve, but may shift the stimulus-activation response curve leftward and upward; a process consistent with the hypersensitive state. Indeed, numerous K^+ channels have been shown to be downregulated in models of neuropathic pain, whilst an overexpression of channels such as Kv1.2 can induce neuropathic pain symptoms (Kim et al. 2002; Zhao et al. 2013). Suggesting that dysregulation of K^+ channels may contribute to neuronal hyperexcitability.

On the other hand, a facilitation of voltage gated sodium channel currents would also lead to a similar lowering in activation threshold of the neurone and a hypersensitive state - indeed it has been shown that Nav 1.7 activity/ expression is increased during some inflammatory and neuropathic like states (Black et al. 2004; Shields et al. 2012). Indeed, in the presence of PGE2 Nav 1.7 may be activated, most likely through lowering of activation thresholds (Vanegas and Schaible 2001). For example, MAPK1 and -3 mediated phosphorylation of Nav 1.7 enables smaller currents to activate the channel (Vanegas and Schaible 2001; Nassar et al. 2004). Interestingly, an R1105W variant of the channel is associated with an increase in C fibre activity, which may render carriers 'predisposed' to developing hypersensitive states. This variant is already associated with OA, and it may be useful to further explore its role in other inflammatory conditions (Reimann et al. 2010; Estacion et al. 2011). Additionally, a gain of function mutation G1662S in Nav 1.8 has been found to be associated with small fibre neuropathy (Han et al. 2013). Taken together, genetic evidence supports the theory that a facilitation of Na^+ channel currents may lead to hyperexcitability associated with chronic pain conditions.

1.1.10. The role of TRPV1 in enhanced pain sensing

The role of TRPV1 has been extensively studied in persistent pain states. In general, the thermal hyperalgesia that develops in several pathological states depends critically on sensitisation of TRPV1 (Huang et al. 2006; Kanai et al. 2007). A growing list of peripheral mediators - including BK, histamine, NGF, PGs, protons, IL-1β, IL-6, CCL2 and CCL3 - can modulate TRPV1. TRPV1 actions may be potentiated through phosphorylation of the receptor resulting in altered channel kinetics, or by increasing the surface expression of receptors (Ji et al. 2002; Moriyama et al. 2005; Huang et al. 2006; Gold and Gebhart 2010). The latter may be most clinically relevant and occurs either through an increase in transport or in number of receptors produced and inserted into the membrane.

Protons are able to both directly activate and potentiate activity of TRPV1. During a state of tissue injury or ischemia, where proton levels may be elevated, hydrogen ions are thought to act at an extracellular site to increase the potential of channel opening (Jordt et al. 2000). PGs such as PGE2 and PGI2 act at EP1 or IP receptors respectively. They have been demonstrated to interact with TRPV1 through both PKC and PKA dependent pathways, resulting in phosphorylation of the receptors, and thus lowering of the temperature activation threshold to as low as 35°C (Moriyama et al. 2005). BK acts on the B1 and B2 receptors, which are believed to activate the diacylglycerol (DAG)-PKC pathway, and thereby the phosphorylation of TRPV1 (Vellani et al. 2001; Vellani et al. 2004). ATP is released from injured cells and its actions at the P2X/P2Y2 have been implicated in TRPV1 sensitisation via a PKC-dependent pathway (Moriyama et al. 2003).

A second method of potentiating the actions of TRPV1, as mentioned, is by increasing the surface expression of receptors either through an increase in trafficking and/ or receptor density (i.e. the number of receptors produced or inserted into the membrane) (Ji et al. 2002; Zhang et al. 2005). In inflammatory conditions, such as OA, NGF is released and contributes towards pain through actions at TrkA receptors, which are expressed on specific sensory neurones such as C and Aδ fibres. NGF is able to recruit a number of intracellular pathways and the importance of this mediator is highlighted by the fact that a loss of function of TrkA leads to an insensitivity to pain all together (Indo et al. 1996; Shu and Mendell 1999). Injection of complete Freund's adjuvant (CFA) and the monosodium iodoacetate (MIA) model of OA have been shown to result in increased TRPV1 expression in the DH (Ji et al. 2002). Zhang et al demonstrated that TrkA induced activation of PI3K/ Src kinase causing phosphorylation of the Y200 residue, which resulted in increased membrane expression of

TRPV1 (Zhang et al. 2005). NGF also increases transcription of TRPV1, and may induce translation via p38 MAPK activation (Ji et al. 2002; Xue et al. 2007). Peripheral sensitisation of TRPV1 receptors, which results in lowering of the activation temperature (perhaps even to body temperature), may occur in a number of inflammatory pain conditions.

Although the real contribution of TRPV1 in chronic pain remains unclear, a small number of patients do exhibit heat hypersensitivity (Maier et al. 2010; Soni et al. 2013). Furthermore some patients, for instance those with inflammatory bowel disease and irritable bowel syndrome, have increased expression of the receptor (Akbar et al. 2010). The same has been claimed for some chronic cough patients, which is known to have similar underlying pathophysiology to chronic pain (Groneberg et al. 2004; Mitchell et al. 2005; O'Neill et al. 2013). In addition, the fact that chronic cough patients are more sensitive to inhaled capsaicin suggests that TRPV1 sensitisation may be a contributing factor underlying some hypersensitivity, whilst children carrying the TRPV1 single nucleotide polymorphism (SNP) I585V (decreasing channel activity) appear to be at a lower risk of developing asthma related cough (Cantero-Recasens et al. 2010). Taken together, these findings suggest TRPV1 could hold potential as a therapeutic target for relief of symptoms in chronic pain. Sensitisation of TRPV1 can be induced by application of capsaicin. This allows further study of the consequences, mechanisms and pharmacological sensitivity. As such this model will be explored further in this thesis in chapters 3 and 4.

1.1.11. Primary afferents synapse with second order projection neurones in the dorsal horn

PAFs enter the DH of the spinal cord via the dorsal root entry zones, where they synapse with cells such as second order neurones or spinal interneurones. DH neurones receive convergent sensory input from a number of neurones responding to different modalities (Todd 2010). The distribution pattern of afferents terminating within the DH is determined by their sensory modality and the body region innervated (Todd 2010). Within the spinal cord the signal is integrated and processed - the initial input may be modulated by other incoming peripheral fibres, through a number of interneuronal connections both within or between, laminae, and additionally by fibres descending from the midbrain (D'Mello and Dickenson 2008). Non-neuronal cells such as astrocytes and microglia may also impact the output of DH

neurones (McMahon and Malcangio 2009). All of these processes that contribute towards the modulation of spinal neurone activity are plastic, as will be discussed later.

The DH is divided into 10 laminae (LI-X), which receive and process inputs. Within the each laminae PAFs synapse with second order neurons. Whilst, Aδ and peptidergic C fibres terminate in LI of the DH and the outermost part of LII (substantia gelatinosa), non peptidergic C fibres terminate in the inner part of LII (Todd 2010). The majority of Aβ fibres are believed to terminate in the deep DH (LIII-V), and some Aδ also terminate here in LV. Furthermore, in addition to local spinal connects there are also indirect connections are made between LI and LV, and neurones residing in LI are able to modulate LV through the recruitment of descending control mechanisms (Bee and Dickenson 2008). Thus LV receives input from all types of sensory fibres and can respond to all types of stimuli from a range of non-noxious to noxious, mechanical, chemical and thermal (figure 1-2).

Depolarisation of peripheral sensory fibres leads to the activation of voltage gated calcium channels such as Cav2.2 within their central terminals, which is believed to be pertinent in the pain pathway (Woolf and Ma 2007). This further initiates the release of neurotransmitters from the presynaptic terminals – in the case of sensory fibres all release glutamate as the main excitatory transmitter, but peptidergic C fibres may also release CGRP, substance P, BDNF, somatostatin and galanin (Dickenson 1995). Glutamate activates post-synaptic N-methyl-D-aspartate (NMDA) receptors, as well as alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors. Acute transmission of nociceptive information relies on the ionotropic AMPA and kainate receptors, however the NMDA receptor has been the focus of many pain studies as it has been shown to be upregulated and sensitised in many chronic pain states (Brown and Krupp 2006). Activation of post-synaptic receptors leads to the activation of second order neurones and allows transmission of the nociceptive signal to higher centres.

Many DH neurones respond to noxious stimuli applied to specific body sites. Cells that respond selectively to noxious stimuli are known as nociceptive specific neurones (NS), whilst those that are activated by innocuous as well as noxious stimuli are known as wide dynamic range neurones (WDR). Some of the NS and WDR neurones have axonal projections to supraspinal sites, while others are interneurones. These interneurones may be excitatory (releasing glutamate) or inhibitory (releasing GABA or glycine) and play an important role in local processing of sensory information. NS cells are mainly located in LI, whilst LIII-IV respond mainly to non-noxious tactile stimuli (D'Mello and Dickenson 2008). The majority of

WDR cells are located in LV (D'Mello and Dickenson 2008), which will be the main area of study in this thesis. In addition to producing graded responses to a wide range of stimuli, LV WDR neurones display a unique characteristic known as wind-up (Dickenson and Sullivan 1987).

The phenomenon of wind-up is a frequency dependent increase in central neuronal responses, whereby repetitive stimulation of peripheral fibres enhances the output of spinal cord neurones. Such activity dependent plasticity depends on the co-release of neuropeptides from afferent C fibres, which leads to a slow membrane depolarisation that summates to release the Mg^{2+} block of the NMDA receptor. Silencing of NMDA receptors is usually mediated via Mg^{2+} block of the ion channel pore. Since wind-up may be blocked by the administration of AP5 and ketamine, it is accepted that the process relies on recruitment of the NMDA receptor (Davies and Lodge 1987; Dickenson and Sullivan 1987). Notably, wind up is both frequency and intensity dependent, since the stimulus must be above C fibre intensity and applied at 0.3Hz or more (Mendell and Wall 1965). The perceptual correlate of wind up can also be measured in humans, whereby it is known that brief, repetitive stimulation to a train of stimuli results in a greater pain responses at the end (Price et al. 1994; Magerl et al. 1998). This is often referred to as temporal summation, and is also blocked by NMDA antagonism suggesting overlapping mechanisms (Price et al. 1994). These features of wind-up and intensity coding make WDR cells attractive to study as responses are comparable with human pain ratings (Dickenson and Sullivan 1987; LaMotte et al. 1991; Simone et al. 1991; Price et al. 1994; Sikandar et al. 2013).

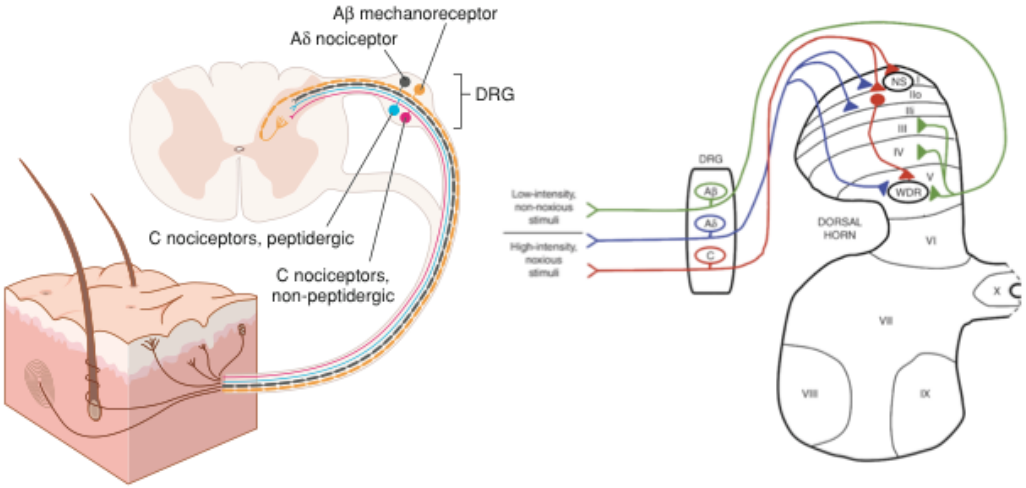

Figure 1-2 Primary afferents synapse in the DH of the spinal cord. Afferent fibres enter the DH via the DRG, where their cell bodies are located. C fibres mainly terminate in LI-II, as do Aδ although some also extend into deeper lamina. In contrast, Aβ principally penetrate into LIII-V. Whilst NS cells receive direct input from Aδ and C fibres, WDR cells receive convergent input both directly and indirectly from all primary afferent fibre types. LII contains many interneurons, which connect peripheral fibres and WDR cells in addition to governing much of the intraspinal processing. Adapted from (D'Mello and Dickenson 2008; O'Neill et al. 2013)

1.1.12. GABAergic interneurones control spinal inhibition

The DH also contains interneurones, which may synapse with both PAF terminals and second order projection neurones. These neurones typically serve as inhibitory controls and thus contain the transmitters GABA or glycine. Melzack and Wall proposed the gate control theory, whereby inhibitory interneurones synapse with peripheral Aβ fibres and NS cells of LI to inhibit them from relaying pain signals (Melzack 1965). These synapses are therefore referred to as being silent as they do not activate second order projection neurones. Furthermore, it has been proposed that low threshold Aβ fibres can activate inhibitory interneurones, which in turn inhibit release of transmitters from Aδ and C fibres, acting as an endogenous modulator of nociceptive signals.

Yaksh demonstrated that administration of GABA/ glycine antagonists resulted in pain from tactile stimuli such as light touch; it is therefore believed that loss of spinal inhibition contributes to the development of allodynia (Yaksh, 1989). A disinhibition of Aβ fibre input and therefore an activation of LI NS cells may result in their conversion to WDR cells. This in turn could results in non-noxious tactile stimuli causing a painful sensation (Torsney and MacDermott 2006). The proposed mechanisms that may contribute to the development of this

phenomenon include a down regulation of the KKC2 transporter, or the release of endogenous endocannabinoids (Coull et al. 2003; Keller et al. 2007; Pernía-Andrade et al. 2009).

The KCC2 transporter is required for the maintenance of internal K^+ and Cl^- concentrations; Cl^- is usually found at a higher concentration outside of the neurones and thus upon activation of $GABA_A$ receptors, Cl^- enters the cell. This results in a hyperpolarisation of second order neurones, and on pre-synaptic neurones a decrease in transmitter release. A downregulation of this transporter could disrupt the usual ionic balance and result in a higher concentration of Cl^- inside the cell. Therefore, activation of $GABA_A$ would result in an efflux of Cl^- from the neurone and depolarisation of the second order neurone or a reduction in GABA release. Overall, resulting in a reduction in spinal inhibition (Keller et al. 2007).

Additionally, it has been suggested that peripheral fibres may release endogenous endocannabinoids in the DH, which act at cannabinoid 1 receptors (CB1R) located on both glutamatergic and GABAergic interneurones (Pernía-Andrade et al. 2009). Hegyi et al found that in LI and LII of the DH around 20% of spinal GABA-ergic interneurones express the CB1R, in addition to being located on the post synaptic membrane of second order neurones (Hegyi et al. 2009). Activation of CB1R on both the pre and post synaptic membranes may suppress GABA mediated neurotransmission and thus result in a depression of inhibitory synaptic activity. As such, these mechanisms are an important area of study as either could contribute to the development of mechanical allodynia experienced by patients, and hold potential for future therapies.

1.1.13. Ongoing input into the DH may result in wind up and central sensitisation

In the 1980s evidence began to accumulate suggesting that sensory processing of noxious stimuli in chronic pain states might not only be amplified by the process of peripheral sensitisation, described above, but also by parallel changes in central processing. These plastic changes have been identified and studied at multiple sites within the CNS, including the cortex, in descending supraspinal controls (described later) and, most extensively, in the spinal processing of painful stimuli. In the spinal cord, the process is often referred to as central sensitisation, by analogy with its peripheral counterpart. It has become clear that there are multiple forms of central sensitisation with different features and mechanisms.

In its most basic form, ongoing stimulation of peripheral C and Aδ fibres, due to peripheral inflammation or nerve injury, can result in the previously mentioned wind up, in addition to central sensitisation and long term potentiation (LTP). As with wind up, the increased input into the DH causes an increase in release of glutamate and neuropeptides such as substance P and CGRP at the synaptic terminal. This in turn results in an increased activation of post synaptic receptors and binding of SP and CGRP to neurokinin 1 (NK1) and CGRP1 receptors, respectively, leading to a slow depolarisation of second order neurones, thus relieving the usual block of NMDA receptors (Dickenson and Sullivan 1987; Latremoliere and Woolf 2009). Sustained depolarisation of projection neurones results in removal of this Mg^{2+} block, and thus Ca^{2+} entry to the neurone. Therefore, once NMDA and CGRP1 receptors are activated, changes may occur within the second order neurone including an increased production and surface expression of post synaptic receptors, in addition to enhanced probability of channel opening (Latremoliere and Woolf 2009). This may be due to a number of mechanisms involving changes in transcription, translation and post-translational modifications.

Many animal studies have explored the detailed molecular mechanisms underpinning central sensitisation (Latremoliere and Woolf 2009; Kuner 2010). One major consequence for the second order cell is a mobilisation of calcium and recruitment of multiple second messenger systems such as protein kinases, p38 and extracellular signal-regulated kinase (ERK). These processes reinforce the central sensitised state both by driving posttranslational changes in receptors in the cell and, over a longer time frame, by altering gene expression in second order neurones. Such changes lead to a significant increase in synaptic efficacy.

Intracellular events in the second order neurone lead to the activation of enzymes such as PKA, PKC, Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) and Src kinases. These in turn lead to phosphorylation of serine and tyrosine residues in GluR1 and GluR2 subunits of AMPA receptors, and NR1 and NR2A/B subunits of NMDA receptors (Zou et al. 2000; Guo and Huang 2001; Guo et al. 2002; Latremoliere and Woolf 2009; Wang et al. 2010). Phosphorylation of GluR1 targets AMPA receptors to the membrane and increases trafficking, whilst phosphorylation of NR2 subunits enhances the probability of channel opening and prevents endocytosis (Latremoliere and Woolf 2009). Furthermore, activation of enzymes such as ERK, result in cAMP response element-binding protein mediated transcriptional changes, increasing the expression of receptors such as NK1 and TrkB, which produce longer lasting changes in excitability (Latremoliere and Woolf 2009). In addition, a number of

heterosynaptic mechanisms may sensitise surrounding cells (including a reduction in spinal inhibition and changes in descending controls).

This plasticity, which causes hyperexcitability of DH neurones, is responsible for the symptoms that are associated with hyperalgesia and allodynia in humans and pain related behaviours in animals (Campbell and Meyer 2006; Schaible et al. 2006; Latremoliere and Woolf 2009). Activation thresholds of spinal neurones are lowered and responses to peripheral stimuli are enhanced (Suzuki et al. 2000). Whilst wind up is only a short-term homosynpatic phenomenon that is reversible, central sensitisation and LTP induce long-term changes across a number of synapses, which may be associated with the development and maintenance of chronic pain (Magerl et al. 1998). LTP is specifically associated with protein synthesis and thus involves structural changes at the level of the synapse (Bailey et al. 2004). The contribution of LTP to chronic pain is debated, since the frequency required for induction is very high (Rygh et al. 1999). Further discussion of LTP is out of scope of this thesis, since this mechanism is not explored.

The importance of these mechanisms is highlighted by the fact that receptor antagonism or knock-out of GluR1/ NR1 inhibits the development of central sensitisation (Chizh et al. 2001; South et al. 2003; Hartmann et al. 2004). Furthermore, as previously mentioned a loss of spinal inhibition may also contribute to the state of spinal hyperexcitability. For example, it has been shown in OA that there is an inhibition of glycine receptors, which prevents the endogenous inhibitory signals (Ahmadi et al. 2001). Persistent noxious stimulation also leads to widespread genetic and epigenetic changes in spinal cord (Denk and McMahon 2012; Crow et al. 2013) but the significance of these changes remains in large measure only partially defined. Many detailed reviews of all the contributing processes identified exist within the field and will be discussed further in chapter 6 (Latremoliere and Woolf 2009; Woolf 2011).

More recently, the proliferation and activation of resident immune cells in the spinal cord (microglia) have been identified as contributors to central sensitisation (Marchand et al. 2005; McMahon and Malcangio 2009). These cells are required under normal conditions to perform a surveillance function of the tissue they populate, and are only activated when that tissue is damaged or compromised in order to participate in innate immunity. However, in some chronic pain states (particularly neuropathic states) it has been shown that spinal microglia are activated and begin to release a number of inflammatory mediators such as TNF-α and IL-1β (McMahon and Malcangio 2009). These microglial responses appear to be an important regulator of spinal excitability, targeting both pre- and post-synaptic elements (Gao

and Ji 2010; Trang et al. 2012). Furthermore, several strategies that block microglial activation, such as broadly acting inhibitors like minocycline or propentophylline or blockers of specific receptors on microglia such as P2X7, prevent the development of many neuropathic pain symptoms (Guasti et al. 2009; Trang et al. 2012). Since all the current data relates to experimental studies in animals the relevance of this process to clinical conditions is not yet established, however further discussion on this topic is out of scope of this thesis.

The presence of central sensitisation is revealed in a number of clinical settings, with the most compelling being the case of referred pain and hyperalgesia (Gwilym et al. 2011). Additionally, in a number of visceral pathologies, such as cardiac ischemia, pain is often felt initially deep within the chest, but with time the pain may radiate to superficial structures, most often the arm (McMahon 1997). The area of referral can also become hyperalgesic. The explanation is that spinal circuits receiving inputs from both visceral and somatic tissues are sensitised by the former, so that the sensitivity of the latter is increased. Another example of this process in humans is seen when focal experimental painful stimuli (such as noxious heat or algogenic chemicals) are applied to the skin of volunteers. The stimuli elicit pain at the site of stimulation but in addition, an area surrounding the stimulus where mechanical stimuli are perceived as being more painful than normal (Raja et al. 1984). The area of so-called secondary hyperalgesia can be large and arises because activity in nociceptors at the site of the stimulus induces a state of spinal hyperexcitability that manifests itself as hyperalgesia. Manipulations that block peripheral activity lead to reversal of the hyperexcitable state of this type of central sensitisation, although high frequency nociceptor activity can trigger longer lasting central hyperexcitability known as spinal long-term potentiation (Sandkühler and Liu 2001).

It is important to note that the two pathophysiological phenomena discussed thus far - central and peripheral sensitisation - are not mutually exclusive and may exist in tandem. An initial peripheral sensitisation may indeed be the driving force behind the development of central sensitisation in some instances. As such, it may be difficult to fully dissect the specific central and peripheral contributions of underlying symptoms of chronic pain.

1.1.14. Projection neurones carry the signal from the DH to higher centres of the CNS

In order to consciously perceive nociceptive signals as pain these messages must reach higher centres in the CNS. Neurones projecting from the DH carry nociceptive signals in ascending tracts to specific areas of the brain. These neurones are found in either LI or LIII-VI and their axons cross the midline before ascending to centres such as the midbrain, cortical structures and the thalamus (Todd et al. 2002; D'Mello and Dickenson 2008). One feature of these projection neurones is that many express the Substance P receptor NK1; the highest concentration of which is found in LI where around 80% of projection neurones in rats are believed to express NK1 (Nakaya et al. 1994; Todd et al. 2002). $NK1^+$ LI projections to the brainstem are imperative for the integration of nociceptive information with arousal, mood and context in order to mediate changes in perception (Tracey and Mantyh 2007). These neurones are particularly important in controlling spinal excitability, since ablation reduces both formalin responses and CFA related hypersensitivity (Suzuki et al. 2002). Indeed hyperalgesia associated with persistent pain states appears to depend critically on these LI projections (Mantyh et al. 1997; Suzuki et al. 2002).

There are a number of pathways through which second order neurones can transmit pain-related signals from the spinal cord. These include the spinoparabrachial, the spinothalamic and spinomesencephalic tracts (Tracey and Mantyh 2007). Most supraspinal projections from the spinal cord originate either in LI, or more deeply from LIV, V, and VI. These two systems may engage different brain regions, with the latter activating regions of the thalamus, insular, anterior cingulated cortex, and somatosensory cortex, which are important for sensory discrimination (Tracey and Mantyh 2007). On the other hand, the LI projection neurones project to parabrachial nuclei and activate the amygdala, hypothalamus, rostral ventromedial medulla (RVM) and periaqueductal grey (PAG). These areas are involved in the affective emotional components of pain perception (Tracey and Mantyh 2007).

1.1.15. Cortical representation of pain

Functional magnetic resonance imaging (fMRI) studies in humans have identified multiple areas that are commonly active when painful stimuli are applied. These include the aforementioned thalamus, mid/rostral anterior cortex, primary and secondary somatosensory cortex, insular cortex and prefrontal cortices, as well as numerous brainstem

nuclei and parts of the basal ganglia (Tracey 2011). Originally these areas together were referred to as the 'Pain Matrix', however the term has since been abandoned because of continued debate about whether these different areas are actually associated with perceptions or pain (Legrain et al. 2011). Furthermore, this signature is plastic and can be influenced by numerous cognitive, emotional, contextual and physiological factors. Such factors are known to influence the reported pain experience and this is reflected in the brain, where different situations result in an increase or decrease in activity in particular regions (Tracey and Dickenson 2012). Therefore it seems reasonable to conclude that the 'pain matrix' is not only nociceptive-specific activity, but rather is a reflection of brain activities involved in processing both nociceptive and non-nociceptive salient sensory input (Iannetti and Mouraux 2010; Legrain et al. 2011).

1.1.16. Descending controls modulate pain processing

While there has been considerable interest and emphasis on the processes of peripheral and central sensitisation, it is important not to overlook the descending pathways from the midbrain and brainstem, which also play a pivotal role in the modulation of pain processing. Pathways descending from the PAG, RVM and the pontine nuclei (most notably the locus coeruleus (LC), but also A5 and A7) have been identified as key players in this modulatory system - with the release of 5HT from the RVM playing an overriding pronociceptive role and noradrenaline (NA) from the LC an antinociceptive one (Bannister et al. 2009). Other neurotransmitters such as dopamine, opioids, substance P, dynorphin and GABA may also be involved in these processes (Millan 2002). The fine balance between excitatory and inhibitory controls may indeed dictate overall excitability of DH neurones.

This descending modulatory system is believed to be activated through a spino-bulbospinal loop, whereby activated NK1 receptor expressing LI/III projection neurones send signals to the parabrachial area and onto the limbic system (Suzuki et al. 2002). Inputs driving the circuits in the amygdala, hypothalamus, frontal lobe, and anterior cingulate cortex activate areas of PAG, which in turn feeds into both the RVM and LC, modulating release of 5HT and NA, respectively. Removal of the projection neurone drive can decrease (via supraspinal circuits) excitability of DH neurones in chronic pain states suggesting an overriding pronociceptive action of descending controls contributing to hypersensitivity (Suzuki et al. 2002). Furthermore, injection of lidocaine directly into the RVM reverses nerve injury

induced behavioural hypersensitivity (Burgess et al. 2002), suggesting that during chronic pain states the balance is tipped towards an increase in descending facilitation/ decreased descending inhibition.

This facilitation is largely due to the actions of 5HT at the 5HT3 receptor, which is expressed both pre- and post-synaptically on neurones in the DH, in addition to on interneurones. 5HT3 receptors are excitatory – allowing an influx of cations, which in turn can result in increase in second order cell excitability. Using ondansetron to block spinal 5HT3 receptors, it was shown that descending influences became facilitatory in models of neuropathy and OA (Suzuki et al. 2004; Rahman et al. 2009).

In opposition to these mechanisms is the release of NA acting at $\alpha 2$ receptors, also expressed pre- and post-synaptically and on intrinsic interneurones in the DH. Stimulation of the LC is antinociceptive, but the effect is reversed with an $\alpha 2$ antagonists (Jones and Gebhart 1986). While the actions of 5HT appear to be enhanced in certain chronic pain states, it would appear that the inhibitory mechanisms are simultaneously down-regulated (Rahman et al. 2008). Given the networks that exist between the PAG and the limbic brain, it is unsurprising that a number of psychosocial factors are able to modulate the pain experience, such as anxiety, depression, context and past experiences. Chronic pain is in fact inextricably intertwined with co-morbidities such as anxiety, depression and sleep disturbances in a vicious cycle as one exacerbates the other (Gormsen et al. 2010; Rehm et al. 2010). It is co-morbidities such as these that make the search for adequate treatment even more pressing, and also highlights these areas as potential drug targets.

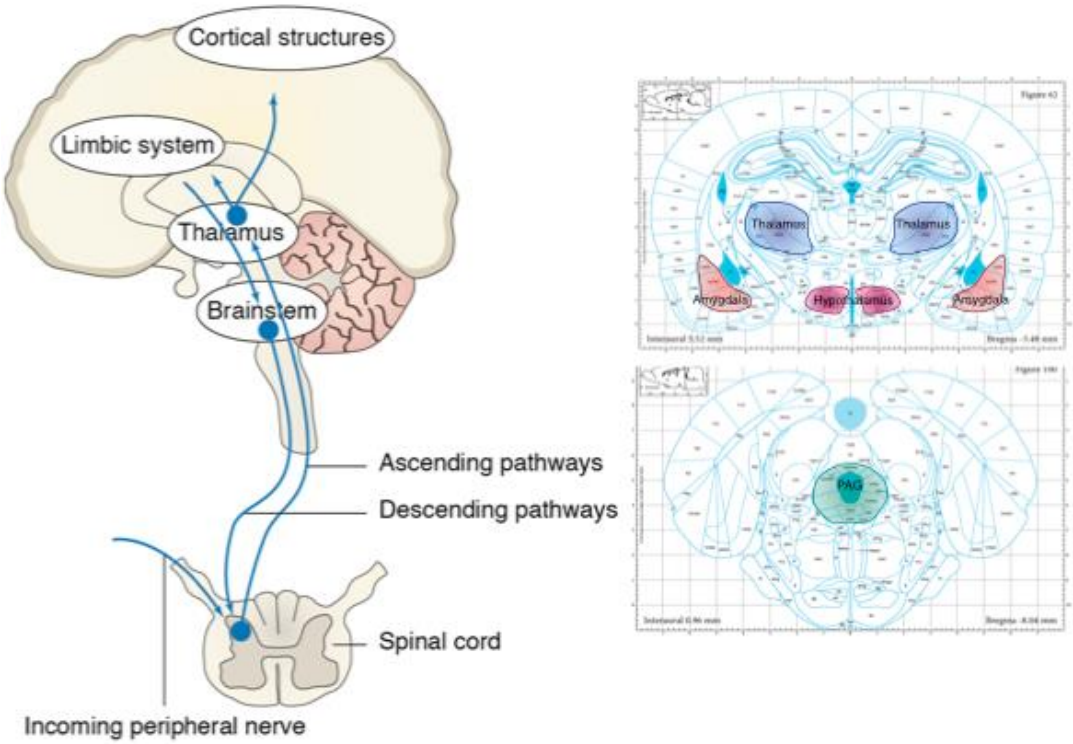

Figure 1-3 Ascending and descending pain pathways link the spinal cord, midbrain and higher centres. Spinal projection neurones carry nociceptive information to the thalamus to allow for sensory discrimination, in addition to conveying signals to brainstem structures such as the PAG, involved in emotional pain processing. Further connections to limbic areas such as the hypothalamus and amygdala are also involved in the emotional component of pain. Serotonergic and noradrenergic descending controls arise mainly in the RVM and LC, contributing to overall spinal excitability. Adapted from (O'Neill et al. 2012).

1.1.17. The transition from acute to chronic pain

As previously mentioned, chronic pain is generally accepted as pain ongoing for a period greater than 3 months (McMahon et al. 2013). It is clear there must be some forms of predisposing factors that render certain individuals more susceptible than others, as only a small proportion of patients with peripheral nerve pathologies develop signs of chronic pain (Kehlet et al. 2006). Whilst up to 26% of diabetes patients may develop a neuropathy, traumatic nerve injury leads to neuropathic pain in less than 5% (Haanpää et al. 2009). Predisposing factors may be of genetic origin, though are likely to also have environmental influences.

A number of risk factors for the development of chronic pain have been identified, which are outlined in table 1-2, below. These factors can be subdivided into those attributed to the psychosocial status of the patient, and those that have medical/physiological underpinnings.

It is important to note that psychosocial factors are likely to play a larger role than perhaps was previously thought (Macrae 2008) – potentially due to the interaction with the descending modulatory controls. It has been shown in animals that the balance between facilitation and inhibition is a key process in the development of chronic pain, and factors that influence this are of course mood, anxiety and sleep (De Felice et al. 2011).

Psychological Factors	Medical Factors
Past Experiences	Genotype
Beliefs/Expectations	Medical History
Anxiety/Depression	Intensity
Catastrophising	Surgery
Social Circumstance	Medications/Anaesthesia

Table 1-2 The potential risk factors associated with chronic pain. There are a number of risk factors, both medical and environmental (psychosocial), which may be associated with the development of chronic pain. It is difficult to ascertain their distinct contributions, but most likely a number of different factors will be involved in each individual case. Adapted from (Macrae 2008).

Past experiences can shape beliefs and future expectations in either positive or negative ways. If a patient has had a negative experience in the past they may be more pessimistic regarding future treatment. However, such beliefs can also arise without any previous experience; with regards to surgery it has been suggested that feeling post-operative pain may result in the patient believing something has gone wrong and may blame the surgeon. This has been associated with poor treatment response and lowering of pain thresholds (DeGood and Kiernan 1996; Turk and Okifuji 1996).

Anxiety and depression are likely to play a key role in susceptibility for the progression of acute to chronic pain, as previously discussed the neuronal networks and pathways involved in both are inextricably intertwined. Catastrophising also appears to correlate with chronic pain (Lamé et al. 2005). It is possible that this may be the result of disrupting the balance between descending facilitation and inhibition. De Felice et al showed that nerve injury induced pain may rely on descending modulation and that inhibition may protect from acute pain progressing to chronic (De Felice et al. 2011). Therefore, a disruption resulting in decreased inhibition/increased facilitation may be a critical factor in the transition to chronic

pain. However, it is unlikely that these psychosocial factors alone are enough to cause this transition and thus physiological and genetic mechanisms may act in synchrony.

Indeed it may be the case that genetic make-up determines susceptibility, or epigenetic modulations predispose an individual to chronic pain (Sikandar et al. 2013). Both animal and human studies suggest that there are existing genetic factors that influence the development of neuropathic pain (Seltzer et al. 2001; De Felice et al. 2011; Sikandar et al. 2013). Identification of genetic predisposing or protecting factors dictating who will, or will not, develop chronic pain is likely to be critical to determining the success of treatment. Could one answer be related to a propensity to develop peripheral sensitisation mechanisms? The TRPV1 SNP I585V variant renders the channel 30% less active and is associated with a decrease in heat and pinprick hyperalgesia (Cantero-Recasens et al. 2010; Binder et al. 2011), thus begging the question of whether this leads to an inability to sensitise or provide enough drive to lead to further central changes? Maybe the key lies in sodium channel SNPs such as R1105, decreasing activity of the afferent fibres? However, given that volunteers who do not develop secondary hyperalgesia report the same initial pain upon capsaicin injection as those who do, this seems unlikely. Many candidate genes have in fact been identified, however they are yet to provide solid evidence of any hereditary variants that are responsible for predisposing individuals to chronic pain. It is likely there are many more important genetic variants that may be involved but are yet to be identified – in particular regarding genes involved the descending controls.

It has been suggested that some animal strains possess certain protective mechanisms, rendering them less susceptible to chronic pain (Mogil et al. 1999; De Felice et al. 2011). After two strains of rats were identified, which developed tactile allodynia in significantly different proportions (85% vs 50%), De Felice and colleagues could find no outstanding discrepancies between the two in terms of markers in either the periphery or the spinal cord. Thus they looked to higher centres and found that blocking the RVM in neuropathic rats decreased signs of hypersensitivity, whereas in the rats that developed no signs of chronic pain, this block actually induced allodynia (De Felice et al. 2011). The results suggest that perhaps there is a failure to engage/loss of descending inhibitory pathways, or an excessive descending facilitation, leading to the development of chronic pain symptoms such as tactile allodynia. Or perhaps an excess of descending inhibition will protect against the development. These finding may be useful groundwork to further assess these mechanisms in patients with chronic pain.

1.2. Experimental pain models in animals and humans further our insights into the mechanistic underpinnings of chronic pain

A number of experimental preclinical models have been developed to further our understanding of the pivotal pathophysiological mechanisms involved in the induction and maintenance of chronic pain. Such models aid identification of potential drug targets and in order to investigate the efficacy of possible new pharmacological interventions. Animal models are one of the most important tools in drug discovery for chronic pain and they range from the induction of inflammatory pain, to neuropathic pain, to a combination of the two in numerous disease specific states- such as cancer induced pain (Wang and Wang 2003; Mogil 2009). The models are able to sensitise the pain signalling system at different points through a number of different mechanisms, which are likely to be relevant to clinical conditions.

Broadly speaking there are two approaches to animal models of chronic pain. This includes the induction of particular symptoms/ mechanisms believed to be important in the clinic, and the induction of specific disease states creating models with 'face validity', such as cancer-induced bone pain (Mantyh 2013). The induction of specific symptoms/ mechanisms may use inflammatory or neuropathic like states. Inflammatory pain may be induced through the use of substances such as CFA, formalin, carrageenan, MIA, and mustard oil, whereas neuropathic pain is induced by injuries to a peripheral nerve such as the L5/L6 spinal nerve ligation (SNL), spared nerve injury, chronic constriction injury (CCI) and partial sciatic nerve ligation/ the Seltzer model (Wang and Wang 2003). Animal models such as these have allowed the elucidation of distinct molecular and cellular mechanisms underpinning chronic pain symptoms, including: peripheral sensitisation, central sensitisation, enlargement of receptive fields in DH neurones and impaired descending controls (Arendt-Nielsen et al. 2007).

Another animal model, capsaicin, is able to induce short term changes that mimic both inflammatory and neuropathic like symptoms and as such is also a popular model for chronic pain. The chilli pepper derived chemical is able to induce a robust peripheral sensitisation, and the barrage of input into the DH also results in central sensitisation. As such, it has already been a very useful tool for elucidating a number of molecular events involved in chronic pain and will be explored further in Chapter 3 of this thesis (for a full review see O'Neill et al, 2012).

More recently, the use of disease-specific models, with face validity, such as HIV-related neuropathy, cancer induced bone pain and DPN have become increasingly popular (Ueta et al.

2005; Rice et al. 2009; Schmelz 2010; Mantyh 2013). The reason behind this is that the different aetiologies are likely to engage different mechanisms at both the stage of induction and maintenance of chronic pain, and as such it is important to study specific states to truly understand their individual physiological underpinnings and uncover the most suitable drug targets. These models have increased our knowledge of conditions such as HIV-associated sensory neuropathy. The stavudine (d4T – a nucleoside reverse transcriptase inhibitor) model has been used to investigate the behavioural changes and specific neuropathology associated with the condition (Huang et al. 2013). Revealing distinct pathologies such as a reduction in peripheral and central terminals of DRG neurones and increases in myelinated and unmyelinated axon diameters (Huang et al. 2013). Therefore, such studies are critical for the future of chronic pain research and drug development.

In addition to disease specific models, further approaches can be taken in order to increase the clinical meaningfulness and predictive value for patients of the preclinical studies. Surrogate human models are aimed at bridging the gap between basic animal science and the clinic. Experimental models allow activation and sensitisation of the pain system and measurement of specific evoked responses (Arendt-Nielsen et al. 2007; Schmelz 2009). Similar to the animal models discussed, they imitate the signs and symptoms of chronic pain under highly controlled conditions, whilst in addition exploiting the human capacity for verbal communication and allow further investigation into the intensity and quality of pain (see figure 1-4 for the full advantages of surrogate models). These studies obviously face many more limitations with regards to ethical and safety concerns and thus can address only a few clinically relevant signs and symptoms. Most concentrate on inflammatory models using the application of topical or injected algogens such as mustard oil or capsaicin. To avoid the use of injections models such as ultraviolet B irradiation (UVB), the thermal burn and freeze lesion have also been developed. Such models are able to consistently induce mechanisms such as peripheral and central sensitisation in order to study the consequences and possible modulation of such phenomenon (Schmelz 2009).

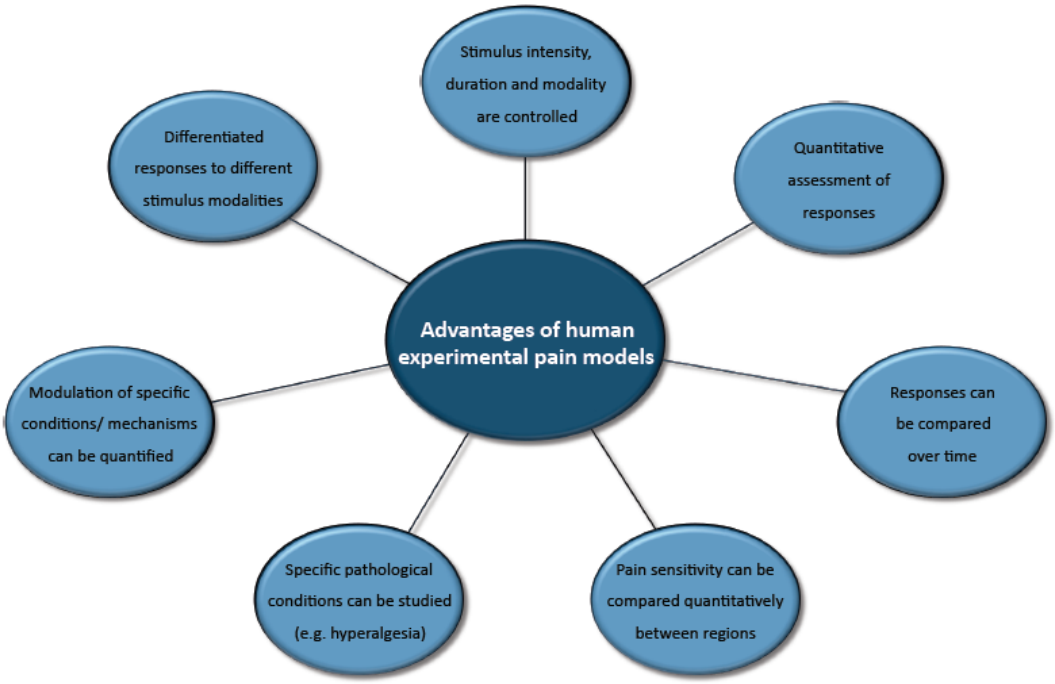

Figure 1-4 Advantages of the use of surrogate models. Numerous advantages of adopting human experimental models exist, from the exploitation of verbal capacity and quantitative assessment of responses, to comparing responses at different sites and over time. Adapted from (Arendt-Nielsen et al. 2007).

However, even using animal and human models it is of course impossible to replicate the exact pathophysiologies and the full complexity of chronic pain syndromes. Using animal and human studies in tandem is one of the most powerful tools currently available in research. Much of our knowledge around pain processing and the mechanisms of induction and maintenance of chronic pain states have arisen from years of preclinical research in animals and humans and it is undoubtedly true that basic research is required still to further our understanding of the complex pathways the pathophysiology of chronic pain. The human studies are imperative for improving the clinical translation of early work by identifying mechanisms and modulation relevant to the human pain processing system. In addition they exploit the ability of subjects to verbally communicate, which adds a qualitative dimension to the studies. On the other hand, many chronic pain syndromes cannot be modelled in humans and the cellular and molecular underpinnings can often only be extracted from the animal models (Mogil 2009). Therefore, it is likely that the most efficient studies will use a combination of animal and human studies in order to reap the benefits of both (Dawes et al. 2011). For example, although it was first noted in humans that Gabapentin could reduce symptoms of neuropathic pain, animal studies elucidated the potential mechanisms (Luo et al.

2002; Bennett 2010). This thesis aims to characterise translational models of chronic pain in order to best exploit the potentials of studies across species.

1.2.1. The question of translational pain research

One challenge faced in the field of chronic pain research is to understand the true predictive value of preclinical animal models. The value rests upon the degree of overlap between the models and patients. The debate has arisen due to the fact that very few analgesics developed will ever enter this pain market. In fact it is estimated that only 16% of pain drugs entering phase I clinical trials will eventually gain approval for use in patients (Kola and Landis 2004). Furthermore, of those that do enter the market, many do not provide adequate treatment; in a meta-analysis of 174 clinical trials it was found that from the therapies that do exist the majority of patients did not receive adequate pain relief, and (Finnerup et al. 2010; Berge 2011). This limited success in translation of basic discoveries into clinical therapies has therefore raised the question as to what is driving this disparity (Mogil 2009). Although there are many explanations that could be behind this, one suggestion is that the preclinical models are to blame. Current problems of translating research into patients include potential species-specific pain processes and efficacy of analgesics, as well as the undeniable difficultly in interpreting pain associated behaviours in animals and the wide variety of aetiologies responsible for chronic pain in the clinical (Blackburn-Munro 2004; Bennett 2010; Schmelz 2010).

It may be argued that at a very basic level, the nervous system is what sets us aside from other species. Indeed, as humans, our nervous system has developed into a unique and sophisticated machine that has subtle differences from that of say, a rat or a mouse and indeed this could raise the question of how applicable are the animal studies to the human conditions (Bennett 2010). However, it is important to note that pain is a basic function necessarily for survival and is unlikely to have changed over time. It is well known that areas of the brain involved in pain processing are not necessarily highly evolved brain regions, but in fact the primitive regions which are conserved through time, such as the primary and secondary somatosensory cortices, the anterior cingulated cortex and the insula are imperative to pain sensing (Iannetti and Mouraux 2010). These areas have been identified using human brain imaging, but were previously identified in animals (Bennett 2010). Simple behavioural responses are believed to be highly conserved through evolution and as such are

most likely comparable in animals and humans. In fact not only has the acute pain system of animals and humans shown to have a great deal of overlap, it has also been shown to be sensitised and modulated in the same way, therefore suggesting there is a reasonable degree of overlap (D'Mello and Dickenson 2008; Dawes et al. 2011; O'Neill et al. 2012). Indeed, stimulation of the PAG in rats and humans was shown to produce analgesia, thus suggesting fundamental similarities (Bennett 2010). It has also been pointed out that the nervous systems do not need to be identical, but simply display a 'functional degree of similarity', which current data certainly suggests and thus warrants the study of pain in preclinical animal models (Bennett 2010).

One difference that presents a challenge to preclinical research is the subjective and emotional nature of the pain experience. In humans we can be certain that pain is not simply a sensory phenomenon but can in fact be influenced by affective and cognitive processing. Pain is a very subjective experience and modulation by higher brain centres may be a function unique to humans, or at least its existence is difficult to quantify in animals (Berge 2011). It is therefore accepted that we cannot model fully the complex pathophysiology of chronic pain as such there is a limited use for animal models as they can only cover certain aspects of the true conditions (Arendt-Nielsen et al. 2007; Schmelz 2010; Berge 2011). One important way to get around this issue is of course the use of translational models, where the human surrogate may also be explored and can provide more information around the full sensory and emotional pain experience.

A second factor which suggests that there may be some species-specific differences in the development of chronic pain is that whilst less than 50% of patients with neuropathies will develop signs of chronic pain (such as hyperalgesia and allodynia), they are usually present in all animal models; suggesting possible differences in the pathologies (Kehlet et al. 2006; Rice et al. 2009; Dickenson and Baron 2011). The low level of variability is important for standardisation of preclinical models, but does perhaps suggest a disparity with the true human conditions. There are a number of risk factors for the development of chronic pain, as previously discussed, which may be unique to humans. On the other hand, this disparity may simply reflect the reduction in variability of the initial insult responsible for the pain or less variability in the animal genetics (Rice et al. 2009). Whilst the animal models are standardised and all receive the same severity of injury, this clearly varies between patients. However, the important point to note is that it is clear that some patients experiencing a

neuropathy will not develop signs of chronic pain and an understanding of the reasons behind this will be of great interest.

The possibility of species-specific mechanisms, and thus drug efficacy, could impact the design of new drugs may and render some promising candidates useless in the clinic. A prime example of this is the NK1 antagonist, which appeared to reduce pain behaviour in pre-clinical models, but failed once it reached human clinical trials (Hill 2000). As previously mentioned, species-specific pain processes seem to be related to the fact that human pain involves the integration of emotional and contextual (Berge 2011). Pain is an experience unique to the individual, which is difficult to exactly recreate and measure with animal models alone and this limitation must be accepted. Furthermore, this highlights the importance of investigating the subjective component of the pain experience.

An additional issue raised with regards to preclinical animal studies is around the choice of model used, as it is questioned to what degree do the mechanisms studied in experimental animal models reflect the true pathophysiologies of clinical pain states (Rice et al. 2009; Berge 2011). Indeed, there are a limited number of preclinical models in use, many of which do not in fact recreate the exact pathophysiologies occurring patients (Rice et al. 2009; Schmelz 2010). Furthermore, the myriad of aetiologies underlying different chronic pain conditions in patients mean it is difficult to relate the findings in one particular model to different conditions in the clinic. For example, Schmelz suggests that animal models of neuropathic pain where a nerve is ligated or injured are unlikely to mimic non-traumatic neuropathic pain states and certainly cannot be used as a surrogate for non-neuropathic pain conditions (Schmelz 2010). In agreement with this sentiment, Rice notes that models of peripheral nerve injury have become an 'industry standard', yet only around 9% randomised controlled trials are actually conducting in patients with a peripheral nerve injury (Rice et al. 2009). It is important that when choosing a preclinical model, thought is given to the clinical condition that will be implicated (Rice et al. 2009). Although disease specific models with greater face validity begin to overcome this issue, it is important to note that such an approach is not without drawbacks. Whilst some diseases do not have a preclinical model: of those available none replicate the true timelines of the disease pathology. Conditions may develop in patients over many years, whereas models are induced in a number of weeks. For example, whilst disease duration of patients entering trials for PHN is 4 years, the animal models develop over a maximum of 10 weeks (Rice et al. 2009).

Given the broad range of underlying aetiologies in patients it is unsurprising that there is no 'one model fits all' and it is impossible to suggest that there may be a single model of chronic pain which will suffice as a model to predict the mechanisms and efficacy of analgesics for a range of different conditions (Berge 2011). Rather, disease and symptom specific models should be used to overcome this issue to investigate mechanisms and modulation relevant to specific conditions. It is important to understand which mechanisms each model can address and one way to do this is by fully characterising and phenotyping a model in both animals and humans, where possible. By gaining knowledge of all the symptoms induced by a specific model we can begin to gather insight into the potential mechanisms at play. In the absence of being able to recreate disease pathologies, it is useful to develop models that induce particular symptoms for mechanistic studies. This thesis aims to fully characterise a number of translational chronic pain models in order to help understand their relevance to clinical conditions.

Finally, one may ask can we really measure pain in animals (Blackburn-Munro 2004; Bennett 2010)? In the absence of the capacity for verbal communication, most animal studies rely on reflexes, such as the tail flick or paw withdrawal, which cannot necessarily be equated to the human pain sensation and certainly does not mimic a clinical situation. Although they may involve a level of supraspinal processing and modulation, on the whole these are thought of as spinal phenomenon, which clearly do not engage all areas integral to the full human pain experience. In order to initially trigger these reflexes the same peripheral fibres and receptors involved in the conscious pain experience are necessary and thus it is at least partially relevant, however the spinal cord mechanisms may be different and the full affective and cognitive processing is not apparent (Mogil 2009; Bennett 2010). In particular, focus falls on the engagement of motor neurones that are involved in this reflex, which poses a particular problem for drug development since it is possible that certain drugs may interact with these interneurones driving the withdrawal reflex, rather than in the pain pathway itself. One way to overcome this issue is with the use of additional tests to explore motor function, which would indicate if the drug is in fact affecting motor neurones, rather than those integral to pain signalling.

An additional downfall of these reflex measures is that they often only test a restricted number of modalities (mechanical and thermal) and assess a gain of function. Since patients with chronic pain display a range of sensory phenotypes, of which sensory gain is only one feature, this limits the usefulness of these reflex measurements (Rice et al. 2009).

Behavioural measures of pain are also subject to a high degree of variability. The measures are sensitive to a number of extrinsic factors that are often difficult to control for, such as habituation and the test environment. Furthermore, there may be a degree of subjectivity between experimenters (Chesler et al. 2002). One way to address this issue is to use more objective measures, such as electrophysiology. This thesis uses in vivo electrophysiology in order to produce more objective characterisation of translational models.

Additional measures explored in preclinical have also received criticism, since pain behaviours in animals such as guarding and licking may simply be signs of other dysethesias and not necessarily what we experience as pain (Blackburn-Munro 2004). As such there is a clear need for more operant measures of pain in animals, in addition robust translational models that allow the human counterpart to be explored for the qualitative aspects confirming the real location and intensity of pain, in addition to capturing the full sensory, emotional and perceptual experience. Furthermore, these endpoints often measured in animals differ from those in the clinical trials (which involve pain questionnaires and reporting's of spontaneous pain); it is important to try and make sure that endpoints measured in preclinical studies are relevant to those in the clinic (and vice versa) (Rice et al. 2009). This thesis aims to address the disparity in outcome measures, by using an identical range of endpoints in both animal and human models.

As previously mentioned, much insight has come from preclinical studies. It is without doubt that a number of animal models have helped our understanding of mechanisms contributing to chronic pain and their respective modulation, which should not be overlooked (D'Mello and Dickenson 2008; Baron et al. 2013; Sikandar and Dickenson 2013). Indeed, much of our understanding of the pivotal mechanisms underpinning central sensitisation and the importance of the fine balance in descending controls has come from preclinical animal models (Bannister et al. 2009; Sikandar and Dickenson 2013). Genetic engineering and the use of KO mice has also been particularly fruitful when elucidating the function of particular proteins involved in pain signalling.

Furthermore, drugs are available to patients today that have come from these studies, such as the N-type calcium channel blocker ziconotide, which has been shown to be as effective in patients as the preclinical studies in animals (Williams et al. 2008). It is particularly effective in refractory malignant pain, reducing patient's pain scores on average by 53.1% (Williams et al. 2008). More recently the introduction of tapentadol has the potential for great benefit to patients, since the requirement for opioids may be lowered – which was originally

demonstrated in acute inflammatory and neuropathic pain models (Prommer 2010). Indeed studies have already reported analgesia in patients suffering with lower back pain (Hartrick and Rozek 2011). It has also been noted that most successful analgesics that were not discovered through this traditional route do still succeed in reversing thermal and mechanical hyperalgesia in these rodent models (Kontinen 2002). Furthermore, retrospective investigation of the mechanisms of certain pain drugs that were discovered by 'accident' has also benefited from animal studies, such as the elucidation of the actions of gabapentin in chronic pain (Luo et al. 2002; Sikandar and Dickenson 2013). Future success will most likely be dependent on the combination of animal and human preclinical studies. However, an integrated approach in translating knowledge obtained from animal models into human research and clinical trials is imperative for progression in the field of pain management.

1.2.2. Limitations of human surrogate models

Although many of the issues raised with the animal studies can be addressed by using human models, they too are not without their own criticisms. The usefulness of existing human surrogate models are questioned despite the clear translational benefits and the ability to investigate human pain under controlled conditions. Especially since some clinically relevant symptoms have not yet been modelled. For example, there are currently there are no reliable surrogates for symptoms such as spontaneous pain and cold allodynia/ hyperalgesia. Spontaneous pain is one of the most important clinical symptoms that chronic pain patients present with, since it is common across virtually all patients regardless of the underlying aetiology (Mogil 2009; Rice et al. 2009). The development of preclinical models that can assess this symptom is therefore of great importance for further understanding of its pathophysiology and the screening of candidate analgesics.

Another limitation is the inability to model nerve injury, as well as other long-term changes. While some inflammatory conditions may be modelled in humans (for instance using UVB irradiation), neuropathic or disease specific pain states cannot be induced experimentally. Instead, existing efforts in human models focus on mimicking sensory signs and symptoms of chronic pain disorders. They supplement animal models with data detailing the exact duration, intensity and quality of pain. However, as such, one major limitation is that the current models do not necessarily engage clinically meaningful mechanisms. All the changes induced are short-term, for obvious ethical reasons, whereas the pain associated with various

conditions appear to manifests over many years. Therefore most models may not necessarily reflect the true pathophysiology. That is to say, it is impossible for ethical reasons to induce nerve injuries and other initiating factors in human volunteers to precipitate long-term changes in the spinal cord and supraspinal sites that underpin chronic pain. Even current models such as intradermal injection of capsaicin can be very painful, and the UVB-sunburn model has the clear risk of inducing heat lesions or blisters on the skin of subjects so must be used with caution.

As a result of these limitations, the current human models are also not necessarily aligned with the animal models. Whilst inflammatory mediators used in animal models such as formalin, carrageen or CFA induce a robust sensitisation, these are not suitable for use in humans due to the risk of damage to nerve fibres. In order to truly benefit from the use of animal and human studies in parallel it is useful to initiate the same models, which are likely to engage similar mechanisms, where the data collected from each will complement the other.

Studies to date have shown that there certainly are some clinically meaningful overlapping models resulting in chronic pain like symptoms in both animals and humans. This was originally demonstrated with the use of intradermal capsaicin to induce central sensitisation in both animals and humans (O'Neill et al. 2012). The basic animal models give a good insight into pathophysiological mechanisms that contribute towards the central changes, whereas the human surrogate models have confirmed that induction of central sensitisation can mimic sensory signs and symptoms of chronic pain and thus may be useful for testing new drug candidates. In addition, the UVB model of inflammatory pain has also been shown to have a high translational impact. This second model has come to be one of the most successful translational studies so far, being used in a number of laboratories and producing similar effects in both animals and humans (Bishop et al. 2007; Bishop et al. 2009; Dawes et al. 2011). It was recently used to identifying a number of candidate molecules that may be involved in inflammatory pain such as the chemokine CXCL5 (Dawes et al, 2011). This model will be explored further in this thesis in chapter 5. The success of such studies would suggest that it might be useful to develop further human surrogate models that can compliment mechanistic animal data.

1.2.3. Poor clinical trial design may impact translation from basic science

A final concern in the field of translational pain research suggests that preclinical models are not completely to blame for the previous drug failures. Misinterpretation of preclinical data and the poor design of subsequent clinical trials may also hinder the progress of basic science discoveries into clinical practice. If the initial research is carried out in animal models that do not accurately reflect the symptoms experienced by the group of patients the drug is subsequently tested in it is unsurprising that no efficacy is found (Woolf 2010). For example, a drug target may be identified that demonstrates efficacy alleviating mechanical hyperalgesia in the SNL model, whilst progressing forward into clinical trials the drug may then be given to a group of patients from various polyneuropathies suffering from a spontaneous ongoing pain (Rice et al. 2009). These two symptoms are of course not comparable and preclinical data against one symptom should not be expected to work against another. Any candidate drug target identified from preclinical studies should eventually lead to the development and testing of compounds in patients specifically selected on the basis that they show similar signs to the original preclinical model used in order to address this mismatch between preclinical models and diseases in clinical trials (Rice et al. 2009).

Furthermore, most clinical trials currently select groups of patients based on the criterion of having a certain disorder or underlying pathology, such PHN or DPN. However, not all of these patients will suffer from the same symptoms. Indeed, it has been noted that patients suffering from the same disorders can be split into subgroups of those suffering from the same symptoms (Baron et al. 2009; Maier et al. 2010). Thus, the clinical trials should also focus on conducting studies in the group of patients that are suitable for the particular compound of interest. That is to say, clinical trials should organise patients into subgroups who suffer from the same collection of symptoms since it is likely that the symptoms may relate to a common underlying mechanisms (Jensen and Baron 2003). Candidate analgesics should then be allocated to subgroups where it has been proven to show efficacy in the equivalent animal model, rather than simply testing on all patients who may suffer from the same disorder but between them exhibit a myriad of different symptoms (Jensen and Baron 2003; Rice et al. 2009; Attal et al. 2011). Poorly designed clinical trials may mean that promising candidate drugs are wasted and in fact it is possible that compounds that previously failed in clinical trials, may be shown to be more effective than first thought if they were to re-test them and examine specific symptoms and modalities. Asking a patient to simply 'rate' their pain is an incredibly broad measure and a reduction in a particular quality of pain could be masked.

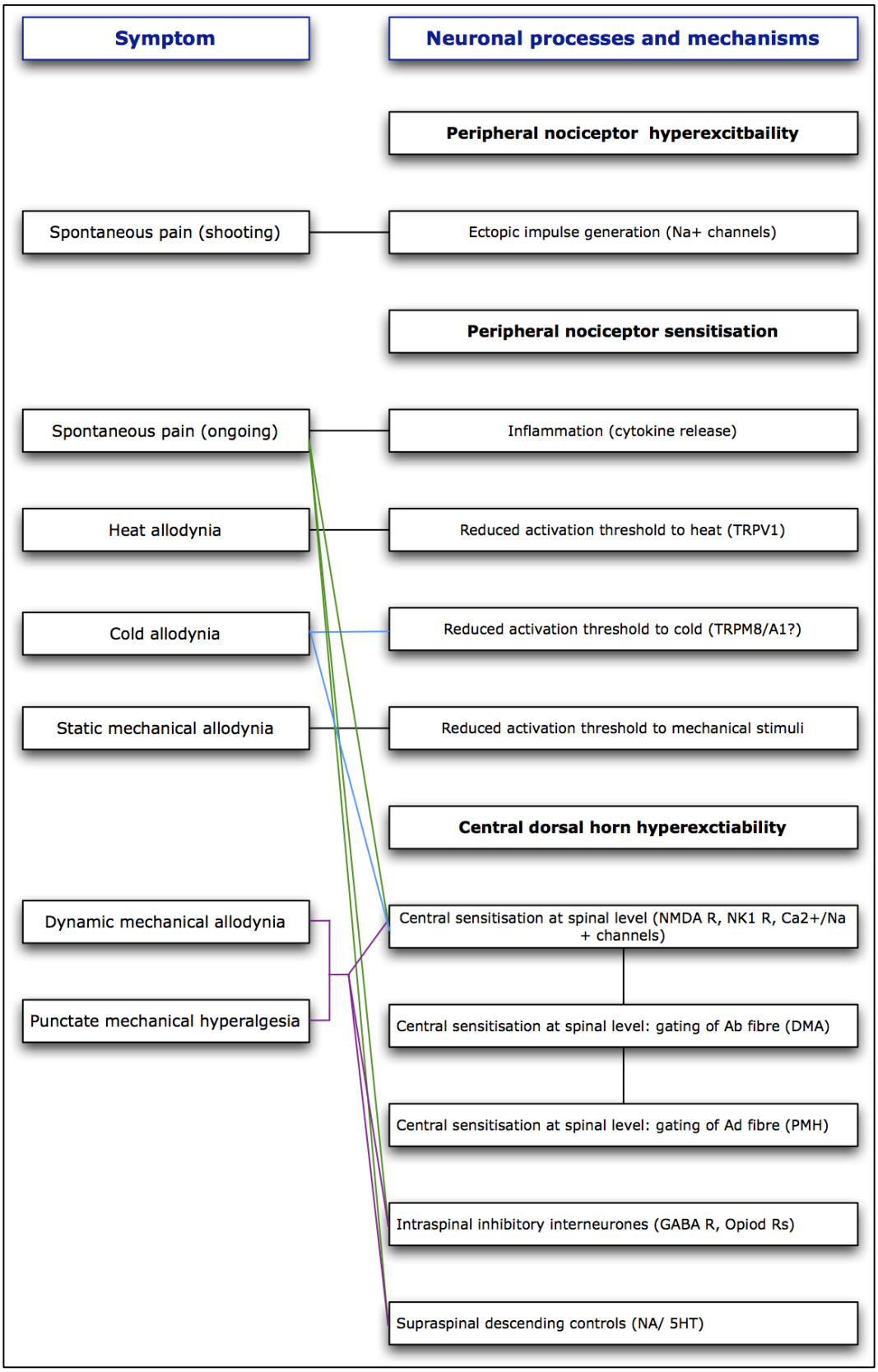

Figure 1-5 Proposed mechanisms for symptoms associated with chronic pain. Different mechanisms underlie different symptoms, although each symptom may arise from a number of mechanisms. Adapted from (Baron 2006).

Although it is out of the scope of this thesis to address the complex issues surrounding the clinical trial design, basic recommendations may be adapted. Using preclinical models with detailed phenotypes will aid the development of a mechanism-based approach to pain classification and treatment. Developing preclinical models that mimic specific symptoms/ mechanisms will enable the testing of drugs designed to act against these particular abnormalities. If we know that a certain drug acts at a specific target that is engaged in a particular mechanism, the most appropriate preclinical model can be chosen for the initial screening (i.e. a hypothesis driven screening). Furthermore, patients suffering from the same symptoms may be selected for the clinical trial based on the fact they would be predicted as more likely to respond. The outcome measures used in preclinical studies may also be used in clinical trials in order to address this disconnect. Finally, such preclinical work can help select the most appropriate endpoints for the clinical trials. Since the models explored in this thesis are symptom specific, rather than being applicable to a particular disease state, recommendations will only be able to be adapted based on symptom profiles. Overall this thesis aims to bring together preclinical data from animal and human models aimed at modelling and treating specific symptoms, in order to improve the path from basic science to the clinic and to aid the design of clinical studies.

In summary, it is clear that animal models based on mechanisms or specific disease states, as well as human surrogate models, all have a number of benefits and drawbacks. However, they have all played important roles in our understanding of pain mechanisms and the development of analgesics. In order to address some of the drawbacks discussed, a combined approach may be undertaken whereby the benefits of all may be exploited. Discovery of analgesic targets and analgesic efficacy should be extrapolated based on specific mechanisms in order to identify the most suitable patient population who may benefit.

1.3. Objective assessment of nociception and pain in experimental models

As previously mentioned, many in vivo models rely on behavioural outcome measures of reflex thresholds to certain stimulation to infer pain in animals, however assessing spontaneous (or ongoing) pain is very difficult. Additionally, since is a subjective measure, there is the possibility that different results may be obtained depending on the researcher undertaking the experiment (Chesler et al. 2002). The same is true of the reporting of pain by human test subjects, which can be hugely variable due to individual subjectivity. In order to try to combat this, more objective measures of pain are being sought out. In animal models this includes the use of measures such as conditioned place preference (CPP), which has started to be used to assess spontaneous pain (King et al. 2009; De Felice et al. 2011). This is an important step forward in the field of pain research, as it begins to relate basic science directly to problems highlighted in the clinic. Other useful techniques include the use of in vivo electrophysiology, which is an objective measure of neuronal activity allowing the study of both sub and supra threshold stimuli. This process is detailed in the methods and will be the main technique used for animal studies in this thesis, as it provides an unbiased and quantitative measure of neuronal activity in response to a number of different painful conditions/stimuli.

1.3.1. Advantages of in vivo electrophysiology

In vivo electrophysiology allows direct objective monitoring of neuronal activity. Recordings can be made at any level of the pain signalling system, including evoked potential recordings of peripheral nerves, spinal tracts, and cortical areas, extracellular single neurone recordings of action potential discharges, and intracellular recordings of postsynaptic potentials and action potentials. In particular, measuring from neurones in the spinal cord to assess sensory processing is of great use as they play a pivotal role in pain signalling (D'Mello and Dickenson 2008; Price 2013). They are a key site of relay for nociceptive information and are the integrators of both peripheral input and descending modulation (D'Mello and Dickenson 2008). Furthermore, the circuitry of the DH is well known to be subject to plasticity in chronic pain states. Electrophysiology enables investigation of the alteration on both physiology and pharmacology of spinal cord processing (Stanfa and Dickenson 2004). Indeed, extracellular recordings have aided the characterisation of central changes and evoked responses in

numerous inflammatory and neuropathic preclinical models in addition to enabling clear demonstration of distinctive pharmacological manipulation (Dickenson and Sullivan 1987; Dickenson and Sullivan 1987; Stanfa et al. 1992; Chapman et al. 1998; Urch et al. 2003; Rahman et al. 2009). The ability to measure suprathreshold stimuli is also of great benefit; since these are more likely match the clinical reporting's from patients experiencing intense pain (as opposed to threshold measures, which are the equivalent to much lower, less clinically relevant, pain ratings) (Sikandar and Dickenson 2013; Sikandar et al. 2013).

As previously mentioned, within the DH of the spinal cord there are two main groups of cells involved in pain processing: NS and WDR neurones. Whilst NS neurones respond on the whole to high intensity input, WDR neurones receive input from A and C fibres resulting in responsiveness to a much broader range of stimuli. Of particular importance is the ability of WDR neurones to code stimulus intensity and wind up, parallel to observations in humans (Price 2013). WDR neurones integrate and modulate signals before they are relayed to higher centres and indeed the coding output of these spinal neurones is comparable to psychophysical reports by humans (Dubner et al. 1989; LaMotte et al. 1991; Simone et al. 1991; Dougherty and Willis 1992; Sikandar et al. 2013). That is to say, that the stimulus response curves of WDR neurones are remarkably similar to human reporting's (Dubner et al. 1989; Price 2013). A recent study by Sikandar and colleagues clearly demonstrated using LV WDR neurone recordings along with human quantitative sensory testing (QST) and electroencephalography (EEG), that the response characteristics of these DH neurones parallels responses in humans (Sikandar et al. 2013). Turning to the animal models of chronic pain WDR neurones have once again been shown (figure 1-6) to exhibit changes that parallel the human responses; in models of hypersensitivity, these neurones increase their firing just as human pain reporting's increase (LaMotte et al. 1991; Simone et al. 1991; Dougherty and Willis 1992).

An additional advantage of studying WDR neurones comes from the fact that these neurones have been shown to model numerous drug responses. For example, pregabalin and gabapentin are effective treatments in many chronic pain disorders, which can quite clearly be demonstrated using recordings from LV WDR neurones that these compounds are able to reduce neuronal activity in a comparable fashion (Dworkin et al. 2003). Whilst the compounds shows efficacy in models of altered nociceptive processing such as SNL and MIA, no such effects can be seen in naïve animals (Suzuki et al. 2005; Bee and Dickenson 2008; Rahman et al. 2009). The same is true in case of tapentadol, whereby it has been

demonstrated that it reduces evoked activity in DH neurones post SNL in parallel to its efficacy in chronic pain patients (Prommer 2010; Bee et al. 2011). As such it is clear that WDR neurones are a useful point of study in order to predict clinical efficacy and reduction in pain intensity.

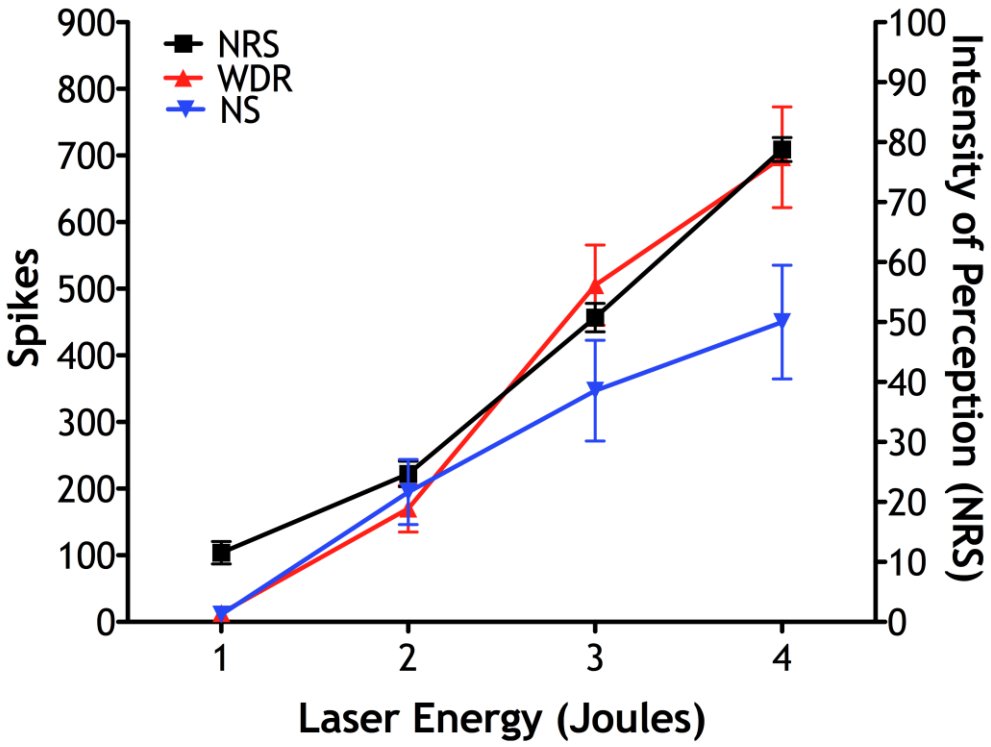

Figure 1-6 Responses to increasing intensities of laser stimuli. There is considerable overlap between responses of WDR cells and human perception. (Sikandar et al. 2013).

1.3.2. Overcoming the subjective nature of assessing human experimental pain

The assessment of chronic pain in experimental human models and the clinic is equally compromised by the subjective nature of the disorder, the limited number of tools that are available, and the time consuming nature of application. Assessment attempts are often made in the form of standardised questionnaires, which are used to aid diagnosis. Since pain is such a subjective experience it difficult to capture and quantify even with such methods. Pain ratings in questionnaires vary depending on the individual and the biological aspects are inextricably intertwined with the social and psychological, which makes analysis complicated. It has been shown that a number of individual and cultural factors such as age, gender, upbringing, personality, can all influence a patient's response to pain (Frederiksen et al. 1978; Chapman 2004). This lack of linear relationship between the underlying cause of the pain and

the patient reports hinder the possibility to extrapolate the potential mechanisms and thus could limit the usefulness of these questionnaires. The wide variety of different questionnaires used also makes comparison between studies difficult. Furthermore, as stated, these provide very different outcome measures than those produced in preclinical the studies, making is very difficult to draw comparisons.

However, that is not to say that such methods provide redundant information – verbal pain descriptors provide important information about the patient phenotype and have been shown to identify up to 90% of cases of neuropathic pain (Haanpää et al. 2011). They are quick and easy to use, providing immediate information. The painDETECT questionnaire has been validated for repeat testing and assesses measures that begin to approach the preclinical studies (i.e. examining different modalities) (Freynhagen et al. 2006). Furthermore, questionnaires can also assess the emotional aspects of chronic pain, which may be seen as equally as important to the patient and thus it should also be monitored as to how a treatment affects co-morbidities such as sleep disturbance, anxiety and depression (Rehm et al. 2010).

In order to address the issue of subjectivity there are a number of objective techniques that have been investigated and hold some potential to be adopted for use in experimental and clinical examination. These range from fMRI and diffusion tensor imaging to magnetoencephalography and EEG. fMRI has been particularly useful in the elucidation of areas of the brain involved in central sensitisation observed in both preclinical pain models and patients, in addition to quantifying pharmacological manipulation (Iannetti et al. 2005; Zambreanu et al. 2005; Lee et al. 2008; Gwilym et al. 2009). Whilst this has driven a lot of interest in fMRI in the past few years, including many successful studies it must be noted that this technique does not directly sample neural activity, but rather the consequential haemodynamic changes. It is also restrictive in terms of providing information that has limitations of both the spatial and temporal domains. As Tracey and Mantyh emphasise, it is imperative to understand the temporal integration within spatially defined areas (Tracey and Mantyh 2007). In addition to this, since the 'pain matrix' is not in fact a unique signature of pain and the same patterns of activation can be evoked by other sensory modalities it could be influenced by other sensory or factors (Legrain et al. 2011). The procedure is also costly and time consuming, which further hinders uptake into the clinic.

In order to address the poor temporal resolution of fMRI, EEG may be used alongside. EEG, although lacking spatial resolution, can provide much better information regarding temporal events. EEG measures ongoing electrical brain activity, which is a reflection of the summation

of low frequency neuronal activity - i.e. postsynaptic potentials - in cortical neurones (Speckmann 1999). A number of sensory, motor, or cognitive stimuli (or events) can be recorded using EEG and are known as event related potentials. Stimulus elicited changes in the EEG waveforms, and may be phase locked or non phase locked to the specific event (Iannetti 2010). The largest wave is known as the negative-positive complex (N2-P2), and is detected mostly at the scalp vertex. A smaller preceding wave is known as N1 and is detected around the temporocentral region on the contralateral side to stimulation. Taken together it is believed that N1, N2 and P2 reflect activity that is generated in the primary and secondary somatosensory cortices as well as the insula and anterior cingulated cortex, in response to sensory stimuli (Garcia-Larrea et al. 2003). With regards to pain, the magnitude of the wave appears to be positively correlated with perceived intensity of the stimuli (Garcí-Larrea et al. 1997; Timmermann et al. 2001). They can therefore be used to assess the functional significance of brain processing in response to specific nociceptive stimuli.

However, as with fMRI, EEG is very time consuming and provides limited information as to the underlying mechanisms. Greater uptake has instead been seen with since the recent introduction of a standardised form of QST designed by the German Research Network on Neuropathic Pain (DFNS) (Rolke et al. 2006; Rolke et al. 2006). The detailed protocol will be described in chapter 2, and despite not being purely objective the technique has many benefits; not least the minimal costs and quick nature of the tests. QST enables the examination of sensory processing, across a number of modalities, under both normal and pathological conditions. A standardised protocol was introduced in 2006, which has allowed the collection of large amounts of data from both healthy human volunteers and patients – all of which is directly comparable.

The QST battery involves a comprehensive list of validated short form tests across relevant somatosensory modalities (Rolke et al. 2006; Rolke et al. 2006). These tests range from mechanical and thermal detection thresholds, to pain thresholds and symptoms of hyperalgesia and allodynia. Whilst assessment of mechanical detection threshold (MDT) using von Frey filaments, or vibration detection using a tuning fork, indicate the function of Aβ fibres, determining the mechanical pain threshold (MPT) using pinpricks assesses Aδ fibre function (Hansson et al. 2007). Abnormalities such as hyperalgesia or allodynia picked up in the stimulus/ response function (S/R function) may also indicate changes in Aβ or Aδ fibres, and could denote the presence of central sensitisation. Assessment of thermal detection and pain thresholds indicate the function of Aδ and C fibres, and thermal hypersensitivity is

believed to be a useful marker of peripheral sensitisation. Finally, pressure pain thresholds (PPT) are testing using an algometer and may be used to gauge the function of muscle Aδ and C fibres. Overall, these symptom profiles give a good indication of the potential underlying pathology. Importantly, these evoked measures can be compared more easily with preclinical studies; for example numerical pain ratings and thresholds can easily compared with animal behaviour and electrophysiological data. This allows for preclinical studies to be run in tandem to collect complimentary data sets.

The QST protocol examines small and large fibres, assessing both gain and loss of function. These functions are explored in cutaneous, and to some extent, deep pain. Furthermore, the tests measure symptoms associated with peripheral and central mechanisms and therefore may aid a more accurate diagnosis than simply asking the patient to rate their pain, and base treatment on this rating and disease state. Different symptoms are believed to be the result of differing underlying pathological mechanisms – therefore a thorough analysis of these sensory profiles allows identification of relevant components, reflecting the underlying aetiologies and guides diagnosis of chronic pain conditions.

For patients in particular, this could allow the determination of particular subgroups with similar somatosensory phenotypes within chronic pain. The patterns of symptoms emerging amongst groups of patients are likely to reflect particular underlying mechanisms. Indeed, it has already been shown that within neuropathic pain patients, 5 subgroups can be defined, based on their symptom profiles (Baron et al. 2012). This suggests that members of each group may have similar underlying mechanisms and thus may benefit from the same treatments. Most likely a mix of these groups will have previously been included in clinical trials and therefore it is not unsurprising that many drugs have failed to show any efficacy across a range of mechanisms not necessarily suitable for the particular compounds. By incorporating experimental human models into preclinical studies, evoking known mechanisms, we can begin to attribute particular symptoms to particular causes. This not only aids diagnosis, but also management of chronic pain - facilitating the move towards the concept of a mechanism based approach to treatment.

However, even QST is limited in the scope of phenotypes measured (Rolke et al. 2006). QST relies on accurate reports from the subject or patient, which will always produce a reasonable amount of variation. As such there would be great benefit to the introduction of more objective assessment. Yet, despite its high clinical relevance, an exclusively objective measure of pain does not yet exist. Rather, as it stands currently QST is one of the best tools available

to fully characterise and compile detailed sensory profiles of patients, and its uptake in preclinical models will increase its usefulness. Using the same tools in the clinic and in early studies is particularly important for translation. If QST is used to characterise preclinical models, with known mechanisms we can begin to understand what the patient profiles mean with regards to the underlying pathology and potential responsiveness of subgroups to novel analgesics. Furthermore, using animal models in addition to QST allows the study of detailed molecular underpinnings. This thesis will explore the use of characterising translational models of chronic pain in animals and humans in order to bridge the gap between basic research and the clinic.

1.4. Thesis aims

This thesis aims to characterise possible translational models of chronic pain using analogous standardised procedures in animals and humans to allow a comparison of results. The models include: capsaicin cream, UVB and UVB heat rekindling. By using both animals and humans, this thesis aims to overcome potential species differences and align the preclinical models that are used. Furthermore, the use if human subjects enables the exploitation of verbal capacity and a thorough investigation of the exact location, intensity and quality of the pain.

Full characterisation of the models will involve the use of an objective outcome measures in animals: in vivo electrophysiology. The use of such measures helps overcome the issues discussed around subjective behavioural measures. The concordance in outcome measures between animal and human studies aims to assess the ability to evoke the same changes/signs/symptoms in animals and humans, suggestive of the induction of overlapping mechanisms. Furthermore, by using QST as the outcome measure in humans this thesis aims to create full sensory profiles of the above models, in order to draw comparison with patient profiles.

This thesis also aims to investigate a novel translational pain mediator: CXCL5. The consequence of intraplantar injection of CXCL5, a chemokine believed to be involved in sensitisation post UVB irradiation, will be assessed in animals.

Finally, this thesis aims to assess the ability to modulate translation models using the same drugs, and using a mechanism based approach to treatment. The ability of ADO to prevent capsaicin induced hypersensitivity will be investigated in both animals and humans, whilst a more in depth study of the mechanism will involve the use of CPA in animals.

2. Materials and Methods

All experimental procedures were approved by the UK Home Office and were performed in accordance with the guidelines provided by the International Association for the Study of Pain for the care and use of Laboratory animals. Human studies were approved by The Kings College Research Ethics Committee and all participants gave written informed consent before commencing the study.

2.1. Animals

Male Sprague-Dawley rats were obtained from the UCL Central Biological Unit. The rats were housed in cages under a 12-hour light/dark cycle with readily available food and water. The weight of the rats used in these experiments was consistently between 220 and 250g.

2.2. In vivo Electrophysiology

2.2.1. Animal set up

The following protocol has been well established and is detailed in full by Urch and Dickenson (Urch and Dickenson 2003).

Rats were anaesthetised in an induction box using 4% isoflurane (carried in 66% N_2O and 33% O_2). Once the rat was fully unconscious and checked for absence of reflexes (by pinching the toes of the hindpaw) a tracheotomy was performed. This procedure involved exposing the trachea and making a small incision with a scalpel horizontally across. A polyethene cannula was then inserted around 80-100mm into the trachea and fastened in place using 3-0 silk thread. Isoflurane was delivered through this cannula for the full duration of the experiment.

Once this procedure was completed, the rat was placed in a stereotaxic frame and secured using ear bars. The anaesthesia was then dropped to 2-3% to perform a laminectomy. Within the stereotaxic frame the rat was placed onto a homeothermic heat mat, controlled using a rectal temperature probe, to maintain core temperature at 37°C.

An incision was made into the skin along length of vertebra, to expose connective tissue and muscle. Connective tissue was removed and the bottom of the rib cage was identified – where the lower ribs meet is the approximate position of the T12 region. Above this, two incisions

were made either side of the vertebral column to enable clamping and stabilisation of the cord to facilitate the laminectomy.

Muscle and vertebrae were removed from around L1-L3 using rangeurs in order to fully expose the L4-5 segments of the spinal cord; where recordings from WDR cells were made and drugs can also may be applied. Watchmaker forceps were used to remove the dura from the cord to improve recordings. Below this region two further incisions either side of the column were made, once again to enable clamping and stabilisation, as well as to aid positioning of the cord for optimum recording. It was important that the cord was straight and no movement occurred with respiration. Further to this the isoflurane was dropped to 1.75-2% for the remaining duration of the experiment. This was raised to 5% upon completion of the experiment in order to overdose the rat before ensuring death via cervical dislocation of the neck.

2.2.2. Electrophysiological recording

Recordings were made from single WDR neurones located in the deep dorsal horn (DDH) of the spinal cord (500μm ventral to the surface of the spinal cord). The recordings were made using a parylene coated tungsten electrode (125μm in diameter) and the system was grounded through both the animal and the frame. Thus the final recording of the input from the electrode was that from the neurone minus the signal from the animal, in order to reduce interference. The electrode was secured into a head stage attached to a 3-axis manual micromanipulator. Recordings were obtained using an AC recording system (NeuroLog System, Digitimer, UK) and were amplified and filtered, before being displayed on an oscilloscope, as well as heard through a sound amplifier.

In order to identify an individual neurone, the ipsilateral plantar surface of the rat's hindpaw and toes were tapped. Spikes must be clearly distinguished from background noise and of a uniform shape and amplitude. It was sometimes necessary to distinguish the cell from neighbouring ones by slightly adjusting the electrode up or down. Once a cell was easily differentiated and counted, the response to different stimuli were recorded. Data was captured using Spike 2 software on a Pentinum computer, which is coupled to a CED 1401 interface. Responses were recorded to natural stimuli (mechanical and thermal) – including brush, von Frey filaments of graded forces (2g, 8g, 15g, 26g, 60g) and different temperatures

of water (35°C, 40°C, 45°C, 48°C). These stimuli were applied to the receptive field for 10 seconds.

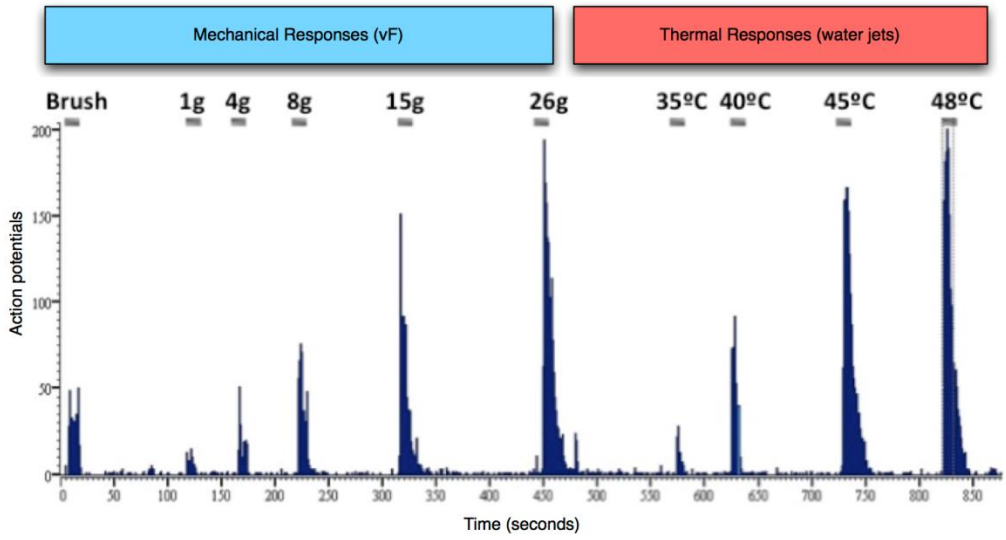

Figure 2-1 Example responses of a single WDR neurone. Stimulus histogram showing WDR neuronal responses to a variety of graded natural stimuli. Mechanical stimuli include dynamic brush and vF filaments and thermal stimuli are water jets.

2.2.3. Transcutaneous electrical stimulation

In order to induce wind up, two fine stimulating needles were inserted under the skin within the cells receptive field. Single electrical pulses were then delivered at graded mA intervals (beginning at 0) in order to find the threshold of Aβ and C fibres. These were identified by the presence of an action potential in the correct latency band of each fibre; i.e. evoked potentials from C fibre stimulation must lie within the latency range of 90-300ms. A train of 16 stimuli were then delivered (2ms wide pulse at 0.5Hz) at 3 times the C fibre threshold. A post stimulus time histogram was constructed in order to classify the responses evoked from the Aβ (0-20ms), Aδ (20-90ms) and C (90-300ms) fibres, in addition to calculating the post-discharge (PD) – this is the activity which occurs after C fibre latency, around 300-800ms. This separation of fibres relies on assuming the conduction distance is approximately 10cm. A predicted 'no wind up' response (e.g. a lack of change in the evoked responses over the 16 stimuli) was calculated by multiplying the number of action potentials elicited by the first single stimulation by 16 (number of action potentials after the first stimulus x 16). This hypothetical number is termed input. The wind up was then calculated by using the formula: Wind up = total number of action potentials after 16 stimuli – input

This paradigm was repeated 3 times, every 20 minutes, within the space of one hour, in order to confirm a stable baseline response of the cell. Depending on the experiment, this may have been further repeated after the application of a drug or sensitising chemical stimuli.

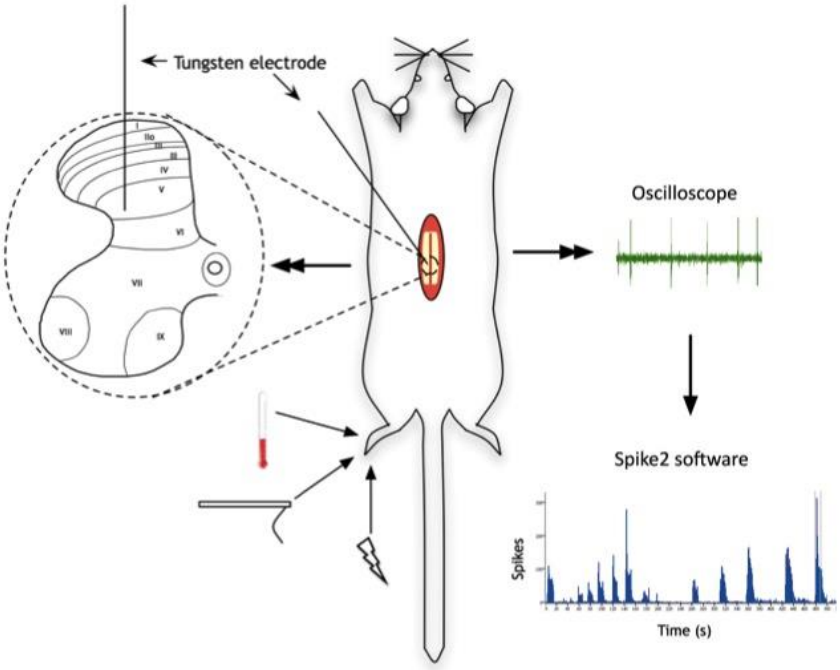

Figure 2-2 In vivo electrophysiology set up. A laminectomy was performed to expose L4-5 segments of the spinal cord and recordings were taken from LV neurones of the DH using a tungsten electrode. Natural and electrical stimuli were applied to the RF on the hindpaw. Action potentials were visualised on an oscilloscope and captured using spike 2 software (Asante 2009).

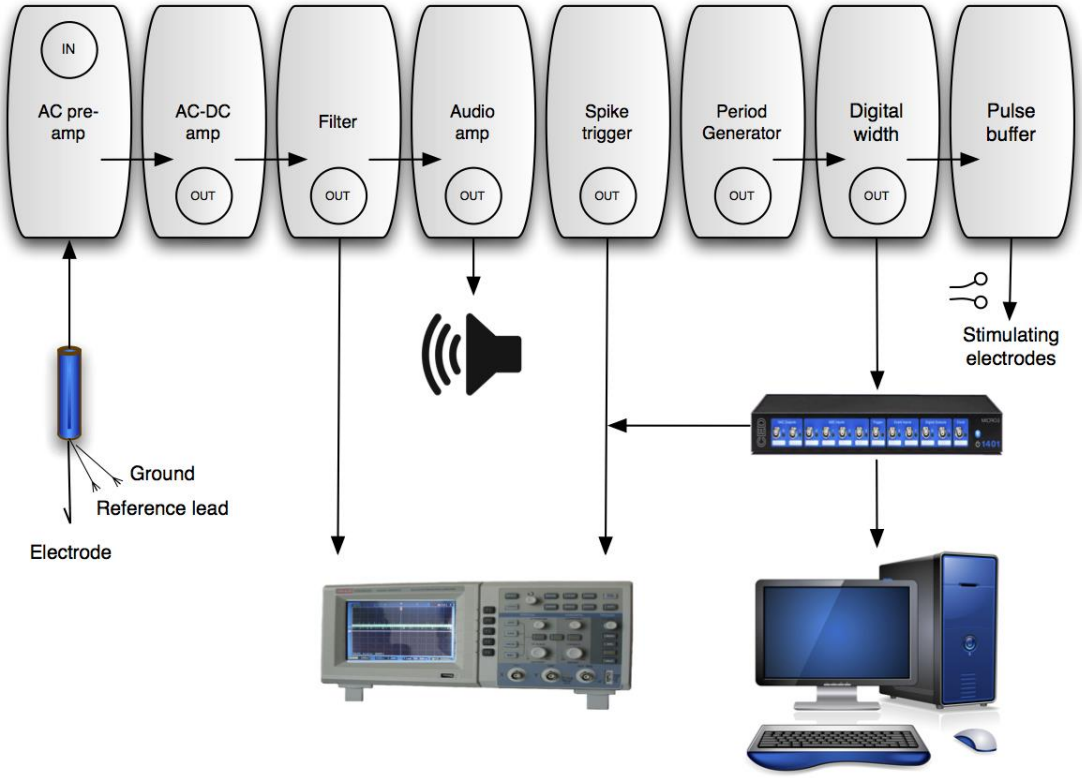

Figure 2-3 Schematic of neurolog recording system. The recording electrode was inserted into the spinal cord and input enters the recording system via the headstage. Grounding was achieved through a lead connecting to the stereotaxic frame. The reference lead recorded interference/ basal electrical activity and subtracted this from the signal, which was then amplified and filtered before being fed into the audio speaker and oscilloscope. These visual and audio representations of the action potentials allowed for isolation of single unit WDR neurones. Action potentials with an amplitude above a set threshold were fed into the CED1401 interface and were quantified by the computer. Electrical stimuli were delivered into the receptive field using two stimulating electrodes and a post-stimulus time histogram was generated depending on the latency of evoked potentials. Natural stimuli were also displayed on a rate histogram and the number of action potentials evoked in 10 seconds were quantified.

2.3. Subjects

Healthy volunteers between 18 and 59 years old with no history of chronic pain were recruited for the studies detailed in this thesis. All subjects were in good health at the time of study, and were advised to avoid painkillers, caffeine and alcohol up to 12 hours prior to the study, since these substances may interfere with the results. Additionally, all female subjects confirmed they were not pregnant at the time of the study. Any volunteers with skin conditions or inflammation such as eczema or dermatitis were excluded from taking part in any study. Once subjects had been selected to take part in any study, they were familiarised with the sensory testing procedure detailed below, so they knew what to expect during the study.

2.4. Human Quantitative Sensory Testing

Subjects were sat comfortably in a temperature controlled, quiet room with no external visual disturbances. After obtaining written informed consent and explaining the protocol, seven tests, measuring 13 different parameters were then undertaken as described by Rolke and colleagues (Rolke et al. 2006; Rolke et al. 2006). The same testing equipment was used for all studies. Before beginning each study, the subject was familiarised with each of the tests on an independent area.

2.4.1. Mechanical detection threshold (MDT):

A standardised set of vF filaments (Optihair$_2$-Set, Marstock nervtest, Germany. 0.5mm diameter rounded tip to avoid nociceptor activation to low force vF), ranging between 0.25 and 512mN were used to assess the average MDT. The subject sat with their eyes closed and their hand, or arm, placed comfortably in front of them. The hairs were carefully applied to the point of bending, over a small area, one at a time using the 'up-down'/ 'method of limits'. Beginning with 16mN the subject was asked to report if they felt any touch sensation. If so the force was then decreased until the subject reported they were no longer able to feel anything. Increasing forces then were then applied until the subject reported they were able to feel the vF. Overall 5 series of ascending and descending stimulus intensities were applied and the geometric mean was calculated to work out the MDT. This threshold is most likely mediated by Aβ fibres (Hansson et al. 2007).

2.4.2. Mechanical pain thresholds (MPT):

A standardised set of custom-made pinprick stimulators (Pinprick, MRC Systems GmbH, Heidelberg, Germany. 0.2mm diameter) ranging between 8mN and 512mN were used to assess the average MPT. The subject once again closed their eyes with their hand, or arm, placed comfortably in front of them. The pinpricks were carefully applied perpendicularly at a standard force, using the 'up-down'/ method of limits. Each pinprick was applied for one second over a small area. The test began with 8mN and increasing pinprick forces were applied until the subject reported that the sensation changed from 'blunt' to 'sharp'. Once the subject reported the pinprick to be 'sharp', the force was decreased until the subject reported the sensation felt 'blunt'. This process was repeated 10 times and the geometric mean was calculated to work out the MPT. The threshold is most likely mediated on the whole by Aδ, with a contribution from C fibres (Hansson et al. 2007; Iannetti et al. 2013).

2.4.3. Stimulus-response-functions (S/R functions) - mechanical pain sensitivity for pinprick stimuli and DMA for stroking light touch:

The seven pinprick stimuli, ranging from 8mN-512mN, were used along with a cotton wisp (3mN), a cotton wool tip fixed to an elastic strip (100mN) and a standardised brush (Somedic, Sweden. 200-400mN). Unlike the determination of thresholds, this allowed for the detection of hyper and hypoalgesia to supra-threshold stimuli. The different stimuli were applied 5 times each in a specific order, as determined by the DFNS protocol. For each stimulus the subject was required to give a rating from 0-100, where 0 = no pain, and 100 = worst pain imaginable. The tactile stimuli were integrated with the pinpricks, and were applied with a single stroke across a 1-2cm length of skin. These functions are mediated by Aβ, Aδ, and C fibres (Hansson et al. 2007).

2.4.4. Wind-up ratio (WUR):

The 256mN pinprick stimulator was used to assess the WUR, which is a measure of the perceptual correlate of temporal summation to repetitive stimuli. The subject was once again asked to close their eyes and place their hand, or arm comfortably in front of them. A train of 10 stimuli was applied at the same force at over an area of $1cm^2$. The stimuli were delivered at a rate of 1/s. The subject was asked to give a rating from 0-100 for the first and last of the

train of stimuli. This test was repeated 5 times over different areas within the same region of the body. The WUR was then calculated from the average pain rating of the final stimuli in the train, divided by the average rating of the initial single stimuli.

2.4.5. Vibration detection threshold (VDT):

A Rydel-Seiffer tuning fork (64hZ, 8/8 scale) was used to assess the VDT. The tuning fork was placed over the nearest bony prominence to the test area and the subject was asked to report when they felt the vibration stop. The threshold was determined by averaging 3 trials. VDT is mediated by Aβ fibres (Hansson et al. 2007).

2.4.6. Pressure pain threshold (PPT):

An algometer pressure gauge device (FDN200, Wagner Instruments USA) with a rubber probe area of $1cm^2$ was used to assess the PPT. The probe was applied to the hand, or arm, at an increasing ramp of 50 kPa/s, until the subject reported the sensation was painful (up to a maximum force of 2000 kPa). The threshold was determined by averaging 3 trials. PPT is also mediated by Aβ fibres (Hansson et al. 2007).

2.4.7. Thermal detection, thermal pain thresholds and paradoxical heat sensations:

The TSA thermal sensory testing device (TSA 2001-II; Medoc Ltd, Ramat Yishai, Israel) was used to assess both thermal detection and pain thresholds. The subject is instructed to sit with their hand, or arm, in front of them and a thermode (area = between5x5 mm and 16x16mm largest is 30x30mm) was fixed in place using a Velcro band. The subject was not able to see the computer screen for the duration of the tests, but was instructed when the test would begin. Thresholds were recorded using the medoc TSA system (WinTSA 5.3 NeuroSensory Analyzer). All thresholds were obtained using a ramped stimulus (1°C/s), which was cut off as soon as the subject clicked a mouse. Testing began at 32°C and the temperatures were cut of at 0°C and 52°C to avoid injury.

The subject was first asked to click the mouse as soon as they perceived a cooling sensation (CDT) i.e. the first time they felt the temperature decrease. This was followed by detection of a warming sensation (WDT) i.e. the first time they felt the temperature increase. Each of these

detection thresholds were repeated 3 times, with an inter-stimulus interval of 10s, and an average value was calculated. A normal detection threshold is within 1-2°C from baseline (32°C), and whilst CDT is believed to be mediated by Aδ fibres, WDT is predominantly mediated by C fibres (Arendt-Nielsen and Chen 2003; Rolke et al. 2006; Hansson et al. 2007).

Next, paradoxical heat sensations were measured using a thermal sensory limen procedure for alternating cold and warm stimuli. The subject was instructed to click the mouse as soon as they perceived a cooling or warming sensation, in addition to also verbally reporting which sensation they felt.

Finally the cold pain, and heat pain thresholds were measured (CPT and HPT, respectively). Subjects were informed that they would feel a cooling, cold and finally a cold pain sensation and that they must click the mouse as soon as they felt the device become painfully cold. Similarly, to measure HPT they were instructed that they would feel a warming, warm and finally a heat pain sensation and that they must click the mouse as soon as they felt the device become painfully hot. Once again, these pain thresholds were measured 3 times and an average was taken. CPT is mediated by both Aδ and C fibres and is highly variable, on the other hand HPT is always around 45°C and mediated on the whole by C fibres with a small Aδ fibre contribution (Arendt-Nielsen and Chen 2003; Rolke et al. 2006; Hansson et al. 2007).

In the original DFNS description of the QST battery the thermal tests preceded the mechanical. However, in this thesis the protocol was reversed since it has been shown that by applying heat first a slight sensitisation may be induced, significantly increasing mechanical pain sensitivity (Gröne et al. 2012).

2.4.8. Data evaluations and z-transformation

Data was first assessed for normality using the Kolmogorov-Smirnov. Any data that did not pass this test was log-transformed and re-tested. It is generally accepted that most psychophysical measures will not be normally distributed, with the exception of heat and CPTs, and VDTs. Negative QST scores (CDT), were be multiplied by -1, before they were logged. Additionally, a small constant of +0.1 was added to all numerical ratings before logging, to avoid a loss of zero values (Magerl et al. 1998; Rolke et al. 2006). In order to compare the data collected from the surrogate models to the baseline responses across all

QST parameters, Z-scores were then calculated using the following formula:

$$\text{Z-score} = (X_{\text{Single Subject}} - \text{Mean}_{\text{Controls}})/\text{SD}_{\text{Controls}}$$

Where X = the value for any parameter of a given individual subject. When presented graphically, the algebraic sign of the Z-score value was adjusted so that Z-scores above '0' indicate a gain of function, while those that are below represent a loss. Post-model scores can be compared to baseline controls using the 95% confidence intervals:

$$95\%\ \text{CI} = \text{Mean}_{\text{Controls}} \pm 1.96\text{SD}_{\text{Controls}}$$

2.5. Chronic Pain Models

2.5.1. In vivo Electrophysiology – Capsaicin

Topical capsaicin cream was used in order to induce sensitisation of the pain pathway, altering both peripheral and central processing mechanisms. Capsaicin was applied only once a cell had been characterised, using the above protocol involving mechanical, thermal and electrical stimuli, and stable responses have been achieved. Following this between 0.1-0.2 ml of 1% capsaicin (Pharmasol & Pharmaserve NW, UK) was applied onto the receptive field (depending on it's size) using a 1.0ml syringe. Once the receptive field was covered, the area was covered with parafilm to ensure there was no loss of cream during the 30 minutes it was applied for.

The capsaicin cream formulation also contains purified water, sorbitol solution, isopropyl myristate, cetyl alcohol, white soft paraffin, glyceryl stearate, PEG-100 stearate and benzyl alcohol. The vehicle was not studied since this has been previously been shown to have no effect on sensory measures and thus, in line with the NC3Rs, these experiments were not repeated (Simone and Ochoa 1991; Altman et al. 1994; Magerl et al. 2001).

30 minutes post application the cream was carefully removed using an alcohol wipe. Since mechanical pressure could affect the degree of sensitisation induced, this procedure was conducted as gently as possible and in the same manner each time, in order to reduce variability. Subsequently the natural (mechanical and thermal) and electrical responses were then re-tested at 30, 60 and 90 minutes post application.

If the experiments were aimed at modulating the effects of capsaicin, the drug was administered prior to capsaicin application. The method of drug administration is detailed in section 2.6.

2.5.2. In vivo Electrophysiology – UVB

UVB is a stimulus believed to cause an entirely peripheral sensitisation of the pain pathway. Rats were first anaesthetised in an induction box using 4% isoflurane (carried in 66% N2O and 33% O2). Once the rat was fully unconscious and checked for absence of reflexes they were placed on-to a heat mat and fully covered with UV resistant material. After exposing the plantar surface of the right hindpaw, the UVB light source (Dermfix 1000MX UV-B Lamp fitted with a 9 Watt fluorescent UVB tube, λ max = 311nm) was placed at a set distance from the paw, ensuring the correct dose is delivered. The irradiance of the lamp was determined using a calibrated photometer (Solartech Inc Solarmeter 6.2 UVB Meter, Merlin Lazer). This reading was used to determine the length of time required to deliver a set dose of 1000mJ/cm^2. The dose used in these studies was chosen on the basis of previous studies, which have found this to have the greatest effect without resulting in any signs of skin damage such as blistering (Bishop et al. 2007). Post irradiation the rats were placed in a temperature controlled recovery box until the effects of the anaesthetic had completely reversed. In vivo electrophysiology was performed 24-30 hours post UVB.

2.5.3. In vivo Electrophysiology – UVB Rekindling

UVB irradiation was carried out as described above in 2.5.2, however only the upper half of the hindpaw was exposed to the light source, rather than the entire paw. 24-30 hours later in vivo electrophysiology was performed. Once a cell had been isolated and characterised with 3 stable baselines the rekindling procedure was initiated. A heat source of a constant temperature of 40°C was then applied to the irradiated area for an initial 5 minutes. A second identical rekindling was then performed after an interval of 15 minutes. Subsequently the natural (mechanical and thermal) and electrical responses were re-tested at 30, 60, 90, 120 and 150 minutes post rekindling.

2.5.4. QST – Capsaicin

Administration of capsaicin cream to volunteers was similar to the protocol described for the animals. Once full sensory testing had been completed, 0.5ml of 1% capsaicin cream was applied to the test area (16x16mm) marked on to the skin. This was then covered with a transparent film dressing (Tegaderm Film, 3M Health Care) ensuring no cream was lost or removed during the 30 minutes it was applied for. Once again, care was taken when removing the cream to ensure it was in a gentle and uniform manner, in order to minimise any effects on the level of sensitisation induced.

2.5.5. QST – UVB

Volunteers were irradiated in a similar protocol as described for the animals. However, the dosing was calculated on an individual basis depending largely on skin type. An initial screening was conducted on each subject to determine their minimal erthymal dose (MED); this is defined as the time required to produce a uniform reddening of the area at 24 hours post irradiation. 3 times the MED was then used for the final experiment to irradiate a 16x16mm area - the surrounding area must was covered with a UV resistant material to ensure a uniform burn.

2.5.6. QST – UVB Rekindling

24-30 hours post UVB irradiation subjects returned for the heat rekindling procedure and full QST profiling. The procedure was carried out using the TSA thermal sensory testing device (TSA 2001-II; Medoc Ltd, Ramat Yishai, Israel), as used for the thermal testing in the QST protocol. The thermode (16x16mm) was placed directly over the UVB burn and held in place with a Velcro strap. The rekindling procedure carried out was the same as that described for the animals – the thermode was kept at 40°C for 5 minutes, followed by a 15 minute interval and a subsequent second rekindling identical to the first.

2.6. Drug Administration

2.6.1. In vivo Electrophysiology - ADO/ CPA

50 μL of adenosine (ADO) (26μg) or N6-cyclopentyladenosine (CPA) (5μg) in a saline solution was injected into the receptive field of the cell (adjacent to the testing area) using a Hamilton syringe. In the paradigm exploring the ability of the drugs to prevent capsaicin induced sensitisation the injection was given 10 minutes before the application of capsaicin over the receptive field.

2.6.2. QST - ADO

A 1ml syringe was used to inject 26μg/50μL ADO in a saline solution into the test area (5x5mm). Successful injection was confirmed by the appearance of a bleb. As with the animals, 10 minutes post injection capsaicin is applied over the treated area (5x5mm).

2.7. Statistical Analysis

All statistical analysis was performed using SPSS software (IBM SPSS Statistics v21). Data was assessed for normality using the Kolmogorov-Smirnov test to determine further methods of analysis. Detailed statistical analysis is found in each individual chapter.

2.8. Control Experiments

All compounds used in this thesis were in solution with 0.9% saline (ADO/CPA/CXCL5). Therefore we first tested the effect of intraplantar/ intradermal saline on WDR cell responses and human QST.

2.8.1. Control methods

In vivo electrophysiological recordings were performed as previously described to obtain baseline responses. Once stable responses had been characterised for each individual cell, 50μL saline was injected using a Hamilton syringe, into the receptive field of the cell, distal from the point at which natural stimuli were applied. The train of electrical and natural stimuli was repeated at every 30 minutes, up to 4 hours post injection. The maximum change was calculated once results had been collected.

Full QST was performed as described in chapter two to obtain baseline responses. The area was then cleaned with an alcohol wipe before intradermal injection of 50μL 0.9% saline. Thresholds and ratings we retested at every 30 minutes, up to 180 minutes post application. The maximum change in subject responses was calculated.

2.8.2. Intraplantar injection of saline has no effect on LV WDR cell responses

Natural and electrical evoked responses of WDR cells were recorded both pre and post intraplantar saline. There was no difference in responses to any of the stimuli tested, thus suggesting that any effects in the following chapters can be attributed to the drug rather than vehicle.

2.8.3. Intradermal injection of saline has no effect on human psychophysical responses.

Mechanical and thermal thresholds, in addition to NRS ratings were tested pre and post intradermal saline. There was no effect on any of the measures, thus suggesting that as with the animals, any effects in the following chapters may be attributed to the drugs tested.

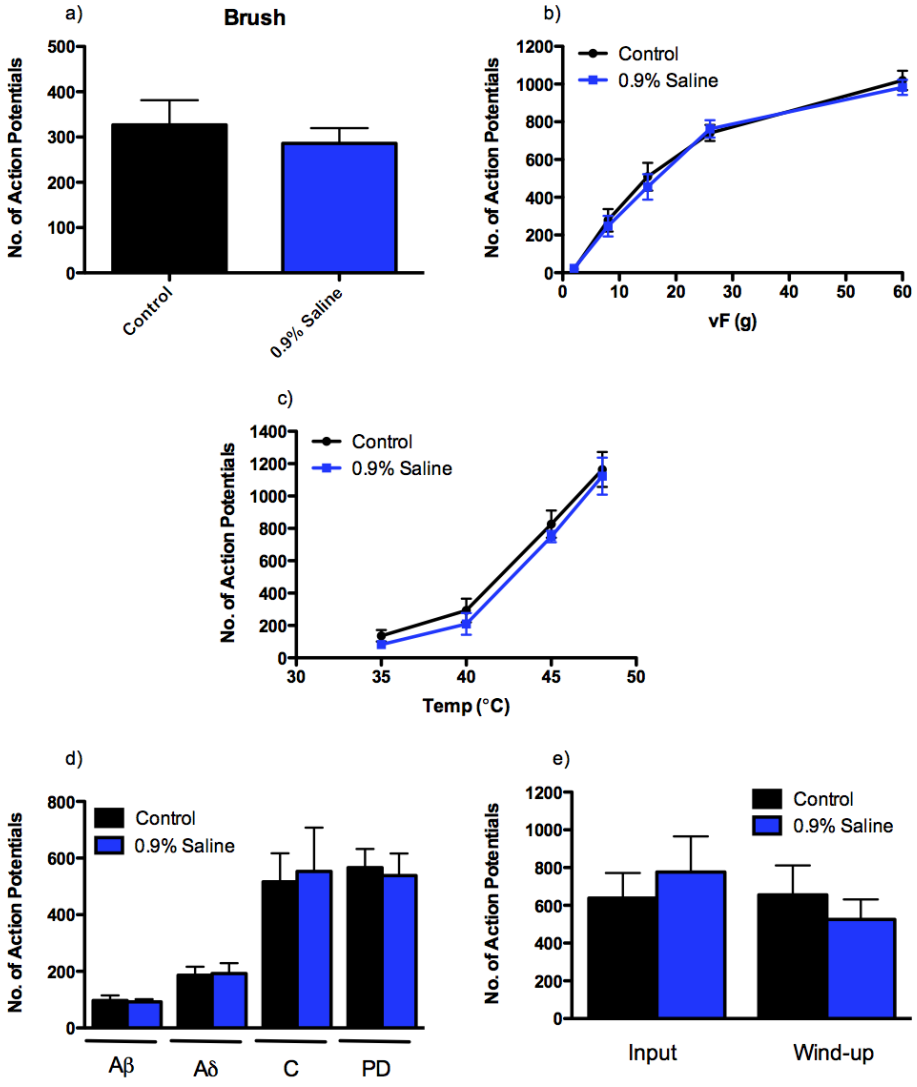

Figure 2-4 Natural and electrical WDR cells responses are unchanged by intraplantar saline. Using the protocol described in chapter 2.2 in vivo single unit recordings of responses of LV WDR cells to natural and electrical stimuli. Natural stimuli (mechanical and thermal) were applied for 10s and included brush, von Frey filaments of graded forces and different temperatures of water. Transcutaneous electrical stimulation was used to measure the input and wind up, in addition to calculating the responses driven by different fibre types. These responses were unchanged up to 90 minutes post intraplantar administration of saline into the receptive field. Such measures are taken as a control for drugs administered via intraplantar injection (as in chapter 4 and 6).

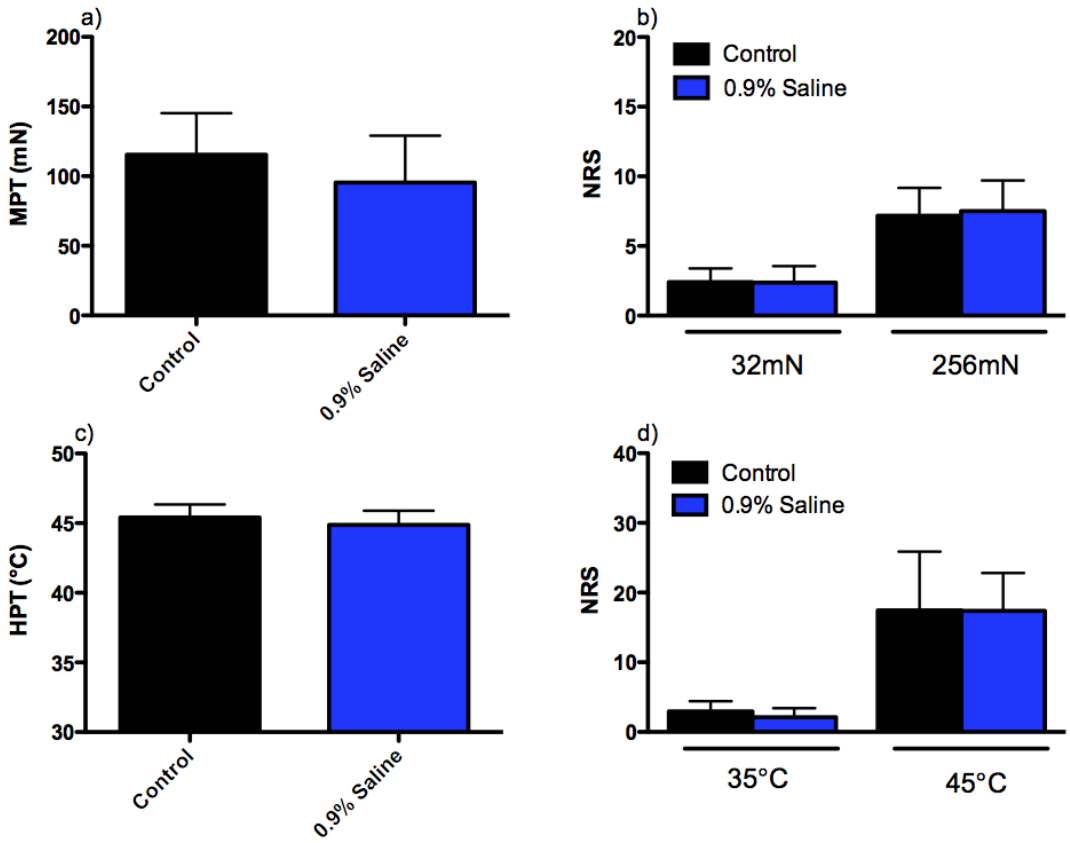

Figure 2-5 Psychophysical human responses are unchanged by intradermal saline. Using the protocol described in chapter 2.3 standardised QST was undertaken to determine the subject's mechanical pain threshold (MPT) and heat pain threshold (HPT), in addition to obtaining numerical ratings (NRS) to graded mechanical and thermal stimuli. These responses were unchanged up to 90 minutes post intradermal injection of saline. Such measures are taken as a control for drugs injected intradermally (as in chapter 4).

3. Capsaicin Cream

3.1. Introduction

One approach in establishing experimental pain models is to mimic the events that produce pain in patients. This is most often undertaken in laboratory animals. One example of such is the pioneering work from the Mantyh lab in creating a model of bone cancer pain in rodents that has considerable face validity in mirroring the pathology in human patients (Bloom et al. 2011; Mantyh 2013). However, one important, and highly criticised, limitation of these models is the possible confound of species differences. Furthermore, it is difficult to fully explore the human condition, since sensory phenotypes and profiles cannot be identified from patients with concomitant therapies and analgesics.

Therefore, a second key approach is to implement models where the same protocols are undertaken in animals, as well as in otherwise healthy volunteers, in order to approach this possible species issue and explore possible human sensory phenotypes. Understandably it is usually not feasible, or ethical, to attempt to recreate the actual pathology occurring in pain patients. Instead one can attempt to mimic the signs and symptoms exhibited by chronic pain patients with the aim of modelling as closely as possible the mechanisms in healthy humans of the relevant underlying pathophysiologies in patients. Pivotal mechanisms of chronic pain include peripheral and central sensitisation, which can indeed be mimicked in healthy volunteers using a number of surrogate models, such as with the use of capsaicin. This chapter explores the use of electrophysiology and QST in order to objectively characterise the use of topical capsaicin cream as a translational model of chronic pain.

Capsaicin is the chemical found in chilli peppers and is responsible for the hot and spicy flavour upon consumption. The molecule is able to depolarise nociceptors and increases their cytosolic free Ca^{2+} concentration. This action is exerted through the TRPV1 receptor, which is expressed on small sensory neurones, including peptidergic and non-peptidergic C fibres (Michael and Priestley 1999; Kobayashi et al. 2005). In addition to immunohistochemistry studies, capsaicin has been shown to selectively activate C fibres and the ablation of TRPV1 positive fibres has been found to result in a reduction in C fibre, but not A fibre related activity (Culp et al. 1989; Brenneis et al. 2013). Thus it would appear that the majority of TRPV1 receptors in the periphery are expressed on nociceptive C fibres. Activation of the receptor with capsaicin results in a flare and burning pain, which can further leads to the development of hypersensitivity to mechanical and thermal stimuli; a phenomenon which has been extensively explored with regards to the relevance of these symptoms in chronic pain.

3.1.1. Capsaicin has been widely explored as a surrogate model of hypersensitivity

The use of capsaicin as a surrogate pain model in human subjects has been explored since the 1960's (Jancsó 1960). It was first introduced by Jansco and colleagues and it is now well described that administration of capsaicin through various means can lead to the hallmarks of chronic pain, such as hyperalgesia and allodynia (O'Neill et al. 2012). The model has been extensively used in healthy volunteers to understand the peripheral and central mechanisms that underpin such symptoms, in addition to screening novel analgesics. It can be administered intradermally, topically, or in combination with a heat source (heat/capsaicin model); it is well established that both intradermal and the heat/capsaicin model induce robust central sensitisation – a key mechanism believed to be involved in chronic pain (Modir and Wallace ; LaMotte et al. 1991; Petersen and Rowbotham 1999; Baron et al. 2013). For a full review on the use of heat/capsaicin and intradermal capsaicin as surrogate models, see O'Neill et al (O'Neill et al. 2012).

On the other hand, topical application alone has not been as thoroughly investigated, although it is also believed to lead to both peripheral and central modifications of the pain pathway. This model has mainly be used in humans, whereby the previous studies have on the whole followed a uniform paradigm consisting of topical application of 0.1-1% capsaicin to the skin for 30 minutes before sensory testing is conducted. A variety of endpoints have been examined although many studies focus on the changes in the secondary area (see table 3-1 for full details)(Carpenter and Lynn 1981; Kenins 1982; Culp et al. 1989; Koltzenburg et al. 1992; LaMotte et al. 1992; Kilo et al. 1994; Liu et al. 1998; Mohammadian et al. 1998). Overall, the general consensus is that topical capsaicin evokes an initial C fibre discharge and subsequently leads to the development of thermal and mechanical hypersensitivity, with both pinprick and brush hypersensitivity extending into the non-treated, secondary area (Kenins 1982; Koltzenburg et al. 1992; LaMotte et al. 1992; Kilo et al. 1994). Although one study has confirmed the activation of C fibres, and another the development of secondary mechanical hypersensitivity in rodents, there have been few investigations conducted in animals (Kenins 1982; Moylan Governo et al. 2006). Furthermore, as stated, very few studies have fully characterised the primary area of injury, where there is likely to be a mix of peripheral and central changes. Since no area of desensitisation is created with the use of low dose topical capsaicin, as with the intradermal injection, it is possible to fully explore the effects of a peripheral sensitisation leading to central modifications in the primary area of injury and therefore, it can also be used to study drugs that modulate both the peripheral and central components. Examination of this primary area may be relevant as there are likely to be

painful patient conditions that encompass both phenomenon. Indeed, this could be the case for patients suffering from OA, post-surgical pain or even fibromyalgia (Gwilym et al. 2009; Baron et al. 2013).

Study	Capsaicin	Treated Area	Time Frame	Thermal Hyper-sensitivity	Mechanical Hyper-sensitivity	Other Observations
Carpenter et al, 1981	1% capsaicin solution painted topically on to slightly abraded skin	Forearm (area 6 to 28cm2)	Retesting 10 minutes post application, capsaicin reapplied every 2 hours until 7 applications	✓	✓	
Kenins et al, 1982	1% capsaicin solution	Rat: Hind leg (square area 1cm2)	30 minute application, followed by multiple applications to cause desensitisation	Not Tested	Not Tested	Topical capsaicin evokes polymodal C fibre discharge and alters activation thresholds (initially decreased, followed by an increase post desensitisation)
Culp et al, 1989	Up to 6% capsaicin solution soaked gauze pad	Forearm or palm (area 1.6x2.5cm2)	30 minute application	✓	✓	A fibre block does not abolish mechanical hypersensitivity
LaMotte et al, 1992	1% capsaicin applied topically to the skin inside a dam with a cotton-tipped applicator	Around the peroneal nerve	Retesting 15-60 minutes post application	✓	✓	Topical capsaicin evokes C fibre discharge
Koltzenberg et al, 1992	1% capsaicin solution in a plaster	Volar forearm/ hairy skin of hand dorsum	30 minute application	Not Tested	✓	Reduction in PPT, DMA, block of large myelinated fibres abolishes hypersensitivity
Kilo et al, 1994	1% capsaicin soaked filter paper under an occlusion dressing	Forearm (square area 1.5cm2)	30 minute application	✓	Not Tested	Pressure pain hypersensitivity
Liu et al, 1998	1% capsaicin soaked cellulose adhesive patch	Volar forearm (square area 4cm)	30 minute application	Not Tested	Not Tested	DMA and pinprick hyperalgesia in secondary area
Mohammadian et al, 1998	1% capsaicin cream	Volar forearm (square area 16cm2)	15 minute application	Not Tested	Not Tested	DMA and pinprick hyperalgesia in secondary area
Moyalan Governo et al, 2006	0.1% capsaicin cream	Rat: Left hindpaw	Retesting after 30 minutes, capsaicin cream is left on throughout the experiment	Not Tested	Not Tested	Secondary mechanical hypersensitivity, BOLD signal intensity is increased in the thalamus and PAG
Bishop et al, 2009	1% capsaicin soaked filter paper under an occlusion dressing	Volar forearm (square area 10.24cm2)	30 minutes application	✓	✓	Pinprick hyperalgesia in secondary area

Table 3-1 Studies of topical capsaicin cream. Sensory changes evoked in previous studies exploring topical capsaicin in animals and humans.

Further advantages of using the topical cream model include avoiding confounding the experiment with an intradermal injection, which may itself result in pain and irritation of the skin. Additionally, anxiety could potentially be associated with the use of needles, and since the cream is less invasive it also overcomes this hurdle. On the other hand, if necessary, it enables the use of an intradermal injection to administer peripherally acting drugs, as two injections in close proximity would be a further confound to any study and would require extensive controls within the experiment. Given the wide use of capsaicin to explore the secondary consequences of central sensitisation, it is of value to further explore the changes in the primary area, in addition to the use of the topical model in rodents.

Here, objective in vivo electrophysiology is used to characterise the model in rodents, since many animal studies rely on behaviour and outcome measures based on nociceptive thresholds (Chapman et al. 1985; Sikandar et al. 2013). To fully explore the signs and symptoms of chronic pain like models, it is important to assess both sub and supra threshold stimuli and recording from spinal WDR neurones enables exploration of the full range coding to stimuli of varying intensities and modalities. Importantly, it enables the study of suprathreshold stimuli which may be relevant to the higher intensities of pain described by patients.

3.1.2. Application of topical capsaicin cream induces peripheral sensitisation

Since capsaicin activates TRPV1 an obvious consequence of this is the development of a peripheral sensitisation of both the receptor itself, and a more general increase in excitability of the fibres on which it is located. Indeed, activation of TRPV1 by capsaicin is to sensitise the receptors/fibre through a number of intracellular pathways, leading to decreased activation thresholds and an increased frequency in action potential discharge when confronted with supra-threshold stimuli. Activation of TRPV1 resulting in this sensitisation of ion channels is likely to be relevant to certain pathological conditions since a number of endogenous inflammatory mediators are also able to result in this phenomenon. Indeed, the pivotal role of TRPV1 in the development of such states is demonstrated by the phenotype of TRPV1$^{-/-}$ mice, who are unable to develop thermal hypersensitivity post inflammation (Caterina et al. 2000; Davis et al. 2000).

Numerous studies have explored the mechanisms underpinning this peripheral sensitisation of TRPV1, whereby the mediators act through second messenger cascades to increase the excitability of the afferents (Kanai et al. 2007). This sensitisation is usually associated with inflammation, as numerous innate and adaptive immune cells including mast cells, macrophages, neutrophils and T lymphocytes release/induce the release of mediators such as BK, histamine, NGF, PGs, protons, and numerous cytokines (including: IL1β, IL-6, CCL2). All of which may result in activation of a number of intracellular signalling cascades that result in phosphorylation and upregulation of ion channels such as TRPV1.

During a state of tissue injury or ischemia protons are able to both directly activate and potentiate activity of TRPV1, as these hydrogen ions act at an extracellular site to increase the potential of channel opening (Jordt et al. 2000). On the other hand, mediators such as PGs, including PGE2 and PGI2, act at EP1 or IP receptors, respectively, which are coupled to Gs. They have been demonstrated to interact with TRPV1 through PKA dependent pathways, resulting in lowering of the temperature activation threshold to as low as 35°C (Smith et al. 2000; Moriyama et al. 2005). BK, ATP and endothelin act at Gq coupled receptors - B1/B2, P2Y2 and ETA, respectively. This is believed to activate the DAG-PKC pathway (Moriyama et al. 2003; Vellani et al. 2004) and therefore once again resulting in phosphorylation of TRPV1. PKCε has been implicated in phosphorylation of TRPV1 at serine residues 502 and 800 as cells containing the mutations S502A or S800A are unable to sensitise (Numazaki et al. 2002; Kawamata et al. 2008). Finally, the influx of Ca^{2+} through TRPV1 and the release from intracellular stores can result in the activation of CaMKII.

TRPV1 actions may also be potentiated by increasing the surface expression of receptors; either through an increase in transport or in number of receptors produced and inserted into the membrane. In inflammatory conditions, such as OA, NGF is released and contributes towards increased pain through actions at TrkA receptors, which are expressed on specific sensory neurons such as C and Aδ fibres. TrkA induced activation of PI3 kinase/ Src kinase causes phosphorylation of the Y200 residue, which leads to an in increased membrane expression of TRPV1 (Zhang et al. 2005). Additionally, Xue et al have showed that NGF can increase transcription of TRPV1, whilst Ji and colleagues found that it could also induce translation via p38 MAPK activation (Ji et al. 2002; Xue et al. 2007). Therefore suggesting that NGF released in conditions, such as OA, may lead to an upregulation and potentiation of TRPV1. Furthermore, after nerve injury it has also been found that TRPV1 is upregulated on

uninjured C-fibres, which may also contribute to the symptoms experienced by patients (Ma et al. 2005).

Overall these studies highlight that peripheral sensitisation of TRPV1 receptors, which results in lowering of the activation temperature and manifests as heat hypersensitivity, may occur in a number of inflammatory, and possibly neuropathic, conditions. Thus the capsaicin model induces clinically meaningful changes. The resulting thermal hypersensitivity does indeed appear to develop in pathological states; for example, it is suggested that patients with OA and up to 25% of patients with a post-traumatic nerve lesion suffer from heat hyperalgesia (Baron et al. 2010; Soni et al. 2013). Furthermore, the burning pain experienced by certain subgroups of patients with neuropathic pain may be underpinned by sensitisation and activity of TRPV1 containing afferents (McMahon and Wood 2006; Biggs et al. 2008; Baron et al. 2012).

It is important to note that the intracellular signalling pathways that may be activated through the accumulation of Ca^{2+} inside the neurone are also able to phosphorylate other ion channels, such as Nav channels (Nassar et al. 2004). This may lower the activation threshold of the afferent fibres and increase overall excitability. Therefore a peripheral sensitisation caused by the activation of TRPV1 may not only lead to a thermal hypersensitivity through changing the properties of the receptor itself, but it may also alter how these TRPV1 expressing afferents respond to all stimuli.

3.1.3. Ongoing activity from capsaicin alters central processing

Although capsaicin certainly does lead to a strong peripheral sensitisation, this cannot account for all the sensory changes evoked by the model, such as the development of secondary hyperalgesia and allodynia mentioned in the human studies. Whilst Lewis had originally postulated that an axonal reflex causing a release of neuropeptides to the area surrounding injury was responsible for the sensory changes, Hardy suggested it was more likely the result of a sensitisation of central neurones (Lewis 1942; Hardy et al. 1950). Indeed, LaMotte and colleagues were able to show that intradermal injection of capsaicin resulted in a hyperexcitability of second order neurones (LaMotte et al. 1991; Simone et al. 1991). Further unravelling of the mystery of these changes in the untreated area post capsaicin has mainly been through the use of intradermal administration, and the heat/ capsaicin model - although the handful of studies regarding topical capsaicin application previously mentioned also

assessed sensory changes in the secondary area. These studies quite clearly conclude that certain changes are due to central mechanisms (LaMotte et al. 1991; Simone et al. 1991; Ziegler et al. 1999; Iannetti et al. 2005; O'Neill et al. 2012).

It is now well established that following a barrage of peripheral input post intradermal capsaicin, an increase in mechanical sensitivity is observed in the surrounding secondary area (LaMotte et al. 1991; Willis W.D 1997). The ongoing peripheral activity from the treated area is believed to sensitise spinal neurones through a number of homo- and heterosynaptic mechanisms. In a state of central hyperexcitability enhanced neurotransmission through activation of the NMDA and NK1 receptors leads to complex intracellular events involving phosphorylation, receptor trafficking and transcriptional changes (Latremoliere and Woolf 2009). Consequently, there is an increase in membrane excitability, increased synaptic strength and a reduction in spinal cord inhibition. As such the thresholds of spinal neurones are lowered and activation kinetics are altered. Thus it is accepted that ongoing activity into the spinal cord is a core driver for the development of central sensitisation (Baron et al. 2013).

A reduction in this drive has also been noted to be able to decrease the area of secondary hyperalgesia usually observed post capsaicin (Dirks et al. 2000; O'Neill et al. 2012). Therefore it appears that the key to developing a stable central sensitisation/ secondary hyperalgesia in experimental models is simply an ongoing peripheral drive of adequate strength, most notably from C-fibres (McMahon et al. 1993; Baron et al. 2013). Proof that this input and subsequent sensory changes are of central origin has come from the fact that the symptoms of pinprick hyperalgesia and allodynia are dependent on non TRPV1 expressing Aδ and Aβ fibres and that they may be modulated by centrally acting drugs, which have no effect in the naïve state (Ziegler et al. 1999; Magerl et al. 2001; Iannetti et al. 2005; Iannetti et al. 2005). Furthermore, fMRI has revealed altered brain processing and a role for the brainstem in capsaicin induced central sensitisation (Iannetti et al. 2005; Lee et al. 2008).

Intradermal capsaicin is not only associated with the signs and symptoms of central sensitisation, but it is also known to be underpinned by the induction of numerous pivotal molecular mechanisms for altering spinal cord processing (for a full review see O'Neill et al. 2012). Given that the previous studies with topical capsaicin also found secondary hypersensitivity it is likely that this model also induces such changes. These are thought to be particularly important with regards to chronic pain disorders and thus there is clinical

relevance of this model (Baron et al. 2013). As previously described, here the activation of fibres by capsaicin itself results in an ongoing drive into the DH, which alters central processing. However, it has been further suggested that in chronic pain states a peripheral sensitisation of TRPV1 could allow activation of the receptor at body temperature and thus result in an ongoing pain, and the model may therefore have more clinical relevance that initially anticipated (McMahon and Wood 2006). Although this is yet to be proven, whatever the cause of the ongoing peripheral input in patients, the resulting alteration in the properties of central neurones is likely to be involved in many chronic pain conditions and thus is an important area of study.

3.1.4. Capsaicin cream application causes primary mechanical hypersensitivity

The presence of pinprick and brush hypersensitivity in the primary area of insult may also be reflective of central rather than peripheral sensitisation – although this is a contentious matter, and perhaps benefits from a discussion of a current topic in pain research – the existence of modality specific subsets of neurones in the periphery.

Since many C fibres are believed to be polymodal, it is widely accepted that distinction between modalities is made at either a spinal or supra spinal level. However, recent evidence has begun to reveal the possibility of modality specific subpopulations of peripheral afferents. Whilst it was clear that the original theory of modality specific labelled lines was too simplistic, a modified hypothesis dubbed 'population coding' encompasses the idea of both modality specific afferents, and cross talk among them at higher levels (Ma 2012). A number of pivotal studies (described in table 3-2) suggest distinct functions for TRPV1+ and IB4+ sensory neurones, respectively (Cavanaugh et al. 2009; Mishra and Hoon 2011; Brenneis et al. 2013; Zhang et al. 2013). It appears that whilst TRPV1 expressing afferents are required for sensing of noxious heat and pressure, IB4+ afferents lend themselves to both mechnosensation and noxious mechanical sensing. Furthermore, TRPV1+ afferents appear essential for the development of heat, cold and mechanical hypersensitivity post inflammation or nerve injury (Brenneis et al. 2013).

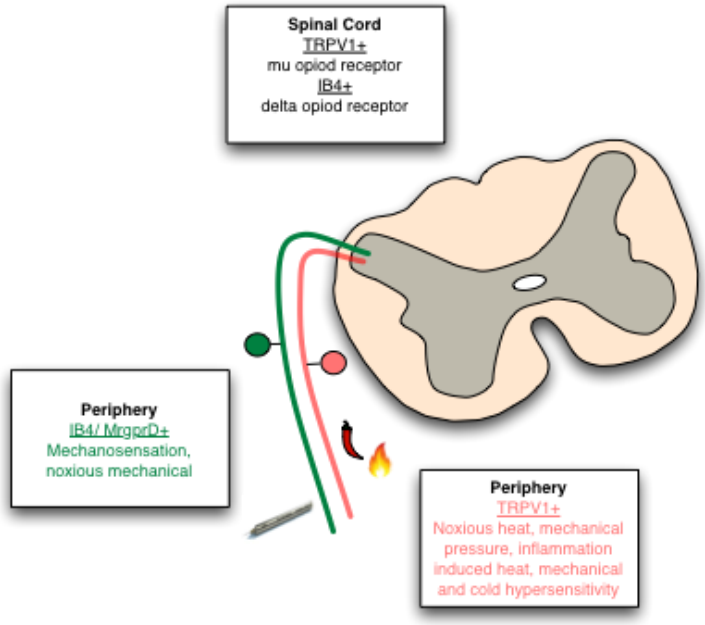

Figure 3-1 Modality specific peripheral afferents. Recent studies have suggested that afferent fibres can be subdivided by function, depending on whether they express TRPV1 or IB4. Whilst ablation of peptidergic TRPV1+ fibres results in a loss of noxious heat sensation, ablation of IB4+ afferents results in a selective loss of mechano/ noxious mechanical sensation.

Study	TRPV1+ Peptidergic Afferent Function
Cavanaugh, 2009	IT capsaicin ablates TRPV1 expressing fibres, near complete loss of response to noxious heat, no change in noxious mechanical or cold
Mishra, 2011	Pharmacological ablation of TRPV1+ neurones from embryo, reduction in noxious heat and cold responses
Zhang, 2013	Pharmacological ablation of TRPV1+ neurones, LI and LV neurones lose responsiveness to noxious heat
Brenneis, 2013	TRPV1 silencing, reductions in C fibre, but not A fibre mediated action potentials, deficits in heat and mechanical pressure, no difference in pinprick or light touch, abolishes heat, mechanical and cold hypersensitivity post inflammation, abolishes tactile and cold allodynia post nerve injury
Study	**IB4+ Non-peptidergic Afferent Function**
Cavanaugh, 2009	Genetic ablation of Mrgprd+, selective reduction in noxious mechanical sensitivity
Zhang, 2013	Ablation of Mrgprd+ neurones, LI NS neurones had reduced responses to noxious mechanical stimuli, proportion of mechanosensitive WDR neurones was reduced

Table 3-2 Studies exploring the possibility of modality specific subpopulations of afferent fibres. Both genetic and pharmacological ablation techniques have been used to help elucidate the distinct functions of TRPV1 and IB4+ afferents.

Given that capsaicin activates TRPV1+ fibres it may be assumed that a direct peripheral sensitisation of these fibres discussed above would therefore result in a hypersensitivity to thermal and pressure stimuli if both these modalities are transmitted by TRPV1 afferents alone. Since none of the studies found that ablation of TRPV1 expressing fibres had any effect on touch or pinprick sensitivity, it is unlikely that TRPV1 is co-expressed with the molecular transducers of these stimuli and therefore could be directly sensitised by capsaicin as other ion channels may be. Induction of a mechanical pinprick, or dynamic brush sensitisation must therefore result from central changes. Additionally, ablation of the TRPV1 expressing population was only found to reduce C fibre mediated action potentials, rather than A fibre mediated activity, which is believed to be responsible for mechanical hypersensitivity post capsaicin (Ziegler et al. 1999; Brenneis et al. 2013). However, since ablation of these fibres prevents the development of hypersensitivity to inflammation it can be inferred that ongoing input from these TRPV1 expressing fibres, for example due to topical application of capsaicin, is required development of this mechanical hypersensitivity. Indeed the requirement of this peripheral drive for development of certain types of hypersensitivity is well established (McMahon et al. 1993; Baron et al. 2013). This theory certainly suggests that the development of DMA and pinprick hypersensitivity post capsaicin are on the whole reflective of the engagement of central, rather than a peripheral mechanisms.

Despite numerous studies using capsaicin, the primary area of treatment is yet to be fully characterised. Additionally, the translational arm in animals as well as humans, exploring the central neuronal consequences of temporary topical capsaicin cream application has not been examined. In humans, the full QST profile in the primary treated area is also yet to be studied. Here, this chapter explores the true translational nature of the use of capsaicin cream as a surrogate model in animals and humans. In particular, focusing on use of paradigms encompassing the same time points and measured end points. Furthermore, this chapter uses objective measures of spinal neuronal activity in rats and full QST in humans in order to fully characterise primary area of treatment of this model.

3.2. Methods

3.2.1. In vivo electrophysiology:

Adult male Sprague-Dawley rats, between 220-250g, were obtained from the UCL Biological Services Unit. All procedures were approved by the UK Home Office and were performed in accordance with the guidelines provided by the International Association for the Study of Pain.

In vivo electrophysiological recordings were performed as previously described to obtain baseline responses. Once stable responses had been characterised for each individual cell, 0.1-0.2ml of a 1% capsaicin cream was applied to the receptive field of the cell on the hindpaw for 30 minutes. Capsaicin was then removed, taking care not to further stimulate the treated area. The train of electrical and natural stimuli was repeated at 30, 50, 70 and 90 minutes post application of capsaicin and the maximum change was then calculated.

3.2.2. Human Quantitative Sensory Testing:

Experiments were conducted in 10 healthy human volunteers aged between 19-59 years old. Individuals were familiarised with the experimental protocol beforehand and gave written, informed consent. The study was approved by The Kings College Research Ethics Committee.

All subjects were free from pain and medical conditions which may otherwise interfere with the results of the study. They were advised they must avoid pain medication such as non-steroidal anti-inflammatory drugs (NSAIDs) and caffeine in the 24 hours prior to the study.

Baseline thresholds were obtained at marked sites (16x16mm) on the ventral forearms for MPT and HPT as previously described. In addition to MPT, subjects were asked for numerical ratings for the 32mN and 256mN pinprick devices; and in addition to HPT, subjects were asked for numerical ratings to 35°C and 45°C. For 6 of the subjects full QST was performed as described in chapter two. The area was then cleaned with an alcohol wipe before topical application of 0.5ml 1% capsaicin cream. A transparent film dressing was placed over the area to ensure no capsaicin was accidentally removed during the 30 minutes it was applied. The test and control arms were alternated. Capsaicin was then carefully removed after 30 minutes before re-testing took place. Thresholds and NRS ratings we retested at 30, 50, 70 and 90 minutes post application. The maximum change in subject responses was calculated.

3.2.3. Statistical analysis:

All analysis was undertaken using SPSS software (IBM SPSS Statistics v21). Data was assessed for normality using the Kolmogorov-Smirnov test to determine further methods of analysis. Electrophysiological data was analysed using either a paired or unpaired t-test, or a 2 way ANOVA accordingly. Psychophysical data, with the exceptions of HPT and CPT, were logged and re-tested for normality. A paired t-test or 2 way ANOVA was then carried out. HPT and CPT were found to be normal without logging, and thus the raw data was used for analysis with a paired t-test. All graphs were plotted to show the mean ± SEM.

3.3. Results

3.3.1. In vivo electrophysiology

Objective electrophysiological recordings of LV WDR cells to a range of natural and electrical applied stimuli were examined pre and post application of topical capsaicin cream to the hindpaw. The results highlighted a clear hypersensitivity to thermal stimuli post capsaicin, in comparison to baseline responses in the same animals. A negligible effect was noted regarding mechanical stimuli. These changes seen in the primary area of treatment post capsaicin are akin to the results observed previously in human psychophysical experiments (Culp et al. 1989; LaMotte et al. 1992; Bishop et al. 2009), however this is the first full characterisation of the model in animals. This effect primarily found regarding thermal responses is most likely due to the well-known ability of capsaicin to sensitise TRPV1.

3.3.1.1. Topical capsaicin application significantly enhances dynamic brush evoked baseline WDR cell responses in naïve animals.

Post capsaicin dynamic brush responses were significantly increased above baseline from 326.0 ± 25.77 to 680.4 ± 47.52 action potentials/ 10s (figure 3-2; p= <0.000) in the primary treated area. Both pre and post capsaicin, coding to the increasing forces of mechanical stimuli is observed. There was no overall significant difference in the evoked responses to innocuous or noxious vF post capsaicin, however there is a clear increase in the number of action potentials/ 10s to 8g from 149.7 ± 21.96 to 335.1 ± 27.3 (figure 3-2).

3.3.1.2. Topical capsaicin cream significantly enhances both innocuous and noxious thermally evoked WDR cell responses in comparison to baseline responses.

Once again, there is a clear coding of WDR cell responses to increasing thermal stimuli, both pre and application of topical capsaicin. A facilitation of neuronal responses was observed in response to all temperatures tested post capsaicin cream (figure 3-3; p= <0.001). The greatest increase was seen at 35°C, where an 827% increase in the firing was observed (p= 0.002). Firing to supra threshold stimuli (45°C and 48°C) were also significantly enhanced by 106.9% and 45.6%, respectively (p= 0.000, 0.022). Overall, there appeared to be a parallel shift in the stimulus-response curves, indicative of a peripheral sensitisation and reduced C fibre threshold.

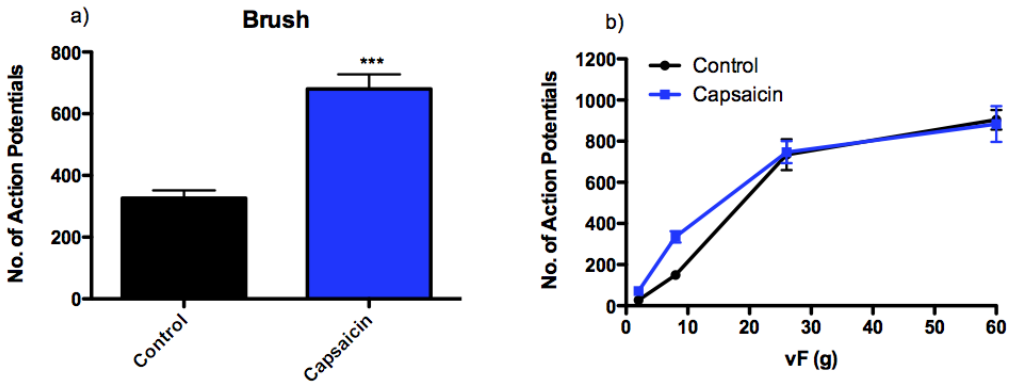

Figure 3-2 Effects of capsaicin on mechanically evoked baseline WDR cell responses. a) Using the protocol described in chapter 3.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of mechanical stimuli, including brush and graded vF, applied to the receptive field for 10s. There is a clear facilitation of mean dynamic brush responses from 326 to 680.4 action potentials/ 10s ($p < 0.000$). b) Responses to vF on the other hand were unaffected, although neuronal responses to innocuous 8g appear enhanced. (Overall 2-way ANOVA $p = 0.27$). n= 10

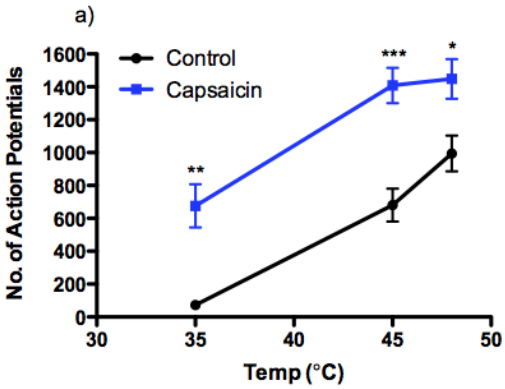

Figure 3-3 Effects of capsaicin cream application on thermally evoked WDR cell responses. a) Using the protocol described in chapter 3.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of thermal stimuli, applied to the receptive field for 10s. Post capsaicin, evoked responses to both innocuous and noxious temperatures were elevated when compared to pre-treatment baselines (Overall 2-way ANOVA $p = 0.001$; 35°C $p = 0.002$, 45°C $p < 0.000$, 48°C $p = 0.022$). n= 10

3.3.1.3. Topical capsaicin cream significantly increases Aδ fibre responses recorded from WDR cells when compared to baseline responses.

Whilst no significant difference was observed between post capsaicin responses and baselines with regards to electrically evoked input, wind up, Aβ fibres and PD, in contrast responses in the Aδ fibre range were significantly facilitated from 178.5 ± 22.83 to 193.5 ± 32.63 action potentials/ 10s (figure 3-4; p=0.004), and responses in the C fibre range were reduced by 25.9% (p=0.02). Such changes are likely driven by a simultaneous central facilitation of Aδ input and peripheral C fibre desensitisation. Furthermore, although not found to be significant, wind up also appears reduced post capsaicin. Since this measure is quantified by calculating the difference between the overall response observed and the baseline response to the first stimulus it is directly effected by a change in input. Examination of the wind-up graphs reveals that the small increase in the initial responses in the capsaicin group was responsible for the apparent reduction of wind-up. In fact, the neuronal responses started from a level close to that normally elicited when wind-up is produced.

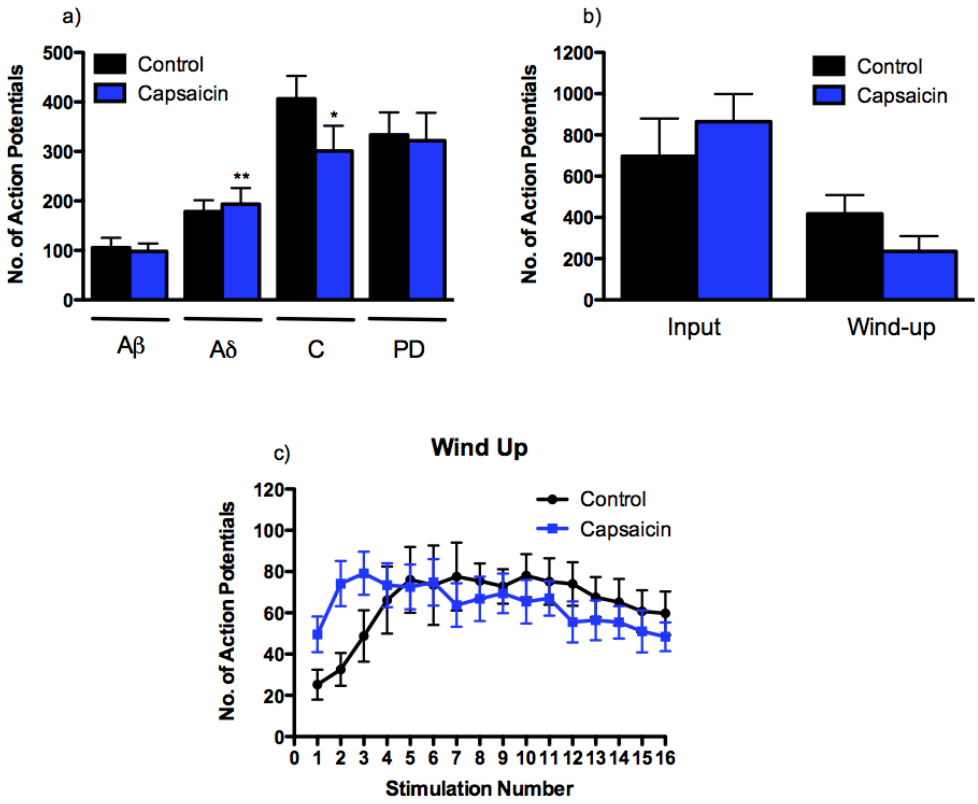

Figure 3-4 Effects of capsaicin on electrically evoked WDR cell responses. Using the protocol described in chapter 2.2 and 3.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli. Transcutaneous electrical stimulation was used to measure the input and wind up, in addition to calculating the responses driven by different fibre types – depending on the latency. Post capsaicin treatment a) there was no significant effect on electrically evoked Aβ mediated transmission nor post-discharge, however responses within the Aδ fibre range were significantly enhanced (p= 0.004) and responses within the C fibre range were reduced (p= 0.02); b) electrically induced input appears to be enhanced, although this was not found the be significant; c) overall wind up was also unaffected. n= 10

3.3.2. Human Quantitative Sensory Testing

Using a standardised QST procedure, human subjects were also found to exhibit sensory changes indicative of hypersensitivity post capsaicin treatment, including both mechanical and thermal hypersensitivity.

3.3.2.1. Topical capsaicin significantly reduces MPT and increases numerical ratings to innocuous and noxious punctate stimuli.

Unlike the rodent studies, in humans responses to pinprick were measured (whereas vF stimuli are used in the animals), here post capsaicin treatment there was a significant drop in the average 50% pain threshold to pinprick stimulation from 128.2 ± 23.2 to 36.0 ± 13.1 (figure 3-5; p= 0.003) within the primary treated area. This is likely underpinned by the facilitation of Aδ fibres observed in the animal model. Furthermore, numerical ratings to dynamic brush were significantly increased (figure 3-5; p= <0.000). Ratings to both sub and supra-threshold mechanical stimuli were also elevated, although this was not found to be statistically significant. There was also no difference found in perceptual wind up post capsaicin.

3.3.2.2. Topical capsaicin induces a thermal hypersensitivity

Average HPT was significantly reduced in the primary treated area from 45.29 ± 0.73°C to 34.45 ± 0.48°C (figure 3-6; p= <0.000). Furthermore, ratings to both sub and supra-threshold temperatures were significantly increased post capsaicin treatment (p< 0.000). Conversely, CPT was reduced highlighting a cold hypoalgesia (figure 3-6; p= 0.003).

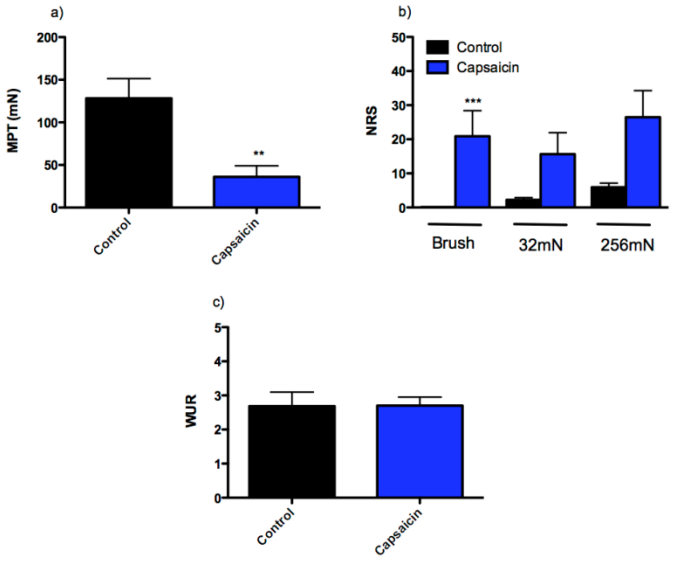

Figure 3-5 Effects of topical capsaicin on psychophysical MPT and NRS ratings. Using the protocol described in chapter 3.2 standardised QST was undertaken to determine the subject's mechanical pain threshold (MPT), in addition to obtaining numerical ratings (NRS) to graded mechanical stimuli. a) Average MPT was significantly lower in the primary area post capsaicin treatment in comparison to pre-treatment baselines (p= 0.003). b) NRS rating to dynamic brush significantly increased (p< 0.000). Ratings to both 32mN and 256mN appear increased, although this was not significant (p= 0.084) n= 9

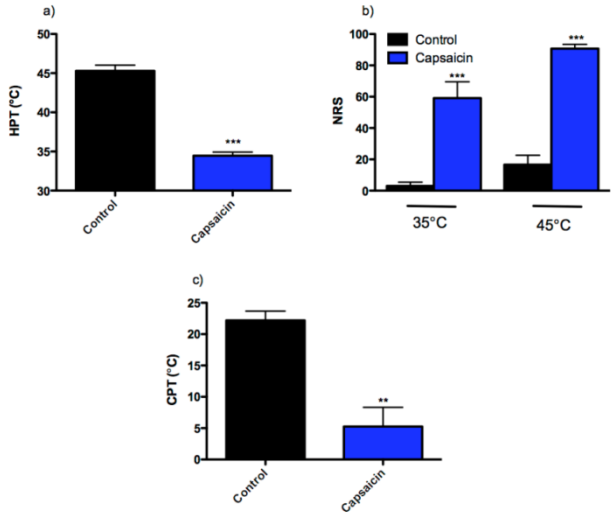

Figure 3-6 Effects of topical capsaicin on psychophysical HPT, NRS ratings and CPT. Using the protocol described in chapter 3.2 standardised QST was undertaken to determine the subject's heat and cold pain thresholds (HPT/CPT), in addition to obtaining numerical ratings (NRS) to graded thermal stimuli. a) Average HPT was significantly reduced in the primary area (p< 0.00). b) NRS ratings to previously innocuous and noxious temperatures are significantly increased (p< 0.000; 35°C < 0.000, 45°C p< 0.000). c) CPT was significantly decreased (p= 0.003). n= 8

3.3.2.3. Sensory profiles post topical capsaicin illustrate a unique combination of hyper- and hyposensitivities

Full sensory profiling using a standardised, comprehensive QST procedure confirmed a sensitisation in the primary area across a number of modalities including: HPT, MPT and mechanical pain sensitivity (figure 3-7). Conversely, there is a clear cold hyposensitivity. This is the first study to fully characterise the primary treated area post topical capsaicin and thus provides a novel profile indicative of a peripheral and central sensitisation, which may be compared to clinical patient profiles in order to assess the potential relevance of the model.

a)

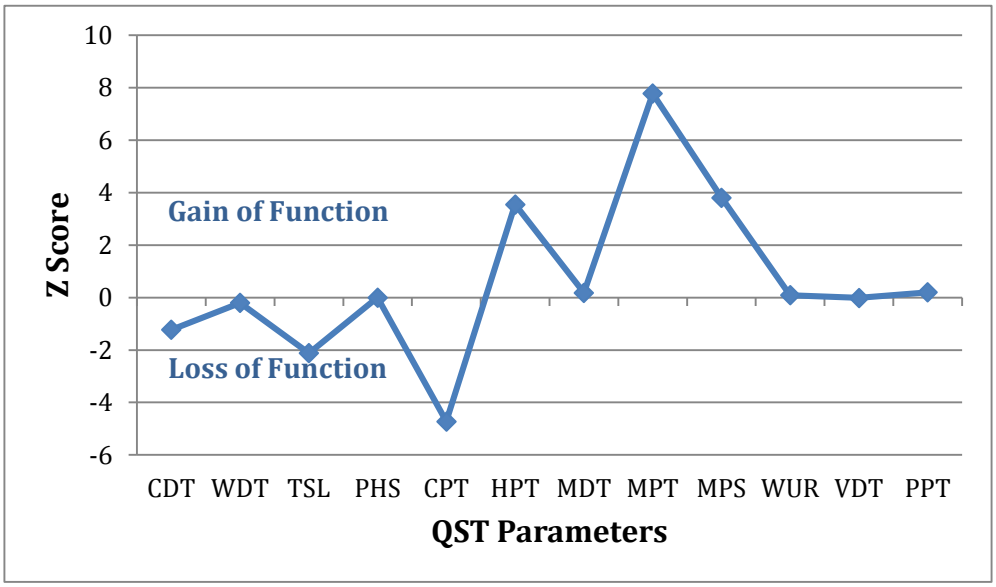

Figure 3-7 Somatosensory changes produced by application of topical capsaicin. Using the protocol described in chapter 2.4 full QST profiling was undertaken. a) A variety of parameters were tested both pre and post capsaicin treatment, the magnitude of the changes are expressed here as Z-scores which highlight specific gains or loss in function. Hypersensitivity to heat, pinprick and dynamic mechanical stimuli are demonstrated here by the gain of function in heat pain threshold (HPT), mechanical pain threshold (MPT), and mechanical pain sensitivity (MPS). On the other hand, there is a hyposensitivity to cool and painful cold, highlighted by the loss in function of cold detection threshold (CDT), cold pain threshold (CPT) and thermal sensory limen (TSL)

3.3.3. Capsaicin induced somatosensory changes in rats and humans show considerable overlap

a)

Stimulus	Hypersensitivity	
	Animal	Human
Brush	✓	✓
Subthreshold Mechanical	✓	✓
Suprathereshold Mechanical	No change	✓
Subthreshold Thermal	✓	✓
Suprathreshold Thermal	✓	✓
Input	✓	✓
Wind up	No change	No change
Fibre count	Reduction in C fibre, increase in Aδ	Not tested

Table 3-3 Comparison of animal and human characterisation. a) There is a remarkable similarity in the sensory changes post capsaicin across species, highlighting the translational nature of this model. Both animals and humans show heightened responses to brush, subthreshold mechanical stimuli and thermal stimuli, whilst wind up is unchanged. The only difference noted between species is regarding suprathreshold mechanical stimuli, whereby human pain ratings were increased, whilst there was no changed observed in WDR cell evoked responses. Fibre count was only assessed in the animal model, this term refers to a change in the number of action potentials elicited from each fibre type; there was a clear reduction in action potentials elicited by C fibres, whilst there was an increase in Aδ mediated activity. Since capsaicin may also desensitise fibres, this reduction in C fibre count was not unexpected, whilst an increase in Aδ fibre count is likely to underpin the observed mechanical hypersensitivity in animals and humans.

3.3.3.1. Lowered HPT in human volunteers post capsaicin corresponds to the number of action potentials evoked in WDR cells

When comparing the animal and human data in terms of the neuronal activity produced by the temperatures which correspond to the HPTs, before and after capsaicin, a remarkable correspondence can be observed (figure 3-8). It can be seen that the number of action potentials evoked by 45.3°C (the average human HPT under normal conditions) was 681, which is very similar to the 642 action potentials that would be evoked by 34.5°C the reduced human HPT post capsaicin (prior to the treatment, this would only evoke around 72 action potentials).

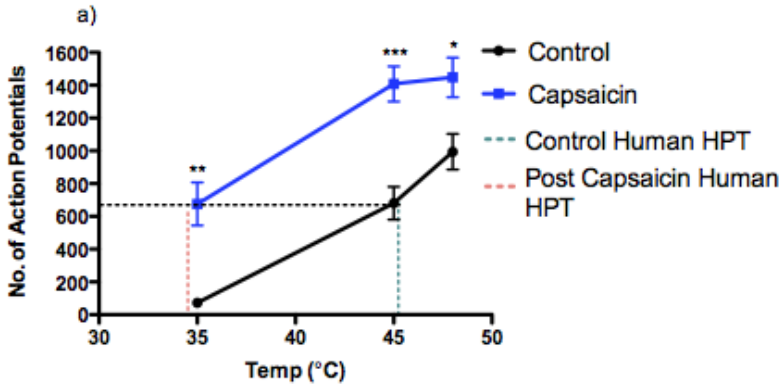

Figure 3-8 Overlap in animal and human data. Comparing changes in thermally evoked neuronal activity to the shift it human HPT, post capsaicin treatment, reveals a remarkable similarity: a) Pre capsaicin the average human HPT was 45.3°C, which corresponds to 681 action potentials/ 10s, post topical capsaicin application this dropped to 34.5 °C, which corresponds to 642 action potentials/ 10s.

3.4. Discussion

Using objective in vivo electrophysiological single unit recordings from LV WDR cells, and human QST, this study assessed the sensory changes evoked by topical capsaicin cream in the primary area of treatment. Increased responses to thermal stimuli post topical capsaicin application were clearly demonstrated in both animals and humans. This sensory abnormality indicates the induction of a peripheral sensitisation, as hypothesised. Tests also revealed the presence of a primary brush hypersensitivity, which was once again observed in both animals and humans. Heighted responses to pinprick stimuli were observed in humans, which are thought to be an Aδ mediated function; indeed in the rodents Aδ responses were facilitated. Although overall mechanical coding in the rodents did not change, 8g vF responses were clearly increased and this force represents a possible drop in threshold since this is the minimum force that elicits behaviour in naïve animals. This is most likely reflective of the engagement of central, rather than peripheral mechanisms. Overall there is a remarkable similarity in the sensory changes post capsaicin across species, highlighting the translational nature of the model. Furthermore this study reinforces the relevance of using WDR cells in order to predict the outcome of human behavioural pain reports.

3.4.1. Topical capsaicin cream produces a consistent primary thermal hypersensitivity in animals and humans

This is the first study to objectively quantify the change in response to thermal stimuli in the primary area of capsaicin treatment. As expected, the human HPT is dramatically reduced post capsaicin treatment, whilst numerical ratings to 35°C and 45°C are increased. In parallel to this shift in stimulus-response function, the rodent counterpart also revealed evidence of a strong thermal hypersensitivity. LV WDR cells show a large increase in firing across temperatures, once again ranging from sub-threshold 35°C to supra-threshold stimuli 45°C and 48°C. The translational nature of this model is further highlighted by the fact that the number of action potentials corresponding to the human thresholds before and after capsaicin are remarkably similar. That is to say, the threshold temperature in humans before capsaicin evoked a certain number of action potentials, which was roughly equal to the number of action potentials elicited by the lowered threshold post capsaicin. Consequently, thermal sensitisation in humans appears to almost identically alter coding in spinal neurones.

This phenomenon has been observed in previous human studies of topical capsaicin application (Carpenter and Lynn 1981; Culp et al. 1989; LaMotte et al. 1992; Bishop et al. 2009). A study of the responses of C-fibre units in humans pre and post topical capsaicin revealed that the activation threshold for such dropped from around 45°C to 35°C, which corresponded exactly to the perceptual thresholds noted in the study in addition to with the drop in HPT observed in human subjects here (LaMotte et al. 1992). Whilst the threshold and responses of C fibres units were altered for thermal stimuli, this was not the case for mechanical stimuli despite the presence of mechanical hypersensitivity (LaMotte et al. 1992). This provides sound objective data supporting the hypothesis that capsaicin application results in primary heat hypersensitivity.

As noted, this symptom is most likely a reflection of a peripheral sensitisation, since capsaicin is able to sensitise TRPV1 and lower the activation threshold, which would explain the drop in HPT and increased firing across temperatures (Moriyama et al. 2005). Indeed, since peripheral recordings have demonstrated that afferent C fibres show reduced thresholds and increased activity to thermal stimuli this confirming there is at least a peripheral component to this phenomenon (LaMotte et al. 1992). Taken together, the results here and the previous studies confirm that topical capsaicin is able to induce a robust peripheral sensitisation in both animals and humans, which may be useful for exploring drug modulation of thermal hypersensitivity. Furthermore, it is interesting to note that this phenomenon can still be picked up even when not recording peripherally, but from DH neurones. In the future it would be interesting to examine if topical TRPV1 antagonists or NSAIDs were able to alter these measures since this would directly address whether this is truly a peripheral phenomenon.

3.4.2. Brush hypersensitivity is apparent in the primary treated area post topical capsaicin of both animals and humans

Once again, in both animals and humans, evidence was found for the induction of dynamic brush evoked hypersensitivity in the primary treated area. Whilst humans reported a novel pain sensation evoked by dynamic brush, LV WDR cells showed enhanced firing to this same stimuli post capsaicin treatment. In agreement with the observations made here, earlier studies of topical capsaicin cream have been found to induce primary and secondary dynamic brush evoked allodynia in humans, although this finding is previously unreported in the

animal model (LaMotte et al. 1992; Bishop et al. 2009). This finding further highlights the translational nature of the model, evoking signs of brush hypersensitivity across species.

Dynamic brush responses are more commonly assessed in the secondary rather than primary area (Koltzenburg et al. 1992; Andersen et al. 1995; Liu et al. 1998; Mohammadian et al. 1998). Similar to the changes noted here in the primary area, Andersen and colleagues noted that a subset of volunteers develop pain (allodynia) to brush stimuli in the secondary area post topical capsaicin, whilst others report an unpleasant sensation (Andersen et al. 1995).

Brush evoked allodynia is believed to be a symptom indicative of central changes, resulting from plasticity in the spinal cord, allowing synaptic reorganisation, and the ability of Aβ fibres to transmit nociceptive signals (Andersen et al. 1995; Latremoliere and Woolf 2009). Since capsaicin evoked allodynia does not develop in the absence of Aβ fibres, this would suggest that such mechanisms are imperative (Treede and Cole 1993). Furthermore, pharmacological evidence involving manipulation of mechanisms involved in central sensitisation using drugs such as ketamine, alfentanil and remifentanil (whose actions include spinal mechanisms) are able to reduce brush hypersensitivity, thus suggesting the engagement of central mechanisms (Park et al. 1995; Sethna et al. 1998; Petersen et al. 2001). Additionally, brush hypersensitivity is not sensitive to topical NSAID treatment, unlike other peripherally mediated symptoms, once again suggesting a role for central modifications (Kilo et al. 1995; Bishop et al. 2009). It is possible that similar mechanisms may be involved in both primary and secondary brush hypersensitivity. Bishop and colleagues noted that the primary brush evoked allodynia spread into the secondary area, suggesting that they may indeed be mediated on the whole by the same (central) mechanisms (Bishop et al. 2009). Further studies using an A fibre block and central drug modulation are required to fully elucidate the mechanisms behind the changes in the primary area.

In light of recent discoveries regarding modality specific subsets of primary afferents, it seems unlikely that peripheral sensitisation would result in hypersensitivity to dynamic brush. Since it has been suggested that light touch is not affected by TRPV1 expressing fibres, it may be inferred that they do not play a large role in conveying this sensation. Therefore, a peripheral sensitisation of these fibres may not affect dynamic brush responses. Taken together with previous evidence of central mechanisms for brush hypersensitivity, the presence of this symptom in the capsaicin model could therefore suggest that central sensitisation is indeed induced.

Given WDR cells respond to dynamic brush under normal conditions, a sensitisation of the WDR cell directly recorded from could indeed lead to a facilitation of brush evoked responses. However, since the changes observed here are in the primary area it is difficult to fully rule out peripheral mechanisms. It is believed that mechanical hypersensitivity in the capsaicin treated area does indeed have a peripheral component, since the symptom is not always abolished by blocking A fibre conduction (Culp et al. 1989). It is also suggested that certain subgroups of C fibre afferents may respond to low threshold inputs, which could be hypothesised to also become sensitised to brush stimuli post capsaicin, whilst non-brush responsive C fibres have been found to develop a small sensitivity post topical capsaicin (LaMotte et al. 1992; Olausson et al. 2002; Löken et al. 2009). These studies implicate a potential peripheral component of brush hypersensitivity, which cannot be fully ruled out.

3.4.3. Sensory changes post topical capsaicin cream application are suggestive of peripheral and central sensitisation

The results discussed so far, including the development of thermal and brush hypersensitivity suggest that there are most likely a number of peripheral and central mechanisms that are engaged post topical capsaicin application. However, the inferences made based on the potential underpinnings of the brush hypersensitivity are inconclusive and further evidence is required to support the notion of capsaicin induced central sensitisation.

Ongoing activity into the DH is a key driver of central sensitisation. Although it was not assessed here, it is widely reported that both intradermal and topical capsaicin result in ongoing activity, highlighted in human psychophysical reporting's and recordings from afferent fibres (Kenins 1982; LaMotte et al. 1991; LaMotte et al. 1992). Furthermore, an increase in basal cFos levels (a surrogate marker of increased input into the spinal cord) has also been associated with intradermal capsaicin (Mitsikostas et al. 1998). Ongoing activity of primary afferents that converge in the DH can modulate NMDA receptor function, as seen in wind-up of spinal neurones and heterosynaptic central sensitisation (Dickenson and Sullivan 1987; Haley et al. 1990; Lewin et al. 1994). As such, many models of altered pain processing or chronic pain states are sensitive to a blockade of this receptor (Woolf and Thompson 1991; Stubhaug et al. 1997). Indeed capsaicin evoked mechanical allodynia is sensitive to such modulation, including ketamine, MK-801, protein kinase inhibitors and gabapentin, thus

suggesting the engagement of central mechanisms(Sluka and Willis 1997; Mitsikostas et al. 1998; Sethna et al. 1998; Dirks et al. 2002).

Further evidence for the presence of central sensitisation in the topical model, comes from the responses of WDR cells to electrical stimulation. A potentiation in the range of Aδ fibres to electrical stimulations was identified post capsaicin (which could underlie the changes in pinprick sensitivity observed in human subjects). Given that central sensitisation may be associated with the recruitment of increasing numbers of afferent fibres it could be hypothesised that this would be reflected in the selective increase in neuronal firing to stimulation of these fibres. Furthermore, it has recently been shown that in the MIA model of knee OA, which has a clear central component since there is no injury in the primary area tested, there is also a potentiation of electrically evoked responses in the Aδ fibre range from the paw which may be taken as a sign of central changes (Burnham 2012; Thakur 2012). A non-significant increase in Aδ has also been found after carrageenan inflammation, which is believed to evoke central changes (Rahman et al. 2004). Given that models with proven central modifications also show this facilitation of Aδ fibre responses, it can be concluded that this may be a sign of engagement of such mechanisms post topical capsaicin, and in addition may help explain the drop in human MPT.

Turning to the C fibres, it was observed that the fibre count here was reduced post capsaicin treatment. This is most likely a consequence of the fact that capsaicin is also able to cause temporary desensitisation of afferents. Desensitization is a dose-dependent phenomenon, whereby repeated application of low dose or a single high dose of injected or topical capsaicin can lead to levels of immediate desensitization (LaMotte et al. 1991; Simone et al. 1998; Kennedy et al. 2010). There are a number of ways in which capsaicin can result in desensitization of TRPV1-positive afferents, from an acute rapid desensitization to long-term tachyphylaxis, and withdrawal of intraepidermal nerve fibres. Both types of desensitization occur in the area localized to the site of injection/ application; however, the time frame for each varies; the initial acute desensitization occurs almost immediately after agonist binding and is thus most relevant in this case, whereas tachyphylaxis and intraepidermal nerve fibre withdrawal occur over much longer periods up to 72 h after application (Simone et al. 1998; Touska F 2011). Rapid desensitization first involves capsaicin binding of TRPV1, one consequence of which is the release of neuropeptides from the C fibre terminals. Once released, the terminals are depleted and the nerve may become desensitized (Maggi and Meli

1988; Simone et al. 1998). Second, voltage-gated ion channels are inactivated, which also results in a rapid, short-lasting, desensitization as further action potentials cannot be generated (Simone et al. 1998). This desensitization of fibres is believed to be a protective mechanism to inhibit excessive calcium influx, which leads to excitotoxic cell death. Since there was also heat hypersensitivity, as previously discussed, this provides further evidence for the peripheral sensitisation of C fibres. If the overall count was reduced, in order for heat hypersensitivity to manifest, it is likely that the remaining fibres must have been sensitised.

It is also interesting to note that wind up is unchanged in both animals and humans. With regards to the animal data, it is clear that the initial input is much larger than in untreated animals. This is despite the apparent desensitisation in C fibres post capsaicin, previously discussed. Since wind up is dependent on C fibre input, a reduction in fibre count could have a knock on effect with regards to wind up. Therefore, the overall increase in input that is observed could suggest once again that the remaining peripheral fibres are sensitised. Thus they are able to evoke greater activity and compensate for any desensitisation. This initial increase allows the WDR cells to reach a plateau more quickly than normal, representing what would have happened after further stimulation in untreated animals. Alternatively, it could be inferred that central sensitisation is present and that the increase in input is due to a lowering in threshold of the WDR neurone. A combination of peripheral and central sensitisation would also explain the results observed here. The wind up ratio is also similar in humans, thus suggesting that the output of WDR neurones reflects human perception.

3.4.4. The discrepancy in mechanical hypersensitivity between animals and humans may be a result of the nature of the tests

Although the majority of sensory changes post topical capsaicin were found to be analogous across species, there was a discrepancy in the results regarding responses to mechanical stimuli. As reported in previous human studies, there was a drop in MPT post capsaicin in the primary treated area, however LV WDR cell responses to a range of increase vF stimuli were overall unchanged (Carpenter and Lynn 1981; Culp et al. 1989). However, it is important to note that there is a clear increase in responses around sub threshold stimuli such as 8g, despite the overall ANOVA not being found to be significantly different from baseline. Given that this difference is seen around what is believed to be the normal withdrawal thresholds, it is possible that if only these stimuli were tested a significant change may have been found.

Therefore the results would be in line with the human experiments that suggest topical capsaicin may alter the mechanical threshold for pain. This finding highlights the importance of testing with stimuli around threshold and in future studies, a 15g vF should be incorporated since this is commonly found to induce a withdrawal response. Previous studies have found a strong concordance in coding to mechanical stimuli in animals and humans, both pre and post sensitisation (LaMotte et al. 1991; Simone et al. 1991; Dawes et al. 2011).

Furthermore, although human mechanical responses are tested with pinprick devices, animals are tested with vF, which may not activate the exact same pattern of peripheral fibres. The responses to higher pinprick forces are mediated on the whole by Aδ fibres (Iannetti et al. 2013), and indeed an increase in Aδ fibre mediated activity was recorded in the rodents, which could underlie the results observed in humans. On the other hand, evoked responses vF filaments could involve a higher proportion of C fibre units, some of which may be sensitised but others are desensitised and thus the overall effect is no net change. Alternatively, if mechanically sensitive C fibres do not express TRPV1, a peripheral sensitisation or desensitisation of these fibres is unlikely (Cavanaugh et al. 2009). Future animal studies could include pinprick devices in order to ensure the same mechanisms are tested.

3.4.5. LV WDR cell recordings highlight changes that are reflective of human psychophysical reporting

Despite the discrepancy observed regarding mechanical stimuli, there are many similarities in the changes produced by topical capsaicin in animals and humans apparent from the responses of LV WDR neurones and QST results. It is well known that responses of second order WDR cells correlate well with human psychophysical reporting's (Dubner et al. 1989; Maixner et al. 1989). Indeed a recent study by Sikandar and colleagues found that reported pain intensities to both sub and supra threshold laser stimulation are highly correlated with the evoked firing of WDR cells, rather than NS cells (Sikandar et al. 2013). Whilst WDR cells continue to code for suprathreshold stimuli, NS cells reach a ceiling and plateau and fire considerably less than the WDR cells. The study found that when comparing human NRS responses and WDR cell firing there was no significant difference between the two, highlighting the high translational nature of DH electrophysiology. The ability of second order spinal neurones to encode stimulus intensity may therefore be related to the perceptual outcome through the graded discharge frequency being relayed to higher centres.

A second advantage of this use of electrophysiological recordings from second order spinal neurones is that it enables the study of a range of stimuli of differing intensities, most importantly to those of a suprathreshold nature. Mechanisms involved in processing these high intensity stimuli may be important in the high levels of pain reported by patients. This is particularly important for screening of analgesics, since many may have modulatory effects only at the higher intensities. The results here, in addition to previous reports, highlight the important information that can be extrapolated from WDR cell recordings and translated to human sensory perception.

3.4.6. Topical capsaicin cream produces a cold hypoalgesia in healthy human subjects

Few studies have examined the consequences of topical capsaicin application on cold sensitivity, although perhaps the resultant hypoalgesia is to be expected, given that it is known that cooling may alleviate other capsaicin induced hypersensitivities (Culp et al. 1989). Human QST results here clearly demonstrate a dramatic reduction in CPT post topical capsaicin in the primary area of injury. These findings do replicate that of one previous psychophysical study, where it was found that average CPT dropped from 25.1°C to 0°C (Callsen et al. 2008). The authors suggest that given the phenomenon is localised to the site of injury, and does not spread into areas of secondary pinprick hyperalgesia, it is the result of a peripheral change (Callsen et al. 2008). Capsaicin has also been shown to reduce cold activated currents in cultured neurones, which would support the results observed in this study and the previous (Reid et al. 2002). The mechanisms underpinning this are still unclear, although Callsen and colleagues suggest that the co-expression of TRPV1 and TRMP8 intracellular interactions may reduce the activity of the cold sensing receptor (Callsen et al. 2008). Further investigation of this symptom will be necessary to elucidate the true mechanisms. During electrophysiological recordings, cold responses were attempted, however reproducibility was poor and so it was not possible to quantify the responses to cold in the animal studies. It would be interesting to assess any changes in cold sensitivity in animals if this technique could be refined.

3.4.7. QST characterisation of the topical capsaicin model produces a novel somatosensory profile

Full sensory characterisation post capsaicin indicates a strong heat and mechanical hypersensitivity, in addition to a cold hypoalgesia, in the primary treated area. The true clinical relevance of this model depends on the occurrence of such features in patients. Certain patients with neuropathy are reported to have burning pain, dynamic allodynia and mechanical hypersensitivity (Baron et al. 2012). In particular many patients with oxaliplatin-induced neuropathy suffer from both heat and mechanical hypersensitivity (Binder et al. 2007). In addition, there also appears to be some overlap of symptoms being shared with inflammatory conditions such as OA (Farrell et al. 2000). However, it would be interesting to see if there are any subgroups of patients whose profiles match that observed with capsaicin. The closer the overlap in symptoms, the greater the indication that capsaicin may mimic some of the underlying mechanisms associated with these conditions, and thus allows them to be modelled pre clinically.

Capsaicin induced primary heat and pinprick hypersensitivity are already well documented, although primary brush hypersensitivity and cold hypoalgesia are less frequently mentioned. The creation of a sensory profile from these evoked symptoms increases the relevance of this model for future studies, since it may be used to match the symptoms profile with those created from various abnormal pain states in order to assess which conditions the model most well reflects. The mechanisms underlying the thermal changes are well understood and thus drugs that alter the transduction of heat stimuli could be screened in this model. However, further exploration of the mechanisms underpinning the sensory abnormalities such as brush and pinprick hypersensitivity in animals could help understand the true clinical usefulness of the model; indeed the use of drugs with peripheral or central actions will help to evaluate the contribution of each phenomenon with regards to mechanical hypersensitivity post capsaicin.

3.4.8. Sensitisation of TRPV1 may be relevant in chronic pain conditions

Although the real contribution of activation/ sensitisation of TRPV1 in chronic pain states remains unclear, it is known that patients with gastro-esophageal reflux disease suffer from heat hypersensitivity and it has been shown that in cases of IBS and inflammatory bowel disease patients have upregulated TRPV1 (Reddy et al. 2007; Akbar et al. 2010; Krarup et al.

2011). In addition, the fact that chronic cough patients (which shares many overlapping mechanisms with chronic pain) are more sensitive to inhaled capsaicin, suggests that TRPV1 sensitisation may be underlying some of their symptoms of hypersensitivity (O'Neill et al. 2013). Furthermore, children carrying the TRPV1 SNP I585V (decreasing channel activity) appear to be at a lower risk of developing asthma related cough (Cantero-Recasens et al. 2010). Taken together, these findings suggest that an upregulation and sensitisation of TRPV1 may be an important mechanism in states of hypersensitivity, and thus modulation of this receptor could hold potential as a therapeutic target for relief of symptoms in both chronic pain. Additionally, since many chronic pain states may arise from damage to peripheral tissues, leading to ongoing activity and central sensitisation, this feature of the capsaicin model may be of importance (Baron et al. 2013). The model may be used to test drugs that manipulate the ongoing pain and if successful at reducing this symptom and further signs of central sensitisation, it may indicate their potential use in the clinic. Since it has been hypothesised that ongoing burning pain may arise from TRPV1 activation, this model could be particularly relevant (McMahon and Wood 2006).

However, it is important to note the specificity in induction of mechanisms, such as peripheral sensitisation of TRPV1, may limit the relevance to patients. Additionally, it is a short-term model whereby the changes reverse after a number of hours and therefore does not mimic any long-term modifications, which are likely to be involved in most chronic pain syndromes. Therefore, it is likely only relevant to the early developmental stages of many conditions.

3.4.9. Topical capsaicin is a reliable translational model of hypersensitivity

Translational models are useful to explore both pain mechanisms and validate new treatments, however since a number of mechanisms of capsaicin induced sensitisation are quite well described, the main use of the model in the future is most likely the use of assessing novel therapies.

The study conducted here used relatively low n numbers, since it is important to keep the use of animals to the minimum required to show significance. However, in larger human studies it has been noted that there may be a variation in responses to the model, which may be a further limitation to the usefulness of this model (Klein et al. 2008). It is possible that TRPV1 polymorphisms may explain some of this variation and such genetic factors may be a

potential source of variability in this model. Of note, this previous observation did not appear to affect the results in this study.

3.5. Concluding remarks

Overall, this is the first study to fully characterise the primary area of injury, and compare the results in animals and humans in order to assess the translational impact of this model. A full sensory examination of the primary area post topical capsaicin cream in animals and humans revealed the presence of thermal and mechanical hypersensitivity, most likely due to a peripheral sensitisation of C fibre afferents, and a central sensitisation including a facilitation of Aδ fibre responses. In addition, in humans a cold hypoalgesia was also noted. Overall this study found an analogous set of sensory abnormalities across species. Therefore, the use of topical capsaicin cream as a translational model of peripheral sensitisation, leading to central modifications, could be useful in assessing novel treatments for early stage chronic pain. With regard to the heat hypersensitivity, it is well known that this symptom is the consequence of TRPV1 sensitisation and thus this model is particularly suitable for assessing drug modulation of this well-defined mechanism. On the other hand, further pharmacological manipulations will help fully elucidate the mechanisms underpinning the dynamic brush hypersensitivity.

4. Modulation of Capsaicin Induced Hypersensitivity

4.1. Introduction

Many of the current treatments for chronic pain, including drugs such as Pregabalin, Tramadol, Amitriptyline, target the pain pathway at spinal or supra spinal sites (Sindrup and Jensen 1999; Field et al. 2006). Not only must we assume they reach their destined site of action through crossing the blood brain barrier, but activity at central pain targets will also be accompanied by potential undesirable side effects through interactions with other CNS functions that utilise the neuropharmacological targets of these drugs. These can range from sedation, dizziness and fatigue, to the less frequently reported memory impairment, hallucinations and other subtle actions which are extremely difficult to gauge in nonhumans (Finnerup et al. 2010). Treatments targeting the periphery are less common, and other than topical formulations, are often given systemically so may in fact also be acting at a number of locations in the pain pathway and elsewhere. Having said this, it must not be overlooked that the new 8% capsaicin patch has been reported to be effective in both HIV-related neuropathy and PHN (Simpson et al. 2008; Irving et al. 2012). Taking together the limited amount of peripherally acting treatments available (such as the lidocaine patch), and the recent success of 8% capsaicin, it could be concluded that the development of a wider range of such drugs could be useful (Argoff et al. 2004; Dworkin et al. 2007). Here, we examine the possibility of developing locally administered peripheral adenosine 1 receptor (A_1R) agonists. Bearing in mind that different treatments are needed for different pain mechanisms (Dworkin et al. 2003; Baron et al. 2010), we assessed the possible modulation by the A_1R of TRPV1 function and the ability of A_1R agonists to attenuate the specific feature of thermal hypersensitivity – thus focusing on a mechanism based approach to treatment.

ADO is an endogenous nucleotide, which is ubiquitously expressed, and is involved in many physiological processes. It acts at the GPCRs A_1, A_{2A}, A_{2B} and A_3, which dictate its actions accordingly. Whilst A_1 and A_3 receptors are coupled to $G_{i/o}$ and thus are inhibitory, A_2 receptors are coupled to G_s/ $G_{q/11}$ and have excitatory actions (Sawynok and Liu 2003; Gao and Jacobson 2007). It is well known that levels of extracellular ADO are elevated after stress or injury to tissues; such as ischaemia, hypoxia or seizures and is involved in a number of processes through actions at these receptors. The half-life of ADO is short as it is rapidly taken up by erythrocytes and metabolised by adenosine deaminase, and thus it is believed that the actions are localised to the site at which it is released (Kowaluk 1998).

4.1.1. A_1 Receptors have antinociceptive properties

The A_1R is widely expressed, in the periphery and the CNS, including within the DH, cortex, cerebellum and hippocampus (Gessi et al. 2011). Actions at the A_1R are believed to be involved in both pain transmission and modulation. More specifically ADO is believed to exert an endogenous antinociceptive effect at this receptor, through its coupling to $G_{i/o}$ (Dickenson et al. 2000; Fredholm 2010; Lima et al. 2010; Fredholm et al. 2011). In the periphery, it has been shown that A_1Rs are expressed on a significant amount of small- medium nociceptive fibres (Lima et al. 2010). Whilst in the DH of the spinal cord it has been shown to be present both presynaptically on afferent fibres, and on specific sets of interneurons (Schulte et al. 2003). Pre-synaptic inhibitory effects may involve interaction with Q-, P- and N-type Ca^{2+} channels, and a reduction in release of neurotransmitters such as substance P and CGRP (Dolphin et al. 1986; Fredholm 2010). However, it is believed that the majority of A_1Rs in the DH are found post synaptically within the substantia gelatinosa (Geiger et al. 1984). Post-synaptic inhibitory effects are likely to be through interaction with K^+ channels, resulting in hyperpolarisation (Gessi et al. 2011).

A_1Rs have also been shown to be co-localised with TRPV1 on afferent fibres, where a study by Lima and colleagues found 79.55% of A_1Rs positive neurons also expressed TRPV1, whilst 95.6% of TRPV1 positive neurons also expressed the A_1R (figure 4-1) (Lima et al. 2010). Activation of the A_1R has been reported to activate PLC, catalysing the breakdown of PIP2, which may be required for function of TRPV1 and therefore ADO may have an indirect inhibitory effect on this receptor function through interference with a common downstream mechanism (Rohacs et al. 2008).

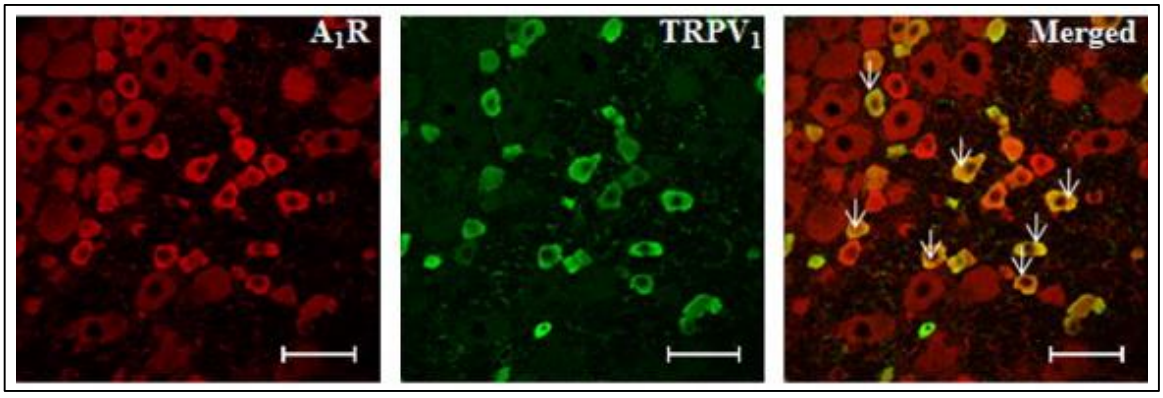

Figure 4-1 Expression and co-localisation of the A1R and TRPV1 on rat primary afferent neurones. Anti-A1R immunoreactivity in subpopulations of DRG neurons labelled using TRPV1 antibodies (Lima et al. 2010).

4.1.2. Activation of the A_1R in the spinal cord reduces acute and chronic pain

The most well documented actions of ADO/ activation of the A_1R are in the spinal cord, where its actions are known to be antinociceptive. Interestingly, it has been observed that ATP is elevated in DRG after nerve injury (which could be broken down into ADO, as the enzymes converting ATP into ADO are highly localised within the DH), suggesting a possible role for endogenous modulation of pain signals (Matsuka et al. 2008). Spinal infusion of ADO has been shown to produce antinociceptive effects in both animal and human models of acute, inflammatory and neuropathic pain (Kowaluk 1998; Dickenson et al. 2000). Reeve and Dickenson showed that IT application of a selective A_1R agonist CPA reduced wind up, as well as decreasing both the first and second phase of the formalin test (Reeve and Dickenson 1995). Further studies in rodent models of nerve injury and inflammation revealed an inhibitory effect on hypersensitivity to both noxious and innocuous stimuli (Sawynok and Liu 2003; Sawynok 2007). For example, spinal ADO was found to reduce mechanical hypersensitivity post nerve injury (Lavand'homme and Eisenach 1999), whilst in the CCI model of neuropathy it was shown that R-phenylisopropyl-adenosine (R-PIA) attenuated scratching behaviour and tactile hypersensitivity (Sjölund et al. 1996; Cui et al. 1997).

Additionally, IT administration of ADO/ A_1R agonists have been shown to have both antihyperalgesic and antiallodynic actions in healthy volunteers (Lynch et al. 2003; Sawynok 2007). Rane and colleagues demonstrated that IT ADO was able to reduce the area of secondary hyperalgesia induced by topical mustard oil, as well as attenuating the reduction in tactile pain threshold usually seen in this model (Rane et al. 1998). In concurrence, Eisenach and colleagues also found that the area of hyperalgesia and allodynia induced by capsaicin were reduced with pre-treatment of IT ADO (Eisenach et al. 2002). As these symptoms are generally attributed to central mechanisms, it can be inferred that ADO is able to suppress this short-term induction of central hyperexcitability.

IT ADO is also able to attenuate both spontaneous and evoked pain, as well as areas of tactile hyperalgesia and allodynia, in patients with neuropathic pain (Karlsten and Gordh Jr 1995; Belfrage et al. 1999). Whilst Eisenach further confirmed ADO reduced the area of allodynia for up to 24 hours in neuropathic pain patients, it is interesting to note that this affect was not seen if given intravenously (Eisenach et al. 2003). Spontaneous pain is also inhibited by a spinal A_1R agonist in a surgical model, and furthermore when give pre-emptively before (and during) surgery it was demonstrated to suppress post-operative pain (Gan and Habib 2007;

Zahn et al. 2007). This suggests there is an important role of spinal ADO via activation of the A_1R in antinociception. Not only is it associated with a reduction in common symptoms of chronic pain such as allodynia, but it also appears to be involved in preventing the development of certain alterations in pain processing.

There is conflicting evidence regarding systemic infusion of ADO, which was originally shown to increase HPTs in healthy volunteers and attenuate ischaemic pain, as well as reduce the area of cutaneous burn induced secondary hyperalgesia (Segerdahl et al. 1994; Ekblom et al. 1995; Rae et al. 1999) (Sjölund et al. 1999). Systemic infusion of ADO is also reported to reduce the area of allodynia in patients with neuropathic pain (Sjölund et al. 2001). However, it has also been found that systemic ADO had no effect on a human model of capsaicin induced central sensitisation (Dirks et al. 2001) and whilst some trials report that IV ADO delivered before surgery resulted in both pain relief and decreased need for opioids, others found it to have no effect (Gan and Habib 2007; Habib et al. 2008). Due to the short half-life of ADO it may be inferred that systemic or IV delivery could be insufficient to reach the target areas (including the DH or peripheral receptors) and thus the modest effects are not necessarily surprising.

4.1.3. Paradoxical actions of peripherally administered ADO and activation of the A_1R

The role of ADO in the periphery is also debated and the specific endogenous actions on the peripheral nociceptive system are unclear. Taiwo and Levine found that intradermal injection of ADO in rats of concentrations up to 1.5μM led to a decrease in mechanical threshold for up to 20 minutes post injection (Taiwo and Levine 1990). In addition, Pappagallo and colleagues found that 2μM ADO delivered intradermally, not only caused pain on injection but also resulted in an area of primary heat and mechanical hyperalgesia (Pappagallo et al. 1993). Sawynok also suggested that peripheral ADO is pronociceptive, though these actions could explained by activation of the A_2R rather than the A_1R. Additionally, the ADO receptors are expressed on various innate immune cells and activation may result in the release of proinflammatory cytokines and tissue destruction pathways, which could contribute to this pronocicpetive action (Haskó and Cronstein 2004). However, since the overriding action of ADO on the immune system is thought to be anti-inflammatory, it is unlikely that there is a

substantial contribution of immune cell mediated sensitisation responsible for the observed hypersensitivity (Haskó et al. 2008).

On the other hand, it has also been found that ADO analogues such as R-PIA and N-ethylcarboxamide-adenosine, when co-administered peripherally with formalin, reduced pain related behaviours in mice (Karlsten et al. 1992). This study went on to show that the compounds had no effect on local blood flow upon injection, which therefore would suggest that the effects are in fact mediated via A_1Rs located on the PAFs themselves (Karlsten et al. 1992).

Local injection of CPA reduces noxious thermal, but not mechanical, sensitivity, in mice. This effect was only seen on the ipsilateral side and was further lost in A_1R KO mice, overall suggesting a local effect of A_1R activation (Hurt and Zylka 2012). This observation of actions specific for the thermal modality is consistent with the theory of A_1R and TRPV1 interaction. Additionally, peripheral ADO is thought to underlie pain relieving effects of acupuncture in both humans and mice, and the A_1R has been demonstrated to be required for the antinociceptive actions of acupuncture in a mouse model of chronic pain (Goldman et al. 2010).

It is clear that the actions of ADO depend on site and respective receptor subtype and density. The expression ratio of A_1 vs. A_2Rs on peripheral fibres for example is unknown, however what is apparent is the high affinity of ADO for the A_1R over the other subtypes. The reported EC_{50} of ADO for the A_1R is as low as 70nM, whereas for the A_{2A} and A_{2B} it is 150nM and 510nM, respectively (Fredholm et al. 1994; Sawynok and Liu 2003). This would suggest that at lower concentrations it is possible to target the A_1R over excitatory A_2 receptors.

It is particularly interesting to further explore this role in the periphery as although positive results have been found using IT and systemic administration of ADO/ A_1R agonists they were also associated with side effects such as back pain, headaches and reduced heart rate (Zylka 2011). If there is an overriding antinociceptive role in the periphery, it could have the potential for exploitation as a local drug target.

4.1.4. Peripheral inhibitory actions of ADO may rely on interactions with TRPV1

More recently it has been demonstrated in animals that peripheral administration of A_1R agonists decrease inflammatory peripheral hypersensitivity through second messenger cascades (Lima et al. 2010). Experiments from Zylka and colleagues have suggested that this may involve PLC, catalysing the breakdown of PIP2, which may be required for function of the heat sensing receptor TRPV1 (Rohacs et al. 2008; Sowa et al. 2010). Animal models have shown that direct application of PIP2 enhanced thermosensation, thermal hyperalgesia and also capsaicin currents (Stein et al. 2006; Sowa et al. 2010). This suggests that the peripheral antinociceptive actions of ADO could be attributed to the secondary interaction with other receptors and ion channels.

4.1.5. Clinical implications and pain relieving prospects of ADO?

Despite the evidence for the pain reducing effects of ADO it is currently not used as a treatment for patients. There are a number of reasons for this, including the undesirable cardiovascular side effects if ADO is given intravenously. Additionally, little is known regarding the effect ADO has on spontaneous pain, which is a critical symptom in many chronic pain conditions. This study investigates the use of ADO as a prophylactic treatment, given pre-emptively before the induction of the model, in order to assess whether it can prevent the development of certain chronic pain symptoms. Although this is not necessarily possible in the clinic, it may be useful to explore the benefits of early treatment, which may prevent the progression of chronic pain conditions – in particular it may prevent/ slow the development of central sensitisation. Since ongoing peripheral activity is often the driver for central sensitisation, a reduction of such activity may indeed prevent its development. Finally, the peripheral actions of ADO currently remain unclear in humans. Therefore further investigation of both the peripheral effects of ADO and the possible ability to reduce the consequences of an ongoing painful stimulus (capsaicin) are required. A clear benefit of peripheral injections is the reduction in cardiovascular and central side effects, however conducting studies with not only ADO but also partial A_1 agonists would also be useful as it has been suggested they also have limited side effects.

Here, this chapter aims to further elaborate on the mechanism of action of ADO through the use of an intradermal injection. It is hypothesised that intradermal ADO should decrease

baseline responses to thermal stimuli in both rodents and healthy volunteers, possibly due to an indirect interaction with TRPV1. Furthermore, the chapter explores the effect of ADO on the response to topically applied capsaicin cream, which also acts at the TRPV1 receptor. To investigate the contribution of the A_1R experiments are repeated using a specific and more stable agonist, CPA, in the animal studies.

4.2. Methods

4.2.1. In vivo electrophysiology:

Adult male Sprague-Dawley rats, between 220-250g, were obtained from the UCL Biological Services Unit. All procedures were approved by the UK Home Office and were performed in accordance with the guidelines provided by the International Association for the Study of Pain.

In vivo electrophysiological recordings were performed as previously described to obtain baseline responses. Once stable responses had been characterised for each individual cell, 26μg/50μL solution of ADO in 0.9% saline was injected using a Hamilton syringe, into the receptive field of the cell, distal from the point at which natural stimuli were applied. (Nb. Control experiments found that intraplantar injection of saline alone causes no significant changes monitored over a time period of 4 hours post injection, see chapter 2). The train of electrical and natural stimuli was repeated at 10, 25, 40, 55 and 70 minutes post injection. The maximum change was calculated once results had been collected.

From this initial experiment it was found that the maximal effects of ADO occurred around 10-25 minutes post injection, thus in the second experiment ADO was injected 10 minutes before application of 1% capsaicin cream for 30 minutes. (Nb. Control experiments found that intraplantar injection of saline alone had no effect on capsaicin induced hypersensitivity, data not shown). Capsaicin was applied as described in chapter 3. Electrical and natural stimuli were then tested at 30, 50, 70 and 90 minutes post capsaicin and once again the maximum change was calculated.

The same protocol was followed for CPA. 5μg /50μL CPA in 0.9% saline was injected into the receptive field, distal to the area of testing. Post intraplantar injection the train of electrical and natural stimuli was repeated at 10, 30, 50, 70 and 90 minutes post injection. The maximal effects of CPA occurred around 30-50 minutes post injection, thus in the second experiment CPA was injected 30 minutes before application of 1% capsaicin cream for 30 minutes. Electrical and natural stimuli were then tested at 30, 50, 70 and 90 minutes post capsaicin and the maximum change was calculated.

4.2.2. Human Quantitative Sensory Testing:

Experiments were conducted in 8 healthy human volunteers aged between 19-33 years old. Individuals were familiarised with the experimental protocol before hand and gave written, informed consent. The study was approved by The Kings College Research Ethics Committee.

All subjects were free from pain and medical conditions which may otherwise interfere with the results of the study. They were advised they must avoid pain medication such as NSAIDs and caffeine in the 24 hours prior to the study. This was particularly important, as caffeine is an antagonist at the A_1R.

Baseline thresholds were obtained at marked sites (5x5mm) on the ventral forearms for MPT and HPT, as previously described. In addition to MPT, subjects were asked for numerical ratings for the 32mN and 256mN pinprick devices; and in addition to HPT, subjects were asked for numerical ratings to 35°C and 45°C. The area was then cleaned for injection of 26μg/ 50μL ADO in 0.9% saline; the test arm was alternated between subjects. (Nb. Control experiments found that intradermal injection of saline alone causes no significant changes monitored over a time period of 180 minutes post injection, see chapter 2). Successful intradermal injections were confirmed by the presence of a bleb beneath the skin. Thresholds and NRS ratings we retested at 10 and 25 minutes post injection.

Secondly, to examine the effects of pre-treatment with ADO on capsaicin induced sensitisation an injection of ADO or vehicle (0.9% saline) was given 10 minutes before topical application of 1% capsaicin cream. Vehicle and treatment arms were alternated. Threshold and NRS ratings were then retested 30 minutes post capsaicin.

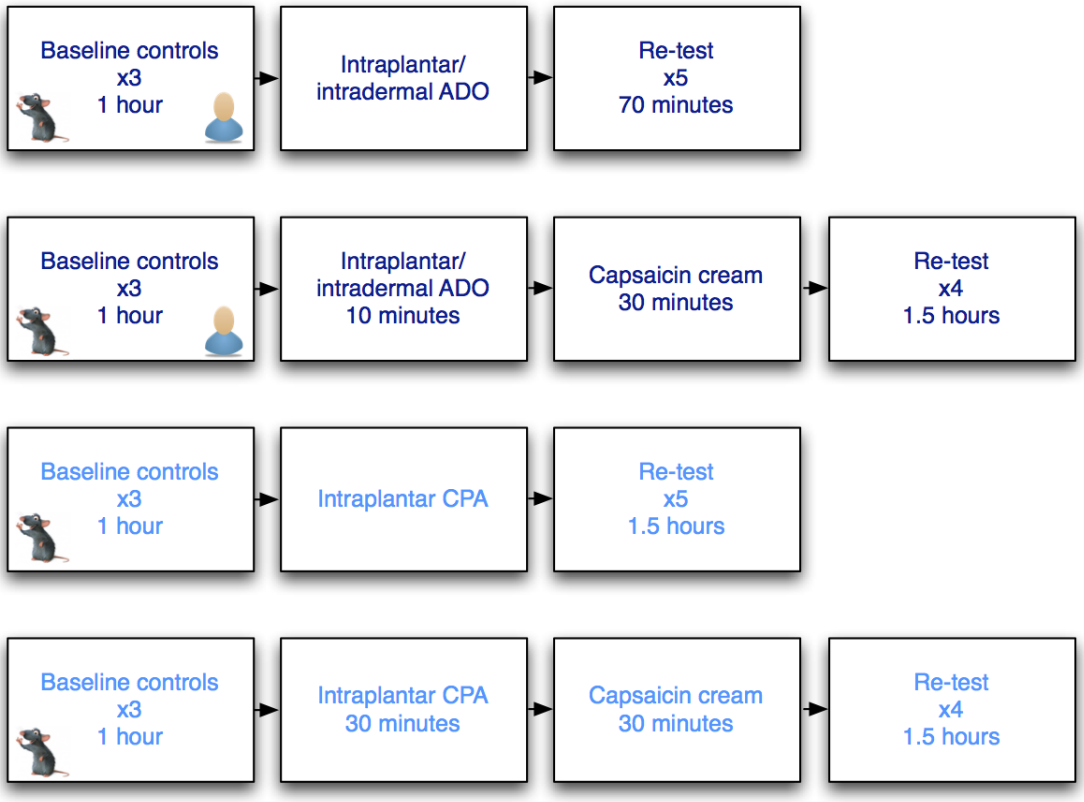

Figure 4-2 Schematic to summarise the experimental protocols conducted in this chapter. Dark blue text describes the protocols undertaken in animals and humans evaluating the effects of ADO. Light blue text describes the protocols investigating CPA in animals.

4.2.3. Statistical analysis:

All analysis was undertaken using SPSS software (IBM SPSS Statistics v21). Data was assessed for normality using the Kolmogorov-Smirnov test to determine further methods of analysis. Electrophysiological data was analysed using either a paired or unpaired t-test, or a 2 way ANOVA accordingly. Psychophysical data, with the exception of HPT, was logged and re-tested for normality. A paired t-test or 2 way ANOVA was then carried out. HPT was found to be normal without logging, and thus the raw data was used for analysis with a paired t-test. All graphs were plotted to show the mean ± SEM.

4.3. Results

4.3.1. ADO In vivo electrophysiology

Using objective electrophysiological recordings, LV WDR cell responses to thermal stimuli were found to be significantly reduced post intraplantar ADO in comparison to baseline responses. Only a small effect was noted regarding mechanical stimuli, and electrical responses were unchanged. This selective effect primarily on thermal responses is suggestive of the expected reduction in activity of TRPV1+ fibres and a possible indirect modulation of TRPV1 itself.

4.3.1.1. Intraplantar injection of ADO leads to small but significant reductions in mechanically evoked baseline WDR cell responses in naïve animals

Post ADO dynamic brush responses were lower than baseline, although this was not found to be significant (figure 4-3). There was also little change in the number of action potentials to innocuous or noxious vF, however the decrease was found to be significant for 2g and 26g. At 2g there was a decrease from 27.7 ± 3.7 to 14.9 ± 4.7 action potentials/ 10s, and the response to 26g decreased from 556.6 ± 80.2 to 440.3 ± 102.5 action potentials/ 10s (figure 4-3; p= 0.025, 0.009).

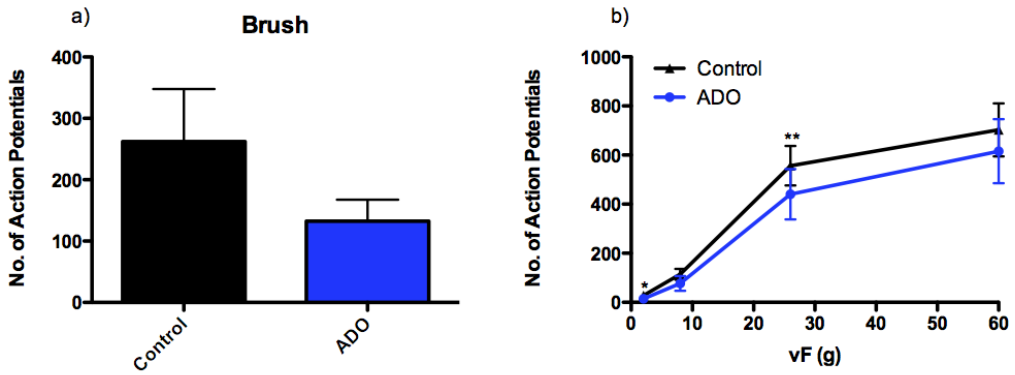

Figure 4-3 Effects of ADO on mechanically evoked baseline WDR cell responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of mechanical stimuli, including brush and graded vF, applied to the receptive field for 10s, before and after intraplantar ADO. a) The mean response to dynamic brush was reduced from 262.1 to 132.6 action potentials/ 10s, although this was not shown to be statistically significant (p= 0.069). b) On the other hand, innocuous 2g vF responses, were significantly reduced, as well as noxious 26g responses (Overall 2-way ANOVA p= 0.025; 2g p= 0.025, 26g p= 0.009). n= 7

4.3.1.2. Intraplantar injection of ADO significantly reduces thermally evoked baseline WDR cell responses in naïve animals.

Decreased firing of WDR cells was observed to all temperatures tested post intraplantar ADO, from warm to highly noxious, in comparison to baseline responses (figure 4-4; p= 0.009). The most significant reduction was seen at 45°C, just above behavioural threshold, from 598.7 ± 78.9 to 285.0 ± 96.5 action potentials/ 10s (p= 0.01). Since TRPV1 is activated at around 42°C this may be the result of A_1R-TRPV1 indirect interactions.

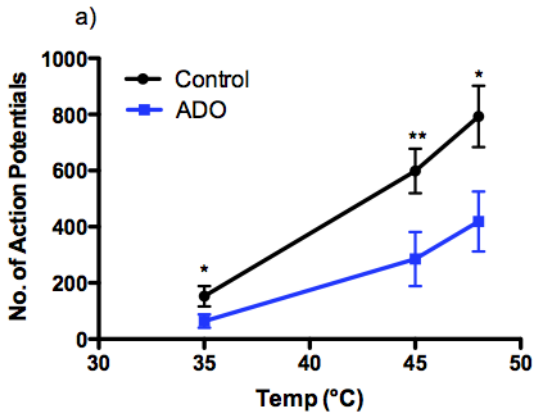

Figure 4-4 Effects of ADO on thermally evoked baseline WDR cell responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of thermal stimuli applied to the receptive field for 10s, before and after intraplantar ADO. a) Both innocuous and noxious thermal responses were significantly decreased post intraplantar injection of ADO (Overall 2-way ANOVA p= 0.009; 35°C p= 0.026, 45°C p= 0.01, 48°C p= 0.015). n = 7

4.3.1.3. Intraplantar injection of ADO has no effect on electrically evoked baseline WDR cell responses in naïve animals

Overall, no significant difference was observed between post ADO and baseline responses to electrical stimuli (figure 4-5). The number of action potentials elicited from each fibre type was unchanged, suggesting that the decrease in responses to natural stimuli is due to an interaction with specific transducers, rather than an overall effect on the excitability of the fibres. Input and wind-up appear reduced, although this was not found to be significant.

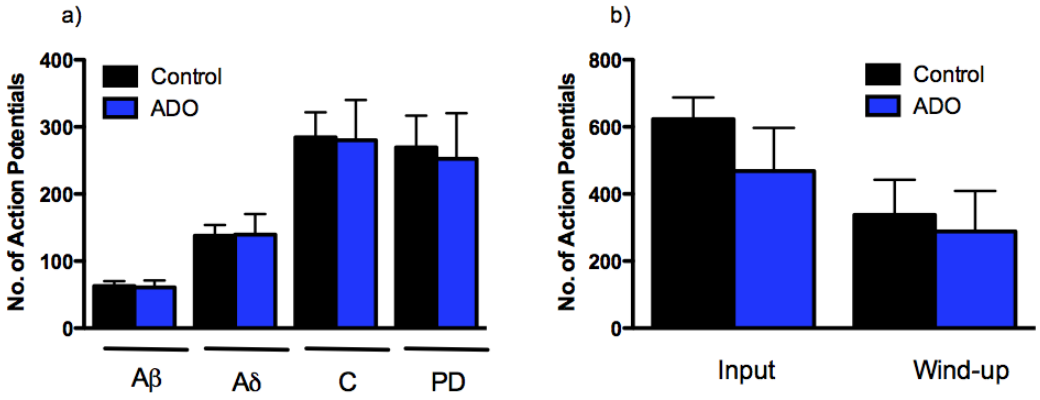

Figure 4-5 Effects of intraplantar ADO on baseline electrical WDR cell responses. Using the protocol described in chapter 2.2 and 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli both pre and post intraplantar ADO. Transcutaneous electrical stimulation was used to measure the input and wind up, in addition to calculating the responses driven by different fibre types – depending on the latency. a) There was no significant effect on electrically evoked Aβ, Aδ, and C fibre mediated transmission, nor post-discharge; b) electrically induced input or wind up. n= 7.

4.3.2. ADO/CAP – In vivo electrophysiology

Overall, pre-treatment with ADO was found to have only a small effect on capsaicin induced hypersensitivity to thermally evoked stimuli. However, there was a clear reduction in capsaicin induced brush hypersensitivity. The results suggest that ADO can partially prevent capsaicin induced sensitisation.

4.3.2.1. Pre-treatment with intraplantar ADO inhibits capsaicin induced sensitisation of innocuous dynamic brush WDR cell responses in naïve animals

1% capsaicin cream results in an increased firing of WDR cells to dynamic brush. Pre-treatment with ADO inhibits this sensitisation, as responses post ADO/CAP are not significantly different from baseline (figure 4-6). Additionally, when compared to cells treated with CAP alone, responses of ADO/CAP cells are significantly reduced from 680.4 ± 47.52 to 360.3 ± 121.2 action potentials/ 10s (figure 4-6; p=0.033).

4.3.2.2. Pre-treatment with intraplantar ADO partially attenuates capsaicin induced sensitisation of thermal neuronal responses in naïve animals

1% capsaicin cream results in a strong sensitisation to thermal stimuli highlighted by a significant enhancement of evoked responses post CAP treatment. Pre-treatment with ADO does not fully inhibit this sensitisation, as responses of ADO/CAP cells are still significantly enhanced from baseline, with the exception of responses to 48°C which appear no different from baseline (figure 4-7; p= 0.049). However, when compared to cells treated only with CAP, overall there is no significant difference. This suggests a partial effect of ADO on capsaicin induced thermal hypersensitivity.

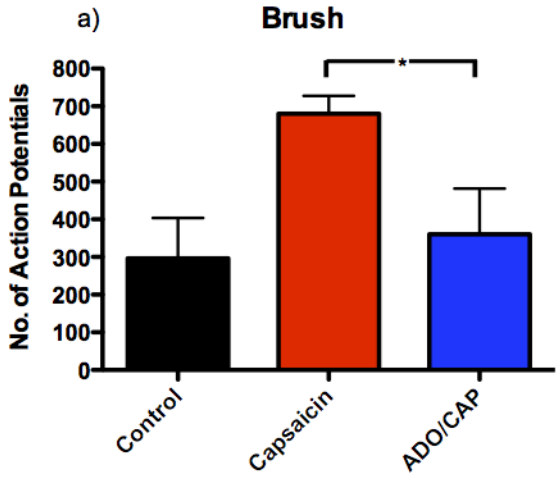

Figure 4-6 Effects of pre-treatment with ADO on capsaicin induced sensitisation of dynamic brush evoked baseline WDR cell responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to brush applied to the receptive field for 10s, before and after pre-treatment with ADO and capsaicin application. a) Brush responses are no longer significantly increased when intraplantar injection of ADO is given 10 minutes before application of topical capsaicin cream. Furthermore, there is a significant difference between the group of cells pre-treated with ADO and those with capsaicin alone (unpaired t-test p= 0.033) capsaicin n= 10, ADO/CAP n= 7.

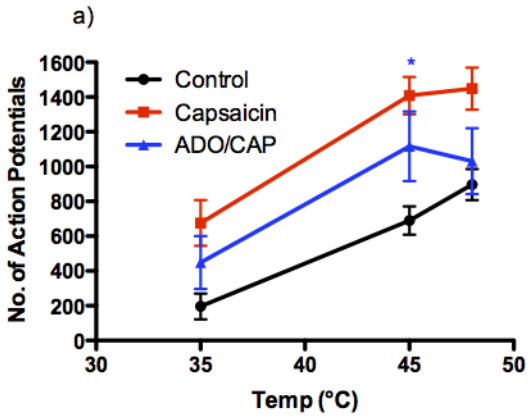

Figure 4-7 Effects of pre-treatment with ADO on capsaicin induced sensitisation of thermally evoked baseline neuronal responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to thermal stimuli applied to the receptive field for 10s, before and after pre-treatment with ADO and capsaicin application. Previously, with capsaicin alone, sensitisation was seen at 35°C, 45°C and 48°C. a) Innocuous and noxious thermal responses (35 and 45°C) are increased despite prophylactic intraplantar injection of ADO given 10 minutes before application of topical capsaicin cream, although 48°C appears unchanged from baseline (Overall 2-way ANOVA p= 0.049; 45°C p= 0.026.) n=7. However, there is no significant difference between the group of cells pre-treated with ADO and those with capsaicin alone.

4.3.2.3. Pre-treatment with intraplantar ADO inhibits capsaicin induced enhancement of Aδ fibre mediated responses and reduction of C fibre mediated transmission in naïve animals

In the previous chapter it was found that 1% capsaicin cream results in a potentiation of Aδ fibre mediated responses through central changes, alongside a reduction in responses mediated in the C fibre range which could be due to peripheral desensitisation. Neither of these differences were seen when cells were pre-treated with ADO (figure 4-8). Since all the results suggest the main effect of ADO is due to an indirect interaction with TRPV1, the respective enhancement and reduction are likely to also be TRPV1 related phenomenon. A modulation of events downstream from TRPV1 by ADO can explain the ability of the purine to inhibit these changes. Alternatively, A_1R induced hyperpolarisation could be counteracting activation of TRPV1. Furthermore, although capsaicin alone results in an increase in input, when pre-treated with ADO the input is significantly decreased, as is wind up, suggesting a possible effect on overall excitability of peripheral fibres (figure 4-8; p= 0.013, 0.005).

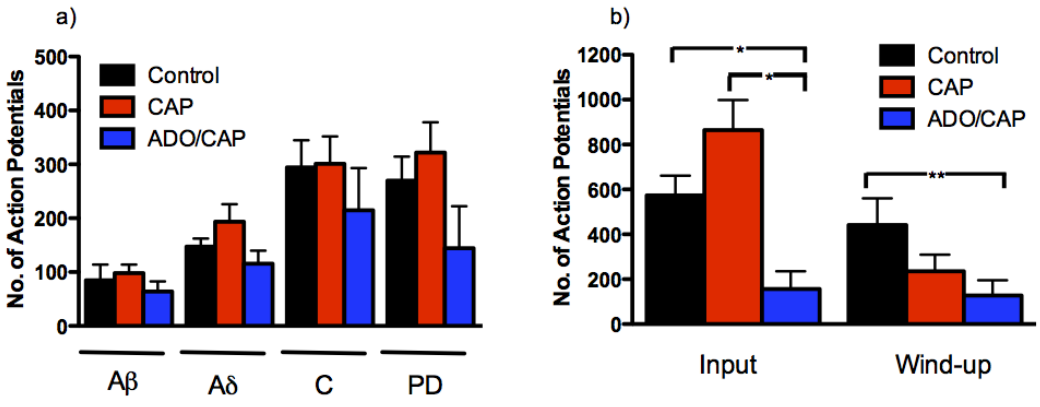

Figure 4-8 Effects of pre-treatment with ADO on electrical neuronal responses post capsaicin. Using the protocol described in chapter 2.2 and 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli. a) There was no significant difference between baseline and ADO/CAP treated neurones with regards to electrically evoked Aβ, Aδ, and C fibre mediated transmission, nor post-discharge, suggesting that capsaicin induced facilitation of Aδ fibres and reduction of C fibre transmission were inhibited. b) Both input and electrically induced wind up were significantly decreased when compared to baseline responses (p = 0.013 and 0.005). n= 6

4.3.3. ADO – Human Quantitative Sensory Testing

Using a standardised procedure, human subjects were also found to have increased HPTs post ADO, with minimal effect on mechanical responses. This is in line with the animal data previously reported, consistent with the theory of a preferential activation of the A_1R on TRPV1+ fibres and a possible indirect interaction of ADO and TRPV1.

4.3.3.1. Intradermal ADO has no effect on MPT or numerical ratings to innocuous and noxious punctate stimuli

Post intradermal ADO there was no significant difference found in either average MPT, or in ratings to innocuous or noxious pinprick in the treated area, suggesting ADO has no effect or normal innocuous or noxious mechanosensation (figure 4-9).

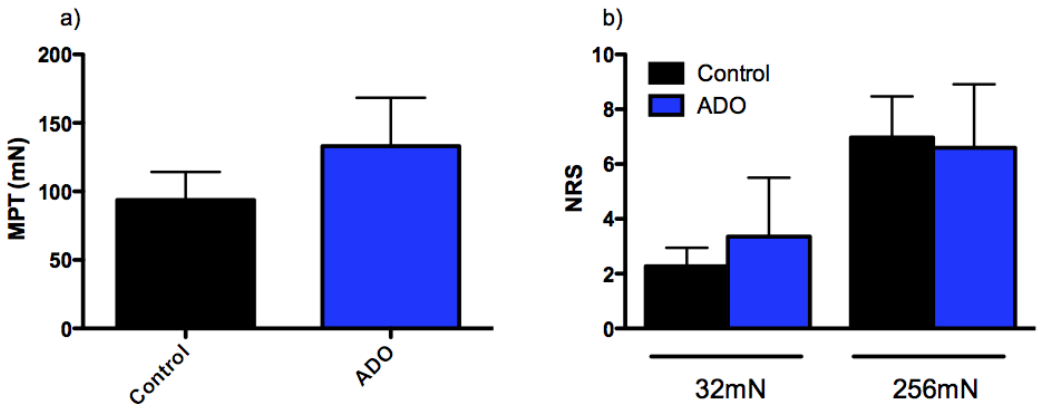

Figure 4-9 Effects of intradermal ADO on psychophysical MPT and punctate mechanical NRS ratings. Using the protocol described in chapter 4.2 standardised QST was undertaken to determine the subject's mechanical pain threshold (MPT), in addition to obtaining numerical ratings (NRS) to graded mechanical stimuli both pre and post intraplantar ADO. a) Average MPT was unaffected by ADO, and b) NRS ratings to 32mN and 256mN were also unchanged post ADO administration. n= 8

4.3.3.2. Intradermal ADO significantly increases HPT

Average HPT was increased in the treated area from 44.9 ± 2.1 °C to 46.9 ± 2.1 °C, and this trend was the case across all subjects (figure 4-10; p≤ 0.0001). Although numerical ratings to 35°C and 45°C appear reduced, this was not found to be significant (figure 4-10). These results are similar to those found in the animals, and are once again suggestive of downstream interactions with the TRPV1 receptor.

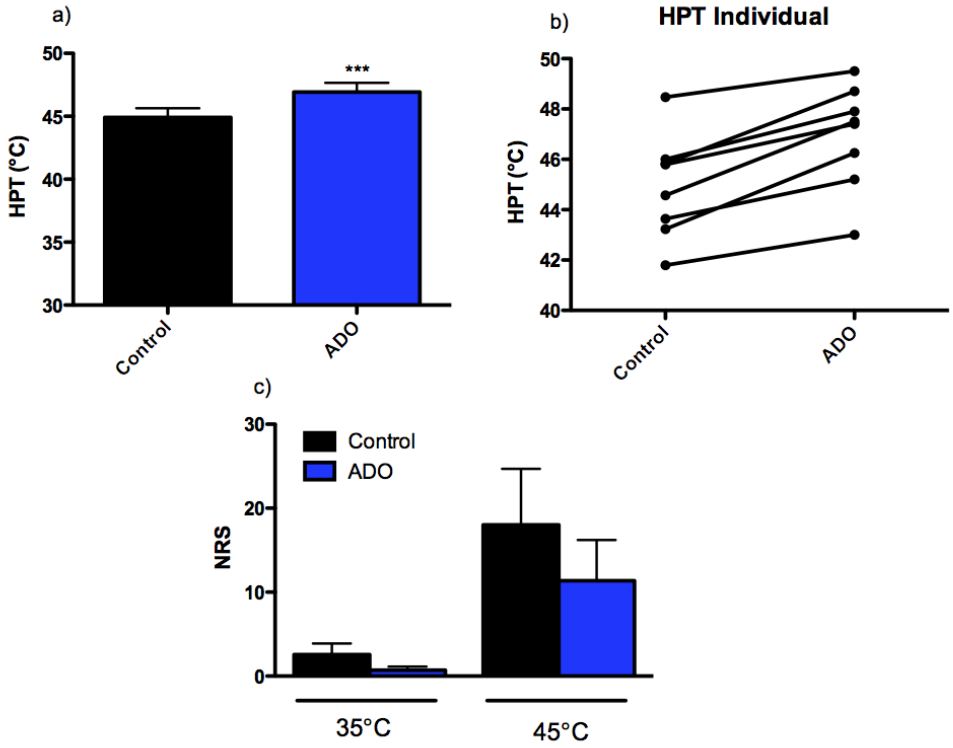

Figure 4-10 Effects of intradermal ADO on psychophysical HPT and NRS ratings. Using the protocol described in chapter 4.2 standardised QST was undertaken to determine the subject's heat pain threshold (HPT), in addition to obtaining numerical ratings (NRS) to graded thermal stimuli, both pre and post intradermal ADO. a) HPT was significantly increased post ADO (p ≤ 0.0001); b) which can be seen consistently in all single subject HPTs. c) NRS ratings to 35°C and 45°C were unaffected, although there was a trend towards a decrease in ratings to 45°C (p= 0.072). n = 8

4.3.4. ADO/ CAP – Human Quantitative Sensory Testing

Since ADO mainly effects thermal stimuli, the ability to prevent capsaicin induced hypersensitivity was examined using HPTs and numerical ratings to thermal stimuli. The results suggest that pre-treatment with ADO is able to partially inhibit capsaicin induced sensitisation of thermal stimuli.

4.3.4.1. Intradermal ADO partially inhibits capsaicin induced reduction in HPT

1% capsaicin cream significantly reduces average HPTs from 42.3 ± 4.0°C to 37.5 ± 4.9°C. This reduction is inhibited by pre-treatment with ADO, where average HPT was only reduced from 42.4 ± 1.6°C to 39.1 ± 2.4°C (figure 4-11). However, although the HPT post CAP treatment alone is lower than when subjects received ADO pretreatment, this was not found to be statisically significant, suggestive of only a partial inhibition of TRPV1 function. NRS ratings revealed a similar picture, since they were not significantly different from baseline control, nor capsaicin treated cells (figure 4-11). Overall it would appear that pre-treatment with ADO is able to paritally reduce CAP induced thermal hypersensitivty.

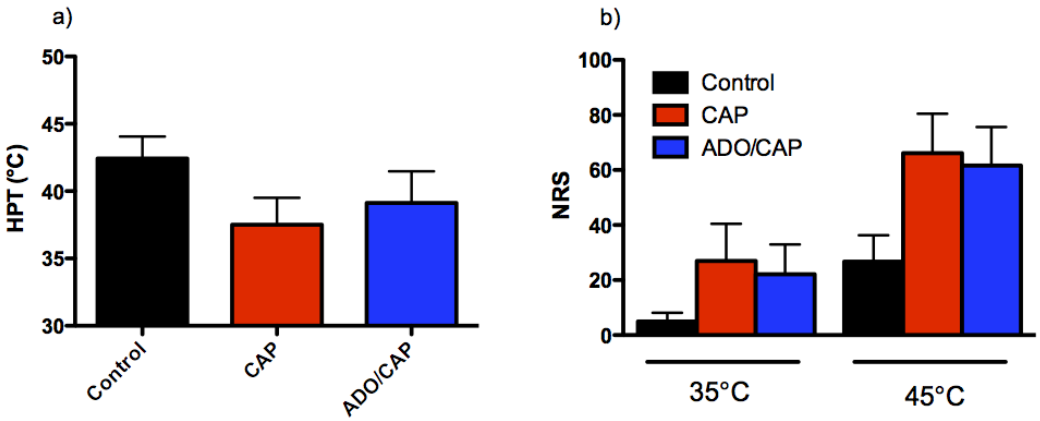

Figure 4-11 Effects of ADO pre-treatment on capsaicin induced sensitisation of psychophysical HPT and NRS ratings. Using the protocol described in chapter 4.2 standardised QST was undertaken to determine the subject's heat and cold pain thresholds (HPT), in addition to obtaining numerical ratings (NRS) to graded thermal stimul**i.** a) Average HPT post ADO/CAP was reduced by 3.3°C, which was not found to be significantly different from baseline (p=0.089). However, no significant difference was found between average HPT post CAP treatment, and average HPT post ADO/CAP. b) NRS post ADO/CAP ratings were not significantly different from baseline, however neither were ratings significantly different from post CAP NRS.

4.3.5. CPA – In vivo electrophysiology

Pre-treatment with ADO was unable to fully inhibit capsaicin induced sensitisation in animals, or humans. This is most likely due to the short half life (~30s) and possibly non specific actions through activation of all receptor subtypes. The A_1R is the major inhibitory subtype of ADO receptor, and has previously been shown to have antinociceptive properties. Therefore, by targeting this receptor spefically it is possible to further investigate the inhibitory actions of the A_1R in the periphery and its interaction with TRPV1. Overall, CPA had a more pronounced effect on evoked responses than ADO, which once again were stronger for thermal stimuli.

4.3.5.1. Intraplantar injection of CPA significantly reduces innocuous and noxious mechanically evoked baseline neuronal responses in naïve animals

Intraplantar CPA reduced WDR cell responses to dynamic brush from 387.9 ± 84.8 to 227.0 ± 151.0 action potentials/ 10s, although this was not found to be significant (figure 4-12). However, responses to both innocuous and noxious vF were reduced at 8g and 26g (figure 4-12).

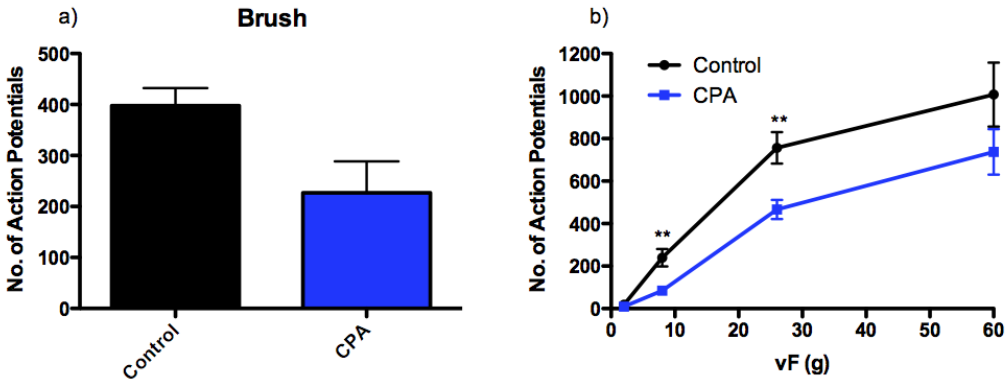

Figure 4-12 Effects of CPA on mechanically evoked baseline neuronal responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of mechanical stimuli, including brush and graded vF, applied to the receptive field for 10s, before and after intraplantar CPA. a) There was no significant effect of CPA on dynamic brush responses; although b) a reduction in mechanical responses was observed (Overall 2 way p= 0.005); both innocuous 8g (p= 0.008) and noxious 26g (p= 0.003) were significantly reduced. n = 6

4.3.5.2. Intraplantar injection of CPA significantly reduces thermally evoked baseline neuronal responses in naïve animals

There is a clear reduction in firing to noxious temperatures post CPA treatment, although coding is unaffected (figure 4-13). A decrease in firing can be observed to both 45°C and 48°C post CPA, from 875.1 ± 142.5 to 309.3 ± 120.8, and 1232.6 ± 162.2 to 545.6 ± 105.7 action potentials/ 10s, respectively (figure 4-13; p= 0,003, 0.001). This is strongly suggestive of the hypothesised interaction with TRPV1+ fibres, or modulating function of the receptor itself.

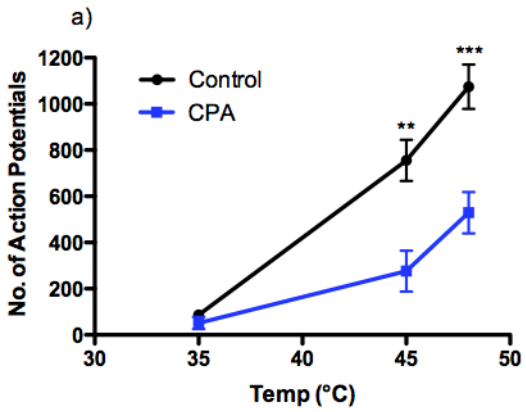

Figure 4-13 Effects of CPA on thermally evoked baseline neuronal responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of thermal stimuli applied to the receptive field for 10s, before and after intraplantar CPA. a) CPA significantly decreased firing to both 45 and 48°C (Overall ANOVA p= 0.001; p= 0.003 and 0.001, respectively.) n=6

4.3.5.3. Intraplantar injection of CPA has minimal effects on electrically evoked baseline neuronal responses in naïve animals

Overall, there was no significant effect of CPA on the number of action potentials elicited from Aβ or C fibres, or post discharge (figure 4-14). Additionally, there was no effect on input or wind-up. This suggests that although the A_1R may interact with TRPV1 to reduced thermal responses, since overall excitability of the neurones is unaffected. However, Aδ fibres mediated transmission was increased from 197.4 ± 119.3 to 230.6 ± 119.5 (figure 4-14; p= 0.032).

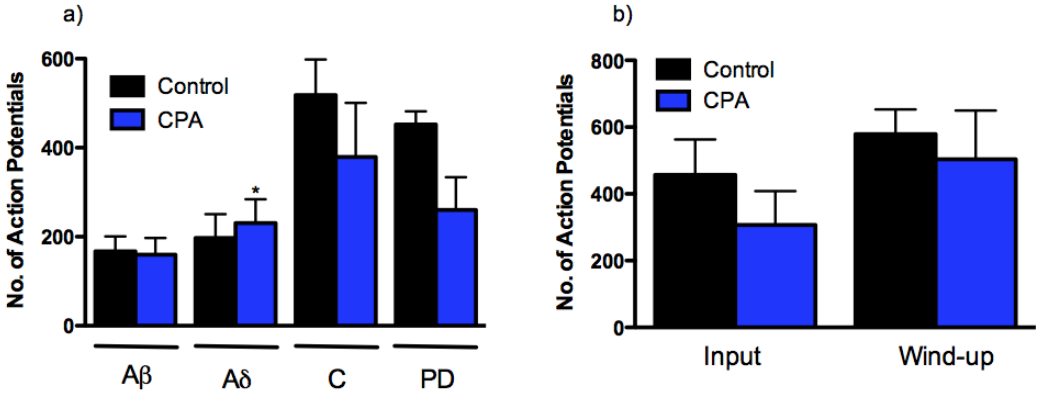

Figure 4-14 Effects of intraplantar CPA on baseline electrical neuronal responses. Using the protocol described in chapter 2.2 and 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli both pre and post intraplantar CPA. a) There was no significant effect on electrically evoked Aβ and C fibre mediated transmission, nor post-discharge, although Aδ mediated transmission was significantly increased (p= 0.032) b) There was no effect on electrically induced input or wind up. n = 5

4.3.6. CPA/CAP – In vivo electrophysiology

Overall, in contrast to previous results with ADO, pre-treatment with CPA was found to inhibit both CAP induced sensitisation to dynamic brush, and thermal hypersensitivity. This strongly suggests that the positive effects of ADO in reducing thermal responses in naïve animals, and partially reversing capsaicin induced sensitisation, is due to activation of the A_1R.

4.3.6.1. Pre-treatment with intraplantar CPA inhibits capsaicin induced sensitisation of innocuous mechanical neuronal responses in naïve animals

As previously mentioned, 1% capsaicin cream results in a clear increase in firing of WDR cells to dynamic brush. Pre-treatment with CPA is able to completely inhibit this sensitisation, as responses post CPA/CAP are not significantly different from baseline (figure 4-15). Additionally, when compared to cells treated with CAP alone, responses of CPA/CAP cells are significantly reduced from 680.4 ± 47.52 to 242.0 ± 56.1 action potentials/ 10s (figure 4-15, p= 0.000). This suggests that CPA is able to completely prevent the development of capsaicin induced hypersensitivity.

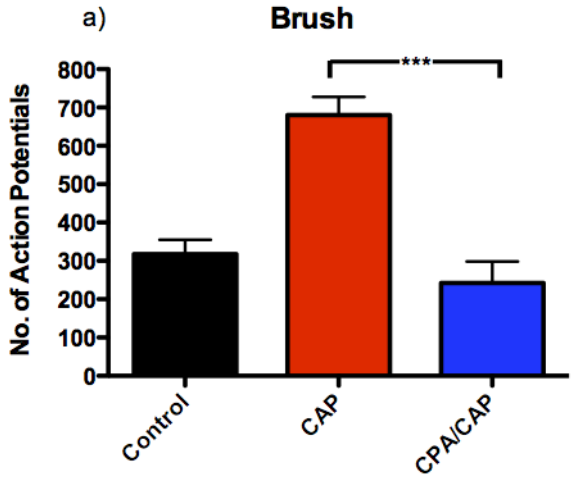

Figure 4-15 Effects of pre-treatment with CPA on capsaicin induced sensitisation of mechanically evoked baseline neuronal responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to brush applied to the receptive field for 10s, before and after pre-treatment with CPA and capsaicin application. a) There is no significant difference between the baseline control and CPA/CPA treated cells, however the latter group are significantly different from those treated with capsaicin alone (p= 0.000). n=6

4.3.6.2. Pre-treatment with intraplantar CPA inhibits capsaicin induced sensitisation of thermal neuronal responses in naïve animals

CPA produces a very similar pattern of effects to ADO when administered before capsaicin, however the inhibition is much more pronounced with the use of a specific and stable agonist. There is only a small degree of sensitisation still apparent with CPA pre-treatment, which is not significantly different from the baseline controls (figure 4-16). Additionally, when compared to the group of cells treated with capsaicin alone, there is a clear significant difference, suggesting that activation of the A_1R by CPA is able to prevent the development of thermal hypersensitivity (figure 4-16; p= 0.013).

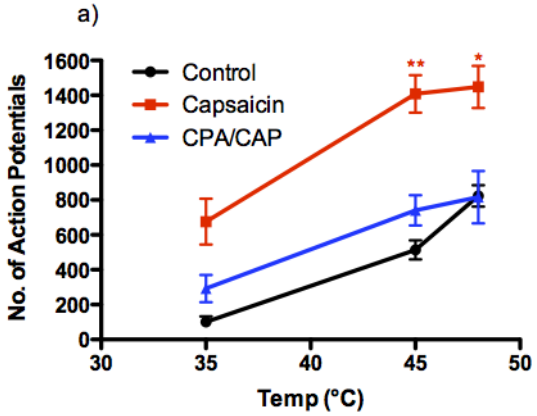

Figure 4-16 Effects of pre-treatment with CPA on capsaicin induced sensitisation of thermally evoked baseline neuronal responses. Using the protocol described in chapter 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to thermal stimuli applied to the receptive field for 10s, before and after pre-treatment with CPA and capsaicin application. Capsaicin treatment leads to a sensitisation of responses to 35□C, 45□C and 48□C. a) Innocuous and noxious thermal responses no longer increased when prophylactic intraplantar injection of CPA is given 10 minutes before application of topical capsaicin cream. Furthermore, there is a significant difference between the group of cells pre-treated with CPA and those with capsaicin alone. (Overall ANOVA p= 0.013; p= 0.008 and p= 0.014, respectively.) n=6

4.3.6.3. Pre-treatment with intraplantar CPA inhibits capsaicin induced enhancement of Aδ fibre mediated responses and reduction of C fibre mediated transmission in naïve animals

In the previous chapter it was found that 1% capsaicin cream results in a potentiation of Aδ fibre mediated responses through likely central changes, alongside a reduction in responses mediated in the C fibre range which could be due to peripheral desensitisation. As was observed with ADO pre-treatment, neither of these differences were seen when cells were pre-treated with CPA (figure 4-17). This suggests that capsaicin induced facilitation of Aδ fibres and reduction of C fibre transmission were inhibited by CPA. Furthermore, although capsaicin alone results in a non-significant increase in input, when pre-treated with CPA there are no signs of an enhanced input (figure 4-17).

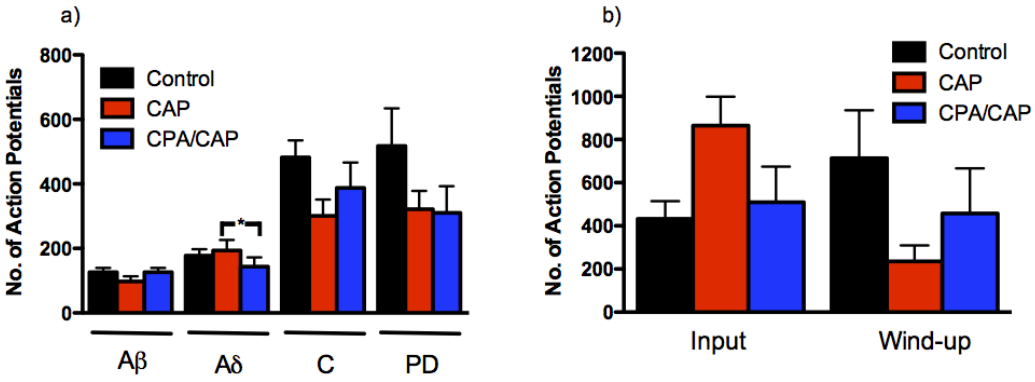

Figure 4-17 Effects of pre-treatment with CPA on electrical neuronal responses post capsaicin. . Using the protocol described in chapter 2.2 and 4.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli. a) There was no significant difference between baseline control and CPA/CAP treated neurones with regards to electrically evoked Aβ, Aδ, and C fibre mediated transmission, nor post-discharge, whilst a comparison between the groups treated with CAP and CPA/CAP show a significant difference in Aδ fibre mediated transmission. b) Furthermore, there was no difference with regards to input and electrically induced wind up between baseline control and CPA/CAP treatment, or between the CAP treated group and CPA/CAP treatment. n= 6

4.4. Discussion

The data in this chapter supports the theory that the A_1R on peripheral fibres is a viable drug target. In this study WDR cell responses were measured post intraplantar administration of ADO; after a combination of ADO pre-treatment followed by capsaicin; post intraplantar CPA; and after a combination of CPA pre-treatment followed by capsaicin. Additionally, QST was undertaken on healthy human volunteers post ADO and after a combination of ADO pre-treatment followed by capsaicin. The key findings were that ADO reduces thermally evoked responses in animals, and increases HPTs in human volunteers. ADO therefore appears to produce similar changes across species with regards to acute nociception. Furthermore, despite being unable to fully inhibit capsaicin induced thermal hypersensitivity, brush sensitivity was reduced, suggesting a partial inhibitory effect of ADO pre-treatment. Finally, CPA was able to reduce both thermally evoked responses in naïve rats, and also attenuate capsaicin induced sensitisation of brush and thermal responses. These results are consistent with the hypothesis that activation of peripheral A_1Rs would be able to modulate capsaicin induced thermal hypersensitivity. As such this receptor may be a useful drug target for future peripheral therapies.

4.4.1. Intraplantar administration of ADO reduces thermally evoked responses of WDR cells and increases HPTs of healthy human volunteers

This is the first study to examine the effects of peripherally administered ADO where the outcome was measured using objective, quantitative, in vivo electrophysiology or QST. ADO led to clear reduction in WDR cell responses to thermal stimuli, which appears to be strongest at around 45°C. Conversely, the effect on mechanically and electrically evoked responses of WDR cells was found to be minor. In line with these observations, the effects of intradermal ADO on healthy human volunteers confirmed an increase in HPT, with modest differences in responses to mechanical stimuli pre/ post ADO. There are relatively few studies that have been conducted in either animals or humans to investigate the local peripheral actions of ADO. However, of those that do exist, few have previously reported this antinociceptive property. In fact, the results of this study are in stark contrast to previous behavioural studies which have suggested that administration of ADO could result in an area of primary heat and mechanical hypersensitivity (Taiwo and Levine 1990; Pappagallo et al. 1993).

When ADO is injected into the skin it may act at peripheral receptors expressed on neurones, or resident immune cells. The subsequent physiological response will thus depend not only on which of these cells ADO acts on, but also the subtype of receptor activated. Activation of the A_1 or A_3 receptors will lead to a reduction of intracellular cAMP, whilst either A_{2A} or A_{2B} activation mediates the opposite biological effect (Fredholm et al. 2011). Upon binding to receptors on peripheral small- medium nociceptive fibres, interactions with K^+ and Q-, P-, or N-type Ca^{2+} channels may lead to an inhibition or excitation. In the case of A_1 or A_3 receptor activation, the lowered levels or cAMP reduce activity of Ca^{2+} channels and decrease neurotransmitter release, and/ or cause an increased conductance of K^+ channels leading to hyperpolarisation. Therefore, binding to the A_1 or A_3 receptors on peripheral afferents could explain the reduction in WDR cell responses to thermal stimuli and elevated human HPTs. Given the reported high expression of the A_1R on TRPV1+ fibres, it is unsurprising that the overriding effects are on thermal responses. On the other hand, activation of the A_2 receptors on peripheral afferents could explain the reported hypersensitivity in previous studies. When using ADO it is important to select an appropriate dose, which appears to produce selective A_1R activation. Responses also depend on the subtype/ density of receptors, which could be affected by the precise location of the injection and therefore could be a further factor that explains the differences between the studies.

A_1R receptor activation has also been suggested to have an indirect modulatory effect on TRPV1 itself, through downstream signalling. In vitro experiments have revealed that ADO can inhibit TRPV1 mediated Ca^{2+} entry to HEK293 cells (Puntambekar et al. 2004). Whilst in vivo it has been hypothesised that activation of the A_1R results in a breakdown of PIP2, which may be required for optimum function of TRPV1, and as such there may be a subsequent reduction in function of the receptor (Sowa et al. 2010). This reported breakdown of PIP2 could explain the lower levels of firing to thermal stimuli and increased HPTs observed here. Indeed, Sowa and colleagues recently demonstrated that PIP2 is required for normal sensing of noxious heat, as administration enhanced thermosensation for up to two hours (Sowa et al. 2010). Given that the experiments here found the actions exerted by ADO were mainly linked to thermal, rather than mechanical, thresholds and responses, it seems plausible that they could be explained by this interaction between TRPV1 and the A_1R through potential modulation of PIP2 levels.

Turning to the immune system, the interaction between ADO and immune cells is highly complex and there is still much to be understood. The actions of ADO once again depend on activation of different receptor subtypes, in addition to the varied functionality of intracellular signalling pathways within different immune cells. It has been proposed that activation of the $A_{2B}R$ mediates mast cell deregulation in humans, and may lead to a release of pro-inflammatory cytokines such as IL-4 (Ryzhov et al. 2006; Haskó et al. 2008). On the other hand, the $A_{2A}R$ is known to be expressed on macrophages, and activation may result in an increased production of the anti-inflammatory cytokine IL-10 and a reduction in levels of TNF-α. In addition, the $A_{2A}R$ is expressed on neutrophils and regulates the production of cytokines such as TNF-α, CCL3, CCL4, CXCL2 and CCL20 (McColl et al. 2006). Thus, there appear to be varied polar roles for ADO within the immune system. However, it has been suggested that overall it appears that the actions of ADO are on the whole regulatory, offering a protective role involving a reduction in inflammation (Haskó and Cronstein 2004; Haskó et al. 2008). The present data show ADO can be antinociceptive under conditions even where inflammation is minimal.

Although there are no previous studies which have found this antinociceptive effect of peripherally administered ADO, when Ekblom and colleagues explored the effect of systemic infusion of ADO on healthy human volunteers they did report an increase in HPT post treatment (Ekblom et al. 1995). Since it is not known exactly where systemic infusions will act, it is impossible to say whether this could be due to a peripheral mechanism, as demonstrated here. However, a peripheral action is certainly one possibility and this study confirms that regardless of the method of administration ADO is able to reduce HPTs. These observations are most likely explained due to the increased expression of the A_1R on TRPV1+ fibres, or through an indirect interaction with TRPV1 itself, involving a combination of the mechanisms discussed above.

4.4.2. Intraplantar administration of ADO has a negligible effect on mechanical and electrical evoked responses

Post ADO WDR cell responses to dynamic brush, sub and supra nociceptive threshold vF appear slightly reduced. Furthermore, human MPT is raised, although this was not found to be significant. Whilst these changes appear in both animals and humans, it is important to note that they are modest in comparison to the changes previously discussed with regard to heat

responses. Indeed, similar observations using CPA have been made, where it is clear that there is a stronger effect on thermal responses rather than mechanical (Gong et al. 2010; Hurt and Zylka 2012). However, regardless of how minor the reduction in activity seen across modalities is this nevertheless suggests that ADO may have an inhibitory effect across modalities on peripheral neurons.

Assuming that the molecular transducer of heat, TRPV1, is expressed on a high proportion of peripheral nociceptive neurones, if the reduction in activity of WDR cell responses to thermal stimuli was due to a generalised dampening of neuronal activity, this effect would most likely be seen to a similar extent across modalities. Since the effect is clearly strongest for thermal stimuli, the main effect of ADO is most likely explained through a more specific interaction with specific channels such as TRPV1. Furthermore, given ADO has no effect on responses to electrically evoked stimuli, it is unlikely that there is an overall decrease in afferent activity.

On the other hand, the overriding inhibition of thermal responses could be the result of a preferential expression or activation of the A_1R on fibres that respond to thermal, rather than mechanical, stimuli. Given that most peripheral afferents are traditionally thought of as polymodal this theory may seem unlikely. However, as discussed in chapter 3, recently a number of cases have begun to be put forwards for modality specific subgroups of afferent fibres. Ablation of TRPV1 expressing neurones results in a reduction in C fibre, but not A-fibre mediated activity, in addition to deficits in heat and mechanical pressure, but not pinprick or light touch perception (Cavanaugh et al. 2009; Brenneis et al. 2013). Since it has been shown that 95.6% of TRPV1 positive fibres also express the A_1R this could suggest that the A_1R is largely expressed on these thermo-pressure-specific neurones and therefore can cause a generalised inhibition of these fibres, through interaction with specific ion channels (Lima et al. 2010). This could explain the heightened drop in thermal responses over mechanical. Further investigation of modality specific fibre populations could therefore be useful to explore and validate this finding.

Additionally, since the A_1R is also expressed on non-TRPV1 positive fibres (which may be responsive to noxious mechanical stimuli), it may simply have less of an effect on these fibres due to a possible reduced density of receptors. It has been shown that 95.6% of TRPV1 expressing fibres also express the A_1R (Lima et al. 2010), however it may be the case that expression of the A_1R is lower on the non-TRPV1 expressing fibres, which would certainly explain the preferential effect on thermal, rather than mechanical stimuli. Furthermore, since

the molecular transducer of mechanical stimuli is unknown, it cannot be predicted as to whether any downstream consequences of A_1R activation could interfere with this receptor.

4.4.3. Pre-treatment with ADO reduces capsaicin induced brush hypersensitivity

Transient application of topical capsaicin cream is able to sensitise WDR cell evoked responses to dynamic brush. As discussed in chapter 3, it is not fully understood whether this is due to an underlying peripheral or central sensitisation, although the latter is more likely. It is clear here that pre-treatment with ADO was able to significantly reduce this capsaicin induced brush sensitisation.

Firstly, as discussed above, intraplantar ADO in naïve animals appears to have a subtle effect on peripheral neuronal activity. The subset of neurones that are modulated by ADO could include peripheral neurones that respond to dynamic brush and are themselves directly sensitised by capsaicin, such as low threshold C fibres, which may express TRPV1. Through binding to inhibitory receptors on these neurones, such as the A_1 or A_3 receptors, ADO could lead to a reduction in overall activity of the neurones and thus directly reduced their ability to be sensitised. Since ADO can reduce WDR cell brush responses in naïve animals, it is possible that pre-treatment reduces baseline responses, and capsaicin is then still able cause some sensitisation but simply to a lesser extent.

However, the inhibitory effects of ADO on peripheral neurones could also directly counteract mechanisms of capsaicin-induced sensitisation. When capsaicin binds to TRPV1, Ca^{2+} enters the neurone and a number of intracellular kinases are activated. These in turn may phosphorylate receptors, targeting them to the membrane and reducing their activation threshold. The candidate receptor for the transduction of dynamic brush responses is unknown. However, it is possible that it could be sensitised in this way. Furthermore, in the same way it has been hypothesised that the A_1R is able to modulate TRPV1, perhaps it is able to interact with and modulate this unidentified receptor. If this transducer requires PIP2 for signalling, the breakdown of this molecule by activation of the A_1R could reduce activity of the receptor. This reduction in activity could also reduce its ability to become sensitised by capsaicin.

As previously discussed, ADO is also able to interact with the immune system to reduce inflammatory responses. Capsaicin cream application causes a clear reddening of the skin

when applied to human volunteers. This indicates a level of extravasation, which may allow immune cells to filtrate out into the tissues. It is therefore possible that ADO could mediate any proceeding inflammatory responses that may play a part in sensitising PAFs. Given that the regulatory actions of ADO are believed to be through the A_{2A} and A_{2B} receptors, this mechanism is quite distinct from the others discussed.

Finally, since the A_1R may interact with TRPV1 function, pre-treatment may reduce ongoing activity caused by capsaicin application (Kenins 1982; LaMotte et al. 1992). Ongoing activity into the DH post capsaicin is thought to drive central sensitisation, which may in turn result in brush hypersensitivity. It has been shown that in vitro ADO is able to mediate Ca^{2+} entry into HEK293 cells expressing receptors for ADO and TRPV1, suggesting ADO does indeed reduce activity of the channel (Puntambekar et al. 2004). Furthermore, inward capsaicin currents in DRG neurones were inhibited, suggesting ADO is able to reduce the activity of neurones post capsaicin application (Puntambekar et al. 2004). Therefore, it is plausible that this reduction on TRPV1 activity could also prevent the development of central sensitisation, and subsequent development of symptoms such as brush hypersensitivity.

Since there is a lack of evidence regarding the peripheral actions of ADO on either chronic pain models, or in patients, inferences can only be made from alternative methods of administration. Several studies have also found that IT/IV ADO is able to reduce both secondary hyperalgesia and brush evoked allodynia, associated with human surrogate models such as topical mustard oil, and also in patients with neuropathic pain – confirming this anti-allodynic property of ADO, as observed here (Rae et al. 1999; Eisenach et al. 2002; Lynch et al. 2003). As ADO is not being administered peripherally in these studies it is not possible to gauge whether ADO is acting against central or peripheral mechanisms. Whilst this effect could be driven through interactions with ion channels on peripheral fibres as discussed above, it is important to note they could also be due to actions at ADO receptors in the spinal cord, which are well known to be antinociceptive. Further studies of peripherally administered ADO/ A_1R agonists are required to investigate the mechanism underlying the effects observed here.

4.4.4. Pre-treatment with ADO inhibits capsaicin induced electrical changes

In naïve animals, capsaicin cream causes an enhancement of Aδ fibre responses, at the same time as a parallel decrease in C fibre mediated transmission. As discussed in the previous chapter, a potentiation of Aδ fibre responses is often seen in models associated with central changes, and may indeed be attributed to a centrally mediated mechanisms induced by ongoing activity as a result of sustained TRPV1 activation. Since this is not observed with ADO pre-treatment, this supports the theory that ADO is able to partially reduce TRPV1 mediated ongoing activity, enough to prevent the development of certain central changes to the pain system. Furthermore, the inhibition of capsaicin induced C fibre desensitisation also suggests that excessive TRPV1 activity is prevented by pre-treating with ADO. Since desensitisation of C fibres is usually associated with excessive Ca^{2+} entry, resulting in temporary desensitisation of fibres and/ or excitotoxicity, these results suggest that ADO inhibits these high levels of TRPV1 activity. Indeed, both the induction of central sensitisation, through ongoing afferent activity, and peripheral desensitisation/excitotoxicity are related to high levels of TRPV1 activity. Thus it may be inferred that ADO is able to partially inhibit the actions of capsaicin, at least to a level where central changes such as an enhancement of Aδ fibre mediated transmission are no longer observed, in addition to reducing excessive Ca^{2+} entry related desensitisation.

4.4.5. ADO partially attenuates capsaicin induced thermal hypersensitivity

Despite the ability to inhibit capsaicin induced brush sensitisation, ADO was not able to fully prevent the development of thermal hypersensitivity. As previously discussed, it is likely that the reduction in thermal evoked WDR cell activity observed when ADO is administrated is due to the interactions between the A_1R and TRPV1, which would suggest that ADO would be able to interfere with the actions of capsaicin. However, since capsaicin is a very strong stimulus, and the actions of ADO are relatively short in duration it is feasible ADO would not be able to fully inhibit these actions. Additionally, responses to heat are large and induced sensitisation enhances these further, therefore a weak effect of ADO is not enough to reduce this. Indeed, given the rather short half-life of ADO, and non-specific actions, it is not entirely surprising that this was the case.

Since thermal hypersensitivity was still induced, despite pre-treatment with ADO, this suggests that a level of TRPV1 activity remained: enough to result in peripheral sensitisation.

That is to say, whilst high levels of TRPV1 activity may be required for the development of central sensitisation and peripheral desensitisation, lower levels may induce peripheral sensitisation. Although the activation of the A_1R and possible downstream interactions between the A_1R and TRPV1 may be enough to prevent any central changes, a low level of activity still remains and leads to the development of peripheral hypersensitivity.

With regards to the results discussed above in relation to the inhibition of brush sensitisation, this may rule out a few of the explanations offered. If engagement of TRPV1 is at a level whereby thermal hypersensitivity is induced, it is likely that other peripheral receptors would also be sensitised during this process. This suggests that if brush induced hypersensitivity was a result of sensitisation of peripheral receptors it would not have been reduced by pre-treatment with ADO. Furthermore, it has been shown that it is possible for activation of receptors to cause peripheral sensitisation, but not necessarily lead to central changes, such as with the use of UVB (Bishop et al. 2010). If a stimulus is not strong enough to result in an ongoing activity into the DH, there is nothing to drive the central sensitisation. Therefore, given that thermal hypersensitivity is still present, it seems most likely that the dampening of peripheral activity by ADO is not enough to fully inhibit peripheral sensitisation, but perhaps does lower the level of activity to below the threshold required to cause central changes. This would suggest not only that ADO is able to prevent capsaicin induced brush hypersensitivity but that this symptom is driven by a central mechanism.

4.4.6. ADO as a possible pain therapy

Several studies have already assessed the possibility of ADO as a chronic pain therapy and indeed IT ADO has been shown to alleviate symptoms of neuropathic pain (Belfrage et al. 1999). However, this is a rather inconvenient mode of administration and due to the undesirable cardiac side effects a systemic therapy is not likely to be possible either. Indeed, a peripheral therapy seems the most plausible option.

Furthermore, methotrexate (which is used in the treatment of RA patients) is believed to exert some of its actions through an ADO mediated modulation of the immune system. Methotrexate inhibits the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide transformylase. By reducing the activity of this enzyme, there is an accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide, which is a competitive inhibitor of AMP

deaminase. The in turn leads to an enhanced release of ADO, which is believed to decrease pain associated with RA (Haskó et al. 2008). This suggests that there may be some possible positive effects of ADO with regards to reducing chronic pain.

However, due to the rather weak and non-specific actions of ADO, and the inability to fully reduce capsaicin induced sensitisation observed here, it may be concluded that peripheral ADO itself would not make a successful chronic pain therapy. Furthermore, since the half-life is so short the duration of action would be very limited, unless given with a complimentary therapy to suppress the breakdown.

Nonetheless, the pain relieving effects of ADO have been explored with regards to alternative therapies and it has been shown that an exercise induced increase ADO can decreases CRPS related pain. Furthermore, this effect was enhanced by suppressing breakdown of ADO and by blocking the A_1R (Martins et al. 2013). Since the short half-life of ADO renders the development of such a treatment unlikely, exploring these possibilities could be one way to exploit the antinociceptive effects of ADO. Additionally, this intrinsic ability to reduce pain should be taken into account by chronic pain patients who consume high levels of caffeine. Since caffeine is a competitive antagonist of ADO receptors it is possible that it could be counteracting the body's own attempts to dampen the pain.

4.4.7. Intraplantar administration of CPA reduces thermally evoked responses of WDR cells

As previously mentioned, intraplantar/ intradermal injection of ADO clearly reduced WDR cell firing to thermal stimuli and increased HPTs, in rats and humans, respectively. This action is most likely explained through the actions of ADO at the inhibitory A_1 or A_3 receptors. In order to further elaborate on these mechanisms underpinning the aforementioned decrease in thermally evoked responses, this study also investigated the specific role of the A_1R. CPA, which binds preferentially to the A_1R, was administered via intraplantar injection to the receptive field of WDR cells. Once again there was a clear reduction in firing of WDR cells to thermal stimuli ranging from 35°C to 48°C. This strongly suggests that the weaker reduction in firing of WDR cells, and increased HPTs, seen post administration of ADO is due to actions at the A_1 receptor.

Several other groups have also noted this specific effect of CPA in reducing responses to thermal stimuli. Gong et al found that intraperitoneal CPA increased paw withdrawal latencies, whilst mechanical thresholds were unchanged (Gong et al. 2010). Additionally, local peripheral injection of CPA has been shown to reduce noxious thermal, but not mechanical, sensitivity (Sowa et al. 2010; Hurt and Zylka 2012). This effect was localised, and furthermore lost in A_1R KO mice. These studies suggest the effect is indeed due to local actions at the A_1R (Sowa et al. 2010). The authors suggest that this is due to a specific interaction of the A_1R with TRPV1. Given that the actions of CPA, like ADO, appear stronger against thermal stimuli this seems like a reasonable explanation. Although, it cannot be ruled out that it may also be due to a hyperpolarisation of neurones, which express both the A_1R and TRPV1. This study confirms that a peripherally administered agonist of the A_1R can dampen thermal responses, though the exact downstream mechanisms are still yet to be proven.

CPA appears to have a stronger effect than ADO, which is most likely due to the stability, and preference to bind to the A_1 receptor. ADO can bind to all ADO receptors, and it is therefore unlikely that at any one time all the A_1 receptors will be saturated. This sub-optimal binding, leaving some A_1Rs left open, along with those that are activated being counteracted by A_2Rs, may explain the modest results observed with ADO. Whereas CPA acts specifically on the A_1R and therefore there is likely to be a higher saturation of these inhibitory receptors with no opposing actions from A_2R activation.

4.4.8. CPA reduces mechanically evoked responses

Overall CPA was able to reduce mechanically evoked responses of WDR cells, whilst having only a small effect on electrically evoked responses. In fact, rather paradoxically given the reduction in mechanical responses, Aδ mediated transmission appeared enhanced post CPA. Given how minor this increase was, it is possible that it may not be biologically significant, or indeed an anomalous result. On the other hand, electrically induced input and C fibre mediated activity were reduced by CPA administration, although these were not found to be statistically significant. Previous studies have also observed the ability of CPA to decrease C fibre, but not A fibre mediated activity, as was observed here (Gong et al. 2010). These reductions in mechanically evoked responses, and electrically induced input could be

explained a general inhibitory effect that activation of the A_1R may exert on peripheral neurones, or a specific effect on mechano-transducers.

As discussed this may involve interactions with voltage gated ion channels, such as Q-, P- and N-type Ca^{2+} and K^+ channels. That is to say, activation of the A_1R could lead to a hyperpolarisation of afferent fibres. Given that both ADO and CPA have relatively minor effects on electrically evoked responses, it seems unlikely that there is on the whole due to indirect interactions with transducers themselves. Since this effect on mechanically evoked responses has not been found before it is difficult to fully ascertain the underpinning mechanisms. As previously mentioned, Gong and colleagues found that CPA in naïve animals reduced thermal, but not MPTs (Gong et al. 2010). Therefore, there are three possibilities that we may consider to explain the results observed here.

Firstly, it is possible that these actions are at A_1Rs expressed on afferent fibres other than those responding to thermal stimuli. Indeed, only 79.55% of A_1R expressing fibres co-express TRPV1, therefore the remaining 20.45% are not TRPV1 positive. This small percentage expressed on non-TRPV1 expressing fibres could be mechano-sensitive afferents, such as Aδ fibres, and therefore and an overall reduction in excitability of these fibres, or an interaction with mechano-transducers on these fibres, would explain the results observed here. Given that fewer A_1Rs are expressed on these fibres, it is unsurprising the effect is less than on thermal responses and perhaps may go unnoticed in some studies. A second possibility is that they are expressed on a population of non-TRPV1 expressing C fibres, which are mechano-sensitive and also express the A_1R (i.e. non-peptidergic C fibres). Once again, an overall reduction in excitability of these fibres, or an interaction with mechano-transducers on these fibres would explain the results here. Thirdly, there may be some TRPV1 and A_1R positive C fibres, which also respond to mechanical stimuli (C-MH fibres) and a reduction in activity of mechano-transducers on these fibres is at the route of these observations. However, this final explanation raises some doubt over the modality specific theory discussed. Indeed, this is yet to be fully accepted, and we cannot rule this out as an explanation. It would be interesting to investigate the actions of CPA after ablation of TRPV1 positive neurones to help understand these results observed here.

4.4.9. CPA is able to reduce capsaicin induced sensitisation of brush and thermal stimuli

Pre-treatment with intraplantar CPA was clearly able to inhibit usual levels of brush and thermal hypersensitivity associated with 1% capsaicin application. Since thermal hypersensitivity is usually attributed to TRPV1 this decrease is most likely explained by the actions on TRPV1+ fibres or the proposed interaction of the A_1R and TRPV1 as discussed above. Lima and colleagues have also noted that peripheral A_1R activation decreases inflammatory peripheral hypersensitivity (Lima et al. 2010). Whist Stein and colleagues have demonstrated that polylysine (an agent which sequesters PIP2) has an inhibitory effect on TRPV1, and Liu et al found that replenishing PIP2 can aid recovery after desensitisation (Liu et al. 2005; Stein et al. 2006). More recently it has also been demonstrated that PIP2 is required for both normal sensing of noxious heat and for the development of sensitisation (Sowa et al. 2010). In addition to enhancing thermosensation for up to two hours, PIP2 also increased thermal hypersensitivity and mechanical allodynia. Therefore it can be inferred that PIP2 is required for function of TRPV1 and a breakdown through activation of the A_1R is the most likely explanation for the results observed in this study.

As previously discussed with regards to ADO, continuous activation of TRPV1 results in ongoing activity into the spinal cord, which is likely to cause central changes that lead to enhanced brush sensitivity or allodynia. A reduction of TRPV1 activity would therefore also explain the ability of CPA to inhibit capsaicin induced brush hypersensitivity, attenuate Aδ facilitation and C fibre desensitisation.

Additional studies have also found that inosine is able to reduce pain-related behaviours, due to possible actions at the A_1R. Nascimento and colleagues demonstrated that inosine decreases responses in the late stage of the formalin test, in addition to CFA induced mechanical allodynia, and PSNL induced mechanical and cold allodynia (Nascimento et al. 2010). These effects were blocked by an A_1R selective antagonist (Nascimento et al. 2010). Thus suggesting that inosine has antinociceptive and antiallodynic properties, related to the involvement of ADO A_1R. Supporting the theory proposed here that the A_1R may be able to modulate such hypersensitivity. However, as inosine was given systemically in these studies, central actions cannot be ruled out.

This analgesic effect of A_1R agonists has also been observed in other models of chronic pain, including the formalin model and DPN (Balasubramanyan and Sharma 2008; Liu et al. 2013).

These models induce very different mechanisms, however A_1R agonists were effective across both. This may be explained by the fact that the A_1R is widely expressed throughout the pain pathway and its inhibitory actions do not require any specific chronic pain related changes, unlike many therapies that act against mechanisms which require the presence of chronic pain induced plasticity. Taken together, the results of this study and previous work, suggest that A_1R agonists may be a useful therapy effective across a number of symptoms/ pain conditions. Whilst previous studies have validated the effects of systemic A_1R activation against signs and symptoms of chronic pain, this study confirms a peripheral action.

The results presented here in this study also highlight the use of objective measures, such as in vivo electrophysiology, as useful pharmacodynamic endpoints to aid drug development. Not only do the recordings of WDR cells produce a clear picture of sensitisation across a range of innocuous and noxious stimuli in response to certain models, such as capsaicin, but they also allow the study of how drugs act across modalities and varying intensities. Behavioural studies, including those with ADO or CPA, have often used threshold measurements and produced conflicting results, whereas here we are able to examine how the drug may effect sub and suprathreshold stimuli. Importantly, since capsaicin is a highly suprathreshold stimulus, this study the extension of the role of ADO/ CPA modulation into these suprathreshold levels of pain related activity that is likely to be relevant to patients.

4.4.10. Potential chronic pain therapies: the A1R receptor and beyond

This study has highlighted the antinociceptive effect of activating the A_1R – an action which is likely to be relevant in animals and humans. Given the positive effects seen here attenuating capsaicin induced sensitisation, and the results of previous studies, it can be concluded that this is a promising drug target. In particular this could be explored as a peripherally administered therapy. Indeed, Giorgi et al have recently noted the potential of A_1R ligands with regard to both efficacy and preferable side effect profiles – in particular for those given by topical administration (Giorgi and Nieri 2013).

Notably, it was observed here that CPA appears to be able to reduce signs of ongoing activity and prevent centrally induced symptoms, such as brush hypersensitivity and increase in Aδ fibre mediated activity. This confirms the importance of peripheral activity, leading to central changes (Baron et al. 2013). Furthermore, it highlights the possibility of developing

peripheral therapies that may be given with the aim of preventing pain from becoming 'central'. Indeed such therapies may be able to prevent both the induction and maintenance of certain pathological changes in chronic pain states.

Another possible therapy, although it was not explored in this chapter, is targeting the A_3R. Since the A_3R actions are similar to that of the A_1R this receptor may also be a useful potential drug target. Chen and colleagues have investigated A_3R pharmacology and demonstrated that several agonists of this receptor were able to reduce CCI induced mechanical hypersensitivity (Chen et al. 2012). The actions were found to be as effective as gabapentin or amitriptyline, which are both widely used chronic pain therapies (Chen et al. 2012). The expression of A_3R on peripheral fibres has not yet been explored, but given the positive results observed regarding systemic therapy it could be useful to explore the peripheral effects of A_3R agonists.

Other than adenosine receptors, studies have also explored alternative indirect modulation of TRPV1 function and the effect on chronic pain models. Fischer and colleagues have recently demonstrated that disruption of A kinase anchoring protein 79 prevents sensitisation of TRPV1 and the subsequent thermal hyperalgesia in the pre-clinical carageenan and formalin models (Fischer et al. 2013). These indirect modulations are particularly useful given the potential role the receptor plays in chronic pain, but direct antagonists often interfere with thermoregulation. Thus, it could be interesting to investigate this further in the translational model of capsaicin to explore the potential in humans.

4.5. Concluding remarks

Overall this chapter has examined the effects of ADO and CPA on acute pain, in addition to exploring the possibility of preventing capsaicin induced hypersensitivity. Both ADO and CPA were able to reduce acute thermal responses, which may be explained through an indirect interaction with TRPV1, or an overall hyperpolarisation of PAFs. Modest effects were also observed regarding reductions in mechanical stimuli, the mechanism of which is not yet clear. Finally, CPA was able to prevent capsaicin induced sensitisation to both brush and thermal stimuli, highlighting the potential of A_1R agonists in chronic pain therapy.

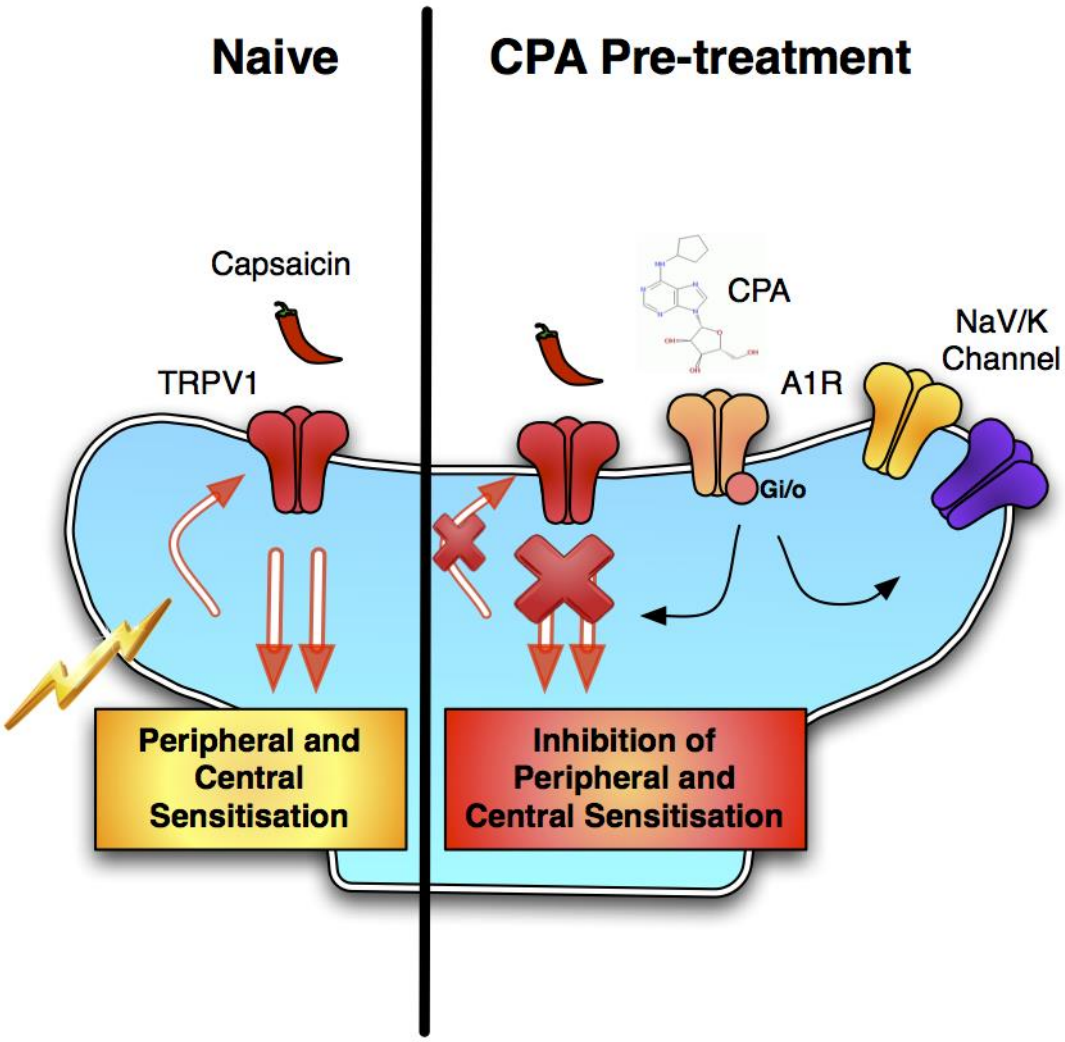

Figure 4-18 Activation of the A1R attenuates capsaicin induced peripheral and central sensitisation. Topical capsaicin leads to the development of both thermal and brush hypersensitivity, however peripheral administration of CPA was able to attenuate the development of both of these symptoms. Activity of TRPV1+ afferents is most likely reduced through interaction with voltage gated ion channels, or an indirect modulation of the TRPV1 receptor. Subsequently, capsaicin was unable to activate TRPV1 at the levels required to drive either peripheral or central changes. As such the A_1R holds potential as a future drug target.

5. UVB

5.1. Introduction

Ideally, surrogate pain models will mimic the signs and symptoms exhibited by chronic pain patients with the aim of modelling as closely as possible the mechanisms in healthy humans of the relevant underlying pathophysiologies in patients. We have gained much insight from the use of models initiated by administration of an exogenous agent such as topical capsaicin application (as described in the previous chapters), which in its acute phase induces a powerful peripheral and central sensitization of pain-signalling circuits. The power of this approach is that it, by definition, studies a particular mechanism. However, the weakness is that it is frequently unknown how important a particular mechanism is in a given pain state. The continuous development and improvement of surrogate models is crucial in furthering our knowledge, in particular with regards to pharmacological interventions that modulate specific key targets brought into play in the model. The closer the mechanisms induced in the models become to the patient reality, the higher the likelihood that they will allow the identification of drug interventions which succeed all the way through to the clinic. Obviously, this excludes models, for ethical reasons, where there is actual marked tissue or nerve damage. This chapter explores the use of electrophysiology in rats and QST in humans in order to further characterise the UVB model of inflammatory pain.

It has long been known that exposure to ultra violet (UV) light evokes sensory changes to the skin. In 1942 Lewis wrote that in this model he had observed a reddening and swelling of the treated area, resulting in *'nerve endings in a state of hyperexcitability'* which he then described as a *'hyperalgesic state'* (Lewis 1942). In more recent years, it has been further established that UV light in the UVB range (290-320nm) is absorbed by the epidermis, and results in an inflammation characterised by the classical features of erythma, hyperalgesia and allodynia - to both mechanical and thermal stimuli (Hoffmann and Schmelz 1999; Benrath et al. 2001; Harrison et al. 2004; Bishop et al. 2007).

5.1.1. UVB irradiation leads to a local inflammatory response

When UVB is absorbed by epidermal cells such as keratinocytes and fibroblasts, the resulting apoptosis and DNA damage leads to the release of a number of neuropeptides, free radicals and inflammatory mediators (Hruza and Pentland 1993; Saadé et al. 2000; Clydesdale et al. 2001; Matsumura and Ananthaswamy 2004; Angst et al. 2008; Dawes et al. 2011). The

sensitisation of nocieptors by these released molecules occurs through a number of intracellular signalling cascades, as discussed in chapter 1. In addition, their release may also recruit immune cells, such macrophages, neutrophils, lymphocytes and mast cells, which work a long side the resident innate cells, further releasing inflammatory mediators such as cytokines and chemokines that act to maintain and enhance the sensitisation of peripheral nociceptors, therefore resulting in a long lasting hypersensitivity of peripheral transduction (figure 5-1). The model has been used to probe the upregulation of such mediators, to compare across rodents and humans with the aim of the identification of potential candidate molecules in inflammatory pain states. In many cases, a skin biopsy was taken (or blood/ CSF) and cytokine levels were measured (table 5-1).

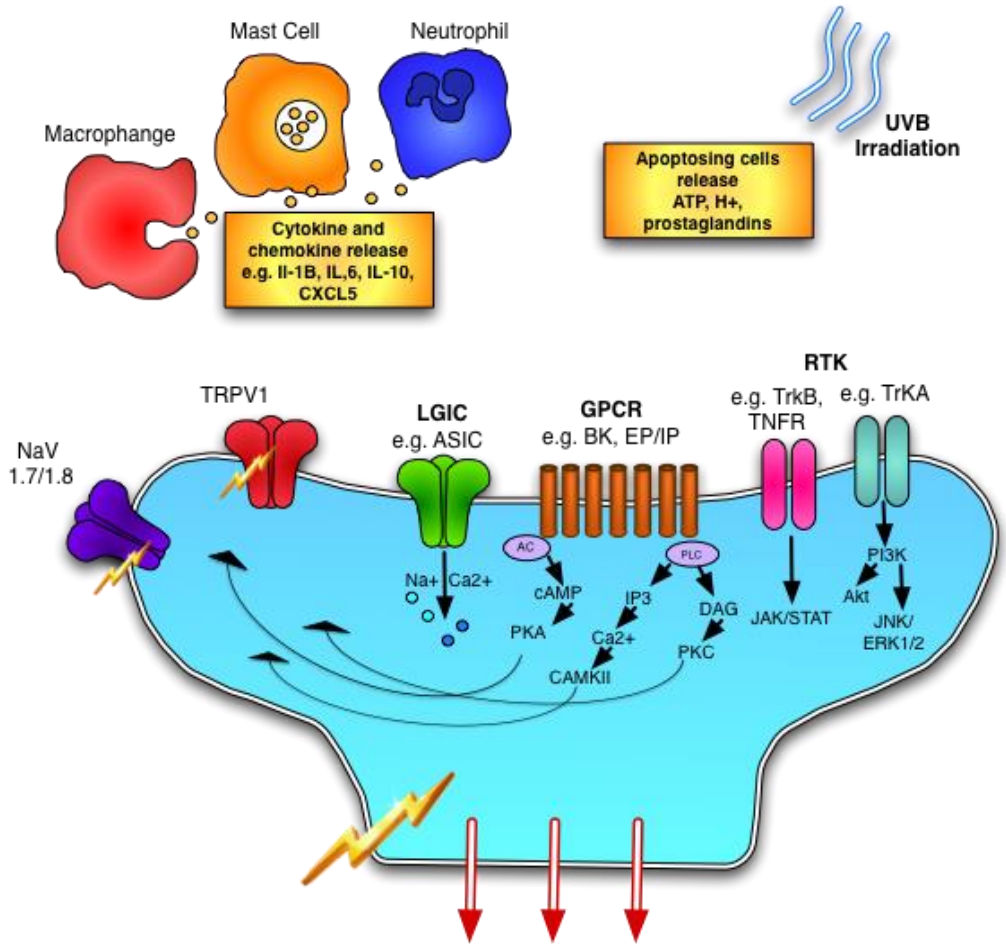

Figure 5-1 UVB induced recruitment of immune cells and peripheral sensitisation. Studies have highlighted the recruitment of neutrophils and macrophages as a result of UVB irradiation. These adaptive immune cells act in synchrony with resident cells to release numerous inflammatory mediators, which are able to sensitise peripheral receptors through intracellular mechanisms.

Mediators	Change in expression post UVB?	Associated with chronic pain?
CXCL5	✓	Unknown, associated with a number of other preclinical models
IL-24	✓	Unknown
CXCL2	✓	Unknown
IL-6	✓	Potential role in peripheral inflammation
CCL7	✓	Unknown
IL-10	✓	Reduced in peripheral neuropathy
IL-1B	✓	Increased in peripheral neuropathy
TNF-A	X	Increased in CRPS, peripheral neuropathy
NGF	X	Increased in interstitial cystitis/painful bladder syndrome, chronic prostatitis/chronic pelvic pain syndrome, OA and DPN
IL-8	X	Increased in PHN

Table 5-1 A selection of inflammatory mediators associated with UVB and/ or chronic pain. Dawes and colleagues found that changes in expression of 90 mediators could be observed post UVB irradiation. IL-1β was upregulated post UVB, whilst IL-10 was found to be downregulated. Both of these mediators have already been recognised as having a potential role in chronic pain and thus highlight the clinical relevance of this model. However, there are also a number of novel mediators identified by this study. TNF-α, NGF and IL-8 are examples of previously indentified mediators believed to play a role in chronic pain, however these were unchanged post UVB. (Sommer et al. 1998; Kotani et al. 2004; Marchand et al. 2005; Üçeyler et al. 2007; Uceyler and Sommer 2007; Backonja et al. 2008; Kumar and Mahal 2012; Dawes 2013).

With regards to the data in table 5-1 it will be interesting to investigate the role of these cytokines in chronic pain, to understand the full relevance of this model to patients. It is important to note, that even if the mediators are not involved specifically in any pain conditions the model may still hold clinical relevance. The downstream mechanisms of inflammatory mediators may indeed have some overlap with those that are involved in chronic pain states.

5.1.2. Peripheral sensitisation is the predominant mechanism of UVB irradiation

Through a number of pivotal studies, using extensive psychophysical and behavioural characterisation, Bishop and colleagues concluded that the inflammatory process occurring in the UVB treated area led to a predominant peripheral sensitisation, that accounts for all the sensory changes recorded (Bishop et al. 2007; Bishop et al. 2009; Bishop et al. 2010). Assessing not only the phenotypic changes induced by UVB - namely the reduced thresholds restricted to the primary area of insult - but also the respective pharmacological sensitivity of the model, in both rats and humans, the group made a strong case. The UVB induced physiological changes were found to be dose dependent and peak around 24-48 hours (Hoffmann and Schmelz 1999; Bishop et al. 2007). Most notably, in the human studies, Bishop and colleagues extensively compared UVB to the capsaicin and thermal burn models; showing that although the latter two evoke pinprick hyperalgesia and allodynia in the secondary area, neither of these manifestations of central changes could be found post UVB, supporting the notion this is on the whole a peripheral model.

The majority of animal data do not support the presence of notable central changes, with only one study finding a significant mechanical hypersensitivity in the secondary area (Davies et al. 2011). One key driver of central sensitisation, that appears to be absent in the UVB model, is spontaneous or ongoing pain; the degree of spontaneous activity is believed to correlate with the level of hypersensitivity (Chu et al. 2004; Baron et al. 2013). Overall the studies in tables 5-2 and 5-3 report no signs of spontaneous pain-like behaviour. Furthermore, recordings from peripheral afferents highlighted no change in spontaneous activity, which is present in other inflammatory models associated with hypersensitivity and central changes, such as OA, CFA and carageenan (Andrew and Greenspan 1999; Hamilton et al. 2001; Chu et al. 2004; Schuelert and McDougall 2009). In addition, there were no reports of UVB inducing an increase in basal c-fos levels (otherwise seen with CFA), which is another marker suggestive of ongoing noxious input linked to central changes (Ma and Woolf 1996). Since peripheral recordings also highlighted heat insensitive C fibres increasing their response to suprathreshold mechanical stimuli, and heat-sensitive C fibres increasing activity to thermal stimuli, all together this suggests a peripheral mechanism for the observed hypersensitivity (Bishop et al. 2010).

However, this proposal of a of an overriding peripheral sensitisation has been opposed by Gustorff, Sycha and colleagues (2013) who presented evidence of additional changes,

including large areas of secondary pin prick hyperalgesia. The details of all animal and human studies are recorded in tables 5-2 to 5-5. Interestingly, this model is one of the few which has been explored further in humans, and it is only in these human studies that clear evidence of secondary changes, and thus a central sensitisation, are present. Though it must be noted that the studies in which this is the case, have a much larger area of irradiation – which may lead to small amounts of spontaneous activity, which could drive central sensitisation. However, in the large majority of studies with relatively restricted UVB irradiation, the changes seem to be confined to the periphery. It may simply be that the way the UVB model is implemented and tested can lead to almost pure peripheral change or peripheral and central change. However, there is enough evidence to show that this model may be used to study the consequences of a strong peripheral sensitisation.

Study	UVB Dose	Treated Area	Time Frame	Evoked Observations	Site of Changes	Spontaneous Activity	Histological Changes
Saadé et al. 2000	Up to 300mJ/ cm^2	Back	3-6 and 48-96 hours	Dose dependent thermal hypersensitivity	Secondary area	—	Upregulation of IL-1β, TNF-α and NGF
Davies et al. 2005	(UV) 52.65mJ/cm^2	Hindpaw (circular area 8mm in diameter)	Maximal changes seen at 24-48 hours	Hypersensitivity to thermal and mechanical stimuli, including both heat and cold	Primary irradiated area only	—	—
Bishop et al. 2007	Up to 1000mJ/cm^2	Hindpaw	Maximal changes seen at 24-48 hours	Dose dependent thermal and mechanical hypersensitivity in hairy and glaborous skin	Primary irradiated area only	No signs of spontaneous pain behaviours	No increase in basal c-fos. Increased c-fos expression in response to 45°C
Bishop et al. 2010	1000mJ/cm^2	Hindpaw	48 hours	Hypersensitivity to thermal and mechanical stimuli	Restricted to the primary site of irradiation	No difference in the degree of spontaneous activity in primary afferents	—
Dawes et al. 2011	1000mJ/cm^2	Hindpaw	40 hours	Hypersensitivity to thermal and mechanical stimuli	Primary irradiated area only	—	Upregulation of numerous inflammatory mediators (including CXCL5, IL-24, CXCL2, CCL4 and IL-6)
Davies et al. 2011	1000mJ/cm^2	Hindpaw	24 hours-72 hours	Hypersensitivity to vF and brush	Secondary area	No signs of spontaneous pain behaviours	—

Table 5-2 Studies of UVB in animals. Sensory changes evoked in previous studies exploring UVB irradiation in animals.

Study	Treated Area	Time Frame	Thermal Hypersensitivity	Mechanical Hypersensitivity	Site	Spontaneous Activity
Hoffmann and Schmelz.1999	Thigh (circular area 1.5cm in diameter)	Maximal changes at 24-48 hours	✓	✓	Primary irradiated area only	—
Koppert. 1999	Ventral forearm (circular area 1.5cm in diameter)	24 hours	✓	✓ (Impact stimuli)	Primary irradiated area only	No spontaneous pain was reported
Benrath et al. 2001	Forearm (circular area 5cm in diameter)	Maximal changes at 24-48 hours	✓	✓ (Pressure pain)	Primary irradiated area only	—
Sycha et al. 2003	Proximal upper leg	20 hours	✓	—	Primary irradiated area only	—
Gustorff et al. 2004a	Lateral side of upper leg (circular area 5cm in diameter)	20-30 hours	✓	✓	Thermal hypersensitivity observed in the primary area, pinprick hyperalgesia observed in the secondary area.	—
Gustorff et al. 2004b	Lateral side of upper leg (circular area 5cm in diameter)	20-30 hours	✓	✓	Thermal hypersensitivity observed in the primary area, pinprick hyperalgesia observed in the secondary area.	—
Harrison et al. 2004	Buttock (6.25cm^2)	Maximal changes at 24 hours	✓	✓	Primary irradiated area only	No spontaneous pain was reported
Sycha et al. 2005	Ventral side of upper leg (circular area 5cm in diameter)	24 hours	✓	✓	Thermal hypersensitivity observed in the primary area, pinprick hyperalgesia observed in the secondary area	—
Chizh et al. 2007	Ventral side of upper leg (circular area 2cm in diameter)	24 hours	✓	—	Thermal hypersensitivity was tested only in the primary area	—
Bishop et al. 2009	Volar forearm (2cm^2)	Maximal changes at 24 hours	✓	✓	Restricted to the primary site of irradiation	No reports of spontaneous ongoing pain
Dawes et al. 2011	Volar forearm (1cm^2)	40 hours	✓	✓	Primary irradiated area only	—
Gustorff et al. 2011	Ventral side of upper leg (circular area 4.2cm in diameter)	24 hours	✓ (Heat and cold hypersensitivity)	✓	Thermal hypersensitivitiy and dynamic mechanical allodynia were observed in the primary area, pinprick hyperalgesia was observed in the secondary area.	—
Ortner etal. 2012	Ventral side of upper leg (diameter 4.2cm)	24 hours	✓ (Heat and cold hypersensitivity)	✓	Heat, cold and pinprick hypersensitivity in the treated area. Surrounding area of mechanical pinprick hyperalgesia and DMA.	No spontaneous pain was reported

Table 5-3 Studies of UVB in humans. Sensory changes evoked in previous studies exploring UVB irradiation humans

5.1.3. The UVB model is sensitive to peripheral NSAIDs and opioids

Pharmacological evidence from both animal and human studies indicate an overriding peripheral mechanism in this model. Since the majority of changes evoked by UVB can be reversed by peripheral administration of both NSAIDs, as well as a TRPV1 antagonist, this suggests there is a strong peripheral component. Whilst NSAIDs block the production of local sensitising mediators such as PGs, opioids may act on the afferent terminals themselves to inhibit activity. Inflammatory processes can lead to up-regulation of peripheral OR's, and the efficacy of opioids in the UVB model suggest that this may occur following irradiation (Stein et al. 2001) although the well-established central analgesic actions of these drugs cannot be excluded. Since morphine binds preferentially to the μOR – which has been shown to affect predominantly thermal, rather than mechanical responses (Cavanaugh et al. 2011) – it is no surprise that the thermal hypersensitivity induced by UVB irradiation is more responsive to morphine administration, and it does not indicate that the mechanical hypersensitivity is centrally mediated. TRPV1 has been shown in numerous studies to play a pivotal role in primary hypersensitivity (O'Neill et al. 2012), thus by blocking this receptor and finding an increase in HPTs and tolerance, it is clear that TRPV1 is involved in the inflammatory sensitisation induced by UVB.

5.1.4. Centrally targeted interventions fail to alleviate UVB induced hypersensitivity

Ongoing activity of primary afferents that converge in the DH can modulate NMDA receptor function, as seen in wind-up of spinal neurones and heterosynaptic central sensitisation (Dickenson and Sullivan 1987; Haley et al. 1990; Lewin et al. 1994). Thus many models of altered pain processing or chronic pain states are sensitive to a blockade of this receptor (Woolf and Thompson 1991; Stubhaug et al. 1997). Since Bishop and colleagues found the changes induced by UVB could not be reversed by the NMDA antagonist MK-801, it is therefore unlikely that any of these central mechanisms underpin the model. Furthermore, gabapentin is effective across numerous models of central sensitisation, whilst showing no effect in naïve animals and healthy individuals, and is thus state-dependent; requiring the upregulation of the α2δ subunit of voltage gated calcium channels and other conditions such as a shift in descending controls and intense peripheral drives (Iannetti et al. 2005; Field et al. 2006; Bee and Dickenson 2008). Once again the UVB model has also been shown to be

resistant to this treatment (Gustorff et al. 2004), suggesting the central changes required for this state dependency are not present post UVB irradiation.

Taken together, the evidence strongly suggests that hypersensitivity resulting from UVB irradiation is a predominantly peripheral phenomenon. Whilst some behavioural studies have concluded there are signs of central components, the majority of pharmacological and quantitative electrophysiological data show little confirmation of this. UVB is therefore a suitable model for assessing mechanisms involved in peripheral sensitisation and investigating peripherally acting analgesics.

Study	Pharmacological Intervention
Saadé et al. 2000	IL-10 and IL-13 (anti-inflammatory cytokines) reduce UVB induced thermal hypersensitivity, and attenuate pro-inflammatory cytokine levels (such as NGF).
Davies et al. 2005	—
Bishop et al. 2007	Systemic and topical ibuprofen reduced thermal and mechanical hypersensitivity. Systemic and peripherally acting opioids reduced thermal and mechanical hypersensitivity. NGF block also reduces thermal and mechanical hypersensitivity, with a greater effect on thermal.
Bishop et al. 2010	The NMDA blocker MK-801 had no effect on mechanical hypersensitivity. Neonatal capsaicin treatment attenuated the development of UVB induced thermal and mechanical hyperalgesia.
Dawes et al. 2011	Intraplantar CXCL5 causes mechanical hypersensitivity. NSAID piroxicam attenuated CXCL5 increase post UVB. CXCL5 neutralising Ab reduces UVB induced mechanical hypersensitivity.
Davies et al. 2011	—

Table 5-4 Pharmacological sensitivity of the UVB model in animals. Hypersensitivity resulting from UVB irradiation is sensitive to NSAIDs and anti-inflammatory cytokines, but not the NMDA antagonist MK-801.

Study	Pharmacological Intervention
Hoffmann and Schmelz.1999	—
Koppert et al. 1999	Peripheral and systemic morphine reduced thermal hypersensitivity, with no effect on mechanical hypersensitivity.
Benrath et al. 2001	—
Sycha et al.2003	Ibuprofen reduced thermal hypersensitivity.
Gustorff et al. 2004a	—
Gustorff et al. 2004b	A systemic opioid (Remifentanil) reduced thermal hypersensitivity and decreased the area of secondary pin prick hypersensitivity. Gabapentin had no effect on either of the sensory changes induced by UVB.
Harrison et al. 2004	—
Sycha et al. 2005	Oral cox-2 inhibitor (Rofecoxib) reduced thermal hypersensitivity and had a modest effect on decreasing the area of secondary pinprick hypersensitivity.
Chizh et al, 2007	A TRPV1 antagonist (SB-705498) increased heat pain thresholds, and tolerance.
Bishop et al. 2009	—
Dawes et al. 2011	—
Gustorff et al. 2011	Pretreatment with 5% lidocaine decreased cold hypersensitivity, the area of pinprick hyperalgesia and secondary mechanical hyperalgesia.
Ortner et al, 2012	Tramadol had a modest effect on primary pinprick hypersensitivity and DMA, it did not reduce the area of secondary hyperalgesia or heat/ cold hypersensitivity in the primary area.

Table 5-5 Pharmacological sensitivity of the UVB model in humans. Hypersensitivity resulting from UVB irradiation is sensitive to NSAIDs, lidocaine, systemic opioids and the TRPV1 antagonist SB-705498. On the other hand there was little effect of gabapentin or tramadol.

Overall, there are several features of this model which make it attractive. Firstly, it is reasonably straightforward to standardise – unlike responses to topical algogens, which are notoriously variable. Secondly, it can be used to cleanly study peripheral sensitisation, in the absence of secondary changes. Thirdly, the multiple lines of evidence discussed show that the model can be implemented in both humans and rodents and that mechanisms are very similar in both. Finally, it does in fact have face validity in that sunburn can be a clinically relevant source of pain. Here we aim to further characterise the UVB model in animals and humans,

using in vivo electrophysiology to assess evoked responses of WDR cells, a long side QST. The same time point was assessed in both animals and humans, and wherever possible similar stimuli were used.

5.1.5. CXCL5 contributes to UVB induced sensitisation

Many of the mechanisms underpinning peripheral sensitisation of afferent fibres are well established, and many of the mediators are mentioned in table 5-1 above, though those which drive UVB induced changes are not definitively established. However, a recent study identified a novel key mediator of UVB induced pain, the chemokine CXCL5 (Dawes et al. 2011). CXCL5 is also known as epithelial-derived neurtophil-activating peptide-78 or lipopolysaccharide induced CXC chemokine in humans and rodents, respectively.

Chemokines are a family of chemotactic cytokines. They are an important part of the immune response, mediating the trafficking and activation of numerous leukocytes at the site of inflammation, thus bridging the gap between the innate and adaptive responses. There are over 50 chemokines, many of which have overlapping functions, which they exert through G protein coupled receptors. ELR^+ chemokines (possessing a glutamic acid-leucine-arginine motif), such as CXCL5, are believed to act at CXCR1 and CXCR2 (Smith et al. 2008; Lüttichau 2010). With regards to CXCL5 in particular, the majority of actions are likely to be through CXCR2 (Lüttichau 2010). CXCL5 is a potent chemoattractant as CXCR2 is expressed on neutrophils, monocytes and endothelial cells (Charo and Ransohoff 2006). CXCR2 is coupled to Gi/o and thus activates multiple signalling pathways, including MAPK/ERK, PI3K/AKT and PLC, which may result in protein translation and gene expression; in endothelial cells this increases the expression of numerous inflammatory mediators (Chandrasekar et al. 2003). Antagonism of the receptor has already shown efficacy in a number of chronic pain models such as carageenan, CFA and collagen induced arthritis (Cunha et al. 2008; Manjavachi et al. 2010).

Dawes et al found that CXCL5 was highly upregulated at the peak of UVB inflammation in both rats and humans (Dawes et al. 2011). There were strong correlations between expression of many mediators in rodents and humans but this mediator topped the list in both species. Subsequently, using intraplantar injection they found it could mimic the mechanical hypersensitivity seen post UVB; however, there was no difference in the latency of

withdrawal to a radiant heat source, thus suggesting that CXCL5 does not mediate UVB induced thermal hypersensitivity. The study also highlighted a pivotal mechanism for CXCL5, through the recruitment of macrophages, which was previously unexplored. Furthermore, by blocking CXCL5 with the use of a neutralising antibody, a reduction was seen in both infiltration of macrophages, and the associated mechanical hypersensitivity. Therefore suggesting that through the recruitment of immune cells such as macrophages and the further release of inflammatory mediators, CXCL5 is able to lead to the observed mechanical hypersensitivity post UVB.

Despite the existence of many studies exploring UVB induced changes, the central neuronal consequences and correlates of a peripheral hypersensitivity have not been examined. Thus, the question of what happens at spinal levels in the face of pure peripheral enhanced drives remains open. Here, this chapter aims to investigate spinal neuronal activity in this model and further characterise and explore this role of CXCL5 in the mediation of hypersensitivity.

5.2. Methods

5.2.1. UVB irradiation - rats:

Adult male Sprague-Dawley rats, between 210-240g, were obtained from the UCL Biological Services Unit. All procedures were approved by the UK Home Office, and were performed in accordance with the guidelines provided by the International Association for the Study of Pain.

Rats were anaesthetised in an induction box using 4% isoflurane (carried in 66% N2O and 33% O2). Once the rat was fully unconscious and was checked for absence of reflexes (by pinching the toes of the hindpaw) they were placed on-to a heat mat and fully covered with UV resistant material. The plantar surface of the right hindpaw was then exposed, and placed at a set distance of 2cm away from the UVB light source, ensuring only this area was irradiated. All experiments were conducted using a Dermfix 1000MX UV-B Lamp fitted with a 9 Watt fluorescent UVB tube, λ max = 311nm. The irradiance of the lamp was determined using a calibrated photometer (Solartech Inc Solarmeter 6.2 UVB Meter, Merlin Lazer). This reading was used to determine the length of time required to deliver a set dose of 1000mJ/cm^2. The dose was chosen on the basis of previous studies, which have found this to have the greatest effect without resulting in any signs of skin damage such as blistering (Bishop et al. 2007). Post irradiation the rats were placed in a temperature controlled recovery box until the effects of the anaesthetic were completely reversed.

5.2.2. In vivo electrophysiology:

24-30 hours post UVB irradiation, rats were anaesthetised and in vivo electrophysiological recordings were performed as previously described, to obtain baseline responses to electrical and natural stimuli. Once stable responses of an individual WDR cell had been characterised, further cells from the same animal were sampled to obtain a thorough population study. Additional cells were contributed by Dr Shafaq Sikandar as part of a collaborative study.

5.2.3. Receptive field mapping:

Receptive fields on the plantar hindpaw were mapped for each cell with an 8g vF, using the methods detailed in (Suzuki et al. 2000). The stimulus was applied repeatedly around the area

of baseline testing until firing was depleted below 0.5Hz. Applications were made at 30s intervals to ensure no wind up was elicited from the testing sequence. The observed receptive field was marked onto a standard diagram of the hindpaw and subsequently digitalised using a Canon MP610 scanner. The size of each receptive field was determined using ImageJ software and calculated as a percentage of the total area of the hindpaw.

Figure 5-2 Receptive field mapping. Receptive fields of single DH LV WDR neurones were mapped using an 8g vF. The exact size was measured using ImageJ software.

5.2.4. UVB irradiation - humans:

Experiments were conducted in 10 healthy human volunteers aged between 22-32 years old. Individuals were familiarised with the experimental protocol before hand and gave written, informed consent. The study was approved by The Kings College Research Ethics Committee.

All subjects were free from pain and medical conditions which may otherwise interfere with the results of the study. They were advised they must avoid pain medication such as NSAIDs and caffeine in the 24 hours prior to the study. This was particularly important as NSAIDs have been shown to be effective against the sensory changes elicited by UVB.

Volunteers were irradiated in a similar protocol as described for the animals. However, the dosing was calculated on an individual basis depending largely on skin type. An initial screening was conducted on each subject to determine their MED; this is defined as the time required to produce a uniform reddening of the area at 24 hours post irradiation. 3 times the MED was then used for the final experiment to irradiate an area of 16x16mm on the volar

forearm, the surrounding area was covered with a UV resistant material to ensure uniform burn.

5.2.5. Mapping area of secondary hyperalgesia:

In line with the animal experiments, subjects were then tested at 24-30 hours post UVB irradiation. Initially, the edges of the primary burn site were marked on the skin and an acetate template was used to mark a spider probe map at 1cm increments along eight spokes (oriented at 45° intervals) radiating out from the primary area (shown below). Once marked on the skin subjects were assessed for the development of both pinprick hyperalgesia and dynamic brush evoked allodynia. Pinprick hyperalgesia was mapped using a 256mN probe (Pinprick, MRC Systems GmbH, Heidelberg, Germany. 0.2mm diameter) - an example stimulation was given on the contralateral arm in order for the subject to familiarise themselves with the sensation. Beginning at 8cm from the centre of the map the stimulation was repeated at 1cm intervals a long each spoke towards the treated area, and the subject was requested to report when this sensation changed. This was usually described as a sharper, or more intense pricking sensation. The stimulus was only applied once to each point, for around 1s. The point at which this change was reported was marked on a standard spider probe map diagram. Adjacent spokes were connected to create 8 triangles, for which the individual areas could be calculated (area of each triangle = 1/2(length a x length b) sin45); the summation of these, minus the primary area (256mm^2), gave the total area of secondary hyperalgesia.

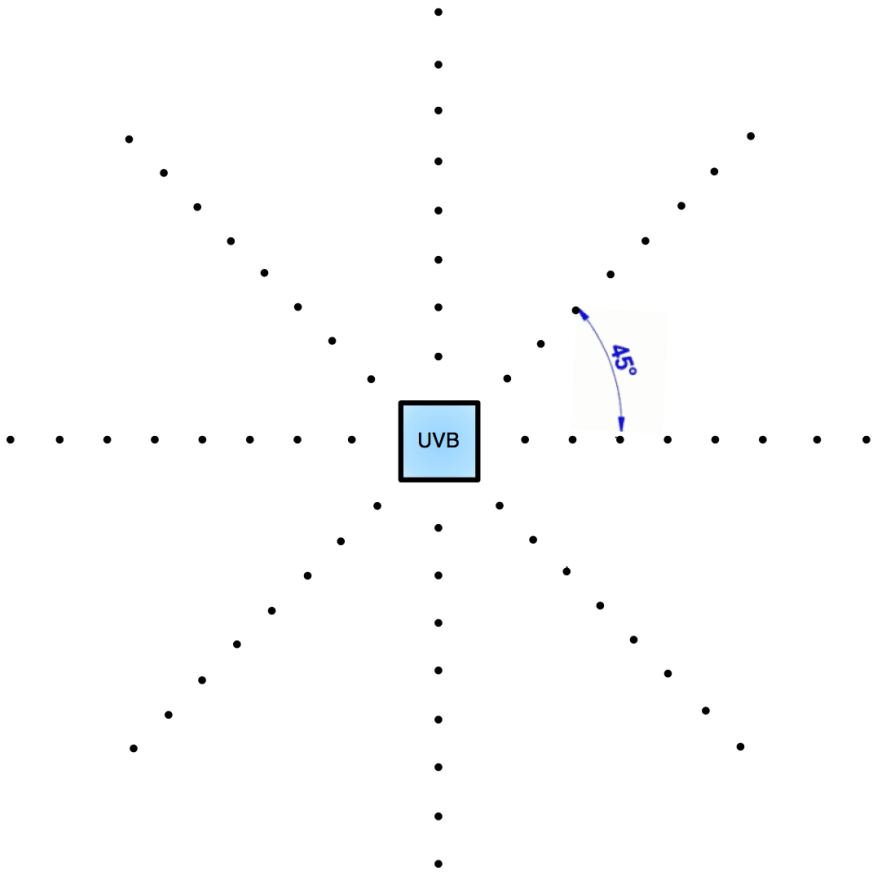

Figure 5-3 Mapping the area of secondary hyperalgesia. A spider probe map consists of 8 radial paths projecting out from the primary treated area. The area of treatment was 256mm^2 in the middle of the map and mechanical stimulation began 8cm from the edge of the burn and continued towards the centre at 1cm intervals until the subject reported a change in sensation.

5.2.6. Human Quantitative Sensory Testing:

Once it was determined if the burn had elicited any secondary changes, full QST profiling was performed as previously described (Chapter 2.4) on the primary irradiated site, and as a control on the contralateral ventral forearm. In addition to the standard QST protocol, subjects were asked for numerical ratings (0-100) to 35°C, 40°C and 45°C.

5.2.7. Administration of CXCL5:

To assess the effects of CXCL5, a previously identified mediator of UVB inflammation, in rodents we used an intraplantar injection of 3μg dissolved in 0.9% saline (Nb. Control experiments found that intraplantar injection of saline alone causes no significant changes

monitored over a time period of 4 hours post injection). This experiment was only carried out in rodents, and not human subjects.

Once a stable cell had been identified and characterised, with at least 3 rounds of baseline testing, a Hamilton syringe was used to inject the solution into the receptive field of the cell, distal from the point at which natural stimuli were applied. The train of electrical and natural stimuli was repeated 30 minutes post injection, and subsequently every 30 minutes up to 4 hours post injection.

5.2.8. Statistical analysis:

All analysis was undertaken using SPSS software (IBM SPSS Statistics v21). Data was assessed for normality using the Kolmogorov-Smirnov test to determine further methods of analysis. Electrophysiological data was analysed using either an unpaired t-test or a 2 way ANOVA accordingly. Psychophysical data, with the exceptions of HPT and CPT, was logged and re-tested for normality. A paired t-test or 2 way ANOVA was then carried out. HPT and CPT were found to be normal without logging, and thus the raw data was used for analysis with a paired t-test. All graphs were plotted to show the mean ± SEM.

5.3. Results

5.3.1. UVB – In vivo electrophysiology

Using objective electrophysiological recordings, LV WDR cell responses to applied stimuli were found to be significantly enhanced when compared to responses observed in naïve rats to the same stimuli, across a range of natural and electrical stimuli. These changes are akin to both mechanical and thermal hypersensitivity observed in previous behaviour experiments, suggestive of an exclusive peripheral sensitisation. The UVB dose (1000mJ/cm2) and time point (24-30 hours post irradiation) were selected from previous studies (Bishop et al. 2007; Dawes et al. 2011) suggesting these resulted in the maximal hypersensitivity.

5.3.1.1. UVB irradiation significantly enhances both innocuous and noxious mechanically evoked WDR cell responses in comparison to naïve animals

24-30 hours post UVB treatment, evoked responses to dynamic brush were significantly enhanced from 331.1 ± 36.6 to 667.6 ±37.7 action potentials/ 10s (figure 5-4; p= 0.000), whilst neuronal responses to low vF forces (8g and 15g) were larger but not significantly changed; thus suggesting that different mechanisms could be involved in the sensitisation of these two stimuli. Responses to higher vF (26g and 60g), which are usually considered noxious in behavioural experiments, were significantly increased by 49 and 54%, respectively (figure 5-4; p= 0.001, 0.000).

5.3.1.2. UVB irradiation significantly enhances both innocuous and noxious thermally evoked WDR cell responses in comparison to naïve animals

Increased firing of WDR cells was observed in response to all temperatures tested post UVB treatment (figure 5-5; p< 0.000). Although this was not shown to be statistically significant at 35°C, there is still an obvious increase from 178 ± 30.8 to 456 ± 45.1 action potentials/10s. The greatest increase was seen just below behavioural threshold, at 40°C, where an increase of 186% in the firing was observed (p= 0.011). Firing to supra threshold stimuli (45°C and 48°C) was also significantly enhanced by 115% and 81%, respectively (p< 0.000, 0.000). Overall, there appeared to be a parallel shift in the stimulus-response curves, indicative of a peripheral sensitisation.

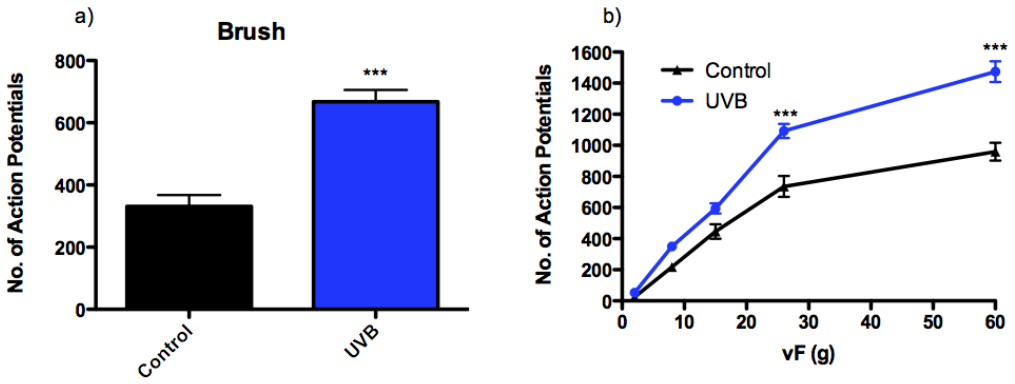

Figure 5-4 Effects of UVB irradiation on mechanically evoked WDR cell responses. Using the protocol described in chapter 5.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of mechanical stimuli, including brush and graded vF, applied to the receptive field for 10s, before and after UVB irradiation. 24-30 hours post treatment both a) dynamic brush and b) noxious vF evoked responses were elevated when compared to naïve animal baselines (brush p=0.000; 26g p= 0.001; 60g p< 0.000). There is a clear coding of mechanical stimuli in naïve animals, which remains in UVB treated animals. n= 38

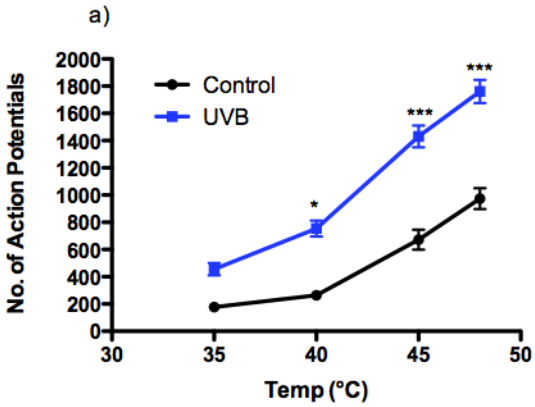

Figure 5-5 Effects of UVB irradiation on thermally evoked WDR cell responses. Using the protocol described in chapter 5.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of thermal stimuli applied to the receptive field for 10s, before and after UVB irradiation. a) 24-30 hours post treatment evoked responses to both innocuous and noxious temperatures were elevated when compared to naïve animal baselines (Overall 2-way ANOVA p< 0.000; 40°C p= 0.011, 45°C p< 0.000, 48°C p< 0.000). There is a clear coding of thermal stimuli in naïve animals, which remains post UVB. n= 37

5.3.1.3. UVB irradiation significantly increases electrically evoked input responses of WDR cells in comparison to naïve animals

Overall, no significant difference was observed between UVB treated and naïve animals, with regards to the number of action potentials elicited from each fibre type (figure 5-6). Although a small difference may be noted in C fibre and PD count – increasing from 405 ± 35.7 to 473 ± 41.9 and 348 ± 37.6 to 430 ± 38.4, respectively, suggestive of a small degree of fibre sensitisation. WU also remained unchanged – the enhanced responses of the spinal neurones remained the same as in normal animals but superimposed upon a greater initial response (figure 5-6). Conversely, C-fibre thresholds were significantly lowered and input was significantly enhanced by 125% (figure 5-6; p= 0.04, 0.01), which is likely due to a peripheral sensitisation reducing thresholds and enhancing pre-synaptic activity of the neurones.

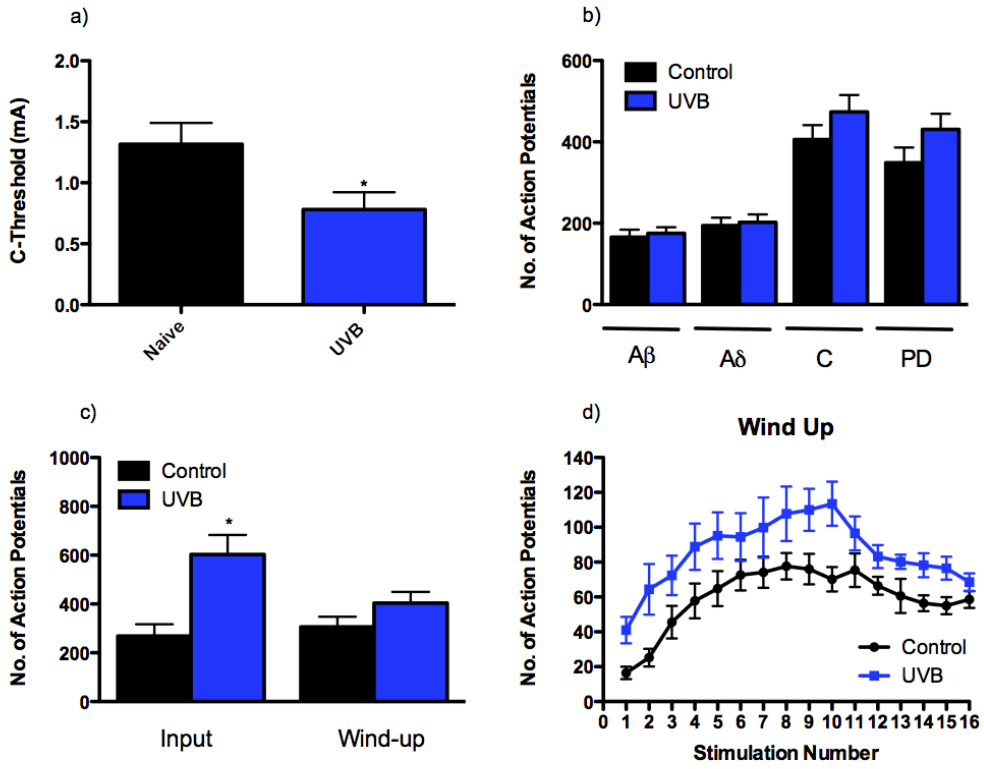

Figure 5-6 Effects of UVB irradiation on electrically evoked WDR cell responses. Using the protocol described in chapter 2.2 and 5.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli pre and post UVB irradiation. Transcutaneous electrical stimulation was used to measure the input and wind up, in addition to calculating the responses driven by different fibre types – depending on the latency. 24-30 hours post treatment a) C-fibre thresholds were significantly lower in UVB treated animals (p= 0.04); b) there were no significant effects on electrically evoked Aβ, Aδ, and C fibre mediated transmission, nor post-discharge; c) electrically induced input was significantly increased (p= 0.01), d) however wind up remained statistically unchanged (graph shows a small sample of example cells) . n= 26

5.3.1.4. UVB irradiation has no effect on receptive field size of WDR cells in comparison to naïve animals

8g vF receptive field size of LV WDR cells in UVB treated animals was not found to be significantly different from the average receptive field size observed in naïve animals (figure 5-7). This is consistent with the evoked responses, which were suggestive of a peripheral, rather than central sensitisation.

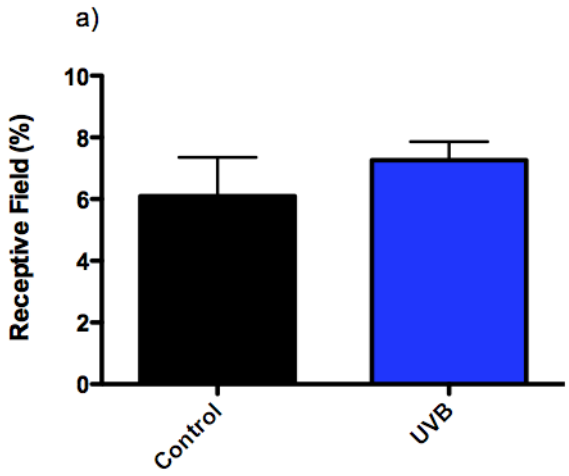

Figure 5-7 Effects of UVB irradiation on receptive field size of WDR cells. Using the protocol described in chapter 5.2 the receptive field was mapped using an 8g vF filament. 24-30 hours post treatment a) there was no significant effect on the size of receptive field in comparison to naïve animals. n= 16

5.3.2. UVB – Human Quantitative Sensory Testing

Using a standardised QST procedure, human subjects were also found to exhibit sensory changes post UVB treatment, such as mechanical and thermal hypersensitivity, once again indicative of a peripheral sensitisation.

5.3.2.1. UVB irradiation significantly reduces MPT and increases numerical ratings to innocuous and noxious punctate stimuli

At the same time-point as the rodent studies, 24-30 hours post treatment, there was a significant drop in the average 50% pain threshold to pinprick stimulation from 103.9mN ± 16mN to 14.9mN ± 3.7mN within the irradiated area (figure 5-8; p< 0.000). Ratings to both sub and supra-threshold mechanical stimuli were increased, whilst perceptual WU remained unchanged (figure 5-8; p< 0.000).

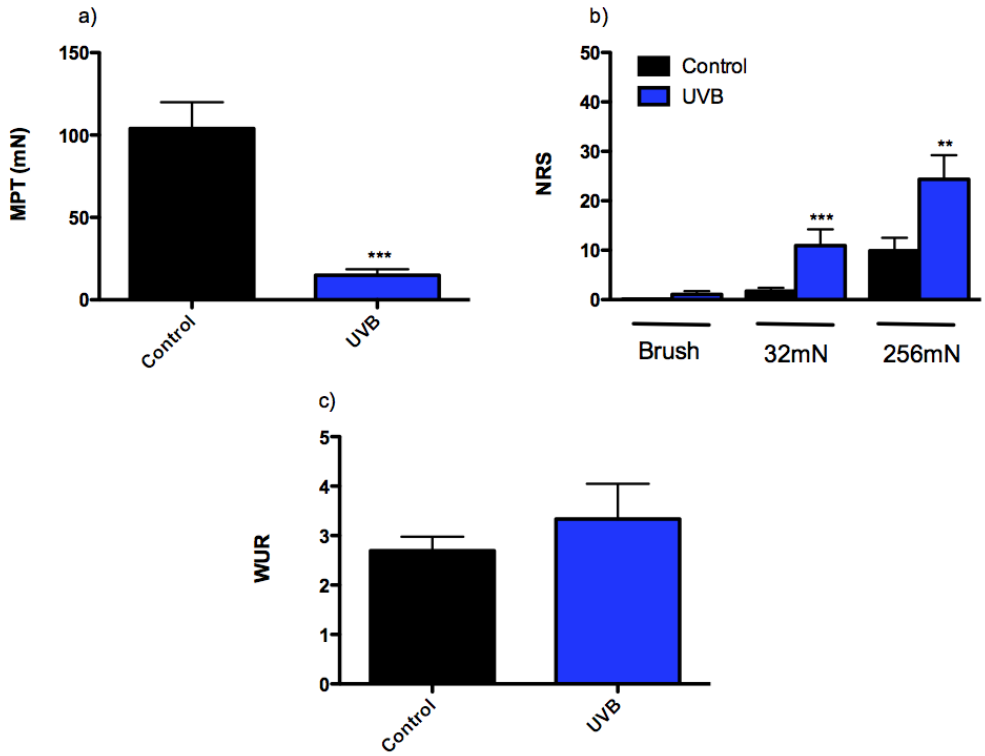

Figure 5-8 Effects of UVB irradiation on psychophysical MPT and mechanical NRS ratings. Using the protocol described in chapter 5.2 standardised QST was undertaken to determine the subject's mechanical pain threshold (MPT), in addition to obtaining numerical ratings (NRS) to graded mechanical stimuli and measuring the wind up ratio (WUR) to repetitive mechanical stimulation. a) Average MPT was significantly lower in UVB treated skin in comparison to pre-irradiation baselines (p< 0.000). b) NRS rating to dynamic brush was unchanged, whereas ratings to 32mN and 256mN were significantly increased (Overall 2 WAY ANOVA p< 0.000; 32mN p< 0.000, 256mN p= 0.004). c) Wind up was unaffected. n= 10

5.3.2.2. UVB irradiation causes a significant primary thermal hypersensitivity

Average HPT was also significantly reduced in the treated area from 45 ± 0.89 °C to 37.4 ± 0.5 °C (figure 5-9; p< 0.000). In line with the rodent data, ratings to both sub and supra-threshold temperatures were significantly enhanced (figure 5-9; p, 0.000). Interestingly, a cold hypersensitivity was also observed, as average CPT was raised from 10.1 ± 3.5 °C to 18.5 ± 2.8 °C (figure 5-9; p= 0.022).

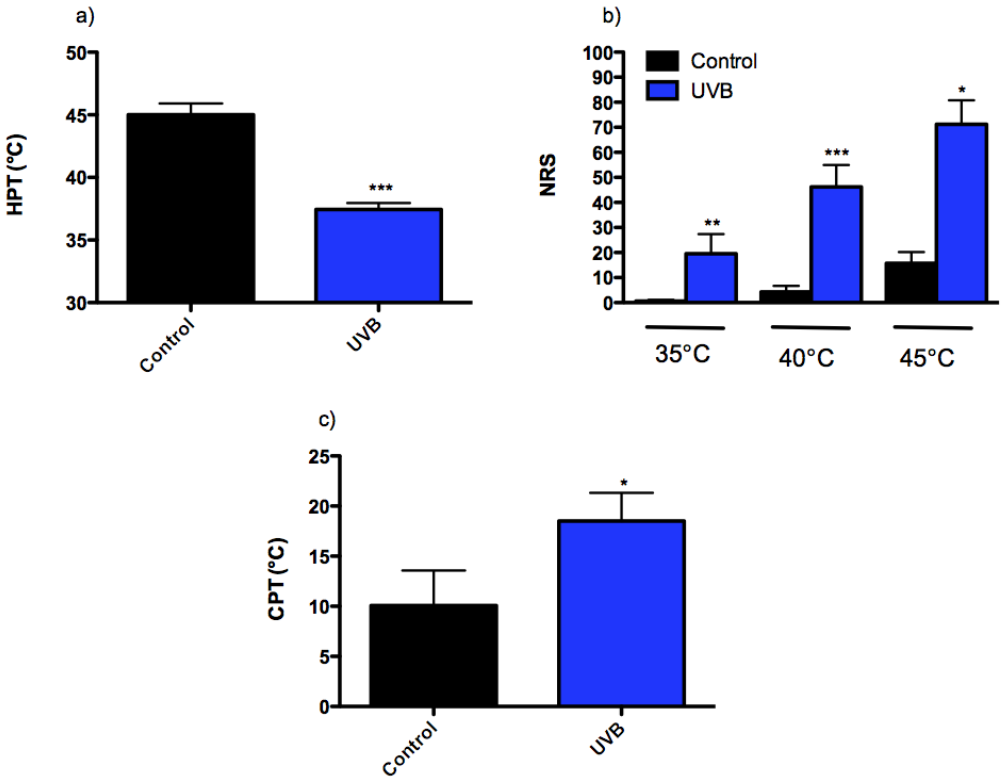

Figure 5-9 Effects of UVB irradiation on psychophysical HPT, thermal NRS ratings and CPT. Using the protocol described in chapter 5.2 standardised QST was undertaken to determine the subject's heat and cold pain thresholds (HPT/CPT), in addition to obtaining numerical ratings (NRS) to graded thermal stimuli. a) Average HPT was significantly reduced in UVB treated skin in comparison to pre-irradiation baselines (p< 0.000). b) NRS ratings to previously innocuous and noxious temperatures were significantly increased (Overall 2 WAY ANOVA p< 0.000; 35°C p= 0.006, 40°C p< 0.000, 45°C p= 0.038). c) CPT was also significantly elevated (p= 0.022). n= 10

5.3.2.3. UVB irradiation causes negligible secondary changes

The area of secondary hyperalgesia was assessed prior to testing of sensory changes in the primary, using a 256mN probe. A relatively small area surrounding the burn was reported by all subjects as being more sensitive (figure 5-10). Although secondary hyperalgesia is generally attributed to central facilitations, an area of this size is more likely driven by infiltration of inflammatory mediators outside the treated area.

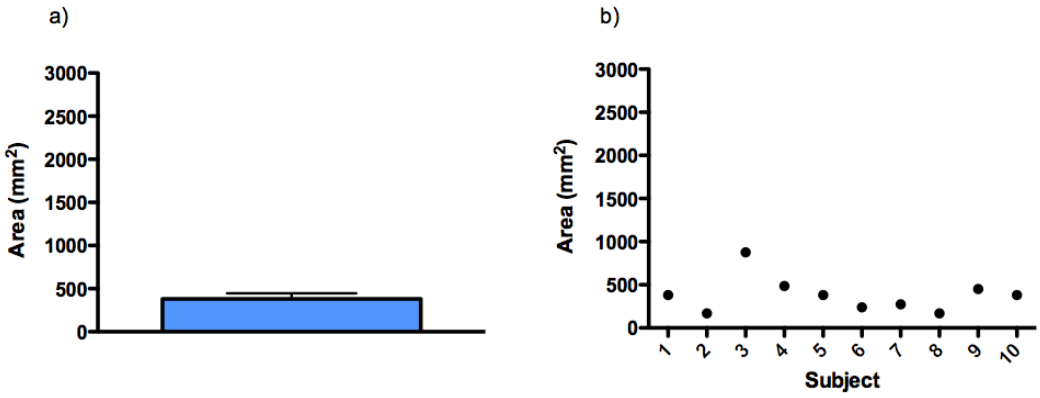

Figure 5-10 The area of secondary hyperalgesia induced by UVB irradiation. As described in chapter 5.2 the area of secondary hyperalgesia was mapped with a 256mN probe. a) 24-30 hours post UVB irradiation a small area (380.3 mm^2) of secondary hyperalgesia was observed in 9/10 subjects. b) The area is negligible across all subjects.

5.3.2.4. Sensory profiles post UVB irradiation illustrate a non specific hypersensitivity.

Full sensory profiling using a standardised, comprehensive QST procedure confirmed a generalised sensitisation in the primary burn area across a number of modalities including: CPT, HPT, MDT, MPT, MPS and PPT. Pinprick and thermal hypersensitivity are previously well documented, however cold and blunt pressure hypersensitivity are new findings (figure 5-11).

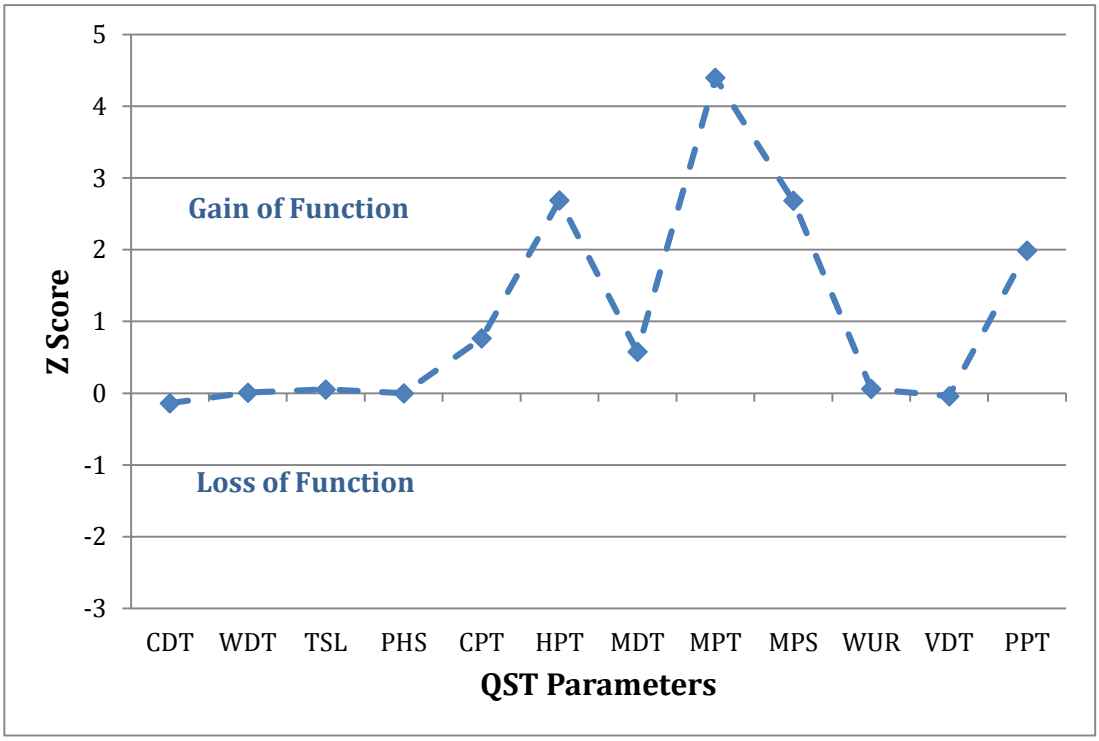

Figure 5-11 Somatosensory changes in UVB irradiated skin. Using the protocol described in chapter 2.4 full QST profiling was undertaken. A variety of parameters were tested both pre and post UVB, the magnitude of the changes are expressed here as Z-scores which highlight specific gains or loss in function. Hypersensitivity to cold, heat, pinprick and pressure are demonstrated here by the gain of function in CPT, HPT, MPT, MPS and PPT.

5.3.3. UVB induced somatosensory changes in rats and humans show considerable overlap

Stimulus	Hypersensitivity	
	Animal	Human
Brush	✓	No change
Subthreshold Mechanical	✓	✓
Suprathereshold Mechanical	✓	✓
Subthreshold Thermal	✓	✓
Suprathreshold Thermal	✓	✓
Input	✓	✓
Wind up	No change	No change
Fibre count	Reduction in C fibre threshold	Not tested
Receptive field/ Area of secondary hyperalgesia	No change	No change

Table 5-6 Comparison of animal and human characterisation. a) There is a remarkable similarity in the sensory changes post UVB across species, highlighting the translational nature of this model. Both animals and humans show heightened responses to mechanical stimuli and thermal stimuli, whilst wind up is unchanged. Fibre count was only assessed in the animal model, this term refers to a change in the number of action potentials elicited from each fibre type. UVB reduced the electrical c-fibre threshold used to elicit the fibre count. Additionally, there was no increase in WDR receptive field size to 8g vF, or an area of secondary hyperalgesia in human subjects. The only difference noted between species is regarding dynamic brush, whereby WDR evoked neuronal responses were increased, whilst there was no changed observed in human perception.

5.3.3.1. Lowered HPT in human volunteers post UVB corresponds to the number of action potentials evoked in WDR cells

When comparing the animal and human data in terms of the neuronal activity produced by temperatures which correspond to the HPTs, before and after UVB, a remarkable correspondence can be observed (figure 5-12). Thus, it was seen that the number of action potentials evoked by 45°C, the average human HPT under normal conditions was 641, very similar to the 614 action potentials that would be evoked by 37.4°C, the reduced human HPT post UVB.

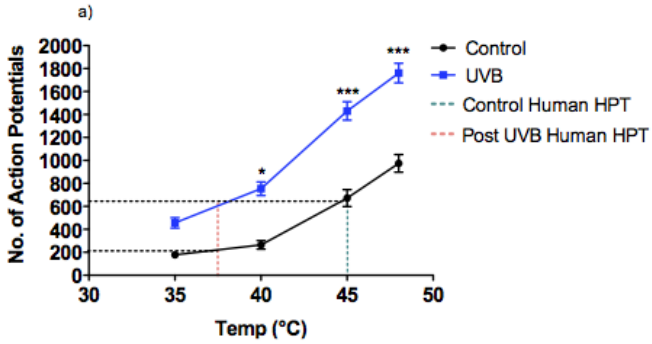

Figure 5-12 Overlap in animal and human data. Comparing changes in thermally evoked neuronal activity to the shift it human HPT, post UVB irradiation, reveals a remarkable similarity a) Pre UVB average human HPT was 45°C, which corresponds to 641 action potentials/ 10s, 24 hours post UVB irradiation this dropped to 37.44 °C, which corresponds to 614 action potentials/ 10s.

5.3.4. CXCL5 – In vivo electrophysiology

Using objective electrophysiological recordings, LV WDR cell responses were found to be significantly enhanced post intraplantar administration of 3μg CXCL5, when compared to baseline responses across a range of natural and electrical stimuli. These data mimicked the changes seen post UVB treatment, as a clear mechanical and thermal hypersensitivity of comparable magnitude was elicited. These results support the notion that CXCL5 may be a key mediator of UVB induced sensitisation.

5.3.4.1. Intraplantar injection of CXCL5 significantly enhances both innocuous and noxious mechanically evoked WDR cell responses in comparison to baseline responses

Dynamic brush responses were significantly elevated from 331.1 ±36.6 to 632.6 ±76.7 action potentials/ 10s post CXCL5 administration (figure 5-13; p= 0.002). Responses to both innocuous and noxious vF were also significantly increased from baseline, suggesting a widespread sensitisation of neurones encoding both modalities (figure 5-13; p= 0.002).

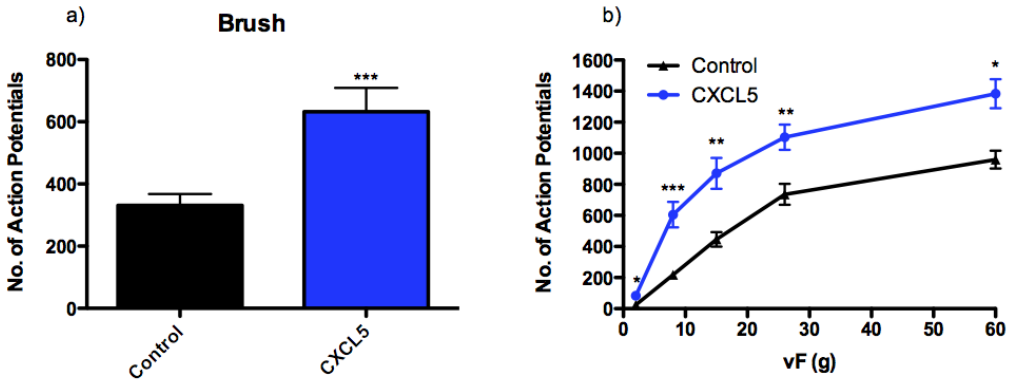

Figure 5-13 Effects of 3μg intraplantar CXCL5 on mechanically evoked neuronal responses. Using the protocol described in chapter 5.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of mechanical stimuli, including brush and graded vF, applied to the receptive field for 10s, before and after intraplantar CXLC5. a) Dynamic brush responses were significantly enhanced post CXCL5 ($p \leq 0.001$). b) Innocuous and noxious punctate mechanically evoked responses were also significantly increased (Overall 2 WAY ANOVA p= 0.002; 2g p= 0.031, 8g p= 0.001, 15g p= 0.003, 26g p= 0.002, 60g p= 0.012). n = 10

5.3.4.2. Intraplantar injection of CXCL5 significantly enhances thermally evoked baseline neuronal responses in naïve animals

There was an increase in firing to all temperatures assessed post CXCL5 treatment (figure 5-14; p≤ 0.001). The greatest change was seen at 40°C, where there was an increase in number of action potentials/10s of 235% (p≤ 0.001). As with UVB this is suggestive of sensitisation of peripheral neurones.

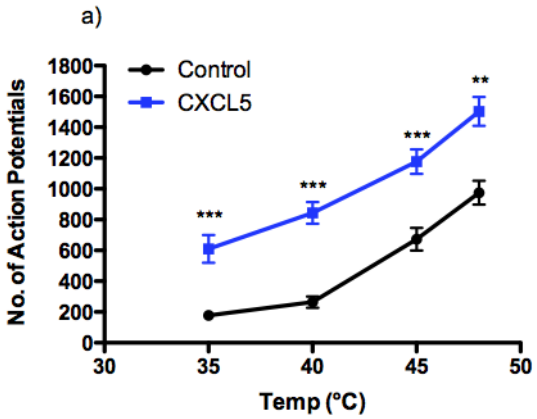

Figure 5-14 Effects of 3µg intraplantar CXCL5 on thermally evoked neuronal responses. Using the protocol described in chapter 5.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of thermal stimuli applied to the receptive field for 10s, pre and post intraplantar CXCL5. a) Innocuous and noxious thermally evoked responses were significantly increased post CXCL5 (Overall 2 WAY ANOVA p≤ 0.001; 35°C p= 0.001, 40°C p≤ 0.001, 45° p≤ 0.001, 48°C p= 0.002). n = 10

5.3.4.3. Intraplantar injection of CXCL5 potentiates electrically evoked input in naïve animals

Overall there was no significant effect of CXCL5 on the number of action potentials elicited from each fibre type, though a small increase can be noted in both the C fibre and post discharge count – from 405.6 ± 35.9 to 488.8 ± 74.8 and 348.9 ± 37.6 to 460.9 ± 112.8, respectively (figure 5-15). However, input was increased from 268.4 ± 48.6 to 705.6 ± 152.9, strongly suggesting a sensitisation of peripheral, or central neurones (figure 5-5; p= 0.005).

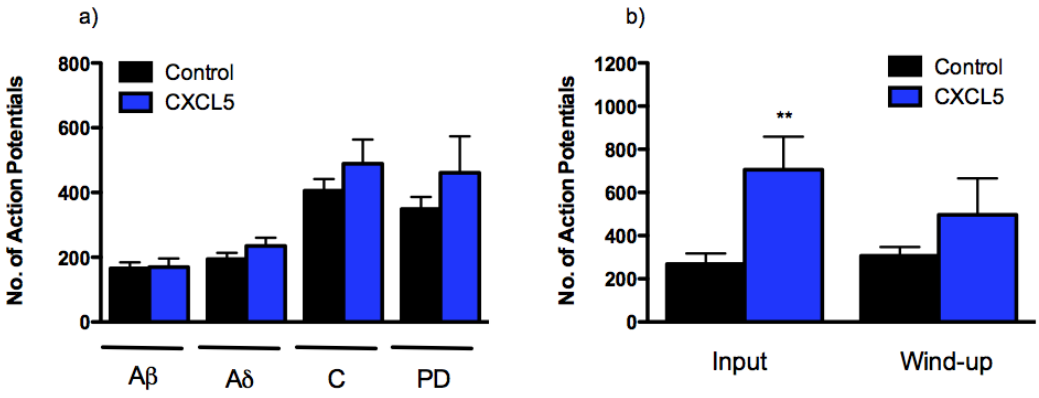

Figure 5-15 Effects of intraplantar CXCL5 on baseline electrical neuronal responses. Using the protocol described in chapter 2.2 and 5.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli pre and post intraplantar CXCL5. Transcutaneous electrical stimulation was used to measure the input and wind up, in addition to calculating the responses driven by different fibre types – depending on the latency. a) There were no significant effect on electrically evoked Aβ ,Aδ, and C fibre mediated transmission, nor post-discharge. b) Electrically induced wind up was unaffected, although input was significantly increased (p= 0.005). n = 10

5.3.4.4. Intraplantar injection of CXCL5 in naïve animals mimics UVB irradiation

When compared with the previous results from UVB irradiated rats, the sensory changes induced via intraplanter CXCL5, are very similar (figure 5-16). The magnitude is also comparable, supporting the theory that CXCL5 is important in UVB induced sensitisation.

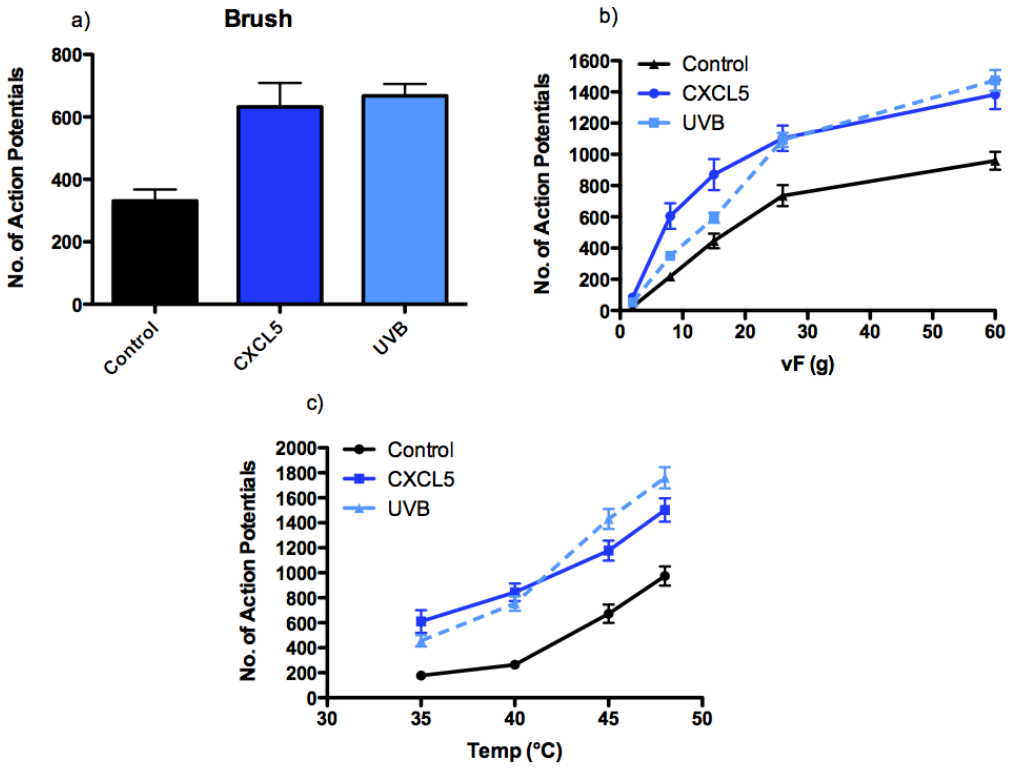

Figure 5-16 Sensitisation of WDR cell responses post CXCL5 to a) brush; b) von Frey and; c) thermal stimuli are comparable in magnitude to those evoked by UVB. Using the protocol described in chapter 5.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of mechanical and thermal stimuli, including brush and graded vF and water jets, applied to the receptive field for 10s. These were compared for control animals with no treatment, and post UVB/ intraplantar CXCL5.

5.4. Discussion

This is the first study to examine the central, spinal, neuronal consequences of UVB irradiation. In this study WDR cell responses were measured 24-30 hours post UVB and after intraplantar administration of CXCL5. Additionally, full QST was undertaken on healthy human volunteers 24-30 hours post UVB. The key findings were that UVB is a reliable translational model of hypersensitivity, producing similar changes in animals and humans. The model induces a strong peripheral sensitisation, with little apparent contribution of central mechanisms. Finally, CXCL5 is able to mimic the sensory changes seen post UVB, and this reinforces the idea that this is a strong candidate as a key mediator of UVB induced inflammatory hypersensitivity and may have relevance in chronic pain states.

5.4.1. UVB irradiation produces a consistent mechanical and thermal hypersensitivity in animals and humans, which can be measured from WDR cells, and with QST

UVB consistently led to a strong mechanical and thermal hypersensitivity in both animals and humans 24-30 hours post irradiation. This is the first full characterisation of WDR cells responses post UVB using in vivo electrophysiology. Evoked activity of spinal WDR neurones was enhanced to dynamic brush and a range of mechanical forces and temperatures. QST revealed a drop in both MPT and HPT, in addition to increased numerical pain ratings to both sub and supra threshold stimuli. Thus confirming, using objective assessment measures, the induction of thermal and mechanical hypersensitivity as previously shown in behavioural studies (Benrath et al. 2001; Harrison et al. 2004; Davies et al. 2005; Bishop et al. 2007; Bishop et al. 2009; Bishop et al. 2010). Furthermore, the results validate the ability to measure hypersensitivity (associated with inflammation) from activity evoked in WDR cells under anaesthesia. This enhanced responsivity has also been shown in alternative models of inflammation such as CFA, thus suggesting WDR cells in the spinal cord, the primary relay site of somatosensory information, are useful for studying the consequences and further characterisation of such models (Marchand et al. 2011).

Also in agreement with previous behavioural reports, the evoked changes in the primary irradiated area are similar in both species (Bishop et al. 2007; Bishop et al. 2009). Interestingly, there is a strong observational parallel between the animal and human thermal responses. Pre UVB the average human HPT was 45°C, which corresponds to 641 action

potentials/ 10s, 24 hours post UVB irradiation this dropped to 37.44 °C, which corresponds to 614 action potentials/ 10s; that is to say, the number of action potentials/10s which correspond to the threshold is very similar both at baseline and post UVB treatment. This was also found in the previous studies detailed in this thesis and is particularly important in support of the value of animal models. Identification of a possible designated 'number of action potentials' which corresponds to human pain thresholds would be a valuable tool for future research and would enhance translation of knowledge from animals studies to humans. This may help bridge the gap between the species and allow better predictions to be made from pre clinical animal data.

The only discrepancy between the animal and human data lies in the dynamic brush responses. There was a marked increase in WDR cell activity in response to dynamic brush post UVB, whereas reports of brush eliciting pain in human subjects were modest. Conversely, previous studies in humans have suggested there is an element of brush-evoked allodynia in both the primary and secondary areas (Bishop et al. 2009; Gustorff et al. 2011; Ortner et al. 2012). However, of note, these studies use a larger area of irradiation, which may result in small amounts of subthreshold spontaneous activity that could drive some central changes. It is clear in this study from the subject reports that there is a negligible brush evoked hypersensitivity, in comparison to models such as capsaicin cream (Bishop et al. 2009).

It is possible that the increasing response of WDR cells to dynamic brush does not necessarily equate to a perceptual pain in volunteers (or rats). It is important to note that WDR cells, by their very nature, are characterised by a response to dynamic brush in naïve animals, since they receive tactile input from both Aβ and low threshold C fibres (Andrew 2010). This suggests that there must be mechanisms in higher centres which allows this signal to be 'filtered out'. While many Aβ afferents travel in the dorsal column, bypassing the spinal cord, to convey the sensation of touch, the Aβ input (and low threshold C fibres) to WDR cells may in fact have very little contribution towards normal tactile sensations (Kandel et al. 2000). This process allowing the selection of which inputs are eventually perceived is not necessarily altered even with these enhanced responses post UVB. Therefore, even if there was a peripheral sensitisation, progressive tactile hypersensitivity, or even small central changes occurring, these may not result in the perception of a painful sensation. It is unknown whether low threshold C fibres express chemoreceptors, however should this be the case, it is certainly possible that they may be sensitised by UVB. While brush stimuli may become

painful in patients with neuropathies whereby function of sensory nerves are altered by disease or a lesion, leading to changes in the processing of the tactile input to WDR cells, may result in allodynia this does not necessarily occur here. In the case of a peripheral sensitisation of low threshold C fibres, perceptual changes may not develop in the absence of any central modifications. Given that any alterations in sensitivity to brush stimuli in the human subjects are not sufficiently large to alter perception, it may be the same in rats that this increase in WDR cell activity does not necessarily equate to a sensation of 'pain'.

A second possibility that cannot be excluded is the engagement of different mechanisms in rats and humans. This could include the development of centrally mediated brush allodynia, or a progressive tactile hypersensitivity, in the animals but not humans. One key difference between the animal and human studies is that while the humans are unlikely to have further strong stimuli come into contact with their burn during the 24 hours between irradiation and tests, the animals continue to walk around during this time. Furthermore, irradiating the whole hindpaw is proportionally speaking a much larger area, which could lead to some spontaneous pain. These differences could lead to a level of afferent input strong enough to evoke some central changes. However, since there is no association with any increase in basal c-fos, in addition to no other signs of central sensitisation in this study, this explanation seems unlikely (Bishop et al. 2007). On the other hand, progressive tactile hypersensitivity has been associated with inflammation and thus may be one possible explanation (Ma and Woolf 1996). The phenomenon is underpinned by phenotypic changes in the primary afferents, rather than requiring central changes. Further behavioural studies, or recordings from PAFs may help further understand this discrepancy.

Overall using objective characterisation methods, UVB appeared to be a reliable translational model of inflammatory hypersensitivity, evoking similar phenotypic changes in both animals and humans, which can be measured using electrophysiology and QST.

5.4.2. UVB induced hypersensitivity is the result of a predominant peripheral sensitisation

In this study, there was no change in the size of receptive field of WDR cells, and only a very modest area of secondary pinprick hyperalgesia was seen in human subjects. Wind-up was not altered in either species and post-discharge, a measure of enhanced evoked activity in

spinal neurones as a consequence of wind-up, was also unaltered. Enlarged receptive fields and increased post discharge of neural activity are key measures of central sensitisation as a result of spinal cord plasticity, which may share overlapping mechanisms. The absence of such here implies the lack of central changes in the UVB model.

An expansion of receptive field of second order spinal neurones indicates the recruitment of additional peripheral fibres, and may result from a number of possible mechanisms. It has been proposed that WDR cell receptive fields are composed of an inner excitatory, 'firing zone' and a surrounding low probability 'firing fringe' (Woolf and King 1989). Input from the firing fringe under normal conditions generates sub threshold excitatory postsynaptic potentials (EPSPs), from which an action potential cannot be generated in the WDR cell. However, when the cord is in a state of sensitisation, and thus activation thresholds are lower, this input is able to activate the second order neurone. Mechanisms that underpin this include engagement and recruitment of NMDA receptors due to enhanced activity into the spinal cord, in addition to being compounded by a loss of inhibitory GABAergic controls, and/or a loss of descending inhibition. Since enhanced receptive fields are sensitive to an NMDA receptor block, and removal of descending controls can generate novel receptive fields it can be assumed these central mechanisms are involved (Schaible et al. 1991; Ren et al. 1992).

The mechanisms discussed above are likely to also be involved in secondary hyperalgesia, whereby the damaged primary area results in ongoing input into the spinal cord, unblocking NMDA receptors and lowering the activation threshold of spinal neurones. Activation of Aδ fibres in the surrounding area then results in an increased activity in the spinal cord, which is perceived by the subjects as more painful than before. Since secondary punctate hyperalgesia can be induced in patients lacking Aβ fibres, and a complete block of A fibres abolishes this symptom, it can be concluded that Aδ fibres are the key mediators of this modality (Treede and Cole 1993; Ziegler et al. 1999).

SNL and CFA are two very different forms of peripheral injury, involving damage to nerves and damage to tissue, respectively, which may therefore produce modifications to pain signalling through different mechanisms; though both are associated with central changes. In both models signs of spontaneous or ongoing pain are present, which are further associated with comparable expanded RFs (Suzuki et al. 2000; Chu et al. 2004). Suzuki et al mapped RFs with a 9g vF and found a clear increase in RF size post SNL surgery, in agreement with previous studies using CCI and chronic inflammation (Ren et al. 1992; Grubb et al. 1993;

Cumberbatch et al. 1998; Suzuki et al. 2000). The expansion in receptive field can be blocked by the NMDA receptor antagonist MK-801, suggesting that such central mechanisms are indeed involved in this phenomenon (Ren et al. 1992). Since hypersensitivity observed in the UVB model was previously shown to be unaltered by MK-801, and in this study there is no increase in receptive field size, it can be concluded this pivotal mechanism of heterosynaptic central sensitisation is unlikely to be present in the model (Bishop et al. 2010).

The lack of considerable areas of pinprick hyperalgesia seen in the human studies here also indicates peripheral, but not central, sensitisation mediating evoked hypersensitivities. Post UVB there was a modest area around the burn that subjects reported as more being painful than normal skin; given the size (380.3mm^2) it is not impossible to postulate that this is the consequence of a spread of inflammatory mediators outside the site of injury itself; although a weak engagement of central sensitisation cannot be fully discounted. In recent studies Gurstoff and colleagues continue to find large areas of pinprick hyperalgesia (Gustorff et al. 2013; Rössler et al. 2013), which were not replicated here.

Despite the evidence against any central changes in both animals and humans found in this study, further research is required to settle this ongoing debate. Since QST still largely relies on subjective reports, a more quantitative measure is needed. Capsaicin induced central sensitisation has been found to be associated with activity in the brainstem, including the mesencephalic pontine reticular formation (Lee et al. 2008). Human imaging of the spinal cord, at present, is restricted. This central activity was found to be correlated to specifically to the state of central sensitisation, rather than an increased stimulus intensity. Therefore, a similar experiment using fMRI to examine the activity post UVB could be a useful tool in resolving this debate. Further pharmacological manipulation of targets implicated in central sensitisation may also be useful.

As previously noted, there were also negligible reports of dynamic brush evoked pain in human volunteers. Brush evoked allodynia is a symptom once again indicative of central changes, resulting from plasticity in the spinal cord, allowing synaptic plasticity, and the ability of Aβ fibres to transmit nociceptive signals. These mechanisms therefore differ from those involved in secondary pinprick hyperalgesia and expanding receptive fields. In contrast to UVB, both capsaicin cream and thermal burn models have been found to induce primary and secondary dynamic brush evoked allodynia (Bishop et al. 2009). Both of these models are

associated with central changes and therefore this is not an unexpected finding. The absence of this in the UVB model suggests that such changes are not present.

5.4.3. UVB produces hypersensitivity to certain electrically evoked responses

In addition to the hypersensitivity observed in response to natural stimuli there is also a clear reduction in C fibre activation threshold and an increase in electrically induced input, indicating hypersensitivity to electrically evoked activity which will bypass peripheral receptor transduction. The recordings are taken from second order neurones, and the pattern of changes observed are highly suggestive of changes due to sensitisation of the primary afferent, and not the secondary neurone itself. Reduced C –fibre thresholds and increased input were the only changes seen in the neuronal responses, with no blanket changes and importantly, no changes seen in wind-up or post-discharges which result from post-synaptic mechanisms. However, a recent paper by Weinkauf and colleagues examined the effect of UVB on electrical stimuli in humans and also found evidence of a sensitisation to this modality (Weinkauf et al. 2013). The results shown are similar to the findings presented here, in that both the electrical pain threshold and the ratings to suprathreshold electrical stimuli increased by 70% (Weinkauf et al. 2013). The authors suggest that as this hypersensitivity is correlated well with the thermal, but not mechanical, hypersensitivity, which implicates axonal hyperexcitability. It is possible that the mediators which sensitise peripheral receptors such as TRPV1, also sensitise ion channels such as voltage gated sodium channels, through downstream intracellular mechanisms. This axonal sensitisation could certainly explain the increase in input seen here, and would be in line with the theory of an overriding peripheral sensitisation.

Electrically evoked responses were not potentiated in the range of any of the fibres, nor was there any change in wind up. Given that central sensitisation may be associated with the recruitment of increasing numbers of afferent fibres it could be hypothesised that this would be reflected in these fibre counts. It has recently been shown that in the MIA model of OA, which has a clear central component, there is a potentiation of electrically evoked responses in the Aδ fibre range which may be taken as a sign of central changes (Burnham 2012; Thakur 2012). A non significant increase in Aδ response has also been found after carrageenan

inflammation, though there is little further evidence of such changes in other models and thus is difficult to draw firm conclusions from the evidence available (Rahman et al. 2004).

With regards to electrically induced wind up, it is well known that this phenomenon relies on the recruitment of NMDA receptors (Dickenson and Sullivan 1987; D'Mello et al. 2011). This mechanism is shared with central sensitisation, which has also been shown to require this receptor (Woolf and Thompson 1991). Thus, if central sensitisation is induced by a particular model, engaging the NMDA receptor and inducing a state of hyperexcitability in spinal cord neurones, it may be expected that their ability to wind up is reduced as the receptor is already close to capacity. That is to say, since they both rely on the same receptor, it may not be possible for the two to occur in tandem. Given that no change in wind up was observed here, this may also suggest the lack of central sensitisation.

5.4.4. UVB produces a cold hypersensitivity in human volunteers

As found in a recently published study, here a cold hypersensitivity was also noted during the human QST sessions. CPT was elevated post UVB from 10.0 ±3.5°C to 18.5 ±2.8°C. This was a previously unreported phenomenon associated with UVB inflammation. Gustorff and colleagues reported similar values, with CPT rising from 15.3 ±2°C to 19.1 ±1.7°C. Little is known about mechanism underpinning cold hypersensitivity, although TRPM8 has been implicated in this since KO mice have impaired development of cold hypersensitivity (Colburn et al. 2007; Dhaka et al. 2007). Given that the data so far indicates an overriding peripheral sensitisation, it may therefore be inferred there is a peripheral component to cold hypersensitivity. This could include the sensitisation of cold receptors, such as TRMP8 or TRPA1, or of ion channels on the axons of the afferent fibres.

Since at least two studies have now confirmed the development of cold hypersensitivity associated with UVB, it can be inferred that this may be a useful model to assess the mechanisms associated with this symptom, and the respective pharmacological sensitivity. Cold hypersensitivity is a symptom associated with conditions such as oxaliplatin-induced neuropathy and has so far been difficult to model in humans (Binder et al. 2007). This finding is therefore important for future studies wishing to assess cold hypersensitivity.

5.4.5. UVB QST profile

Full sensory characterisation post UVB indicates a general hypersensitivity across modalities in the primary irradiated area. A non-specific hypersensitivity such as this is shared with conditions including complex regional pain syndrome, and chemotherapy induced neuropathic pain (Binder et al. 2007; Gierthmühlen et al. 2012). Therefore suggesting UVB may mimic some of the underlying mechanisms associated with these conditions, and thus allows them to be modelled pre clinically.

Pinprick and heat hypersensitivity are already well documented, but sensitisation to cold and blunt pressure represent new findings. These have been replicated by a recent publication, released after the completion of the study described here (Gustorff et al. 2013). These findings increase the relevance of this model for future studies, since both of these symptoms are associated with various abnormal pain states. A study of 1236 patients with varying pain origins found that heightened sensitivity to blunt pressure occurred in 36% patients, and cold hyperalgesia in 19% (Maier et al. 2010). Consequently, further characterisation of these sensory abnormalities in animals could aid the understanding of their respective underlying mechanisms. Additionally, drugs that alter the transduction of cold or blunt pressure stimuli could be screened in this model to evaluate the contribution of relevant transducers in UVB-induced sensitisation.

5.4.6. CXCL5 produces a consistent mechanical and thermal hypersensitivity in animals

The mechanisms underpinning UVB are not yet fully established, however it is generally well accepted that inflammation induced sensitisation is responsible for sensory changes observed in the primary burn area (Møiniche et al. 1993; Saadé et al. 2000; Marchand et al. 2005; Bishop et al. 2010). However, the exact inflammatory mediators involved in this process are still under investigation. Since CXCL5 is upregulated in both rats and humans post UVB it was hypothesised that it may contribute towards nociceptor sensitisation. Here, intraplantar injection of CXCL5 led to a clear mechanical and thermal hypersensitivity. This is in partial agreement with behavioural data, which suggests that CXCL5 mediates mechanical, but not thermal, hypersensitivity (Dawes et al. 2011). Additionally, an increase in input also highlights a previously unreported electrical hypersensitivity. These results suggest that

CXCL5 is able to result in the sensitisation of primary afferent neurones, including peripheral receptors and ion channels in the axon.

The discrepancy between the previous behavioural data and electrophysiology could be due to a number of reasons. Thermal hypersensitivity is tested in behaviour using the latency of withdrawal to a radiant heat source, which would only demonstrate a difference in reflex action to this temperature. Although there was a trend towards a reduction in latency of withdrawal with 3μg CXCL5, this was not found to be significant; it could simply be that this test is not sensitive enough to pick up the changes. The measure is of a reflex withdrawal, which occurs usually in less than 15 seconds and thus may be difficult to pick up significant, yet subtle differences. Since the temperature of the paw after 10-12 seconds exposure to the radiant heat source is unlikely to be very high, it is probable that the temperatures being assessed correspond to the lower temperatures examined with electrophysiology, where the difference is not as highly significant. Thus, if suprathreshold temperatures were assessed with behaviour a difference may have been found. This emphasises the importance of electrophysiology and the ability to assess a range of stimuli from sub to supra threshold (Sikandar and Dickenson 2013).

However, since other chemokines, which may act through similar mechanisms to CXCL5, have been shown to result in thermal hypersensitivity and even direct sensitisation of TRPV1 it seems most likely that the results seen here reflect a true thermal hypersensitivity (Zhang et al. 2005; Dansereau et al. 2008). Assessment of intradermal administration of CXCL5 in humans would be useful to further investigate the role of CXCL5 in thermal and mechanical hypersensitivity.

5.4.7. Chemokines such as CXCL5 are important in the development of altered pain states

CXCL5 is associated with infiltration of both neutrophils and macrophages (Dawes et al. 2011). Therefore it is plausible that a release of inflammatory mediators from these immune cells results in sensitisation of the peripheral neurones. Additionally, chemokines have been shown to directly interact with neurones, via sensitisation of TRPV1 on peripheral afferents, as well as inhibition the μOR (Zhang et al. 2005; Zhang and Oppenheim 2005). This interaction between the nervous and immune system is likely to be involved not just in

inflammatory pain, as evidence suggests it may also play a role in neuropathic pain (Marchand et al. 2005; Uceyler and Sommer 2007). Therefore, chemokines and their receptors may be potential drug targets.

CXCL5 is already known to be upregulated in the joints of arthritic patients, although its associations with other chronic pain conditions are yet to be revealed (Grespan et al. 2008). Since this study has highlighted the ability of the chemokine to induce a strong hypersensitivity, it is possible it will be found to be associated with other inflammatory pain conditions. Should this be the case, these basic mechanistic studies may have identified a new drug target for inflammatory pain. Blocking CXCL5 with a neutralising Ab does indeed reduce UVB induced hypersensitivity, though due to the high levels of redundancy in the roles of most chemokines, it is more logical to target the receptor; in this case CXCR2. This receptor also binds CXCL1 and CXCL8 and thus blocking the receptor would mediate the action of all three inflammatory chemokines. In fact, antagonism of CXCR2 reduces hypersensitivity associated with both carrageenan and CFA, indicating the importance of this receptor in the development of inflammatory induced sensitisation (Cunha et al. 2008; Manjavachi et al. 2010).

5.4.8. CXCL5 evokes similar sensory changes to UVB

Using in vivo electrophysiology we measured evoked responses of spinal neurones to a range of natural and electrical stimuli post UVB and post intraplantar CXCL5. The changes seen in both conditions show a considerable similarity across all modalities, i.e. a potentiation of thermally-evoked and mechanically evoked activity with little effect on electrically-evoked responses. The only discrepancy is seen around the lower, behaviourally innocuous vF. It is feasible that this may be due to the dose of CXCL5 used in our study, as it is higher than naturally upregulated levels post UVB. Therefore this may induce a stronger immune response with subsequent hypersensitivity. The similarities in changes to evoked measures post UVB and CXCL5 suggest an analogous underlying biological mechanism. Thus this electrophysiological evidence supports the theory that CXCL5 is a key mediator of UVB induced inflammation.

5.5. Concluding remarks

Overall using objective characterisation methods, UVB appeared to be a reliable translational model of peripheral sensitisation, evoking similar phenotypic changes in both animals and humans, which can be measured using electrophysiology and QST. Furthermore, in animals CXCL5 produces a remarkably similar set of alterations, confirming its place on the list of strong candidate molecules for mediating UVB induced hypersensitivity.

6. UVB Rekindling

6.1. Introduction

The previous chapters of this thesis have focused on the development and characterisation of possible reliable, translational models of chronic pain. As previously mentioned, although it is one of the most widely recognised and frequently used, the capsaicin cream model has a number of drawbacks with regards to its true clinical relevance. On the other hand the newer model of UVB irradiation could begin to closer reflect clinically meaningful mechanisms. However, it was concluded in the last chapter that this model is mainly underpinned by a peripheral sensitisation, and whilst this is useful for exploring these mechanisms alone, most patients who suffer from chronic pain will most likely have numerous overlapping peripheral and central mechanisms contributing to their symptom profiles (Baron 2006; Gwilym et al. 2009; Latremoliere and Woolf 2009; Thakur et al. 2012).

Central sensitisation differs substantially from peripheral sensitisation, both in terms of the location and molecular mechanisms that underpin the phenomenon and in its manifestation. As such, a model encompassing both peripheral and central changes is likely to best reflect certain underlying aetiologies relevant to patients. Therefore, the continuation in development of translational models is required to further bridge the gap between preclinical and clinical research - ensuring that models continue to approach more and more relevant mechanisms and in the hope of eventually creating particular models that closely reflect specific individual chronic pain conditions. This chapter explores the use of electrophysiology and QST in order to establish and characterise UVB rekindling as a translational model of inflammatory pain.

Given that UVB irritation produces such clear somatosensory changes, which are not only apparent in both rodents and humans, but also may reflect clinically relevant inflammatory mechanisms, it is logical to build on and further explore the potential of this model. It is well established that models of peripheral sensitisation and ongoing activity, may lead to a subsequent central sensitisation given adequate levels of stimulation (Latremoliere and Woolf 2009; Baron et al. 2013). However, it has been discussed in the previous chapter that UVB is unlikely to produce a large enough peripheral drive on its own to establish, or maintain, central changes. Coupled with a second stimulus on the other hand, it may be possible to induce a state of central sensitisation. In fact, other models have already explored the possibility of combining a heat rekindling method in order to enhance central sensitisation.

6.1.1. Heat rekindling enhances capsaicin cream induced central sensitisation leading to robust secondary mechanical hyperalgesia

The central aspects of the pain pathway can be sensitised through a range of different forms of functional, chemical, and structural plasticity. The hallmarks of such central sensitisation include mechanical hyperalgesia and allodynia/ mechanical and brush evoked hypersensitivity in the secondary area – i.e. an area adjacent to that which received the original treatment. This may also be thought of as an area outside the inner excitatory 'firing zone' of the neurones in the treated area. Stimuli to this zone are only able to evoke activity when the spinal neurones are sensitized. Any model that induces central sensitisation is therefore characterised by evoking clear signs of inducing hypersensitivity in this secondary area. As reported in the first chapter, the use of capsaicin cream alone is believed to result in a strong peripheral sensitisation, along with the induction of a degree of central sensitisation (O'Neill et al. 2012). However, it is apparent that the symptoms generated by this model, including secondary mechanical hyperalgesia, are smaller than those seen with intradermal injection of capsaicin.

The idea of strengthening these changes with heat rekindling was therefore pioneered by Petersen and Rowbotham. The colleagues found that by combining the chemical stimuli with a physical stimuli (45°C for 5 minutes, 0.075% capsaicin cream for 30 minutes, 40°C heat rekindling for 5 minutes repeated 3 times with intervals of 40 minutes) it was possible to evoke a more stable secondary mechanical hyperalgesia for a longer duration (Petersen and Rowbotham 1999). Dirks et al confirmed the increased strength of this model was indeed due to the rekindling procedure (Dirks et al. 2003). Given that the changes induced by the model were shown to be responsive to systemic lidocaine, remifentanil, and oral gabapentin it can be concluded that it is sensitive to peripheral and central modulation (Dirks et al. 2000; Petersen et al. 2001; Dirks et al. 2002). Furthermore, highlighting that this is also a model suitable for the assessment of pharmacological interventions.

6.1.2. Preliminary investigation of UVB rekindling reveals enhancement of central changes

Cookson and colleagues adapted the rekindling paradigm described by Peterson and Rowbotham, to explore its potential use in combination with UVB irradiation. Given the dispute as to whether UVB alone could result in central changes, it was suggested that this

might provide a more reliable model of inflammatory pain involving a robust central sensitisation. The model was established in human volunteers, whereby subjects received irradiation at 3 x their individual MED and 24 hours later returned for the rekindling procedure and sensory testing. The rekindling involved 3 rounds, each lasting for 5 minutes at a temperature of ≤45°C, equally spaced by 40-minute intervals. The study concluded that this rekindling procedure enhanced and maintained both secondary mechanical pinprick hyperalgesia and brush evoked allodynia (Cookson 2005; Wang 2005). Therefore the studies suggest that combining an initial stimulus that produces inflammation with a subsequent noxious stimuli, can indeed evoke more robust alterations in central pain processing.

Despite these positive results, the model has not been explored any further in human subjects, most likely due to the inconvenient timing of the study, whereby the multiple rekindling procedures were spaced by 40 minutes each. However, the model has since been tested in animals and initial data published appears to suggest it could be another useful translational model of chronic pain (Davies et al. 2011). More recently, Davies et al employed a similar rekindling procedure in rats to that originally described by Cookson, in order to behaviourally characterise this model. However, for the animals the paradigm involved a maximum of two rekindling procedures, separated by only 15 minutes (Davies et al. 2011). A clear benefit of rekindling, with regards to enhancing the signs of central sensitisation, was also observed in the animals; the study concluded that while a single rekindling procedure alone was able to enhance mechanical hypersensitivity in the secondary area, the effect was even stronger with two procedures as these animals then had the lowest withdrawal threshold to vF filaments (Davies et al. 2011). The effect was found to last up to 10 days in the group receiving 2 rekindling procedures, a duration which exceeds most models currently used. Therefore it appears possible to induce long lasting signs of modifications of central processing in animals, through rekindling of the UVB irradiated area.

When originally characterising the model of UVB irradiation, Bishop and colleagues extensively compared UVB to the capsaicin and thermal burn models; while the latter two evoke pin prick hyperalgesia and allodynia in the secondary area, neither of these manifestations of central changes were found post UVB (Bishop et al. 2009). On the other hand, both the animal and human studies of UVB rekindling mentioned above suggest that brush and mechanical hypersensitivity are present in the secondary area. The somatosensory changes induced by this model therefore appear to resemble those observed post capsaicin

and the thermal burn, both of which are known to evoke central sensitisation. Taken together the initial studies by Cookson and Davies suggest that combining UVB irradiation with heat rekindling in animals and humans results in robust and long lasting secondary changes, reflective of altered central processing mechanisms.

6.1.3. The UVB rekindling model is sensitive to COX-2 inhibition and NMDA antagonism

Pharmacological evidence from both animal and human studies indicate that both peripheral and central mechanisms are indeed involved in this model. Preliminary studies have found that the model of UVB rekindling is sensitive to both oral rofecoxib and intravenous ketamine. Whilst both of the compounds were able to reduce the area of secondary hyperalgesia in human subjects, ketamine had a patently stronger effect (Wang 2005). Given that ketamine is an antagonist of the NMDA receptor, which is involved in central hyperexcitability, this supports the theory that such mechanisms are engaged. It has also been shown in animals that systemic ibuprofen can reverse the secondary mechanical hypersensitivity induced by the model, which could be acting at peripheral or central sites (Davies et al. 2011). These studies indicate that the model is sensitive to both peripheral and central modulation, and thus such mechanisms must be induced by the rekindling procedure.

Both heterosynaptic central sensitisation and wind up of spinal neurones are underpinned by ongoing activity of primary afferents that converge in the DH and modulate NMDA receptor function (Dickenson and Sullivan 1987; Haley et al. 1990; Lewin et al. 1994). Thus many models of altered pain processing or chronic pain states are sensitive to a blockade of this receptor (Woolf and Thompson 1991; Stubhaug et al. 1997). In the model of UVB irradiation it was observed that the NMDA antagonist MK-801 could not reverse the changes induced and it is therefore unlikely that any of these central mechanisms underpin the model (Bishop et al. 2010), in keeping with the observations made in the previous chapter. However, since ketamine is able to reduce secondary mechanical hyperalgesia in humans after UVB rekindling treatment, this would suggest that such mechanisms have indeed been induced in the model. Therefore, overall, the limited evidence available suggests that as hypothesised, somatosensory changes evoked by rekindling are most likely underpinned by sensitisation at both levels of the pain pathway. Further objective characterisation of the model and pharmacological interventions will aid understanding of the mechanisms involved.

6.1.4. Rekindling may lead to a barrage of activity into the CNS, driving excitability and altered central processing

UVB irradiation leads to a local inflammatory response, and subsequent peripheral sensitisation. It has been concluded that this alone does not lead to any spontaneous or ongoing input into the spinal cord. On the other hand, in the model of topical capsaicin it has been suggested that rekindling is able to increase the ongoing input into the DH, which is necessary for induction and /or maintaining the state of central sensitisation. A reduction in this drive, has also been noted to be able to decrease the area of secondary hyperalgesia observed (Dirks et al. 2000; O'Neill et al. 2012). Thus the key to developing a stable central sensitisation/ secondary hyperalgesia in experimental models could simply be an ongoing peripheral drive of adequate strength, most notably from C-fibres (McMahon et al. 1993). It is likely that rekindling the pre-sensitised nociceptive endings post-UVB treatment will also result in an ongoing activity into the second order neurones of the spinal cord. These sensitised primary afferents not only have lowered activation thresholds, but may also generate activity of a greater magnitude than those in untreated skin. In addition previously silent nociceptors may also have been recruited (Schmidt et al. 1995). Overall this may lead to a barrage of activity into the CNS, driving excitability and altered central processing (Baron et al. 2013).

In exploring the transition from acute to chronic pain, Levine and colleagues have described a similar phenomenon known as 'priming'. Exposing neurones to an initial inflammatory stimulus such as TNF-α, prior to exposure to a subsequent pro-inflammatory mediator (PGE2) results in a prolonged mechanical hyperalgesia (Parada et al. 2003). Changes in nociceptor function as a result of the initial insult are believed to be the driving factor behind this observed increase in hypersensitivity. However, given that these studies focus on primary hypersensitivity it is difficult to draw any further comparisons. Additionally, administration of TNF at 24 or 48 hours before the second insult did not induce a prolonged hypersensitivity, and rather it appears that 72 hours are required to establish these 'priming' mechanisms (Bogen et al. 2012). Given the time frame required it would appear this phenomenon is related to genomic changes and thus it seems unlikely that such mechanisms underpin the changes seen post rekindling.

Simply put, an afferent barrage of C fibre activity resulting in the engagement of central mechanisms may underpin this model (LaMotte et al. 1991). This sensitisation as a result of

an afferent peripheral drive is also relevant to patients, since by removing the ongoing activity when replacing a damaged joint the signs and symptoms of central sensitisation may disappear (Malfait and Schnitzer 2013).

This chapter aims to validate the preliminary findings from the studies of UVB rekindling, in order to confirm this as a translational model of inflammatory pain involving modifications to the central pain pathway. The critical intent was to establish a paradigm suitable for use in animals and humans that is convenient to execute and robust in symptom induction, though investigation of spinal neuronal activity in animals, and full QST profiling in human volunteers.

6.2. Methods

6.2.1. UVB irradiation - rats:

Adult male Sprague-Dawley rats, between 210-240g, were obtained from the UCL Biological Services Unit. All procedures were approved by the UK Home Office, and were performed in accordance with the guidelines provided by the International Association for the Study of Pain.

Rats were anaesthetised in an induction box using 4% isoflurane (carried in 66% N2O and 33% O2). Once the rat was fully unconscious and was checked for absence of reflexes (by pinching the toes of the hindpaw) they were placed on-to a heat mat and fully covered with UV resistant material. The upper half of the plantar surface of the right hindpaw was then exposed, and placed at a set distance of 2cm under the UVB light source, ensuring only this area was irradiated. All experiments were conducted using a Dermfix 1000MX UV-B Lamp fitted with a 9 Watt fluorescent UVB tube, λ max = 311nm. The irradiance of the lamp was determined using a calibrated photometer. This reading was used to determine the length of time required to deliver a set dose of 1000mJ/cm^2. Post irradiation the rats were placed in a temperature controlled recovery box until the effects of the anaesthetic were completely reversed.

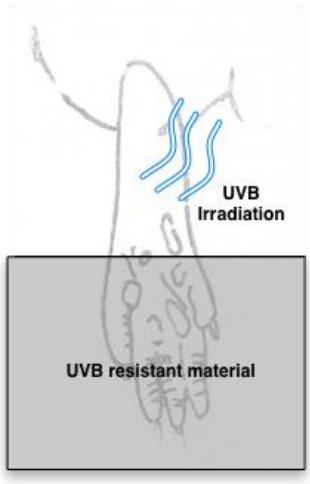

Figure 6-1 Method of Rodent UVB Irradiation. In order to test the effects in the secondary area post rekindling, only the top half of the paw is irradiated. Therefore cells can be found with receptive fields outside the area of treatment. The bottom half of the hindpaw is covered with a UVB resistant material.

6.2.2. In vivo electrophysiology and heat rekindling:

24-30 hours post UVB irradiation, rats were anaesthetised and in vivo electrophysiological recordings were performed as previously described, to obtain baseline responses to electrical and natural stimuli. Cells were only used if they had receptive fields located on the lower half of the hindpaw in the untreated, secondary area. Once stable baselines were established the rekindling procedure was carried out. The treated area of the paw was exposed to a heat source kept at a constant temperature of 40°C for 5 minutes. After 15 minutes, a second rekindling procedure was carried out, once again using a heat source kept at a constant temperature of 40°C for 5 minutes. Following this, natural and electrical responses were re-tested every 30 minutes, up until 180 minutes post rekindling. Additional cells were contributed by Dr Shafaq Sikandar as part of a collaborative study.

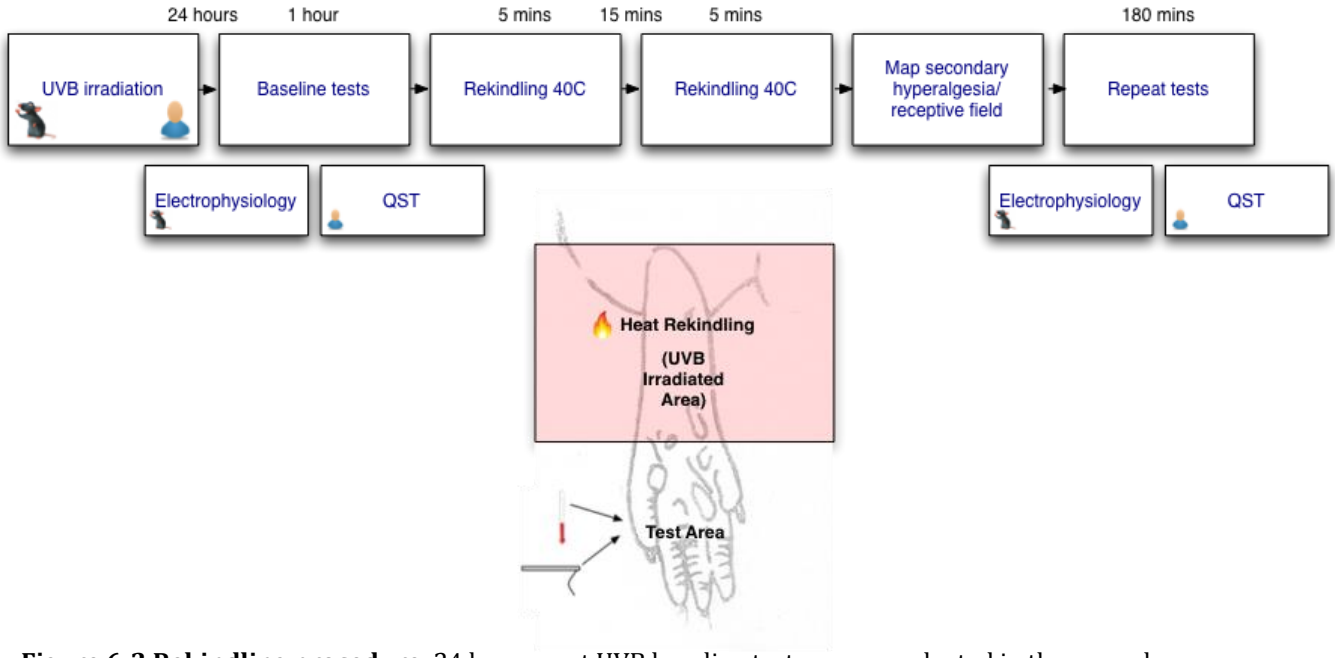

Figure 6-2 Rekindling procedure. 24 hours post UVB baseline tests were conducted in the secondary area, away from the site of irradiation. Two separate rekindling procedures were undertaken on the irradiated area, separated by 15 minutes.

6.2.3. Receptive field mapping:

Receptive fields on the plantar hindpaw were mapped for each cell with an 8g vF, using the methods detailed in (Suzuki et al. 2000). The stimulus was applied repeatedly around the area of baseline testing until firing was depleted below 0.5Hz. Applications were made at 30s intervals to ensure no wind up was elicited from the testing sequence. Receptive fields were

mapped both pre and post heat rekindling, before each round of testing. The observed receptive field was marked onto a standard diagram of the hindpaw and subsequently digitalised using a Canon MP610 scanner. The size of each receptive field was determined using ImageJ software and calculated as a percentage of the total area of the hindpaw.

6.2.4. UVB irradiation - humans:

Experiments were conducted in 10 healthy human volunteers aged between 22-32 years old. Individuals were familiarised with the experimental protocol beforehand and gave written, informed consent. The study was approved by The Kings College Research Ethics Committee.

All subjects were free from pain and medical conditions which may otherwise interfere with the results of the study. They were advised they must avoid pain medication such as NSAIDs and caffeine in the 24 hours prior to the study. This was particularly important as NSAIDs have been shown to be effective against the sensory changes elicited by UVB.

Volunteers were irradiated in a similar protocol as described for the animals. However, the dosing was calculated on an individual basis depending largely on skin type. An initial screening was conducted on each subject to determine their MED; this is defined as the time required to produce a uniform reddening of the area at 24 hours post irradiation. 3 times the MED was then used for the final experiment to irradiate an area of 16x16mm on the volar forearm, the surrounding area was covered with a UV resistant material to ensure uniform burn.

6.2.5. Heat rekindling- humans:

24-30 hours post UVB irradiation subjects returned for the heat rekindling procedure and full QST profiling. The procedure was carried out using the TSA thermal sensory testing device (TSA 2001-II; Medoc Ltd, Ramat Yishai, Israel), as used for the thermal testing in the QST protocol. The thermode (16x16mm) was placed directly over the UVB burn and held in place with a Velcro strap. The rekindling procedure carried out was the same as that described for the animals – the thermode was kept at 40°C for 5 minutes, followed by a 15 minute interval and a subsequent second rekindling identical to the first.

6.2.6. Mapping area of secondary hyperalgesia:

In line with the animal experiments, subjects were then tested immediately after the rekindling procedure, and every 30 minutes up to 180 minutes post rekindling. Prior to the rekindling the edges of the primary burn site had been marked on the skin and an acetate template was used to mark a spider probe map at 1cm increments along eight spokes (oriented at 45° intervals) radiating out from the primary area. After the rekindling procedure had been carried out subjects were assessed for the development of both pinprick hyperalgesia and dynamic brush evoked allodynia. Pinprick hyperalgesia was mapped using a 256mN probe (Pinprick, MRC Systems GmbH, Heidelberg, Germany. 0.2mm diameter) - an example stimulation was given on the contralateral arm in order for the subject to familiarise themselves with the sensation. Beginning at 8cm from the centre of the map the stimulation was repeated at 1cm intervals a long each spoke towards the treated area, and the subject was requested to report when this sensation changed. This was usually described as a sharper, or more intense pricking sensation. The stimulus was only applied once to each point, for around 1s. The point at which this change was reported was marked on a standard spider probe map diagram. Adjacent spokes were connected to create 8 triangles, for which the individual areas could be calculated; the summation of these, minus the primary area (256mm^2), gave the total area of secondary hyperalgesia.

6.2.7. Human Quantitative Sensory Testing:

Once the area of secondary hyperalgesia had been mapped full QST profiling was performed as previously described (Chapter 2.4) within this area of secondary changes, and as a control on the contralateral ventral forearm. In addition to the standard QST protocol, subjects were asked for numerical ratings (0-100) to 35°C, 40°C and 45°C. This was repeated every 30 minutes, up until 180 minutes post rekindling.

6.2.8. Statistical analysis:

All analysis was undertaken using SPSS software (IBM SPSS Statistics v21). Data was assessed for normality using the Kolmogorov-Smirnov test to determine further methods of analysis. Electrophysiological data was analysed using either an unpaired t-test or a 2 way ANOVA accordingly. Psychophysical data, with the exceptions of HPT and CPT, was logged and re-

tested for normality. A paired t-test or 2 way ANOVA was then carried out. HPT and CPT were found to be normal without logging, and thus the raw data was used for analysis with a paired t-test. All graphs were plotted to show the mean ± SEM.

6.3. Results

6.3.1. UVB Rekindling– In vivo electrophysiology

Using objective electrophysiological recordings, LV WDR cell responses to a range of natural and electrical applied stimuli were found to be significantly enhanced when compared to baseline responses in the same animal pre-rekindling (control). The initial baseline responses measured (control) were found to be no different to those recorded from a group of naïve animals with no treatment. (Note. It was not assessed whether the distance from the UVB treated area made a difference to the degree of sensitisation). These changes seen in the secondary area post rekindling are akin to the mechanical hypersensitivity observed in previous behavioural experiments (Davies et al. 2011). The UVB dose (1000mJ/cm2), rekindling procedure and time point (24-30 hours post irradiation) were selected from previous data (Bishop et al. 2007; Davies et al. 2011; Dawes et al. 2011), along with an initial pilot study which suggested these set parameters resulted in the maximal secondary hypersensitivity. These changes are most likely indicative of central sensitisation.

6.3.1.1. UVB rekindling significantly enhances both innocuous and noxious mechanically evoked WDR cell responses in the secondary area, in comparison to baseline responses

There is a patent coding of mechanical stimuli in both naïve, untreated animals and in the secondary area of treated animals pre-rekindling. The control responses recorded in UVB treated animals pre-rekindling are no different to those seen in naïve animals (figure 6-3). Since the UVB irradiation is confined to the upper half of the hindpaw, and testing takes place on cells with receptive fields confined to the lower half (secondary area), this suggests there is no, or very little, spread of inflammatory mediators from the primary irradiated zone. Post UVB rekindling coding is also seen to mechanical stimuli. These evoked responses to both innocuous and noxious vF were significantly increased (figure 6-3; $p< 0.000$). Neuronal responses to both low vF forces (2g, 8g and 15g) and higher vF (26g and 60g - which are usually considered noxious in behavioural experiments) were enhanced (figure 6-3; $p= 0.019$, < 0.000, 0.003, 0.055, 0.005). 26g and 60g vF responses were increased by 20.3% and 19.6% respectively, although this was only found to be significant for 60g (figure 6-3; p=0.055, 0.005). Furthermore, dynamic brush responses in the secondary area were also significantly enhanced by 42.8% from 351.1 ± 49.55 to 613.8 ± 44.72 action potentials/ 10s (figure 6-3; p=

0.002). All of these data are strongly suggestive of the engagement of central mechanisms, such as a facilitation of Aδ fibre responses, and unmasking of Aβ fibres.

6.3.1.2. UVB rekindling significantly enhances both innocuous and noxious thermally evoked WDR cell responses in comparison to baseline responses

Once again, there is a clear coding of WDR cell responses to increasing thermal stimuli, both pre and post rekindling. The baseline responses in the secondary area before rekindling are no different from responses recorded in naïve animals. Interestingly, an increased firing of WDR cells was observed in response to all temperatures tested post UVB rekindling (figure 6-4). The greatest increase was seen at 40°C, where a 40.9% increase in the firing was observed (figure 6-4; p= 0.006). Firing to supra threshold stimuli (45°C and 48°C) were also significantly enhanced by 32.6% and 28%, respectively (figure 6-4; p= 0.004, <0.000). Overall, there appeared to be a parallel shift in the stimulus-response curves.

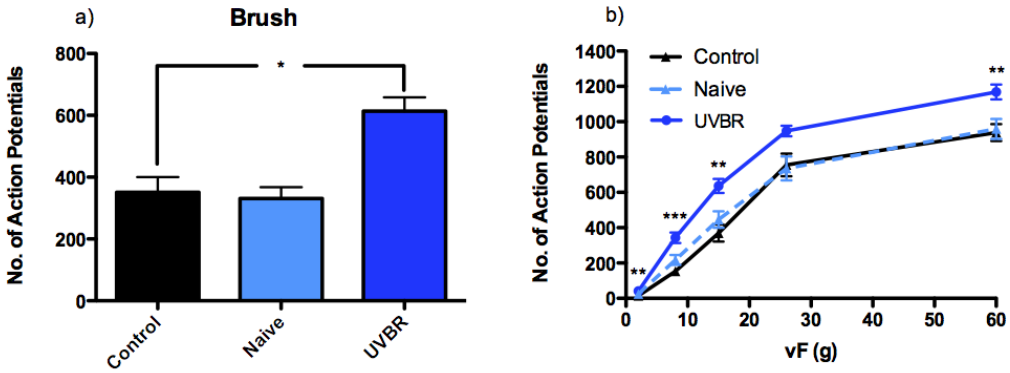

Figure 6-3 Effects of UVB rekindling on mechanically evoked WDR cell responses. Using the protocol described in chapter 6.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of mechanical stimuli, including brush and graded vF, applied to the receptive field for 10s, before and after UVB rekindling. Naïve values refer to animals with no treatment, whereas control values refer to pre UVB rekindling. Post rekindling treatment a) dynamic brush (p= 0.002), b) innocuous and noxious vF evoked responses were elevated when compared baseline controls (Overall 2-way ANOVA p< 0.000; 2g p= 0.019, 8g p< 0.000, 15g p= 0.003, 26g p= 0.055, 60g p= 0.005). n= 30.

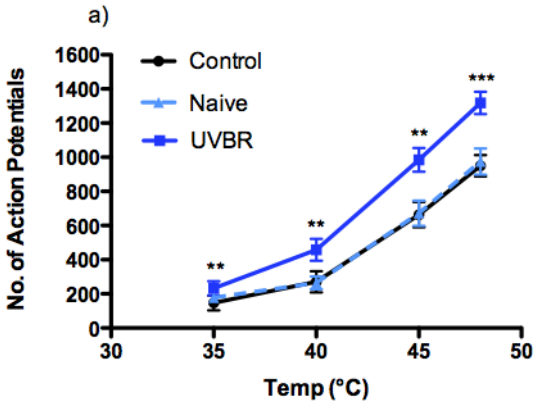

Figure 6-4 Effects of UVB rekindling treatment on thermally evoked WDR cell responses. . Using the protocol described in chapter 6.2 in vivo single unit recordings of LV WDR cells responses were recorded to a range of thermal stimuli applied to the receptive field for 10s, before and after UVB rekindling. Naïve values refer to animals with no treatment, whereas control values refer to pre UVB rekindling. a) Post rekindling, evoked responses to both innocuous and noxious temperatures were elevated when compared to pre-rekindling baselines (Overall 2-way ANOVA $p < 0.000$; 35°C $p = 0.005$, 40°C $p = 0.006$, 45°C $p = 0.004$, 48°C $p < 0.000$). n= 29

6.3.1.3. UVB rekindling significantly increases electrically evoked input and Aβ fibre responses recorded from WDR cells when compared to baseline responses

Overall, no significant difference was observed between UVB rekindled responses and baselines with regards to the number of action potentials elicited from Aδ and C fibres, as well as PD (figure 6-5). Conversely, electrically evoked input was significantly increased from 364.6 ± 56.3 to 761.8 ± 88.6 action potentials/ 10s, and responses in the Aβ fibre range were potentiated by 19.3% (figure 6-5; $p = 0.02$, 0.008). Furthermore, WU was significantly reduced, suggesting the central neurones were already in a state of hyperexcitability and thus responses were unable to be further enhanced (figure 6-5; $p = 0.035$). This measure is quantified by calculating the difference between the overall response observed and the baseline response to the first stimulus. Examination of the wind-up graphs reveals that the large increase in the initial responses in the rekindled group was responsible for the apparent reduction of wind-up. In fact, the neuronal responses started from a level that normally only would be elicited when wind-up is produced, strongly suggestive of enhanced central processing.

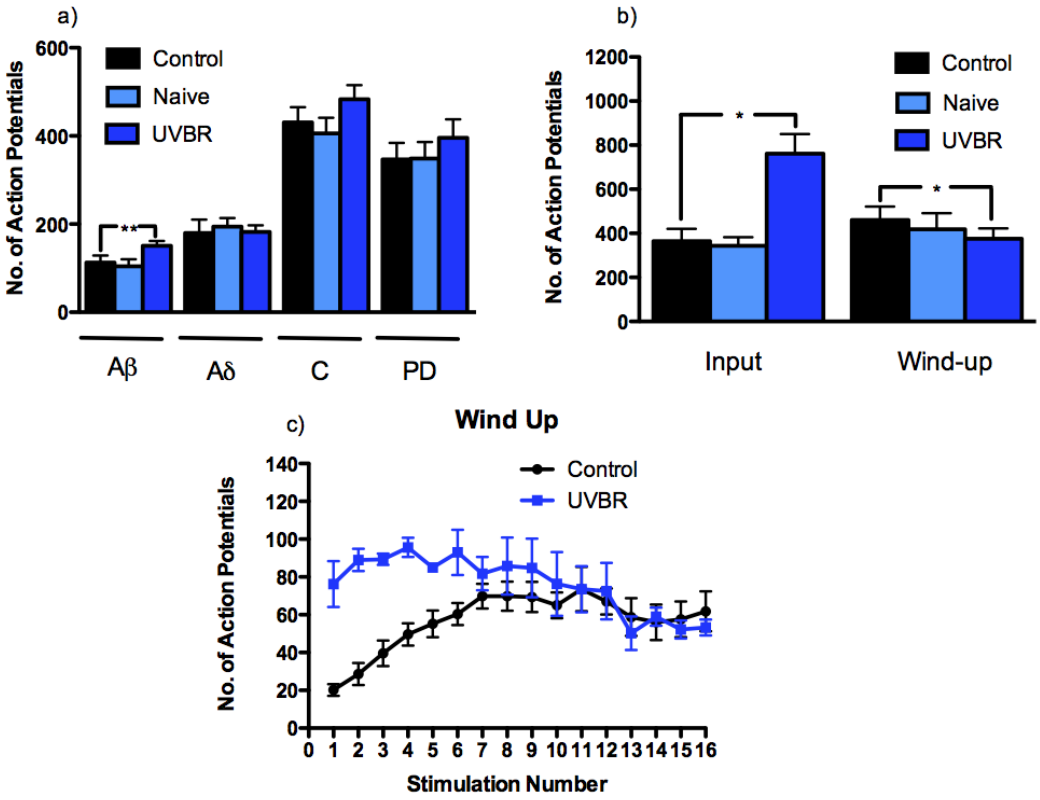

Figure 6-5 Effects of UVB rekindling on electrically evoked WDR cell responses. . Using the protocol described in chapter 2.2 and 6.2 in vivo single unit recordings of LV WDR cells responses were recorded to electrical stimuli pre and post UVB rekindling. Transcutaneous electrical stimulation was used to measure the input and wind up, in addition to calculating the responses driven by different fibre types – depending on the latency. Naïve values refer to animals with no treatment, whereas control values refer to pre UVB rekindling. Post rekindle y treatment a) there was no significant effect on electrically evoked Aδ mediated transmission nor post-discharge, however responses within the C fibre range appear to trend towards an increase (p= 0.08) and responses within the Aβ fibre range were significantly enhanced (p= 0.008); b) electrically induced input was also significantly increased (p= 0.02), whilst on the other hand, wind up appears decreased (p= 0.035). n= 30. c) Sample WU n=16

6.3.1.4. UVB rekindling significantly increases receptive field size of WDR cells in comparison to pre-treatment controls

Receptive field size was mapped using an 8g vF both before and after rekindling. As with humans, the mapping uses mechanical stimuli in order to avoid extra sensitisation that could occur with thermal stimuli. Furthermore, it is not frequently reported that patients suffer from extensive areas of thermal sensitisation outside of the area of injury. The receptive fields of LV WDR cells post rekindling treatment was found to be significantly larger, about 2-fold, than the average receptive field size measured in the same animals before the rekindling (figure 6-6; p=0.01). This is consistent with the evoked responses, which were also suggestive of a central sensitisation.

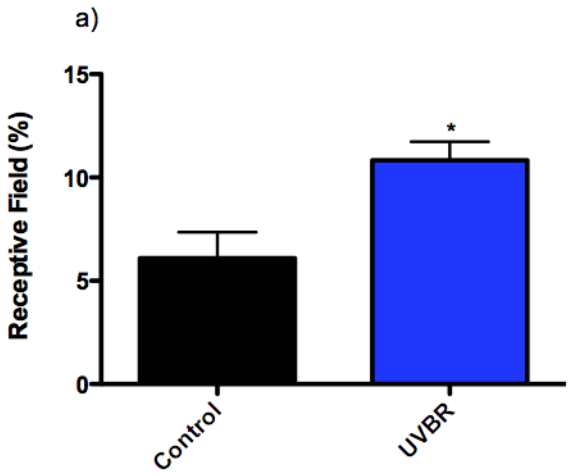

Figure 6-6 Effects of UVB rekindling treatment on receptive field size of WDR cells. Using the protocol described in chapter 5.2 the receptive field was mapped using an 8g vF filament. a) There was a significant increase in the size of receptive field in comparison to pre-treatment controls in the same animals (p= 0.01). n= 16

6.3.2. UVB Rekindling – Human Quantitative Sensory Testing

Using a standardised QST procedure, human subjects were also found to exhibit secondary sensory changes indicative of a central sensitisation post UVB rekindling treatment, including both mechanical and thermal hypersensitivity.

6.3.2.1. UVB rekindling significantly reduces MPT and increases numerical ratings to innocuous and noxious punctate stimuli

Similar to the rodent studies, post rekindling treatment, there was a significant drop in the average 50% pain threshold to pinprick stimulation from 103.98mN ± 15.99mN to 9.75 ± 0.28mN within the secondary, non irradiated area (figure 6-7; p< 0.000). Ratings to both sub and supra-threshold mechanical stimuli were also increased, whilst perceptual WU remained unchanged (figure 6-7; p< 0.000).

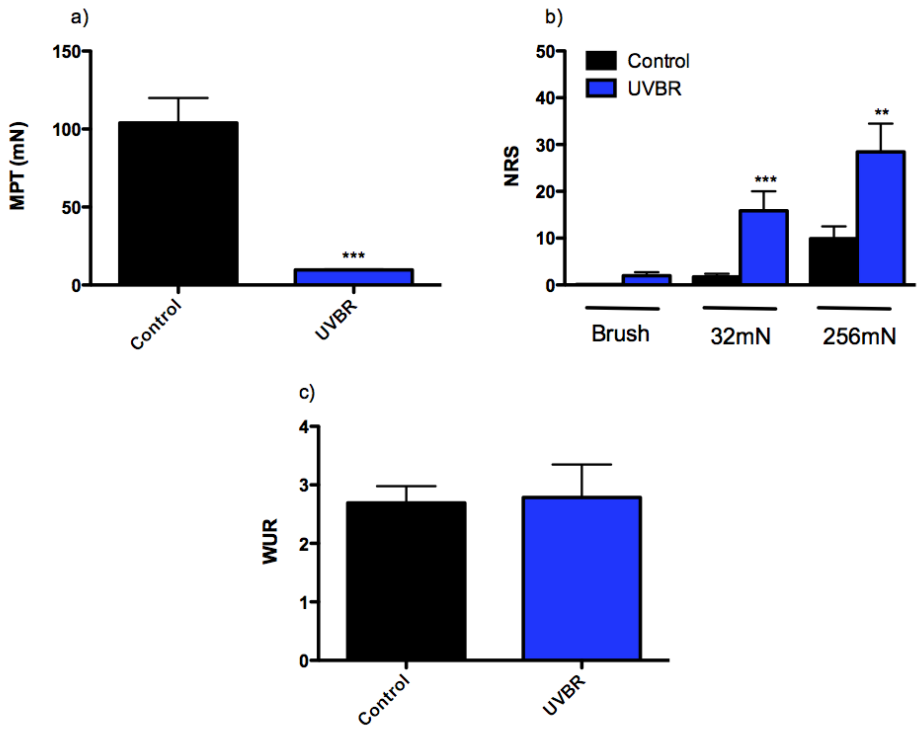

Figure 6-7 Effects of UVB rekindling on psychophysical MPT and mechanical NRS rating. Using the protocol described in chapter 6.2 standardised QST was undertaken to determine the subject's mechanical pain threshold (MPT), in addition to obtaining numerical ratings (NRS) to graded mechanical stimuli and measuring the wind up ratio (WUR) to repetitive mechanical stimulation. a) Average MPT was significantly lower in the secondary area post rekindling treatment in comparison to pre-treatment baselines (p< 0.000). b) NRS rating to dynamic brush were present in the secondary area unlike in normal skin, although this was not found to be significant. Ratings to both 32mN and 256mN were significantly increased (Overall 2 WAY ANOVA p< 0.000; 32mN p< 0.000, 256mN p= 0.004). c) Perceptual wind up was unaffected. n= 10

6.3.2.2. UVB rekindling induces a secondary thermal hypersensitivity

Average HPT was also significantly reduced in the secondary area from 45.0 ± 0.89°C to 40.3 ± 1.1°C (figure 6-8; p= 0.001). However, despite a trend in increased ratings to both sub and supra-threshold temperatures these were not found to be significant. Interestingly, a cold hypersensitivity was also observed, as average CPT was raised from 10.08 ± 3.45°C to 15.01 ± 3.18°C (figure 6-8; p= 0.003).

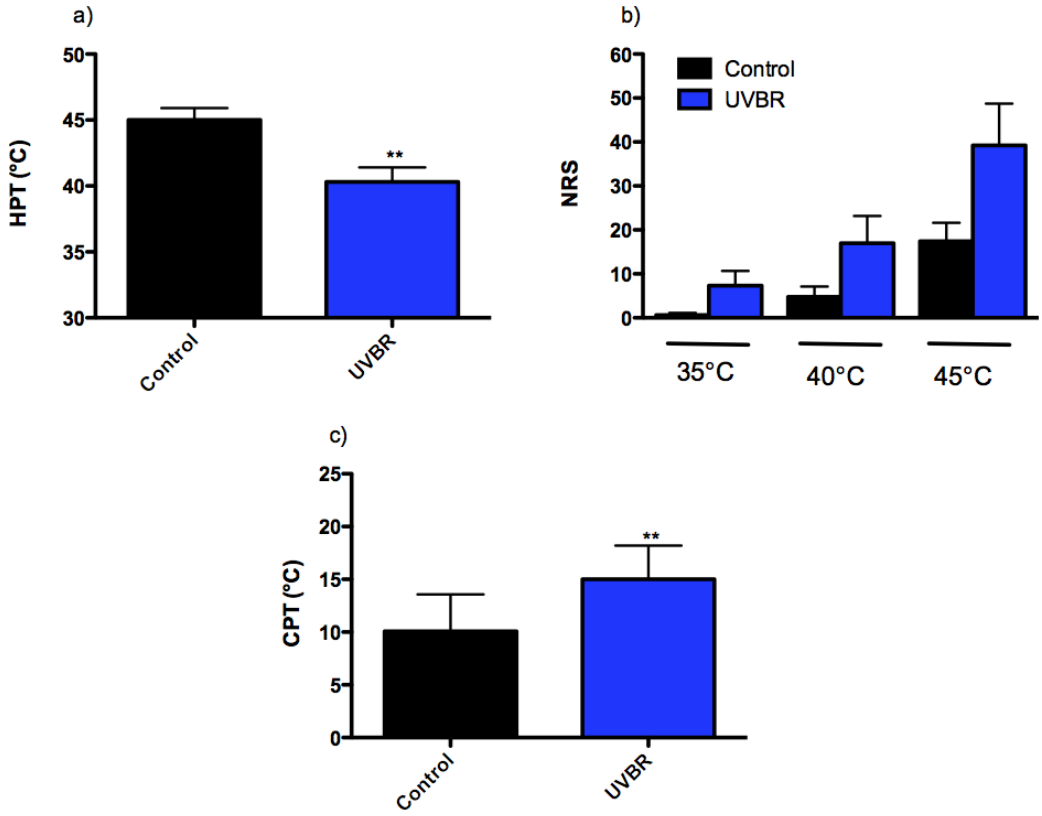

Figure 6-8 Effects of UVB rekindling on psychophysical HPT, thermal NRS ratings and CPT. Using the protocol described in chapter 6.2 standardised QST was undertaken to determine the subject's heat and cold pain thresholds (HPT/CPT), in addition to obtaining numerical ratings (NRS) to graded thermal stimuli. a) Average HPT was significantly reduced in the secondary area in comparison to pre-irradiation/rekindling baselines (p= 0.001). b) NRS ratings to previously innocuous and noxious temperatures appear increased, although this is not significant. c) CPT was also significantly elevated (p= 0.003). n= 10

6.3.2.3. UVB rekindling results in a considerable area of secondary hyperalgesia

The area of secondary hyperalgesia was assessed before testing the secondary area for sensory changes, using a 256mN probe. A large area surrounding the burn was reported by all subjects as being more sensitive than the percept to the stimulus in untreated skin (figure 6-9). Indicative of central sensitisation, underpinned by and expansion of receptive fields.

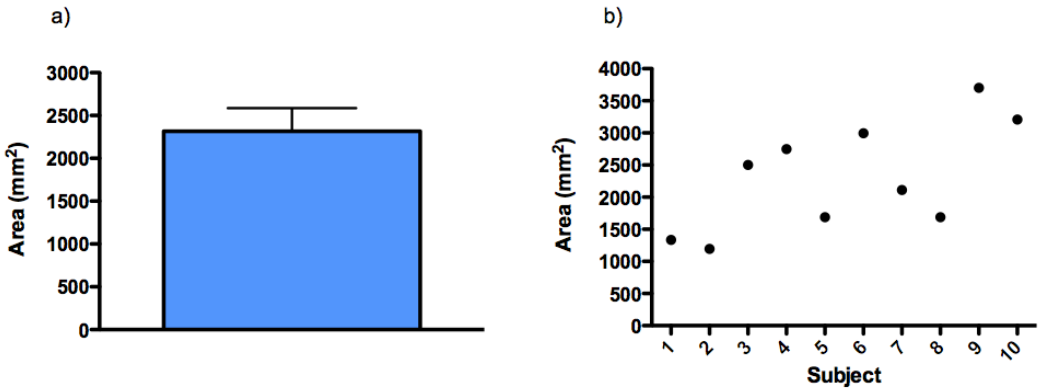

Figure 6-9 The area of secondary hyperalgesia induced by UVB rekindling. As described in chapter 5.2 the area of secondary hyperalgesia was mapped with a 256mN probe. a) Post rekindling a large area (2317.48 mm^2) of secondary hyperalgesia was observed in all subjects. b) The area is variable, yet considerable across all subjects.

6.3.2.4. Sensory profiles post UVB rekindling illustrate a largely mechanical hypersensitivity

Full sensory profiling using a standardised, comprehensive QST procedure confirmed a sensitisation in the secondary area across a number of modalities including: WDT, CPT, HPT, MDT, MPT, MPS and PPT. However, the mechanical hypersensitivity is much stronger than the other modalities (figure 6-10). Previous studies have observed pinprick hypersensitivity, however heat, cold and blunt pressure hypersensitivity represent novel findings of this study.

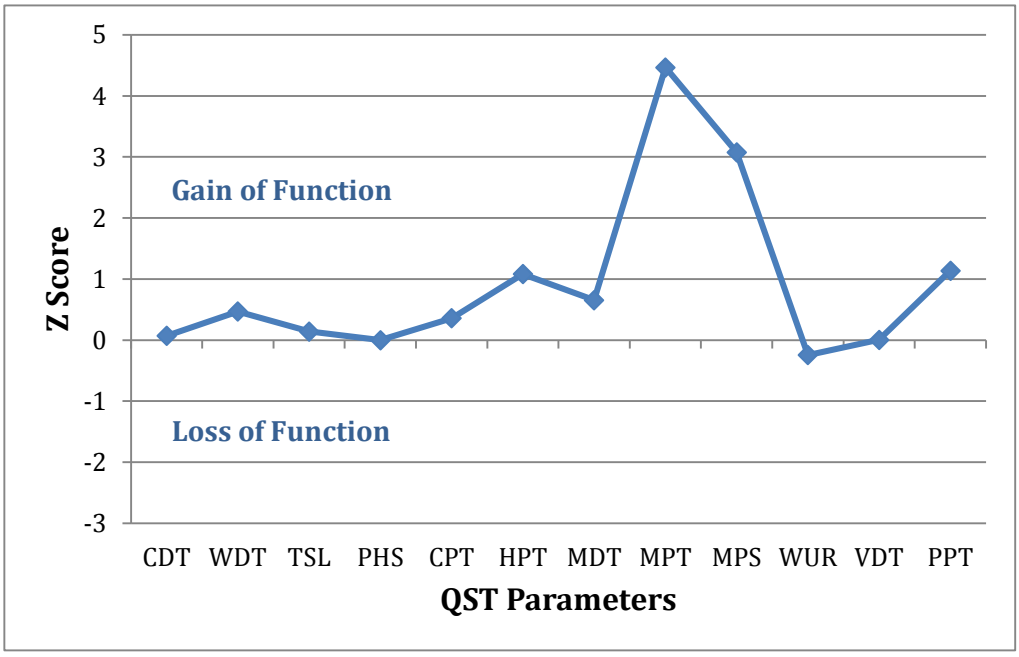

Figure 6-10 Somatosensory changes in UVB rekindled subjects. Using the protocol described in chapter 2.4 full QST profiling was undertaken. A variety of parameters were tested both pre and post UVB rekindling treatment, the magnitude of the changes are expressed here as Z-scores which highlight specific gains or loss in function. Hypersensitivity to warm, cold, heat, pinprick and pressure are demonstrated here by the gain of function in WDT, CPT, HPT, MPT, MPS and PPT..

6.3.3. UVB rekindling induced somatosensory changes in rats and humans show considerable overlap

Stimulus	Secondary Hypersensitivity	
	Animal	Human
Brush	✓	Non significant increase
Subthreshold Mechanical	✓	✓
Suprathereshold Mechanical	✓	✓
Subthreshold Thermal	✓	✓
Suprathreshold Thermal	✓	✓
Input	✓	✓
Wind up	Reduced	No change
Fibre count	Increase in Aβ	Not tested
Receptive field/ Area of secondary hyperalgesia	2-fold increase	2317.48 mm2

Table 6-1 Comparison of animal and human characterisation. a) There is a remarkable similarity in the sensory changes post UVB rekindling across species, highlighting the translational nature of this model. WDR receptive field size to 8g vF were increased post rekindling, and large areas of secondary hyperalgesia were noted in human subjects. Both animals and humans show heightened responses to mechanical stimuli and thermal stimuli in the secondary area. On the other hand responses to dynamic brush were not aligned as WDR evoked neuronal responses were increased, whilst the increase in human perception was not found to be significant. Fibre count was only assessed in the animal model, this term refers to a change in the number of action potentials elicited from each fibre type. The increase in action potentials elicited from Aβ fibres supports the theory that UVB rekindling results in central sensitisation, and may underpin the brush hypersensitivity observed in animals.

6.4. Discussion

In this novel study WDR cell responses were measured and full QST was undertaken on healthy human volunteers, post UVB rekindling. This is the first study to examine this model using these techniques and includes many previously unexplored end points. The key finding is that UVB rekindling is a reliable translational model of secondary hypersensitivity, producing similar changes reflective of maladaptive central modifications in animals and humans. In addition to confirming the previously reported secondary mechanical hypersensitivity, the novel observations made in this study include secondary thermal and cold hypersensitivity, in addition to changes in electrical responses and an expansion in receptive field size of WDR cells.

6.4.1. UVB rekindling produces a consistent secondary mechanical hypersensitivity in animals and humans, which can be measured from WDR cells, and with QST

This is the first full characterisation study post UVB rekindling using in vivo electrophysiology and QST. Rekindling treatment consistently led to a strong mechanical hypersensitivity in the secondary untreated area of both animals and humans. Evoked activity of LV spinal WDR neurones was enhanced to both dynamic brush and a range of mechanical von Frey forces. In the same way, in human volunteers, QST revealed a drop in MPT and increased numerical pain ratings to both sub and supra threshold stimuli. Thus the data provides objective assessment measures of the induction of secondary mechanical hypersensitivity, as reported in the initial behavioural studies (Cookson 2005; Wang 2005; Davies et al. 2011). In line with the WDR cell recordings here, the behavioural study found that post rekindling treatment von Frey withdrawal thresholds were significantly reduced and dynamic brush withdrawal responses were enhanced for up to 10 days (Davies et al. 2011). In agreement with the human QST the previous group also revealed large areas of pinprick hyperalgesia and touch allodynia (Cookson 2005). Overall, secondary mechanical hypersensitivities are clearly induced by the rekindling of UVB irradiated skin.

Since the receptive field of the cells are distal from the treated area, and MPT/ numerical ratings were performed in the area of mapped secondary hyperalgesia distant from the initial stimulated area, it is unlikely these changes can be explained by a peripheral sensitisation as seen with UVB irradiation alone. Rather, this enhancement of responses in the untreated area

is reflective of changes in the properties of central neurones. This is comparable with models such as intradermal capsaicin where it is well established that following a barrage of peripheral input, an increase in mechanical sensitivity is observed in the surrounding secondary area (LaMotte et al. 1991; Willis W.D 1997). The ongoing peripheral activity from the treated area is believed to sensitise WDR neurones through a number of homo- and heterosynaptic mechanisms. In a state of central hyperexcitability enhanced neurotransmission through activation of the NMDA and NK1 receptors leads to complex intracellular events involving phosphorylation, receptor trafficking and transcriptional changes (Latremoliere and Woolf 2009). Consequently, there is an increase in membrane excitability, increased synaptic strength and a reduction in spinal cord inhibition. As such the thresholds of spinal neurones are lowered and activation kinetics are altered. Subsequent stimulation of peripheral fibres in the surrounding untreated may then evoke action potentials of greater amplitude and frequency than under normal conditions, which is perceived by the subjects as more painful than before. For a full review of these events see Latremoliere and Woolf, 2009.

With regards to the model of UVB rekindling many of these mechanisms are likely to underpin the observed secondary hypersensitivity. It has been hypothesised that an area of secondary hyperalgesia may be the result of a barrage of input from afferents in the primary treated area, sensitising the second order neurones directly and these spinal cells may also synapse with afferents in the secondary area (LaMotte et al. 1991). However, since in the animal studies here WDR cells recorded from are selected on the basis that their baseline receptive field is outside of the area of UVB irradiation this rather simplistic model cannot fully explain the changes seen. Rather, when the rekindling procedure is undertaken the ongoing activity initially results in a sensitisation of second order neurons with direct synaptic input, i.e. those with receptive fields in the primary irradiated area. Through a process of volume transmission of neuropeptides such as substance P the surrounding second order neurones may also become sensitised (Sandkühler 1996). Secondly, a loss of inhibition through interneurones and descending inhibitory controls could also contribute towards this widespread sensitisation. Finally, it is possible that WDR cells recorded from have afferent fibres sitting within a low probability firing fringe which encompasses the primary rekindling zone. Whilst under normal conditions stimulation of these afferent fibres may not produce an action potential the rekindling procedure and subsequent central sensitization may result in a summation of EPSPs that does lead to the generation of action potentials and subsequent

sensitisation of the WDR cell. Any combination of these mechanisms could contribute towards in the secondary changes observed in both the animals and humans.

A number of models of chronic pain also report similar changes in animal and human experiments. The enhanced firing of LV WDR cells to both punctate mechanical stimulation and dynamic brush seen here are also observed within the untreated area after intraplantar injection of capsaicin (Simone et al. 1991; T. K. Baumann 1991; Willis W.D 1997). Whilst in humans intradermal capsaicin is known to result in a large area of secondary pinprick hyperalgesia, in addition to the less frequently reported development of brush hypersensitivity (LaMotte et al. 1991; Park et al. 1995; Magerl et al. 1998). Intra-articular injection of CFA and the MIA model of OA are also known to evoke similar electrophysiological changes, whereby an increase in the magnitude of responses of DH neurones to mechanical stimuli applied outside the initial area of treatment is observed (Martindale et al. 2007; Rahman et al. 2009; Burnham 2012). These models are all confirmed to have central components and therefore given that they induce similar changes to those observed here post rekindling, it can be inferred they may evoke overlapping central mechanisms.

This synaptic plasticity involves engagement of different fibre types, which lead to the development of the distinct symptoms observed in this study such as static pinprick and dynamic brush hypersensitivity. Since pinprick hyperalgesia can be induced in the absence of Aβ fibres, but a complete block of A fibres abolishes this symptom, it has been assumed that Aδ fibres are the key mediators of hypersensitivity to this modality (Torebjörk et al. 1992; Treede and Cole 1993; Ziegler et al. 1999). On the other hand it is the large Aβ fibres that are thought to conduct brush evoked hypersensitivity. As mentioned, the development of secondary pinprick hyperalgesia post capsaicin has been observed in a subject found to suffer from a large-fibre sensory neuropathy, however allodynia could not be evoked (Treede and Cole 1993). Therefore suggesting that Aβ fibres mediated brush evoked allodynia. Enhanced responsiveness of DH neurones to this low threshold Aβ inputs is believed to underlie the phenomenon (Simone et al. 1991).

To assess contribution of central mechanisms and fibre types a number of methodologies could be employed. It is possible to inhibit A fibre conduction through a superficial radial nerve block and if pinprick hyperalgesia is unable to develop this would confirm the dependency of this symptoms on A fibres. Additionally, as previously mentioned, the

induction of this enhanced responsiveness of spinal cord neurones is believed to be activity dependent, that is to say it triggered by the ongoing input into the spinal cord (McMahon et al. 1993; Baron et al. 2013). Since secondary hyperalgesia resulting from intradermal capsaicin can be reduced by pretreatment with systemic or local lidocaine, it can be inferred that the induction of the symptom is indeed dependent on the peripheral drive (Dirks et al. 2000). It is likely that rekindling the UVB treated area also results in a strong peripheral drive that is able to alter spinal processing. To further investigate this hypothesis, it would be interesting to treat both animals and humans with lidocaine before the rekindling procedure is undertaken in order to confirm the contribution of this mechanism to the induction of the changes reported here. NK1 antagonists could also be used to assess the contribution of substance P in the induction of this hypersensitivity.

However, the most important finding here is the induction of secondary mechanical hypersensitivity to pinprick and brush across species. In line with the preliminary results from Cookson and Davies previously discussed, the findings of this study also highlight the similarities in changes evoked by the model in both animals and humans (Davies et al. 2011). Notably, recordings from WDR cells correlate particularly well with human QST.

Furthermore, these results validate the ability to measure secondary hypersensitivity from activity evoked in WDR cells under anaesthesia. As discussed, enhanced responses have also been shown in alternative models engaging central mechanisms, such as intradermal capsaicin, CFA and the MIA model of OA (Willis W.D 1997; Martindale et al. 2007; Rahman et al. 2009; Burnham 2012). Thus suggesting that WDR cells in the spinal cord, the primary relay site of somatosensory information, are useful for studying the consequences and further characterisation of such models.

6.4.2. Thermal hypersensitivity is observed in the secondary area in animals and humans post UVB rekindling

One of the most interesting and unexpected observations in this study was the presence of a small, yet significant, degree of thermal hypersensitivity in the secondary untreated area in both animals and humans. There is a general consensus that thermal hypersensitivity is the result of a peripheral sensitisation, and it is not present in areas of secondary hyperalgesia. In fact evidence from most studies investigating the consequences of central sensitisation is

controversial, with the majority suggesting that there is no increase in behavioural or electrophysiological responses to thermal stimuli (Lewis 1942; LaMotte et al. 1991; Serra et al. 1998; Sumikura et al. 2003).

On the other hand, a generalised trend for increased firing of LV WDR cells across modalities, including thermal responses, is also seen in both the early and late phases of the MIA model of OA (Rahman et al. 2009; Burnham 2012). This model is believed to evoke mainly central changes since MIA is injected into the knee and testing may be conducted on the hindpaw, which is therefore not associated with any peripheral damage (Vonsy et al. 2009; Thakur et al. 2012). However, since the enhanced central processing is driven by ongoing activity from a site of peripheral inflammation similarities may be drawn between this model and the rekindling paradigm. The heat hypersensitivity has also been observed in patients suffering from OA, and therefore suggests that secondary thermal hypersensitivity may exist in such conditions (Kosek and Ordeberg 2000). Serra and colleagues also firmly believe that hypersensitivity at least to suprathreshold stimuli may exist, reporting large areas of heat hyperalgesia post intradermal capsaicin (Serra et al. 1998). Whilst Chen and colleagues found that a novel model of chronic pain induced by melittin – a protein found in honeybee venom – is also able to induce a secondary thermal hypersensitivity (Chen and Chen 2000; Sumikura et al. 2003; Sumikura et al. 2006). Therefore, although the topic is controversial and the evidence is limited it appears there is a reasonable argument for the development of this symptom within a site distal to the initial injury.

This is the first reporting of this somatosensory phenomenon being associated with the UVB rekindling model, and therefore can only be compared with similar preclinical models. The model to best compare these results to is the early phase of MIA induced OA. This early inflammatory phase is underpinned by peripheral sensitisation of the joint afferents, with a barrage of ongoing input into the DH leading to a referred hypersensitivity driven by central mechanisms (Vonsy et al. 2009; Thakur et al. 2012). Therefore it is not dissimilar to the rekindling model, and is logical they may evoke similar changes. Confirming that in a state of hyperexcitability, driven by ongoing activating from an area of peripheral inflammation, secondary thermal hypersensitivity may result.

Thinking back to the cellular mechanisms underpinning the central sensitisation involved in these models, it does not seem implausible that a generalised increase in activity across modalities could be induced in either of these models. Heterosynaptic central sensitisation

results in a number of cells that receive input distal from the area of injury in the spinal cord becoming hyperexcitable. In fact, since the WDR neurones recorded have polymodal inputs, it would be expected that any sensitisation of these cells would lead to an enhancement of both mechanical and thermal stimuli. Indeed, both types of responses were similarly enhanced, both below and above the pain threshold, suggestive of post-synaptic changes.

It is possible to further investigate the presence of this symptom and the supporting mechanisms through pharmacological interventions. If it is true that thermal hypersensitivity results from a central sensitisation it stands to reason that the development should be inhibited with drugs targeting such central mechanisms. There are a number of approaches which could be adopted to interfere with the cellular processes underpinning central sensitisation, but as discussed they could include the use of MK-801, or an NK-1 antagonists. Alternatively spinal PKA/C inhibition could be a useful indicator of the mechanisms underpinning this phenomenon. Finally an assessment of the status descending controls could also be useful to both confirm the presence of this symptom and highlight the underlying cause.

The hypersensitivity seen in the MIA model does in fact appears to be partially dependent on a shift in descending controls. By blocking the $5HT_3$ receptor, secondary noxious thermal hypersensitivity may be alleviated (Rahman et al. 2009). This possible increase in descending facilitation arises in the RVM and also appears to be active in the model of topical mustard oil. Whilst ON cells discharge in response to mustard oil application, OFF cells decrease their firing. In parallel to this appears the development of secondary heat hypersensitivity characterised by an increase in paw withdrawal latency, which can be inhibited by blocking ON cell activity (Xu et al. 2007). These studies suggest that an increased facilitatory drive may be required for secondary thermal hypersensitivity. It would be interesting to explore the contribution of descending modulation in the model of UVB rekindling, however given that these models are both associated with an acute inflammation leading to central modifications it is possible similar mechanisms may also be engaged post rekindling.

6.4.3. UVB rekindling results in a secondary cold hypersensitivity in human volunteers

There is a clear increase in CPTs measured in the secondary area post UVB rekindling. This phenomenon has previously been reported post UVB in the primary irradiated area, however

this is the first study to find it present in the area of secondary hyperalgesia (Gustorff et al. 2013). An increased withdrawal to acetone in both the early and late phase of MIA induced OA is also suggestive of a hypersensitivity to cooling stimuli (Vonsy et al. 2009; Burnham 2012). Furthermore, this phenomenon has also been observed in patients with OA (Kosek and Ordeberg 2000). This evidence corroborates the finding here that a cold hypersensitivity may develop in an area distal to the main site of inflammation. Given that it has also been observed in patients this is an important finding with high clinical applicability.

The mechanisms for cold hypersensitivity are quite unclear and from the studies conducted in this thesis there appears to be a role for both peripheral and central sensitisation. However, as discussed at length in this chapter, since the symptom is observed in the secondary area in this case it is most likely of a central origin, involving the mechanisms previously mentioned. It is important to further explore this sensory occurrence since cold hypersensitivity is a symptom not only associated with OA, but is also present in conditions such as oxaliplatin-induced neuropathy (Kosek and Ordeberg 2000; Binder et al. 2007). The identification of a stable model of cold hypersensitivity in humans has been rather illusive and therefore this finding is important for future studies wishing to assess cold hypersensitivity.

6.4.4. UVB rekindling potentiates Aβ fibre responses

A potentiation of responses in the Aβ fibre range was observed in LV WDR neurones post rekindling treatment. This finding would certainly help explain the increased responses of WDR cells to dynamic brush and subthreshold vF, as well as the increased NRS ratings to non-painful mechanical stimuli post rekindling treatment. Indeed it is believed that an enhanced responsiveness of DH neurones to low threshold inputs contributes to dynamic brush hypersensitivity/ allodynia (LaMotte et al. 1991; Torebjörk et al. 1992). Administration of GABA or glycine antagonists results in the recruitment of Aβ fibre input and thus it is thought that the loss of inhibition associated with central sensitisation results in this enhanced responsiveness to large fibres (Baba et al. 2003). This recruitment of Aβ fibres is often thought of as a novel input to the nociceptive pathways, leading to Aβ fibre mediated pain. However, since WDR cells have a small Aβ fibre input under normal conditions it is difficult to interpret this finding with regards to chronic pain. It could be hypothesised that normally this low level of input from Aβ fibres, which is considerably smaller than Aδ and C fibre input, is

somehow filtered out in higher centres and does not result in the conscious perception of pain. However, the recruitment of more fibres and a greater Aβ input may result in the surpassing of a 'threshold' in order for the messages to be perceived as painful.

Inflammation alone may result in a release of neuropeptides from large fibres and a subsequent increase in Aβ fibre input in the DH (Ma and Woolf 1996; Baba et al. 1999). Indeed after UVB irradiation there is a clear increase in brush evoked responses, as found in chapter 5. It therefore appears likely that this potentiation is initiated after UVB inflammation and is further induced and maintained by the rekindling procedure. This treatment most likely engages central mechanisms such as a reduction in spinal inhibition and thus enhanced Aβ fibre input. As suggested, this recruitment of Aβ fibre may lead to the generation of activity such that is passes a threshold and is no longer 'filtered out' by higher centres in the pain pathway. Therefore the potentiated responses in the Aβ fibre range observed here, may explain the brush hypersensitivity/ allodynia that is also induced by the model.

6.4.5. Secondary electrical hypersensitivity is induced by UVB rekindling

The increased input observed post rekindling is most likely the result of the lowering in threshold of the WDR cell recorded from. As discussed, through a number of mechanisms such as volume transmission and disinhibition the cells that are recorded from may become sensitised. This results in both a reduction in activation threshold and an increase in response to noxious stimuli. The electrical responses of WDR cells are the result of stimulation within the receptive field to 3 times the C-fibre threshold. The threshold is obtained during baselining and most likely will drop post rekindling. However, since re-thresholding is not conducted during the experiment in order to compare the results to baseline responses and reduce confounds, it is likely that the newly sensitised cell will have a greater response than at baseline. Given that the receptive field of the cell is distal to the rekindling, it is unlikely that this increase in input is reflective of a peripheral sensitisation.

A reduction in pain threshold to intraneural micro stimulation in the secondary area is observed post capsaicin injection (Torebjörk et al. 1992). Furthermore, comparing this model once again to the early phase of MIA induced OA, an increase in input is seen in the early and late phase (Burnham 2012). Suggesting that inflammation associated with spontaneous ongoing activity results in a central sensitisation and heightened sensitivity of spinal

neurones to electrical stimulation. Electrical stimulation bypasses the traditional transduction machinery that is the receptors located on the afferent terminals. Therefore electrical hypersensitivity could result from a peripheral or central sensitisation, however as discussed, in this case it is most likely a reflection of an enhanced central processing.

6.4.6. Spinal WU is not enhanced post UVB rekindling, and human perceptual WUR remains unchanged

The ability to wind up is a key distinguishing feature of WDR cells, primarily located in the deep DH. Under normal conditions repetitive stimulation of C fibres at a low frequency (0.3-2 Hz) can result in the progressive potentiation of WDR cell responses. Wind up is believed to be a homosynpatic event, whereby a repetitive activation of the peptidergic C fibres results in the release of substance P and CGRP onto neurones synapsing with the peripheral fibres activated. These neuropeptides produce slow EPSPs that allow the removal of the Mg^{2+} block of NMDA receptors. Indeed by blocking this receptor the ability of a cell to wind up is lost (Dickenson and Sullivan 1987; D'Mello et al. 2011).

Post rekindling, there is an enhanced input which results in a decrease in WU, although examination of the AUC reveals an overall enhancement of around 25%. Once again, this is most likely due to the increase in input. The overall total number of action potentials reached is the same both pre and post rekindling. It stands to reason that any given cell must have a maximum capacity – that is to say, it reaches a maximum level of discharge and regardless of an increasing peripheral input and further recruitment of NMDA receptors it is unable to produce a greater amount of action potentials. This mechanism is shared with central sensitisation, which has also been shown to require this receptor (Woolf and Thompson 1991). Thus, if central sensitisation is induced by a particular model, engaging the NMDA receptor and inducing a state of hyperexcitability in spinal cord neurones, it may be expected that their ability to wind up is reduced as the receptor is already close to capacity. That is to say, since they both rely on the same receptor, it may not be possible for the two to occur in tandem. Given that wind up was reduced post rekindling, this may also suggest the engagement of central sensitisation in the model.

Perceptual wind up is the human correlate of a similar engagement of temporal summation. Repetitive low frequency stimulation with a pinprick device also results in an increased pain

rating for the final stimuli, with respect to the first. Dependence on C fibres and NMDA receptors is also a feature of this psychophysical correlate of wind up (Price et al. 1994). However, unlike in the animal experiments, there was no change observed in perceptual wind up post rekindling. It is possible that overall, in a state of central hyperexcitability only some cells would reach capacity, while others are simply primed by unblocking of NMDA receptors are may in fact wind up more readily. The balance of these two opposing mechanisms may explain why overall there is no change in the human psychophysical correlate.

6.4.7. Expansion of receptive fields and notable areas of secondary pinprick hyperalgesia are apparent post UVB rekindling

In this study there is a clear enlargement of receptive fields post rekindling treatment. The receptive field is a malleable feature of WDR cells due to the synaptic plasticity of the spinal cord. Under normal conditions, only a fraction of synaptic inputs terminating in the DH will contribute towards the generation of action potentials as many simply result in subthreshold EPSPs (Woolf and King 1989). These neurones sit within what has been described as a low-probability firing fringe. However, in a state of central hyperexcitability increased synaptic efficacy leads to recruitment of subthreshold inputs. Models confirmed to induce central sensitisation often report expanded receptive fields, such as mustard oil, SNL, CCI and chronic inflammation (Woolf and King 1990; Ren et al. 1992; Grubb et al. 1993; Cumberbatch et al. 1998; Suzuki et al. 2000). Therefore the ability of previously subthreshold EPSPs to generate action potentials seen in this model is most likely reflective of altered central processing as described.

In humans the development of a large area of secondary hyperalgesia is also reflective of changes in properties of central neurones. Ongoing input during the rekindling procedure most likely results in an increase in membrane excitability, increased synaptic strength and a reduction in spinal cord inhibition. As such the thresholds of spinal neurones are lowered and activation kinetics are altered. Subsequent stimulation of peripheral fibres in the surrounding untreated may then evoke action potentials of greater amplitude and frequency than under normal conditions, which is perceived by the subjects as more painful than before. Secondary hyperalgesia most likely reflects both the lowering of activation thresholds and recruitment of neurones that previously sat within the firing fringe, in addition to the increased activity of spinal neurones.

Although an expansion of receptive fields and the development of secondary hyperalgesia are not directly comparable, they are both indirect measures of the induction of central sensitisation and corroborate the theory that this state has been evoked in both animals and humans. Overall suggesting that this model induces a robust central sensitisation across species and may be useful for examining the relevant mechanisms involved, and testing new treatments for chronic pain.

6.4.8. UVBR QST Profile

The QST profile of subjects undergoing UVB rekindling treatment highlights a generalised gain in function of the nociceptive system. As expected there is a clear hypersensitivity to mechanical stimuli such as vF, pinprick and brush. Novel findings include the heightened warm detection, cold and heat hypersensitivity and a sensitisation to pressure. Given that all testing is undertaken within the area of mapped secondary hyperalgesia, these increased responses are most likely reflective of enhanced central processing as described above.

These changes are similar to those seen in OA patients where QST has revealed hypersensitivity to cold, warm, heat, and pressure pain. Furthermore, in agreement with this study despite the cold hypersensitivity there was no difference in cold detection thresholds in patients. All of the abnormalities were found to return to normal after surgery (total hip replacement or osteotomy), suggesting that in this group of patients they had been maintained by an ongoing afferent input, and any changes in the spinal cord were reversible (Kosek and Ordeberg 2000). The similarities between symptoms of OA patients and those observed in this model of UVB rekindling suggest that similar mechanisms may be involved. Indeed, it has already been raised that the most analogous preclinical model appears to be is the MIA model of OA. The advantage of the UVB rekindling model over MIA is simply that is can be induced over a period of 24 hours and it suitable for use in both animals and humans and therefore may have greater translational relevance.

6.4.9. Somatosensory changes observed post UVB rekindling are reflective of altered central processing

In order to induce central sensitisation, a stimulus must be intense, repetitive and sustained. This input leads to an enhancement in the functional status of neurones in addition to altered

circuitry, through increases in membrane excitability, synaptic efficacy, or reduced inhibition (Latremoliere and Woolf 2009). Neuronal and perceptual correlates of central sensitisation include spontaneous activity, threshold reduction, increased responses to suprathreshold stimuli and enlarged receptive fields. Overall, these experiments provide objective evidence of reduced thresholds, increased responses to suprathreshold stimuli in the secondary untreated area, in addition to enlarged receptive fields and large areas of secondary hyperalgesia in animals and humans, respectively. Suggesting that the activation of sensitised afferents during UVB rekindling results in an input of an adequate intensity and frequency to induce changes in properties of central neurones and is thus a robust translational model of central sensitisation.

As previously mentioned, a peripheral sensitisation results in an increase in afferent input and thus may indirectly lead to central sensitisation. In this model the pre-sensitised peripheral afferents create barrage of input into CNS upon rekindling. Under normal conditions it is thought that in order to induce a state of central sensitisation a temperature of above 49°C is required, however since PAFs are already sensitised, it appears that a stimuli of 40°C is able to generate action potentials of same frequency and amplitude as 49°C – as was observed in chapter 5 (Latremoliere and Woolf 2009). Processes underlying central sensitisation likely to be involved in this model include NMDA receptor activation as a result of direct ongoing activating into the spinal cord, or from the summation of EPSPs from the firing fringe such that an action potential is generated from outside of a given cells receptive field. Further heterosynaptic mechanisms likely to be involved in this secondary hypersensitivity include volume transmission of neuropeptides and a disinhibition of interneuron's and descending controls. Engagement of any number or combination of these mechanisms may lead to the changes observed post rekindling.

To further examine the extent of central mechanisms engaged in this model a number of pharmacological modulations could be employed. From an anaesthetic block at site of peripheral injury, to an NMDA receptor block and an examination of the contribution of descending controls. If the underpinning mechanisms are truly of central origin, an anaesthetic block at the site of peripheral injury would be unable to reduce the expansion of receptive fields, and unlike UVB irradiation alone, the model should show sensitivity to NMDA receptor antagonists such as MK-801 (Bishop et al. 2010). Furthermore, since it is well known

that changes in descending controls contribute towards changes in properties of central neurones it would be interesting to investigate what role, if any, they play in this model.

Central sensitisation is a cardinal feature of chronic pain and contributes to a number of conditions, including both neuropathic and inflammatory pain, migraine and IBS (Latremoliere and Woolf 2009). It is therefore essential to be able to model this phenomenon effectively, to understand the mechanisms for induction and maintenance and to assess the pharmacological sensitivity. It is important to note that central sensitisation is made up of two distinct phases, and whilst the early phase is phosphorylation-dependent (changes in receptor and ion channel properties), the latter is transcription-dependent (synthesis of new proteins) (Woolf and Salter 2000). It is believed that the latter is more relevant to patients, and thus it will be important to assess the contribution of both to fully understand the true clinical meaningfulness of the model.

6.5. Concluding remarks

Overall using objective characterisation methods, UVB rekindling appeared to be a reliable translational model of secondary hypersensitivity, evoking similar phenotypic changes in both animals and humans, which can be measured using electrophysiology and QST.

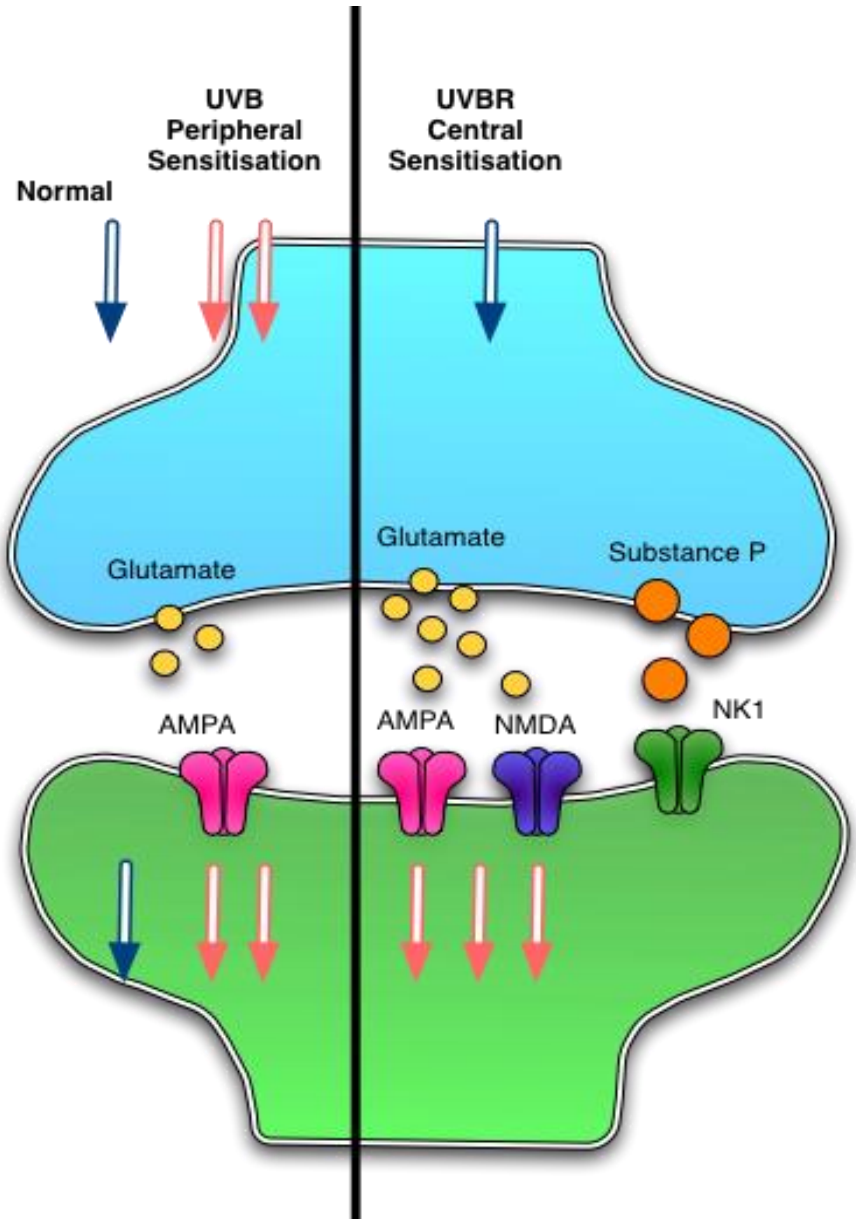

Figure 6-11 The UVB Rekindling Model. It is believed that UVB irradiation leads to a peripheral sensitisation, which increases afferent activity into the DH and thus the hyperexcitability can be recorded from WDR neurones (left panel). When recording from WDR neurones with receptive fields outside of the treated area, heat rekindling increases evoked responses. This is indicative of the development of central sensitisation, involving release of neuropeptides such as substance P and recruitment of the NMDA receptor (right panel).

7. General Discussion

7.1. Translational models induce similar signs and symptoms in animals and humans

This thesis aimed to characterise translational models of chronic pain, in order to bridge the gap between basic science and the clinic, and to address the disparity between the animal and human preclinical models that are in current use. Furthermore, the studies were conducted using similar outcome measures in order to draw comparisons between the animal and human data, whilst exploiting the advantages of each. The development of translational models is imperative since such models can be used not only in basic mechanistic studies, but also in pharmacological studies to assess analgesic efficacy (Arendt-Nielsen and Yarnitsky 2009).

All of the models explored in this thesis were able to induce signs and symptoms of chronic pain in both animals and humans. Table 7.1 below summarises the changes that were observed across species in all 3 models. Overall in each of the studies it became clear that the models were able to induce analogous changes in the animal and human subjects, suggestive of the induction of comparable underlying mechanisms. Thus, highlighting their potential as surrogate translational models.

The topical capsaicin model had previously been used on the whole in human subjects (Carpenter and Lynn 1981; Koltzenburg et al. 1992; LaMotte et al. 1992); therefore this thesis assessed the translational implications in animals. Using DH single unit in vivo electrophysiology and QST it is clear that within the primary area of treatment, capsaicin is able to induce a strong thermal and mechanical hypersensitivity across species. Pinprick hyperalgesia in human subjects was complemented by a facilitation of Aδ fibre responses in animals. These results are suggestive of peripheral sensitisation of TRPV1/ C fibres, in addition to central modifications most likely driven by the afferent barrage of activity during the application of topical capsaicin.

Conversely, the model of UVB irradiation had previously been explored in both animals and humans. However, these studies did not include a full objective characterisation (Gustorff et al. 2004; Bishop et al. 2007). Therefore, this thesis provided the first objective evidence of primary mechanical and thermal hypersensitivity in animals. The lack of receptive field expansion and negligible area of secondary hyperalgesia in human subjects provide further proof of this model inducing dominant peripheral changes.

Finally, very little data previously existed with regards to the model of UVB rekindling (Cookson 2005; Davies et al. 2011). This thesis provided the first fully translational study to reveal the pattern of secondary hypersensitivity in animals and humans. Hypersensitivity was apparent to both thermal and mechanical stimuli. Brush evoked allodynia was complimented by the facilitation of Aβ fibre responses in animals. Finally, the large expansion in receptive fields and area of secondary hyperalgesia in humans confirmed these changes were most likely underpinned by secondary changes.

Stimulus	Capsaicin Induced Hypersensitivity		UVB Induced Hypersensitivity		UVBR Induced Secondary Hypersensitivity	
	Animal	Human	Animal	Human	Animal	Human
Brush	✓	✓	✓	No change	✓	Non
Subthreshold	✓	✓	✓	✓	✓	✓
Suprathereshold	No change	✓	✓	✓	✓	✓
Subthreshold Thermal	✓	✓	✓	✓	✓	✓
Suprathreshold Thermal	✓	✓	✓	✓	✓	✓
Input	✓	✓	✓	✓	✓	✓
Wind up	No change	No change	No change	No change	Reduced	No change
Fibre count	Reduction in C fibre, increase in Aδ	Not tested	Reduction in C fibre threshold	Not tested	Increase in Ab	Not tested
Receptive field/ Area of secondary hyperalgesia	Not tested	Not tested	No change	No change	2-fold increase	2317.48 mm2

Table 7-1 Comparison of the symptoms induced by the translational models

Patients suffering from chronic pain exhibit a myriad of different symptoms. The models explored in this thesis are able induce a range of these sensory changes that are observed in patients, as highlighted in table 7-2. In the model of topical capsaicin symptoms such as pinprick hyperalgesia and DMA were found in the primary area. These symptoms were also observed in the secondary area post UVBR. These are both clinically relevant phenomenon

since DMA occurs in up to 49% of patients with PHN and pinprick hyperalgesia is a symptom suffered by 36% and 30% of PHN and PNI patients, respectively (Maier et al. 2010). Both the UVB model and UVBR induced a cold hypersensitivity, which is also experienced by patients suffering from OA and oxaliplatin-induced neuropathy (Kosek and Ordeberg 2000; Binder et al. 2007). Additionally, all of the models produced a primary heat hypersensitivity, which is found in patients with OA and around 20% of neuropathic pain patients (Maier et al. 2010; Soni et al. 2013). Overall, suggesting that these models are able produce a number of positive sensory symptoms that may be applicable to the clinical conditions.

Topical capsaicin also resulted in a reduced C fibre count, indicative of fibre desensitisation. This was not reflected in most of the animal or human evoked responses, with the exception of the CPT in humans. CPT was significantly lower post topical capsaicin, when compared to baseline responses. This symptom is observed in patients with central pain, PHN and PNI (Maier et al. 2010). Therefore suggesting that this sensory loss may also be relevant to patients.

As apparent from table 7-2, aside from a cold hypoalgesia, one limitation of the models explored in this thesis is that they do not induce a profound sensory loss. Rather, each of the models produced strong positive symptoms in the primary (capsaicin and UVB) and secondary areas (UVBR). This is important to note since negative symptoms indicative of sensory loss are common in many chronic many patients (Rice et al. 2009; Maier et al. 2010). Negative symptoms are often associated with non-nociceptive parameters, and may be a result of damage to peripheral or central neurones (Maier et al. 2010). Therefore, for ethical reasons it is likely that such symptoms could not be modelled in healthy volunteers. However, it is important to note that such damage may be difficult to treat and as such there would only be a limited use of exploring these symptoms further in models. Additionally, one interesting possibility is that peripheral and central hyperexcitability is actually a compensation for a sensory loss associated with neuropathic pain conditions. Therefore, even though the models do not induce negative symptoms, they may still engage relevant mechanisms.

Symptom	Model
Spontaneous pain (shooting)	-
Spontaneous pain (ongoing)	-
Heat allodynia	Capsaicin, UVB and UVBR
Cold allodynia	UVB and UVBR
Static mechanical allodynia	Capsaicin, UVB and UVB
Dynamic mechanical allodynia	Capsaicin and UVBR
Punctate mechanical hyperalgesia	Capsaicin, UVB and UVBR
Sensory loss	Capsaicin (cold hypoalgesia)

Table 7-2 Symptoms induced by experimental models in this thesis. The symptoms listed in the left hand column are experience by chronic pain patients, the right hand column highlights which model can be used to mimic each symptom.

In addition to negative symptoms indicating a sensory loss, many chronic pain patients also report spontaneous or ongoing pain (Baron et al. 2009; Rice et al. 2009). A clear limitation of these studies is the inability to explore this symptom. This is partially due to the methods used in this thesis, since spontaneous pain is not easily assessed with either of the techniques described. However, models such as UVB are not believed to be associated with any spontaneous pain (Bishop et al. 2010; Davies et al. 2011). Since we are unable to effectively model this symptom in translational studies it has been suggested this is better investigated in patients (Schmelz 2009). However, such data may still be supplemented by ongoing research into examining mechanisms and modulation of spontaneous pain in animals using tests such as the CPP (King et al. 2009). Induction of CPP by manipulations that are not otherwise rewarding provides evidence of ongoing pain in neuropathic animals.

7.1.1. Clinical relevance of the mechanisms induced by translational models

The potential underlying mechanisms involved in each of the pain models have been inferred from the complimentary animal and human data. For example, each modality in the QST battery relates to the function of different fibre types (Arendt-Nielsen and Yarnitsky 2009),

whilst electrophysiological changes in fibre counts and receptive fields are useful to assess central changes. Collectively, this information allowed assumptions to be made with regards to the peripheral versus central components for each model. Each symptom may be the result of a number of underlying mechanisms, and therefore it is often more useful to look at the expression pattern of pain-related sensory abnormalities in addition to the sensory phenotypes in order to gather hints as to the overall underlying pathophysiological dysfunctions (von Hehn et al. 2012). Given that the models explored produce symptoms observed in chronic pain patients, it may be assumed that these are reflective of pathophysiological mechanisms involved in chronic pain. Therefore, such translational models can be used to gain a better understanding of the mechanisms that may be involved in chronic pain.

The mechanisms involved in each of the models are discussed in each chapter, however, to briefly summarise: the sensory changes evoked by capsaicin in animals and humans were reflective of both a peripheral and central sensitisation, including mechanical hypersensitivity accompanied by a facilitation of responses in the Aδ fibre range. UVB appeared to be a strictly peripheral model, resulting in no secondary changes or receptive field expansion. On the other hand the UVBR model showed clear signs of engaging both peripheral and central mechanisms, including secondary brush hypersensitivity and a facilitation of Aβ fibre responses.

To fully understand the mechanisms at play it is also useful to be able to compare and contrast between the models explored in this thesis, in particular with regards to the model of UVB versus UVBR. There is much debate as to whether the original model of UVB irradiation in animals led to the development of a purely peripheral sensitisation, or whether it also engaged central mechanisms (Bishop et al. 2010; Gustorff et al. 2013). The somatosensory changes observed here, in both animals and humans, are certainly suggestive of a strong peripheral sensitisation, without the induction of central sensitisation. On the other hand, UVBR resulted in large areas of secondary hyperalgesia and expansion of WDR neurone receptive fields, suggestive of the development of central sensitisation. Furthermore, the development of secondary brush hypersensitivity/ DMA accompanied by a facilitation of Aβ fibre responses also suggests central changes are present. Given such changes are not observed in the model of UVB alone, this provides more support to the theory it is mainly driven by peripheral changes.

Another interesting comparison is that of the CAP model vs. UVBR, both which appear to have elements of peripheral and central sensitisation contributing to the changes observed. It was noted that UVBR treated WDR cells lost the ability to wind up further, which was attributed to the prior induction of a strong central sensitisation. Since wind up and central sensitisation share overlapping mechanisms, it is possible that due to the induction of central sensitisation wind up cannot occur any further, since all NMDA receptors may already be activated. Similarly, it has been found that it is more difficult to induce LTP in SNL rats, compared to naives (Rygh et al. 2000). This may be explained in analogous manner whereby the prior central sensitisation engages mechanisms similar to LTP and thus further induction of excitability is not possible. On the other hand, turning to the model of topical capsaicin cream, there is no change in wind up with regards to WDR cells or human percept. Therefore this could suggest that although central changes may have been induced, the degree of sensitisation is less and therefore wind up can still occur.

As previously mentioned, the model of capsaicin cream allows the study a particular mechanism (TRPV1 activation and sensitisation). However, the weakness of this model is that it is unknown how important this particular mechanism is in any given pain state. The newer model of UVB irradiation could begin to closer reflect clinically meaningful mechanisms, however since it was concluded that this model was mainly underpinned by a peripheral sensitisation this model may also have its limitations. Whilst it is useful for exploring peripheral mechanisms alone, most patients who suffer from chronic pain will most likely have numerous overlapping peripheral and central mechanisms contributing to their symptom profiles (Baron 2006; Gwilym et al. 2009; Latremoliere and Woolf 2009; Thakur et al. 2012). Therefore, it may be concluded that the UVBR model induces the most clinically relevant changes. The experiments in this thesis highlighted a clear primary hypersensitivity from UVB, followed by rekindling induced enlargement of WDR cell receptive fields, and large areas of secondary hyperalgesia in humans. UVBR therefore exhibits signs of engagement of a number of clinically relevant phenomena, such as peripheral inflammation driving a central sensitisation (Baron et al. 2013).

Previous studies have shown that the common inflammatory mediators expressed post UVB irradiation in humans and rats not only highlight the translational nature of the model, but also the clinical relevance. The correlation in gene expression in rats and humans is suggestive similar underlying biological (Dawes et al. 2011). This infiltration of immune cells

and release of mediators may be applicable to other persistent pain states in humans. Pain in OA can arise from damage in the peripheral tissues and is driven by peripheral inflammatory mediators (Malfait and Schnitzer 2013). Although many of these mediators are currently unidentified, it is possible that there may be some overlap with the mechanisms induced here. Most notably, CXCR2-chemokines are believed to be upregulated in arthritic knee joints (Grespan et al. 2008). This peripheral component of the disease is highlighted by the fact that local anaesthetics can reduce OA related pain.

Central sensitisation is also believed to be present in OA, in addition to a number of other chronic pain conditions such as fibromyalgia, neuropathic pain (including PHN and PNI) and post-surgical pain (Woolf 2011; Baron et al. 2013). OA patients develop symptoms such as mechanical, heat and cold hypersensitivity distant from the site of injury, which are explained through central modifications (Gwilym et al. 2009; Lee et al. 2011). Studies further suggest that these centrally mediated symptoms may be linked to a peripheral ongoing input, since a block of afferent activity with lidocaine can abolish symptoms such as allodynia (Gracely et al. 1992). Furthermore, in OA a total joint replacement often eliminates the pain, suggesting that it was driven by the ongoing afferent activity as a result of peripheral inflammation, reducing activation thresholds (Woolf 2011; Malfait and Schnitzer 2013). Similarly, the central changes involved in the UVBR model are driven by ongoing activity produced during the rekindling. Inflammation caused by UVB irradiation reduces the threshold of afferent fibres, thus allowing ongoing activation of nociceptors by a 40°C stimulus. On the other hand, the fact that some patients do not respond to local anaesthetics, or joint replacement suggests the central pain may become independent of the peripheral drive, and thus limits the use of UVBR in modelling such conditions (Lim et al. 2006; Malfait and Schnitzer 2013).

Models that induce only a peripheral or central sensitisation, are useful for investigating peripherally and centrally acting drugs, respectively (Chizh et al. 2007). However, a model including both peripheral and central components can be used to detect efficacy of a wider range of analgesics. It is well known that the UVB model responds to anti-inflammatory drugs, and preliminary work suggests the model of UVBR responds to centrally acting mediators (Wang 2005; Bishop et al. 2007; Bishop et al. 2009). Therefore the UVBR model is likely to be useful in assessing analgesic efficacy of novel analgesics acting at numerous sites in the pain pathway.

It is therefore possible that these models (in particular UVBR) can be used as proof of concept studies, conducted prior to larger clinical trials. Thus, the work described in this thesis has direct relevance and utility within drug discovery. Such a technique appears to have been successful when applied in retrospective studies, whereby drugs have been tested in models after approval for patients. Pregabalin is known to reduce pain in PHN patients, many of whom suffer from pinprick hyperalgesia (Dworkin et al. 2003; Maier et al. 2010). Furthermore, in patients with HIV-related neuropathy post-hoc analysis showed efficacy of pregabalin in a subgroup of patients with pinprick hyperalgesia (Simpson et al. 2010). It has since been shown that pregabalin can reduce pinprick hyperalgesia induced in human subjects by electrical stimulation (Chizh et al. 2007). Furthermore, a similar compound (gabapentin) is able to reduce capsaicin induced pinprick hyperalgesia and the activation in the brainstem thought to be associated with central sensitisation (Iannetti et al. 2005). Therefore pregabalin and gabapentin are able to reduce signs of central sensitisation in experimental models, such as pinprick hyperalgesia, which further translates to efficacy in the clinic. Current trials are also in process to evaluate the efficacy of tapentadol in the models of capsaicin and menthol. It is predicted that Tapentadol will also be able to reduce signs and symptoms of central sensitisation (such as areas of pinprick mechanical hyperalgesia and allodynia), in addition to reducing pain intensity scores (Baron 2013). Pioneering experiments such as these that are run alongside clinical trials will provide important information as to the ability of these models to predict trial outcomes (Baron 2013).

7.1.2. Limitations to preclinical translational models

It is important to note that surrogate models induced in humans produce short-term, reversible changes. As such, there are likely to be mechanisms involved in chronic pain that are not induced by these models. That is to say, since chronic pain often develops over the course of many months or years in patients, certain mechanisms may depend on this long term set up. These experimental models are unlikely to model the full complex clinical conditions experienced by patients (Arendt-Nielsen et al. 2007; von Hehn et al. 2012).

One example is the role of trophic factors such as NGF (Schmelz 2009). Neurotrophic factors are known to regulate long term processes such as survival, growth and differentiated function. NGF is seen to be upregulated in conditions such as OA and DPN, and anti-NGF molecules are able to reduce OA related pain (Lane et al. 2010; Kumar and Mahal 2012).

Preclinical studies have revealed that NGF effects gene expression of ion channels such as TRPV1, ASIC3 and Nav 1.8, resulting in sensitisation of peripheral neurones (von Hehn et al. 2012). Furthermore, NGF believed to be able to alter the distribution of Aδ fibres, enabling greater proportions to respond to nociceptive stimuli (Stucky et al. 1999). These afferent fibres also showed heightened responses to mechanical stimuli (Stucky et al. 1999). Taken together, evidence suggests a potential role of NGF in contributing to chronic pain, which is particularly important to note; firstly as such changes may indeed require longer time periods of induction and secondly because NGF was not shown to be upregulated by UVB irradiation. Therefore these changes are unlikely to be captured by the models in this thesis.

Additionally, it is believed that after nerve injury, Aβ fibres are able to undergo phenotypic changes, such as an increased expression of neuropeptides (Nitzan-Luques et al. 2011). Thus they may acquire the capacity to trigger or maintain central sensitisation. PNI has also been noted to induce changes in dendritic spines of DH neurons, mediated by the G protein Rac1, suggestive of physical changes in spinal cord circuitry (Tan et al. 2011). It is clear that the human experimental models discussed in this thesis cannot replicate such mechanisms. Thus, it is imperative to study the underpinnings of long-term modifications in animal models, and examine the possible consequences in patients.

As previously mentioned, spontaneous pain has not been associated with the UVB model. However, the mechanisms underpinning this symptom are of great clinical importance. A number of candidate molecules have been put forward as mediators of spontaneous pain, including Na^+, K^+ and hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels (von Hehn et al. 2012). Modifications to these ion channels due to inflammation or nerve injury may indeed lead to ectopic activity of peripheral fibres. Ongoing activity could be generated by non-inactivating Nav1.3- and Nav1.6-mediated currents, or by down regulation of K+ channels such as Kv1.2, 1.4 or 2.2 (Herzog et al. 2001; Kim et al. 2002; Dib-Hajj et al. 2010). HCN antagonists are able to alleviate both ectopic discharge in primary afferents and signs of spontaneous pain in nerve injured animals (Chaplan et al. 2003; Lee et al. 2005). Taken together these findings strongly suggest a role for these channels in the generation of spontaneous pain sensations. Given that spontaneous activity does not appear in the models described, it may be inferred that these mechanisms are not captured, highlighting an important weakness of these studies.

7.2. Novel therapies for chronic pain explored in this thesis

This thesis provided the first preclinical assessment of the effect ADO/ CPA on capsaicin induced central sensitisation. Most importantly, it included studies in both animals and humans that also involved supra threshold stimuli. Despite the ability of ADO to reduce thermally evoked responses in WDR cells and human HPT, it was unable to fully prevent the development of capsaicin induced hypersensitivity. However, the A_1R agonist CPA was able to reduce signs of both peripheral and central sensitisation (i.e. thermal and brush evoked hypersensitivity) in rodents. Previous studies have also found that agonists of the A_1R are able to reduce inflammatory pain (Lima et al. 2010), in addition to noting analgesic effects models such as formalin and DPN (Balasubramanyan and Sharma 2008; Liu et al. 2013). This suggests that the A_1R is a viable drug target, and using activation of this receptor as a pre-emptive treatment may be able to reduce peripheral sensitisation and inhibit the development of subsequent central modifications.

An additional target explored in this thesis is the chemokine CXCL5. This chemokine has been shown to be upregulated in both animals and humans post UVB exposure (Dawes et al. 2011). Experiments here found that injection of intraplantar CXCL5 resulted in heighted responses of WDR cells to both thermal and mechanical stimuli. Although this chemokine is yet to be explored in patients, it has also been found to be associated with a number of other preclinical models (Dawes 2013). Therefore an antagonist of its receptor, CXCR2, could also hold potential for future drug therapies, in particular for inflammatory conditions, such as OA (Grespan et al. 2008; Dawes et al. 2011). Indeed, preclinical data suggests that antagonism of this receptor is able to attenuate pain-related hypersensitivity in a number of experimental models, including the collagen-induced arthritis model, carrageenan, and CFA (Cunha et al. 2008).

Most importantly both of these potential targets are in the periphery. There are two main advantages of developing such treatment. Firstly, analgesics acting outside of the CNS should not induce side effects such as sedation, dizziness and fatigue, of which may be debilitating to patients requiring these drugs (Finnerup et al. 2010). Secondly, given that central sensitisation mechanisms are often inextricably linked to ongoing peripheral afferent activity, using a treatment that may inhibit this activity could prevent the development or maintenance of central modifications (Baron et al. 2013). The earlier the treatment is given, the higher the likelihood of interfering with disease progression.

7.3. Use of in vivo single unit DH recordings and QST

This thesis also aimed to address the issues surrounding subjective measures of pain, through the use of objective in vivo electrophysiological recordings from LV WDR cells in animals. Relying on behavioural studies poses a number of difficulties with regards to pain research, in addition to the subjective nature of the tests. Most notably, whether reflex measures truly equate to the human pain sensation and the absence of suprathreshold measurements (Mogil 2009; Bennett 2010). All the studies undertaken in this thesis highlight the strong concordance between the rodent WDR cell activity and human psychophysical responses both pre and post the induction of the surrogate models, as has been observed in previous work. Indeed it has been noted that both the coding properties of WDR neurones, and the ability to wind up, correlate closely with human perceptions (Maixner et al. 1986; Dubner et al. 1989; Sikandar et al. 2013).

Single unit recordings of spinal neurones allow the assessment of responses to both natural and electrical stimuli, in addition to their modulation, allowing full characterisation of preclinical pain models. LV WDR neurones are of particular interest in the study of chronic pain since they are under regulation of both local networks and descending controls (D'Mello and Dickenson 2008). Additionally, one key advantage of DH electrophysiology is that responses to supra threshold stimuli, and their modulation, can be examined - which are likely to relate to the high pain levels which patients report (Sikandar and Dickenson 2013).

When examining thermal threshold measurements, it was observed that the action potentials evoked correlate with human changes in threshold. That is to say, if the baseline threshold in humans evokes a responses ‘X’ number of action potentials in animals, after induction of hypersensitivity the new human threshold now also evokes ‘X’ number of action potentials in the animals. This highlights not only the strong overlap between animal and human studies, but also the usefulness of using electrophysiology in chronic pain studies, in agreement with previous findings (Price 2013; Sikandar and Dickenson 2013). Therefore, it can be concluded that objective recordings of WDR cells are a useful measure that reflect the human pain sensation.

This thesis also explored the potential of WDR responses as a potential endpoint with regards to drug efficacy. Single unit recordings also allow the study of how drugs act across modalities and varying intensities. This thesis attempted to use a mechanism based approach to

treatment, with the use of ADO and CPA pre-treatment in order to inhibit capsaicin induced sensitisation. It has been suggested that activation of the A_1R is able to indirectly reduce the activity of TRPV1, therefore it was hypothesised the ADO and CPA may be able to inhibit the effects of capsaicin through this downstream interaction (Rohacs et al. 2008; Sowa et al. 2010). Behavioural studies, including those with ADO or CPA, have often used threshold measurements and produced conflicting results, whereas here we are able to examine how the drug may effect sub and suprathreshold stimuli across modalities. Importantly, since capsaicin is a highly suprathreshold stimulus, this study extends the role of ADO/ CPA modulation into these suprathreshold levels of pain related activity that are likely to be relevant to patients. It was found that the enhanced responses of WDR neurones produced by topical capsaicin could be partially attenuated by ADO, and fully inhibited by CPA. This confirms the use of WDR cell recordings as a suitable measure of analgesic efficacy. It is important to note that studies of analgesic efficacy without this objective measure could be misled by threshold measuring behaviour.

One interesting observation from the results of this thesis is the possibility that recordings of LV WDR neurones provide a more sensitive and accurate measure of pain, as a predictor of human perception. For example, previous behavioural studies using A_1R agonists have reported an overriding effect on thermal stimuli (Gong et al. 2010). However, the studies conducted in this thesis also reported minor effects on mechanical stimuli. A small, non-significant, increase in human MPT was also observed. Thus suggesting that small changes picked up using the sensitivity of single unit recordings of WDR cells may indeed correlate with human behavioural responses. Studies in this thesis also found that intraplantar CXCL5 resulted in a thermal hypersensitivity, whereas previous behavioural reports suggested it exclusively led to mechanical hypersensitivity (Dawes et al. 2011). It would be interesting to explore the changes in sensitivity in humans after intradermal CXCL5 to assess whether the electrophysiology or behaviour reflects most accurately the human perception.

The use of QST in human subjects provided a comparable data set to that produced in the animals, importantly including tests across modalities to sub and suprathreshold stimuli. QST enables the examination and quantification of alterations in function of the nociceptive system, resulting in a broader understanding of surrogate models, which compliments the data obtained from animal studies. The wide range of tests allows examination of a gain or loss of function in large myelinated fibres, thin myelinated and unmyelinated fibres.

Advantages of QST include the controlled nature of the stimulus intensity, duration and modality, which can be compared over time (Arendt-Nielsen and Yarnitsky 2009). Additionally, standardisation of the procedure, allows comparison between studies. Finally, QST enables the creation of sensory profiles for each of the models to help understand the clinical relevance of the models, and to compare preclinical and clinical studies.

As with the electrophysiological recordings, it was found that QST was sensitive to changes induced by the surrogate models at both sub and suprathreshold levels. Since optimum pain diagnosis and treatment should be mechanism based, a selection of QST tests were used in assessing the modulation of capsaicin induced sensitisation (Woolf et al. 1998). In this experiment QST was also able to highlight the analgesic effect of ADO. This suggests that QST is suitable for use in testing the efficacy of chronic pain drugs, in both preclinical and clinical settings. By using the same tests addressing the same underlying mechanisms, in animals and humans, it may be possible to increase the potential to predict efficacy of drugs in given patient populations (Arendt-Nielsen and Yarnitsky 2009). Importantly, since QST is cheap, reasonably fast and provides reproducible results it could be used in proof of concept studies before drugs enter large clinical trials (Arendt-Nielsen and Yarnitsky 2009).

It is important to note that there are of course limitations to both of these techniques discussed. Notably, neither technique is able to easily distinguish between peripheral and central mechanisms of altered pain processing. Assumptions may be made using the complete data sets from both animals and humans, however firm conclusion require pharmacological manipulation of the models. A further disadvantage of the methodologies used in this thesis is the difficulty in assessing spontaneous/ ongoing pain. With regards to in vivo electrophysiological recordings of single units, when selecting a WDR neurone to characterise it is inevitable that based the criteria used, those with spontaneous activity will be discarded. This is due to the simple fact that it is inherently difficult to produce stable, reproducible baseline recordings when a cell exhibits inconsistent spontaneous firing. This is not to say that it is impossible to study ongoing activity with the technique, as it has been used to demonstrate increased activity post SNL (Chapman et al. 1998). However, even in this instance it is not possible to tell the source or modality of the ongoing activity. With regards to QST, any spontaneous activity could also have a negative effect on subjects reported outcomes with regards to threshold measurements and pain ratings.

Regarding QST, this method relies on subject cooperation and understanding, which could limit its usefulness in subjects who fail to comply with or comprehend the instructions given (Krumova et al. 2012). Furthermore, thresholds can be variable across subjects and therefore it may be difficult to detect small abnormalities in the models (Hansson et al. 2007). This is highlighted by the normative data provided by the DFNS where the value for female HPT on the hand is 42.61°C ± 3. 33, whilst CPT is 16.16°C ±7.08, which may indeed make interpretation of data difficult for small studies or moderate effects (Rolke et al. 2006). Finally, this method is limited in the examination of any models addressing deep somatic pain. However, all psychophysical methods are faced with these limitations and as it has been shown in this thesis, they do not necessarily affect all studies.

QST can only be performed on a small area of body, which is suitable for models such as these where the area of interest is well defined. However, this does limit the use in patients where a small test site may not be fully representative of the affected area (Hansson et al. 2007). This may present a challenge when comparing model profiles to those of patients. As such it may be recommended to perform in the QST in several areas on patients (Krumova et al. 2012). Additionally, as previously noted the DFNS protocol used in this thesis does not assess spontaneous pain, which is a major symptom suffered by many patients. Once again, this could be a potential hurdle for translation of preclinical models to patients. To overcome both of these limitations, QST data sets could be supplemented with questionnaires, to provide additional complementary information. Questionnaires such as the fully validated painDETECT would provide data to help fully determine the phenotype of both models and patients as it captures useful pain descriptors and qualities of pain, giving a good overview of the 'whole picture' (Baron et al. 2012). It has been suggested that these alone and could be used to subgroup patients, and thus would nicely complement QST (Baron et al. 2012). Questionnaires are quick and cheap to implement, although it important to note these are purely subjective and negative symptoms are difficult to analyse, thus they could not fully replace QST.

7.4. A mechanism based approach to treatment

It has long been noted that there is a need to shift focus from clinical trials based on change in pain scores and classifying patients by the underlying aetiology, to viewing chronic pain as a manifestation of pathological neural plasticity that may result in a number of different symptom profiles (Jensen and Baron 2003; Baron et al. 2012; von Hehn et al. 2012). Using a disease-based classification results in heterogeneous groups of patients in which drugs will inevitably struggle to show an overall positive outcome. Distinct pathophysiological mechanisms produce specific sensory abnormalities, whilst individual phenotypes are made up of a number of contributing factors, such as the genotype and environmental factors including diet and life style (von Hehn et al. 2012). Indeed chronic pain patients have diverse genetic and environmental backgrounds in addition to varying degrees of inflammation or nerve damage, all of which will contribute to the complex combination of pathophysiological mechanisms, which in turn manifest as the individual pain phenotype (von Hehn et al. 2012). Thus, it is unsurprising that a group of patients with the same aetiology will not necessarily present with the same signs and symptoms. Since we are unable to test for the presence of specific mechanisms in patients, the symptom profiles can be used as a surrogate marker. Classifying patients by their symptom profiles should reduce heterogeneity and provide clues as to the underlying mechanisms. Therefore serving as a guidance as to which drugs would be most suitable for use in each subgroup.

It has been suggested that identification of the pattern of symptoms present in a patient should be a useful approach for identifying those who are more likely to respond to a particular treatment. As previously discussed, this is due to the fact that the pattern of expression of pain-related sensory abnormalities and the individual sensory phenotype reveals clues of the underlying mechanisms involved (von Hehn et al. 2012). Given that a specific symptom, such as burning pain, may be generated by a number of different underlying mechanisms (peripheral sensitisation – such as a reduced threshold of TRPV1, gain of function mutations in Nav1.7, or ectopic activity due to alteration in HCN channels), it is the overall profile of sensory symptoms and signs that will predict underlying mechanisms (von Hehn et al. 2012). Such profiles can be created by using QST as described in this thesis, in addition to validated questionnaires. Profiles of the preclinical models in this thesis are able to bridge the gap between research and the clinic, since they can help elucidate particular

mechanisms associated with the symptom profiles in addition to testing pharmacological sensitivities.

Classifying patients by their symptoms has already revealed a number of distinct subgroups in patient populations, characterised by their unique sensory profiles (Dworkin et al. 2007; Baron et al. 2009; Maier et al. 2010). Such studies have confirmed that there are no specific pain profiles associated with particular aetiologies, but rather clusters of different sensory profiles within each category (Freeman et al. 2013). Furthermore, similar symptoms profiles are present in the different aetiologies, suggesting that patients can be classified into subgroups based on symptoms, independent of the initiating disease (Freeman et al. 2013). By using the painDETECT questionnaire, Baron and colleagues revealed 5 subgroups of patients with neuropathic pain (PHN, DPN, painful radiculopathy) (Baron et al. 2009). On the other hand, using QST 12 subgroups of patients have been identified (Maier et al. 2010). Since the pattern of symptoms may be indicative of underlying mechanisms, it may be concluded that these subgroups of patients most likely have similar underlying pathophysiologies and therefore would benefit from the same treatment. Introducing this classification of patients will help guide future clinical trials.

These groups, characterised by their pattern of symptoms, can also be compared to the preclinical models, in order to improve translation of knowledge. This relies on preclinical models assessing the same outcome measures in animals and humans as in patients, such as the work presented in this thesis. When preclinical targets are taken forward into clinical development, drugs with preclinical efficacy reducing a specific group of symptoms should be tested in the equivalent patient population.

However, despite more than a decade of academic discussion, it would appear that the majority of clinical trials are still grouping patients by disease and using outcome measures of overall reduction in pain scores (table 7-3) (Woolf et al. 1998; Jensen and Baron 2003; Baron et al. 2012). Not only is this more difficult for drugs to show efficacy in whole cohorts, but it is also difficult to use the preclinical data to guide the trial design. The models explored in this thesis produce specific symptoms and mechanisms, which could be used to screen suitable analgesics. If a drug is found to reduce a particular sign or symptom in the model, this could help guide selection of the group of patients it can be trialled in. However, this is not possible if clinical trials still used disease based classification and do not assess the different symptoms.

Analgesic	Clinical Trial Criteria	Pain Specific Outcome Measures
Quetenza	PHN	Percent change from baseline in the "average pain for the past 24 hours"
	HIV-Related Neuropathy	Percent Change in the "Average Pain for the Past 24 Hours"
	DPN	Change in the average daily pain scor, Question 5 of the Brief Pain Inventory-Diabetic Neuropathy (BPI-DN)
Tapentadol	Chronic Lower Back Pain	Change in the Average Pain Intensity Score, painDETECT, Neuropathic Pain Symptom Inventory
	OA	Change in the Average Pain Intensity Score
	DPN	Change in the Average Pain Intensity Score
	Neuropathic Pain (thermal or mechanical hyperalgesia)	Thermal thresholds, MPT, MPS, DMA, WUR
CNV2197944	PHN	Pain Intensity Numerical Rating Scale, Neuropathic Pain Symptom Inventory
	DPN	Pain Intensity Numerical Rating Scale, Neuropathic Pain Symptom Inventory
MK-6096	DPN	Change in pain score
KW21052	DPN	Numerical pain rating scale (NRS)
NXN-462	PHN	Change from baseline to the last week of treatment in daily pain scores

Table 7-3 Examples of recent clinical trials. A selection of recent clinical trials highlights the difficulty in getting academic work reflected in practice. Despite being raised over a decade ago, very few trials aim to classify their patients by symptoms or test separate modalities.

The work in this thesis should help bring together preclinical and clinical studies and encourage the use of subgrouping in order to move towards a mechanism based approach to treatment. In this thesis it has been shown that models can be used to induce a specific symptom of chronic pain, such as capsaicin induced thermal hypersensitivity. This symptom is attributed to a peripheral sensitisation of C fibres, and most likely TRPV1 modulation. Using a mechanism based approach to treatment, whereby the A_1R is believed to interfere with TRPV1 function, it was shown that it is possible to prevent this symptom. Therefore it may be

predicted that A_1R agonists would have efficacy in subgroups of patients who appear to have a strong peripheral component of their pain.

There is already evidence that using drugs in subgroups may be effective. Although there are no larger studies that have used this method of phenotyping and subgrouping, positive retrospective data does exist (Baron et al. 2012). Using sensory tests it has been shown that patients who suffer from mechanical allodynia appear to respond better to lidocaine treatment, than in those who do not exhibit this symptom (Attal et al. 2004). Additionally, a high baseline HPT and loss of peripheral terminals, correlates with the response to systemic opioids (Edwards et al. 2006). A study of clonidine in DPN patients also found they could predict responses using topical capsaicin cream. It was found that those who had increased responses to capsaicin pre-treatment (i.e. indicative of peripheral afferent sensitisation) responded better to topical clonidine and their pain was significantly reduced (Campbell et al. 2012). Furthermore, as previously mentioned, one important post-hoc analysis of pregabalin in HIV-related neuropathy revealed that despite an overall negative result, by subgrouping the patients it was found that those with pinprick hyperalgesia responded positively to the drug (Simpson et al. 2010). A pooled post hoc analysis of pregabalin trials based on cluster analysis also showed that patients in 3 particular subgroups responded better to the drug (Freeman et al. 2013). Thus overall suggesting that subgrouping patients, based on their symptom profiles may allow prediction of response to different analgesics.

Since the technique is not yet adapted in the clinic, for now a post hoc analysis may be a more realistic option to pursue. Furthermore, it is important to note it is difficult to truly predict responders, since the symptoms only indicate the mechanisms (Attal et al. 2011). However, by using surrogate models to explore the mechanisms underlying different symptoms and confirming their pharmacological sensitivity will help the design of subsequent clinical trials, if this method of profiling and subgrouping is taken up. The tapentadol study in table 7-3 highlights a possible move in this direction, since the outcome measures of the trial include various QST measures.

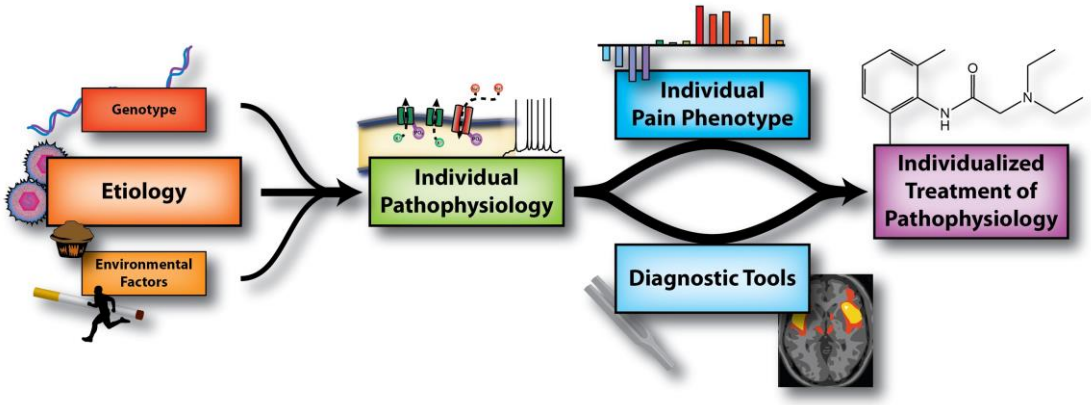

Figure 7-1 Distinct pathophysiologies underling chronic pain phenotypes dictate treatment requirements. A number of factors contribute to individual pain phenoptypes; this includes the aetiology, genotype, and environmental factors. Together these lead to the engagement of a number of chronic pain mechanisms and distinct pain profiles. It is imperative that patients are subgrouped to define their pain phenotype in order to identify the most suitable treatment options. (von Hehn et al. 2012)

7.5. Future studies

This thesis has provided objective and quantitative characterisation of three translational models of chronic pain. Overall, it would appear that the most clinically relevant and useful model to take forward is that of UVB rekindling. This model consists of an initial inflammatory phase, which has been well characterised, followed by the induction of secondary hypersensitivity. It is believed that these secondary changes are the result of engagement of mechanisms of central sensitisation. To fully understand the complex underpinnings of the sensory changes observed it would be useful to undertake some further studies involving pharmacological manipulation. It is well known that central sensitisation involves the activation of NMDA receptors, and thus modulation with antagonists such as ketamine and MK-801 would help confirm the engagement of such mechanisms (Bishop et al. 2010). Furthermore, central changes can also involve a shift in descending modulation. One example of such is an increase in descending facilitation from the brainstem, which has been shown to promote spinal neuronal hyperexcitability and behavioural mechanical hypersensitivity in chronic pain states (Porreca et al. 2002; Suzuki et al. 2002; Rahman et al. 2009). Under normal conditions there is little effect of blocking the 5HT3 receptor with low doses of ondansetron. However, after the induction of models such as SNL and OA even low doses are able to reduce signs of mechanical hypersensitivity (Suzuki et al. 2004; Rahman et al. 2009).

Therefore, it would also be useful to assess the ability of ondansetron to modulate the changes observed post rekindling.

Inhibitory controls can also be measured in humans, through conditioned pain modulation (CPM). CPM, or diffuse noxious inhibitory controls (DNIC) in animals, is the idea that a pain at one site may be able to inhibit another at a distant site. DNIC involves feedback loops similar to the descending controls, most likely reliant on input from LI/III projection neurones (Suzuki et al. 2002). An endogenous control loop involving supraspinal structures such as the caudal medulla and medullary reticular function then facilitate the descending modulation (Bouhassira et al. 1992; Le Bars et al. 1992). Given that lesions to the PAG and RVM do not alter DNIC, it is possible that it may involve reducing the descending inhibitory controls from the LC (Le Bars et al. 1992). A similar phenomenon is observed in humans and may be tested by administration of two simultaneous painful stimuli (Pud et al. 2009)((Yarnitsky et al. 2010). Working to the principle of a mechanism based approach to treatment, it may be assumed that individuals exhibiting an inefficiency of CPM would respond better to drugs that promote an increase in inhibitory circuits. A recent study therefore hypothesised that patients with low CPM would benefit most from an enhancement of descending inhibition. This study investigated analgesic efficacy of duloxetine in a group of DPN patients (Yarnitsky et al. 2012). Duloxetine is a serotonin-noradrenaline reuptake inhibitor believed to augment descending pain inhibition through inhibiting reuptake of spinal NA and 5HT. The study concluded that CPM was indeed a predictor of duloxetine response, since those with lower baseline CPM responded better to duloxetine treatment (Yarnitsky et al. 2012). Thus, it would be interesting to explore any changes in CPM after the induction of experimental models such as UVBR. That is to say, if the models decrease CPM ability, perhaps there is a shift towards descending facilitation/ less inhibition.

Secondly, it may be interesting to investigate the possibility of ongoing pain in the model of UVBR. Previous behavioural data noted that the secondary hypersensitivity post UVBR peaks at 48 hours (Davies et al. 2011), therefore suggesting that an ongoing drive may outlast the rekindling procedure itself. This possibility could be explored further, in humans with the use of questionnaires to be filled in at set time points, and in animals with the use of CPP. Additionally, levels of spinal c-fos could be measured at 24 and 48 hours post rekindling, as this can be taken as a surrogate marker of ongoing activity into the DH.

Thirdly, it is important that translational models engage mechanisms relevant to chronic pain patients. The symptoms can often be taken as indicators of particular mechanisms (Baron et al. 2012; von Hehn et al. 2012). Therefore, it will be of great use to compare the sensory profiles created from the preclinical models, to those from patients in the clinic. Currently the creation of sensory profiles focuses on neuropathic pain, however since the models in this thesis share more in common with non-neuropathic chronic pain it would be useful to compare with more relevant populations. This will help identify which condition or subset of patients that the models best reflect.

Finally, once there is a clear picture of the underlying mechanisms involved in this model and the clinical relevance, it may be used to for drug screening to assess analgesic efficacy. Most importantly, since this thesis has detailed full characterisation of the sensory changes evoked by UVB rekindling, it can be used for hypothesis driven screening/ a mechanism based approach to treatment. The identification of analgesics that are able to reduce specific signs and symptoms can then be tested in the appropriate subgroup of patients.

7.6. Concluding remarks

Despite the potential drawbacks discussed, QST enables the quantitative measurement of a number of signs and symptoms of chronic pain in translational models. This allows for full characterisation of the models and understanding of the possible underlying mechanisms. By using animal models a long side these human studies it is possible to pry further into the distinct mechanisms underlying different symptoms. Taken together, these studies enable us to determine the suitability of each model with regards to testing specific analgesics. This can help bridge the gap between preclinical research and the clinic, in order to help provide better diagnosis and management of chronic pain in patients.

8. Appendices

8.1. Scientific Publications

8.1.1. Published Manuscripts

O'Neill, J., C. Brock, A. E. Olesen, T. Andresen, M. Nilsson and A. H. Dickenson (2012). "Unravelling the mystery of capsaicin: a tool to understand and treat pain." Pharmacological reviews 64(4): 939-971.

O'Neill, J., S. B. McMahon and B. J. Undem (2013). "Chronic cough and pain: Janus faces in sensory neurobiology?" Pulmonary Pharmacology & Therapeutics 26(5): 476-485.

8.1.2. Manuscripts in Preparation

O'Neill, J., S. Sikandar, S. B. McMahon and A. H. Dickenson. "Characterisation of UVB and UVB rekindling induced sensitisation in rodents and healthy human volunteers"

O'Neill, J., S. B. McMahon and A. H. Dickenson. "Activation of the A1R prevents the development of heat hypersensitivity in a translational model of pain"

8.1.3. Abstracts

O'Neill, J., G. D. Iannetti. S. B. McMahon and A. H Dickenson. (2012) "Translational Studies of Pain: Electrophysiological validation of capsaicin induced sensitisation and its modulation in rodents and healthy human volunteers" IASP, Milan.

Lee, M., J. O'Neill, M. Laing, and G. D. Iannett.. (2012) "ERPs recorded in the secondary area post capsaicin sensitisation" IASP, Milan.

Dawes, J.M., J. O'Neill, S. Sikandar, J. R. Perkins, K. Bartus, N. D. James, R. S. Morland, A. S. Rice, E. J. Bradbury, D L. Bennett, A. H. Dickenson, S. B. McMahon. (2013). "Expression and Functional effects of CXCL5 in pain" BNA, London.

O'Neill, J., S. Sikandar, S. B. McMahon and A. H Dickenson. (2013) "Translational Studies of Pain: Characterisation of UVB rekindling induced sensitisation in rodents and healthy human volunteers" SfN, San Diego.

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