THE UNIVERSITY of ADELAIDE

Investigation of a Functional Relationship Between Toll-Like Receptor 4 and Transient Receptor Potential Cation Channel Subfamily V Member 1 in Relation to a Novel Neuroimmune Pain Model

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Abstract

Chronic pain represents a significant global disease burden, affecting 1 in 5 adults worldwide and costing billions of dollars annually. Due to poor efficacy and undesirable side effects including tolerance, nausea, constipation, diarrhoea, dizziness and addiction; current therapeutic options are less than ideal. In order to improve upon current therapeutic options, an improved mechanistic understanding underlying the generation and maintenance of chronic pain conditions is required. Communication between the immune system and the nervous system is an emerging area of interest in pain research. Known as neuroimmune signalling, this interaction is involved in altering key elements of the nociceptive signalling pathway and therefore represents an interesting target for future novel therapeutics. Recently, an interaction between innate immune receptor toll-like receptor 4 (TLR4), and neuronal ion channel transient receptor potential cation channel subfamily V member 1 (TRPV1) has been suggested as a clinically relevant neuroimmune interaction. A clinical model provides evidence that TLR4 agonist lipopolysaccharide (LPS), potentiates responses mediated by TRPV1 agonist capsaicin. We aim to further clarify the nature of this interaction in an *in vitro* overexpression system. And secondly, we aim to replicate the clinical findings in a preclinical model that allows investigation of peripheral and central mechanisms. Our *in vitro* investigations reveal that TLR4 alters TRPV1 mediated calcium influx dynamic and accumulation in HEK293FT cells. The dynamic was not altered by antagonising or activating TLR4 signalling, although both potentiated calcium accumulation. We were unable to back-translate the clinical endotoxin-capsaicin model into BALB/c mice; however, a primed, peripherally targeted LPS challenge potentiated capsaicin-induced mechanical hypersensitivity in a population of BALB/c mice. However, we found that consecutive intraplantar injections result in attenuated capsaicin-induced mechanical hypersensitivity, an effect reversed by both classical and non-classical opioid antagonists (-)- and (+)-naltrexone. Further, (-)- and (+)-naltrexone produced an extended and potentiated capsaicin-induced mechanical hypersensitivity. The effect of naltrexone appeared to be dependent on the state of nociceptive activity at the time of application. We also present a systematic review of the literature analysing capsaicin-induced animal pain models in order to better inform future research decisions. Therefore, we present a study investigating the TLR4/TRPV1 functional interaction *in vitro* and *in vivo*; finding TLR4 alters TRPV1 function. Our results agree with previous studies which postulate both a direct physical interaction and

indirect interaction via intracellular calcium-induced signalling and/or pro-inflammatory mediators. Therefore, we present evidence suggesting the interaction between TLR4 and TRPV1 warrants further investigation as a relevant neuroimmune signalling element in chronic pain states; representing a novel therapeutic target.

Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abbreviations

5-HT	Hydroxydopamine (serotonin)
AEA	Anandamide
AKAP	A-kinase anchoring protein
AMPA	Alpha (α)-amino-3-hydroxy 5-methyl-4-isoxazelopropionic acid
ANOVA	Analysis of variance
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
AP-1	Activator protein 1
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
CaM	Calmodulin
CaMKII	Ca^{2+}/Calmodulin-dependant protein kinase
CB1	Cannabinoid receptor 1
CCI	Chronic constriction injury
CCL	Chemokine (C-C motif)
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene related peptide
CINP	Chemotherapy-induced neuropathic pain
CNS	Central nervous system
COX	Cyclooxygenase
CX	Connexin
CX3C	Chemokine (C-X3-C motif)
CXC	Chemokine (C-X-C motif)
DAB	3,3'-Diaminobenzidine
DAG	Diacylglycerol
DAMP	Danger associated molecular pattern
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified Eagle's medium
DOR	Delta (δ) opioid receptor
DRG	Dorsal root ganglia

Drt	Dorsal reticular nucleus
ELISA	Enzyme-linked immunosorbent assay
EP	Prostaglandin (PGE_2) receptor
EPSC	Excitatory post synaptic current
ERK	Extracellular signal related kinase
GABA	Gamma (γ)-aminobutyric acid
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter
GlyR	Glycine receptor
HEK293FT	Human embryonic kidney 293-FT
HPA	Hypothalamic-pituitary-adrenal
HSP	Heat shock protein
IFN	Interferon
IL	Interleukin
IP	Prostaglandin (PI_2) receptor
iNOS	Inducible nitric oxide synthase
IRF3	IRF regulatory factor 3
JNK	JUN N-terminal kinase
K2P	Two pore background potassium channel
KOR	Kappa (κ) opioid receptor
Kv	Voltage gated potassium channel
LPS	Lipopolysaccharide
LS	Low threshold
LTP	Long term potentiation
MAPK	Mitogen activated protein kinase
MD2	Myeloid differentiation factor 2
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MOR	Mu (μ) opioid receptor
MyD88	Myeloid differentiation primary response protein 88
NaV	Voltage gated sodium channel
NE	Noradrenaline
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells

NGF	Nerve growth factor
NHS	Normal horse serum
NK-1	Neurokinin 1 receptor
NLR	NOD-like receptor
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
NOP	Nociceptin/orphanin GQ receptor
NS	Nociceptor specific
NSAID	Non-steroidal anti-inflammatory drug
NTX	Naltrexone
P2X	Ionotropic purinoceptor
P2Y	Metabotropic purinoceptor
PAG	Periaqueductal grey
PAMP	Pathogen associated molecular pattern
PG	Prostaglandin
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinostiol 4,5-bisphospate
Pirt	Phosphoinositide interacting regulator of TRP
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNS	Peripheral nervous system
PRR	Patter recognition receptor
PSNL	Partial sciatic nerve ligation
mRNA	Messenger ribonucleic acid
siRNA	Small interfering ribonucleic acid
RT	Room temperature
RTX	Resiniferatoxin
RVM	Rostral ventral medulla
SC	Spinal cord
SCI	Spinal cord injury
SIA	Stress-induced analgesia
SP	Substance P

STK	Src tyrosine kinase
TG	Trigeminal ganglia
TIR	Toll/interleukin receptor 1
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adapter inducing IFNβ
Trk	Neurotrophic tyrosine kinase receptor
TRP	Transient receptor potential
TRPA	Transient receptor potential cation channel subfamily A (ankyrin)
TRPV	Transient receptor potential cation channel subfamily V (vanilloid)
TRPM	Transient receptor potential cation channel subfamily M (melastatin)
WDR	Wide dynamic range
VGCC	Voltage gated calcium channel
VLM	Ventrolateral medulla

Chapter 1. An Introduction to pain and neuroimmune signalling

This chapter provides background to pain signalling, highlighting interactions with classically immune systems and their impacts on pain perception. This includes a basic introduction to pain and the mechanisms of nociceptive signal transduction, followed by an introduction to commonly utilised animal models of pain. Neuroimmune interactions will then be introduced in the context of altering nociceptive transmission. The information presented will highlight the consequences of neuroimmune interactions and their importance to chronic pain. This will give relevance to a review of the relationship between toll-like receptor 4 (TLR4) and transient receptor potential cation channel subfamily V member 1 (TRPV1), presented in chapter 2.

1.1 Definition of pain

Pain, and pain related diseases remain a leading cause of disability and disease burden globally (Kassebaum et al., 2017). Defined as an "unpleasant sensory experience associated with actual or potential tissue damage or described in terms of such damage" by The International Society for the Study of Pain (Merskey et al., 1979). As such, pain is viewed as an individual's experience, separating it from the physiological process in which noxious stimuli are processed by the nervous system, known as nociception. Pain generally serves a protective purpose, warning of potential tissue damage. However, pain can become maladaptive, persistent and without purpose; referred to as either pathological or chronic pain, which has profound negative impacts on individuals and society (Cao et al., 2008; Painaustralia, 2019).

1.1.1 Acute Pain

Acute pain is also referred to as physiological pain, and can be further categorised into either nociceptive or inflammatory pain (Cao et al., 2008; Costigan et al., 2009). Acute pain is transient, with a protective purpose, alerting individuals to potentially damaging stimuli, thereby guarding against tissue damage (Cao et al., 2008). As such, acute pain is mediated by high threshold sensory neurons which can respond to external chemical, mechanical or thermal stimuli, as well as internal danger signals, such as those generated following muscle cramp or myocardial infarction (Millan, 1999; Woolf et al., 2007). Inflammatory pain serves to aid healing following tissue damage (Costigan et al., 2009). To achieve this, inflammation results in reduction of thresholds required to activate sensory neurons (Huang et al., 2006). This results in the affected area becoming more

sensitive, and therefore protected (Huang et al., 2006). Typically, both nociceptive pain and inflammatory pain abate following removal of stimulus or resolution of tissue damage (Costigan et al., 2009). However, either long term changes in the sensory pathway, or chronic inflammatory conditions can result in persistent, pathological pain.

1.1.2 Pathological and chronic pain

Pathological pain no longer serves a purpose to protect and/or support healing (Costigan et al., 2009). Pathological pain can be divided further into neuropathic pain (associated with damage or dysfunction to the nervous system, e.g. trauma, diabetic neuropathy, postherpetic neuralgia), cancer pain, dysfunctional pain (no identifiable inflammation or damage, e.g. fibromyalgia, irritable bowel syndrome) and aforementioned inflammatory pain (e.g. rheumatoid arthritis). A number of pain types fall into multiple categories (e.g. HIV pain, lower back pain) (Cao et al., 2008; Chwistek, 2017; Costigan et al., 2009; Feldman et al., 2019; Grace et al., 2014; Hadley et al., 2016; Uebelacker et al., 2015; Urits et al., 2019). Collectively these conditions fall under the overarching umbrella of chronic pain. Chronic pain is often defined in terms of pain duration, commonly, that which lasts longer than three months (Treede et al., 2019). While often the result of disease or injury, it is considered a separate condition which represents a significant personal trauma and wider economic burden (Costigan et al., 2009; Kassebaum et al., 2017; Painaustralia, 2019). Estimated to affect 20% of the global population, in Australia chronic pain affects 3.24 million people (Goldberg et al., 2011; Painaustralia, 2019). In Australia alone, the estimated cost is $139.3 billion annually (2018), accounting for treatment, lost productivity, care/aid, and loss in quality of life; this figure is predicted to reach $215.6 billion by 2050 (Painaustralia, 2019).

Common chronic pain symptoms include spontaneous pain generation (ongoing pain), painful responses to innocuous stimuli (allodynia), and exaggerated responses to noxious stimuli (hyperalgesia) (Milligan et al., 2009). Allodynia generated by dynamic mechanical stimuli, such as putting on clothing or brushing against furniture is considered particularly debilitating and can present in 20 – 40% of neuropathic pain patients (Hansson, 2003; Mogil, 2009). In neuropathic pain patients, cold allodynia is the second most commonly reported form of allodynia (Hansson, 2003). Studies indicate elicited pain sensitivities (allodynia or hyperalgesia) of mechanical and thermal sources present in 64 and 38% of patients respectively (Backonja et al., 2004). However, spontaneous pain, both continuous

and paroxysmal, is the primary complaint in chronic pain patients and consists of stabbing, shooting, and burning sensations which are associated with the highest reported pain scores in routinely administered pain intensity questionnaires (Backonja et al., 2004). Commonly, individuals also report abnormal tingling (paraesthesia) and numbness (Hansson, 2003). As mentioned above, these symptoms along with associated anxiety, decreased motivation, depression and fatigue cause a significant decrease in quality of life (Loeser, 2000).

Unfortunately, current therapeutic options provide limited efficacy; it is estimated only one in four patients experience over 50% pain relief (Nightingale, 2012). Common therapeutics include non-steroidal anti-inflammatory drugs (NSAIDs), antidepressants, anticonvulsants and opioids, which along with questionable efficacy all present side effects making them less than ideal options. Pregabalin and duloxetine, a commonly administered anticonvulsant and antidepressant respectively offer questionable efficacy and side effects including sedation, dizziness, dry mouth, constipation and nausea (Saarto et al., 2010). NSAIDs only offer short term relief and are associated with gastrointestinal symptoms (pain, diarrhoea), dizziness, headaches and tiredness (Roelofs et al., 2008; Ryder et al., 2005). The efficacy of opioids is also questionable in chronic pain, and when administered present challenges in the form of diminished efficacy over time, nausea, sedation, constipation, risk of addiction and opioid-induced pain (Baldini et al., 2012; Reinecke et al., 2015). A number of topically applied agents are also used, examples include lidocaine and capsaicin patches; however repeated and painful application processes respectively result in limited uptake of these therapies (Nightingale, 2012). It is clear that for such a significant health problem, treatment options must be improved with a push for improved efficacy. This is a major catalyst behind increased research effort into understanding the underlying physiology of generation and maintenance of chronic pain states.

1.2 Pain mechanisms

1.2.1 Peripheral and central nervous systems

The somatosensory system is responsible for converting signals from noxious stimuli into pain sensations. Separated into two anatomically separate systems; the peripheral (PNS) and central (CNS) nervous systems communicate in order to detect visceral or peripheral stimuli and process it in higher brain regions (D'Mello et al., 2008; Woolf et al., 2007). The peripheral nervous system is composed of sensory primary afferent neurons known as nociceptors, which convert noxious stimuli into electrical impulses, initiating nociceptive

signalling (Woolf et al., 2007). These neurons are pseudo-unipolar, with cell bodies located in dorsal root ganglia (DRG) or trigeminal ganglia (TG) for skin and visceral or orofacial innovation respectively. Either side of the cell body, long axonal process extend distally to peripheral sites and centrally into the spinal cord from DRG or the spinal tract nucleus from TG (Huang et al., 2013). Primary afferent neurons can be subdivided into C-, Aδ- and Aβ-fibres. The majority of nociceptors are the small diameter, un-myelinated and therefore slow conducting C-fibres; medium diameter, thinly myelinated Aδ-fibres are also involved in nociceptive signalling (Woolf et al., 2007). C-fibres have a high-threshold for activation, while Aδ-fibres have a lower, intermediate threshold which makes these neuron classes ideal for detecting high intensity noxious signals. Aβ-fibres are large diameter, highly myelinated, fast conducting neurons responsible for detection of non-noxious low intensity stimuli including touch, pressure and vibration (Millan, 1999). Non-neural cells considered part of the peripheral nervous system include Schwann cells, responsible for myelination and neural nutrition; and satellite glia located at the ganglion, which communicate with neurons and influence activity by inducing changes in receptor expression and neuropeptide release (Catala et al., 2013; Huang et al., 2013).

Neural components of the CNS include the spinal cord (SC), brainstem and brain; while microglia, astrocytes and oligodendrocytes make up the non-neural components (D'Mello et al., 2008; Milligan et al., 2009). Input from the PNS is transferred to the CNS at the dorsal horn of the spinal cord from the DRG, while the medullary dorsal horns of the trigeminal spinal tract nucleus receives input from the TG (Huang et al., 2013). The grey matter of the spinal cord is subdivided into 10 sections (laminae) containing numerous cell types, including nociceptive-specific (NS), low-threshold (LS), wide dynamic range (WDR) neurons and interneurons (Basbaum et al., 2009; D'Mello et al., 2008; Millan, 1999; Steeds, 2016). Sensory information is processed in the dorsal horn region which includes the first 6 laminae, as well as the tenth (grey matter around central canal) (D'Mello et al., 2008; Millan, 1999). NS cells are primarily found in the most superficial laminae, I and II (specifically the superficial section known as outer laminae II (II_0)) and only respond to nociceptive input from C- and Aδ-fibres; a smaller number are present in deeper laminae; IV, V, VI and X (Basbaum et al., 2009; Millan, 1999; Todd, 2002). These deeper laminae also contain LS cells which only respond to input from Aβ-fibres, and WDR cells which are able to respond to all types of peripheral input from light touch to

noxious mechanical and thermal (D'Mello et al., 2008; Steeds, 2016). Therefore, nociceptive signals alone are processed in the most superficial laminae, while all neuron types can signal through deeper laminae. Excitatory (glutamatergic) and inhibitory (GABAergic, glycinergic) interneurons make up a significant proportion of dorsal horn neurons influencing the activity of NS and WDR cells and are therefore important in output from the dorsal horn (D'Mello et al., 2008; Maxwell et al., 2007). Inhibitory interneurons play multiple important roles; including preventing spontaneous excitatory firing, separating different signalling modalities (e.g. low-threshold vs nociceptive) and preventing spatial spread of nociceptive input to maintain somatotopic representation (Cioffi, 2018). Higher structures involved in nociceptive transmission include the parabrachial nuclei (PB) and periaqueductal grey (PAG) of the brainstem, thalamus (ventrocaudal, medial), and numerous cortical regions (primary and secondary somatosensory, insular, anterior cingulate and prefrontal) (D'Mello et al., 2008). The details of these pathways will be discussed further in section 1.2.2.

Astrocytes are the most abundant cell type in the CNS. Connected by gap junctions, astrocytes support neuron function. They also encapsulate the synapse and are closely associated with capillaries enabling them to become a signalling pathway between neurons, astrocytes and capillaries. This allows modulation of water balance, synaptic transmission, blood flow, and potassium and glutamate homeostasis (Haydon, 2001; Milligan et al., 2009; Ransohoff et al., 2012). Microglial cells are considered macrophage cells of the CNS and constitute approximately 10% of all cells (Ransohoff et al., 2012). The first line of defence against homeostatic changes including invading pathogens, microglia survey their environment by extending and retracting philopodia (Cao et al., 2008; Nimmerjahn et al., 2005). Like astrocytes, microglia are intimately linked with the synapse providing support for synaptic transmission (Cao et al., 2008). Oligodendrocytes are primarily involved in myelination of CNS axons, while also assisting neural communication and modulating neuronal function (Cao et al., 2008; Milligan et al., 2009).

To maintain its delicate chemical composition while preventing entry of neurotoxic plasma components, pathogens and blood cells, the blood brain barrier (BBB) isolates the CNS from peripheral tissues (Sweeney et al., 2019). Endothelial cells form continuous non-fenestrated capillaries and the innermost layer. Pericytes incompletely cover the endothelial layer and are embedded in a basement membrane; to which astrocytes, linked

by gap junctions attach. Peripheral and central immune cells in the form of macrophages and microglia are also present at the BBB (Sharif et al., 2018; Sweeney et al., 2019). Further, epithelial cells at the choroid plexus form the blood-CSF (BCSF) barrier and arachnoid membrane (surrounding brain and SC) (Abbott et al., 2010). These sites are important for the infiltration of peripheral immune cells during injury and/or disease, the importance of which will be discussed in section 1.4.

1.2.2 Nociception – neural pathways

Specific neural pathways receive nociceptive signals and relay them to higher brain centres; each pathway is responsible for different sensory aspects of pain, related to its SC origin and final destination (Tracey et al., 2007). All nociceptive signals begin outside the CNS, synapsing with either NS, LS or WDR cells, known as second order projection neurons. Numerous tracts originate from these second order neurons, with major outputs being from laminae I and V (Basbaum et al., 2009). Major tracts include the spinothalamic and spinoreticular tracts, of which the majority of second order neurons cross-over (decussate) to the white matter on the opposite (contralateral) side of primary afferent stimulation; before projecting to higher CNS sites (Millan, 1999; Tracey et al., 2007; Yam et al., 2018). A majority of the ascending tracts synapse at the thalamus before reaching cortical regions including the primary and secondary somatosensory cortices; the insula, anterior cingulate and prefrontal cortex (D'Mello et al., 2008; Millan, 1999). Sending the signal to multiple cortical areas allows differentiation of nociceptive input, from sensory discriminative aspects (e.g. location, intensity, sharp/diffuse) to autonomic and affected/emotional aspects of pain (D'Mello et al., 2008; Yam et al., 2018). Multiple ascending tracts enter the thalamus only after synapsing or sending offshoots at brain stem sites, including the periaqueductal grey (PAG), reticular formation, rostral ventral medulla (RVM) and tectum (Kendroud et al., 2020; Yam et al., 2018). From these brainstem sites, along with the thalamus, further ascending tracts connect the hypothalamus and limbic system (Kendroud et al., 2020). Nociceptive input from visceral organs follows the same ascending routes, which is often the reason for referred pain, as the brain interprets visceral input as the somatic input entering at the same spinal cord level (Steeds, 2016). Trigeminal nociceptors relay information from the orofacial region (head, face, intraoral), entering the CNS at the brainstem (pons, medulla). From here, second order neurons synapse at the thalamus before projecting to somatosensory cortical regions (Kendroud et al., 2020).

Descending pathways play an important role in nociceptive transmission. Signals from the brainstem influence activity at the SC level, both facilitating and inhibiting nociceptive transmission (D'Mello et al., 2008; Millan, 1999; Tracey et al., 2007). Two important regions are the PAG and RVM. The PAG receives input from the spinothalamic tract, thalamus, hypothalamus, limbic system and cortex (D'Mello et al., 2008; Steeds, 2016). PAG neurons then excite cells of the RVM, an important site for integrating spinal cord descending influence. RVM projections return to the dorsal horn and generally inhibit transmission of nociceptive signals by modulating neurotransmitter release and altering activity of inhibitory interneurons, mechanisms of which are discussed in section 1.2.4. (Millan, 1999; Steeds, 2016). Further to the PAG-RVM system, the dorsal reticular nucleus (DRt) and ventrolateral medulla (VLM) also exert descending modulation (Heinricher et al., 2009). Similarly to the PAG-RVM system, these descending tracts can communicate directly with primary and secondary neurons as well as dorsal horn interneurons to influence pain outcomes (Cioffi, 2018).

1.2.3 Signal generation and transmission

Nociceptors detect noxious stimuli due to the array of surface ion channels and receptors at their exposed peripheral terminals; the type and amount of which determine which stimulus, be it heat, chemical or mechanical, excites the nociceptor (Woolf et al., 2007). Nociceptors can be classified as high-threshold mechanoreceptors, responding to intense mechanical stimuli; thermal receptors, responding to thermal (hot or cold) stimuli; and polymodal receptors, responding to mechanical, thermal and chemical stimuli (Basbaum et al., 2009; Kendroud et al., 2020). Major channel types include transient receptor potential (TRP), acid sensing ion channels (ASICs) and two pore background potassium channels (K2P) (e.g. TRAAK, TREK-1) (Basbaum et al., 2009).

TRP channels are nonselective cation channels, considered the most important family for detection of noxious stimuli due to wide expression profile and polymodal nature. Three of the six subfamilies, ankyrin (TRPA), vanilloid (TRPV) and melastatin (TRPM) play important roles in nociception (Gonzalez-Ramirez et al., 2017). Detection of thermal stimuli is well documented in TRP channels, transient receptor potential cation channel subfamily V member 1 (TRPV1), discovered as the receptor for capsaicin, is found on the majority of heat sensitive nociceptors, while transient receptor potential cation channel subfamily M member 8 (TRPM8) is expressed on the majority of cold sensitive

nociceptors (Caterina et al., 1997; Peier et al., 2002). Multiple other TRP channels are involved in the detection of noxious heat (TRPV2, TRPV3, TRPV4) and cold (TRPA1) (Basbaum et al., 2009; Dhaka et al., 2006). As mentioned, TRP channels are polymodal and respond to numerous chemical stimuli including capsaicin, protons, toxins (TRPV1), mustard, wasabi, cinnamaldehyde (TRPA1) and menthol (TRPM8) (Basbaum et al., 2009). ASICs including ASIC3, ASIC1A and ASIC1B are permeable to cations and implicated in nociception. Known for the response to protons produced by tissue ischemia, activation by mechanical stimuli and non-proton chemical stimuli has also been observed (Basbaum et al., 2009; Wemmie et al., 2013; Yu et al., 2010). Potassium channels present at peripheral terminals play a regulatory, rather than faciliatory role in nociceptive firing. Potassium channels respond to thermal (TREK-1, -2, TRAAK), mechanical (TREK-1, -2, TRAAK) and chemical (TREK-1, TASK-1,- 3.) stimuli (Du et al., 2013; Honore, 2007; Kang et al., 2005).

Increases in cation influx at the afferent terminals has a depolarising effect on membrane potential, in turn activating voltage-gated ion channels critical for propagation of the action potential, the frequency of which determines stimulus intensity (Basbaum et al., 2009). Voltage gated sodium (NaV) and calcium channels are important for this process, while K2Ps and voltage gated potassium channels (Kvs) exert regulatory influence. NaV1.7 and 1.8 in particular are well characterised due to their high expression levels in C-fibres; with altered function resulting in clinical pain disorders erythromelalgia and paroxysmal extreme pain disorder (Basbaum et al., 2009; Nassar et al., 2004). Generally, it is NaV channels which are activated when the appropriate membrane potential is reached, starting the action potential which propagates along the neuron (Yam et al., 2018).

Numerous Kv and K2P channels are associated with nociceptor function; maintaining resting potential and therefore nociceptor threshold, repolarising the cell following action potential, and as mentioned above, responding to noxious stimuli (Tsantoulas et al., 2014). Due to their activity at resting membrane potential, Kv7 (M channels) are considered an important element of maintaining resting membrane potential and overall nociceptor activation threshold. Enhancing Kv7 activity by altering voltage characteristics reduces nociceptive stimulation (Brown et al., 2009). Due to their presence on small diameter primary afferents, alongside TRPV1, Kv1.4 is implicated in nociceptive function (Rasband et al., 2001). Further, Kv1.4 levels are reduced in neuropathic pain models and implicated

in alteration of membrane potential and decreased excitability in glycoprotein 130 deficient nociceptors (Langeslag et al., 2014; Rasband et al., 2001). T-type voltage gated calcium channels (VGCC) present on the cell body and neuronal dendrites are also important to maintenance of resting potential. Knockdown of these low-voltage activated channels results in attenuated nociceptive sensitivity following nerve injury; highlighting their regulatory role in nociceptive threshold maintenance (Cao, 2006).

Upon reaching the presynaptic terminal, an action potential induces neurotransmitter release by activating VGCCs. High threshold N-, Q/T- and R-type VGCCs exist within the dorsal horn, with differing distribution across the laminae (Cao, 2006; Zamponi et al., 2009). Following activation of VGCCs, calcium enters the presynaptic terminal promoting fusion of neurotransmitter vesicles to the neuronal membrane. Typically, Aδ-fibres release glutamate, while C-fibres release various neuropeptide neurotransmitters, including calcitonin gene-related peptide (CGRP) and substance P (SP) (Kendroud et al., 2020). Glutamate exerts its excitatory effect on multiple receptors once crossing the synapse, including; α-amino-3-hydroxy 5-methyl-4-isoxazeloproprionic acid (AMPA), *N*-methyl-D-aspartate (NMDA) and G-protein coupled metabotropic receptors (mGluR), inducing calcium influx (D'Mello et al., 2008; Stanley, 2016). SP and CGRP bind to neurokinin 1 receptors (NK-1) and the receptor activity modifying protein 1 (RAMP1) accessory protein/calcitonin receptor-like receptor (CRLR) complex respectively, inducing intracellular signalling and activating kinases which facilitate glutamatergic transmission (Iyengar et al., 2017; Zieglgansberger, 2019). Neurotransmitters must be removed from the synaptic cleft in order to stop continued signal transmission. This can be achieved by degradation, reuptake by the releasing nociceptor, removal by astrocytes and drifting (Yam et al., 2018). Glutamatergic transmission results in excitatory post-synaptic currents (EPSCs) in the secondary order projections. Summation of ESPCs results in action potential, transmitting the nociceptive signal (Basbaum et al., 2009).

In addition to neurotransmitters, a number of neuromodulatory substances may be released by nociceptors depending on the CNS environment. Fractalkine, a chemokine expressed by nociceptors is cleaved from the membrane in response to overstimulation (Chapman et al., 2000; Verge et al., 2004). Adenosine triphosphate (ATP) is released by damaged cells as well as during synaptic transmission, modulating excitatory signals by binding presynaptic

ATP receptors (P2X), enhancing glutamate release (Li et al., 1998; Nakatsuka et al., 2001). Synaptic vesicle release can also promote δ-opioid receptors (DOR) to the cell membrane, joining μ-opioid receptors (MOR) and cannabinoid receptors (CB1) in an analgesic role at the presynaptic terminal (Guan et al., 2005; Woolf et al., 2007).

1.2.4 Descending influence

As described above, ascending pathways are moderated by descending inhibition and facilitation by a PAG-RVM system (Heinricher et al., 2009). The dorsal horn is a key site for integration of ascending and descending signals; ultimately it is the dynamic interaction of descending inhibitory and excitatory signals which determine nociceptor output to higher brain centres. This balance is altered by multiple factors, including illness, injury, stress and fear (Heinricher et al., 2009). Activity of pain inhibiting OFF-cells or pain facilitating ON-cells in the RVM determines the descending effect on the dorsal horn, with low nociceptive thresholds associated with an active ON-cell population and inactive OFF-cell population (Heinricher, 2016; Heinricher et al., 1989; Ossipov et al., 2010). The modulation of each of these RVM cell types by the PAG is the mechanism of action for a number of receptor systems, importantly, opioids and cannabinoids (Maione et al., 2006; Maione et al., 2009). Outside of the PAG-RVM system, the areas of caudal medulla, DRt and VLM participate in descending modulation. Both systems consist of closed loops with the dorsal horn. The DRt receives ascending input and plays a mainly facilitatory role in descending modulation (Lima et al., 2002). While the VLM exerts a tonic inhibitory function via noradrenergic cells (Tavares et al., 2002).

Endogenous opioids, including β-endorphin, dis-inhibit PAG output neurons, increasing OFF-cell firing while inhibiting ON-cells and exerting an inhibitory tone onto the dorsal horn (Adams et al., 1986; Fields et al., 1991; Heinricher, 2016). This inhibition is associated with an increase in inward potassium conductance at presynaptic GABAergic inputs and postsynaptic PAG and RVM GABAergic interneurons (Chieng et al., 1994; Lau et al., 2014). An interaction with co-expressed TRPV1 has also been observed *in vivo* as non-analgesic doses of either agonist when co-administered produce analgesia to thermal stimuli in rats, inhibited by both TRPV1 and opioid receptor antagonists (Maione et al., 2009).

CB1 receptors are also highly expressed in the RVM descending tracts. Excessive glutamate production activates glutamate receptor mGlu5, releasing endocannabinoids (endogenous cannabinoids), in turn, activating CB1 receptors in the RVM. Activation of CB1 receptors reduces GABAergic tone by presynaptic inhibition thereby dis-inhibiting OFF cell descending tracts (Lau et al., 2014; Maione et al., 2006). The effect of endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG) on OFF-cell activity is dependent on their relative levels in tissue, therefore can have either a facilitatory or inhibitory role (Maione et al., 2006). Their pro-nociceptive effect at low doses is thought to involve their CB1 activation on excitatory (glutamatergic) neurons (Maione et al., 2006; Marsicano et al., 2003). Like opioid expressing cells within the PAG, CB1 receptors are colocalised with TRPV1 which is activated by AEA, resulting in immediate analgesia in rat a thermal pain model by activation of OFF cells. Therefore, the effects of endocannabinoids in the PAG on descending pain modulation is determined by concentrations, relative timing of release and their actions at both CB1 and TRPV1 receptors (Maione et al., 2006).

Descending projections include serotonergic, glycinergic, GABAergic and noradrenergic neurons. Hydroxydopamine (5-HT), also known as serotonin, can be both pro- and anti-nociceptive depending on which receptor it binds; it is unclear which RVM population is serotonergic, or indeed if action is direct or via interneurons (Ossipov et al., 2010). Nevertheless, antagonising spinal receptors 5-HT7 and 5-HT3 has both pro- and anti-nociceptive effects respectively, suggesting relevance of serotonergic tone on the dorsal horn (Dogrul et al., 2009). Noradrenergic neurons are not found at either the PAG or RVM, however these regions connect with noradrenergic sites, including A5, A6 and A7 nuclei which project to the spinal cord (Ossipov et al., 2010). Noradrenaline (NE) is released by descending neurons and targets $\alpha 2$ and $\alpha 1$-adrenergic receptors. $\alpha 2$-adrenergic receptors inhibit pre- and post-synaptic terminals resulting in nociceptive inhibition, while $\alpha 1$-adrenergic receptors depolarise inhibitory GABAergic interneurons (Gassner et al., 2009; Pertovaara, 2006). In addition to the vast literature on 5HT and NE as descending neurotransmitters, there is also evidence of direct GABAergic and glycinergic descending projections from the RVM (Antal et al., 1996; Lau et al., 2014).

1.3 Pathological Pain Models

1.3.1 Neurogenic pain models

Neurogenic compounds are pain producing and do not directly reflect a particular clinical pain state, but recruit nociceptive mechanisms leading to altered pain processing. They can also be considered inflammatory pain models due to their neurogenic inflammatory effect and are often utilised for investigation of nociceptive mechanisms and as tools for screening therapeutic compounds of interest. Capsaicin is widely used in animal and human pain models, directly activating TRPV1 channels resulting in spontaneous pain and longer lasting (up to 120 min) hypersensitivity (Gilchrist et al., 1996; Hutchinson et al., 2013). Further to inducing symptoms relevant to pathological pain states; preclinical capsaicin models respond to commonly utilised opioid and anti-epileptic drugs (Joshi et al., 2006). Similarly, mustard oil is used to elicit spontaneous pain by direct activation of TRPA1 channels in human and animal models (Andersen et al., 2017; Laird et al., 2002). Bee venom is also utilised to produce short lived spontaneous pain and longer lasting hypersensitivity (Lariviere et al., 2000). These compounds directly activate nociceptors and allow greater control of pain outcomes. In addition, the procedures are minimally invasive, and pain induced is transient in comparison to other pain models mentioned here, improving ethical considerations (Joshi et al., 2006). While neurogenic compounds result in relevant behavioural outcomes and are attenuated by known analgesics, the overall clinical relevance to pathological pain is questionable given they do no replicate clinical pathophysiological events.

1.3.2 Inflammatory pain models

Numerous immune stimulating substances are used to induce inflammatory pain. TLR4 agonist lipopolysaccharide (LPS) is commonly used, which does not produce spontaneous pain behaviours but induces pain hypersensitivity in clinical and preclinical animal models (Calil et al., 2014; Maier et al., 1993; Wegner et al., 2014). Similarly, intrathecal human immunodeficiency virus-1 envelope glycoprotein, gp120 is used to induce thermal and mechanical hyperalgesia (Milligan et al., 2001b). Complete Freund's adjuvant (CFA) and carrageen represent commonly administered immune driven pain mediators. CFA and carrageen are commonly administered into the hind-paw producing peak hypersensitivity between 6-8 h post administration; carrageen-induced sensitivity persists compared to CFA (Cunha et al., 2005; Ren et al., 1999). Similarly, zymosan can be administered into the hindpaw for short lasting (6 h) sensitivity, or centrally to induce mechanical

hypersensitivity lasting weeks (Milligan et al., 2003; Sweitzer et al., 1999). Formalin, commonly administered subcutaneously, causes both spontaneous pain and mechanical hypersensitivity lasting 60 min (Choi et al., 2003; Sweitzer et al., 1999). Less common inflammatory mediators include lithium chloride, picrotoxin, and kainite (LaBuda et al., 2000; Maier et al., 1993; Oliveras et al., 1996). Similar to neurogenic compounds, these compounds don't reflect clinical pathophysiology in terms of causation and timeframes. However, they can be useful for understanding mechanisms associated with inflammatory-induced nociceptive signalling changes. Further, many of these models are carried out over longer time periods, a disadvantage to both subject and experimenter compared to neurogenic models.

There are however inflammatory pain models which aim to replicate clinical disease pathophysiology. Arthritis models are often used as a screen for compounds ready for early clinical trials (Berge, 2011). These models include antigen-induced arthritis (AIA), collagen-induced arthritis (CIA), and monosodium iodoacetate arthritis (MIA). Models of arthritis can induce mechanical and thermal hypersensitivity upwards of 30 days (Boettger et al., 2008; Ebbinghaus et al., 2012; Fernihough et al., 2004; Inglis et al., 2007). Multiple behaviours are observed in arthritis models which are potentially relevant in clinical situations, including weight bearing and limb movement (Gregory et al., 2013). Other examples of preclinical inflammatory models aimed at specific clinical pathologies include remifentanil administration to replicate opioid-induced hyperalgesia (OIH), incision models imitating post-surgical pain and meningeal inflammatory stimulation to mimic migraine (Aguado et al., 2018; Melo-Carrillo et al., 2013; Pogatzki et al., 2002).

1.3.3 Neuropathic pain models

Neuropathic pain models are developed to recreate the pathophysiological mechanisms following nervous system damage. Damage to peripheral nerves is a common method of inducing neuropathic pain hypersensitivity, resulting in common clinical sensitivity changes (Campbell et al., 2006). Multiple nerve ligation models exist due to multiple attempts to create a standardised approach (Berge, 2011). Chronic constriction injury (CCI) of the sciatic nerve is commonly performed, inducing thermal and mechanical hypersensitivity up to 4 weeks (De Leo et al., 1997). Ligation of spinal nerve roots (SNL) causes similar hypersensitivity (up to 35 days) (Arruda et al., 2000; Hashizume et al., 2000; Kim et al., 1992). Similar time frames are observed following partial sciatic nerve

ligation (PSNL) (Seltzer et al., 1990). Spinal cord hemisection is a form of spinal cord injury (SCI) used to create mechanical hypersensitivity lasting 6 weeks (Peng et al., 2006). Spared nerve injury (TST), transecting the common peroneal and tibial nerves, results in significantly greater duration of sensitivity, (upwards of 200 days) for mechanical, heat and cold stimuli (Decosterd et al., 2000). Less common techniques, include sciatic cryoneurolysis, sciatic nerve transection and facial nerve ablation albeit with varying timeframes owing to the nature of injury (De Leo et al., 1994; Kiefer et al., 1993; Rotshenker et al., 1992). A significant downside to this approach is that it is in contrast to clinical trials of neuropathic pain, which don't use subjects with comparable lesions. Instead, they more frequently include patients with port-herpetic neuralgia and diabetic neuropathies (Finnerup et al., 2010; Rice et al., 2008). This approach makes it difficult to compare clinical and preclinical outcomes. Finally, these models report elicited behavioural sensitivities, ignoring the significant clinical symptom of spontaneous pain.

Neuropathic pain results from numerous clinical pathologies, the pathophysiology of which are recreated in preclinical pain models in an attempt to overcome the relevance issues of above models which utilise traumatic lesions. Diabetic neuropathy is one such example, including the streptozotocin-diabetic model, numerous diabetic mouse strains and diet-induced diabetes (Ahlgren et al., 1993; Obrosova, 2009; Wuarin-Bierman et al., 1987). Chemotherapy-induced neuropathic pain (CINP) models are widely used, utilising multiple drugs including paclitaxel, vincristine, cisplatin and bortezomib (Flatters et al., 2004; Robinson et al., 2015; Yan et al., 2015). Cancer pain models are also commonly utilised, inoculating tumour cells to specific target areas (Jaggi et al., 2011; Shimoyama et al., 2002). Whether these models reflect clinical symptoms is questionable however (Berge, 2011; Gregory et al., 2013). While the exact mechanisms underlying the generation of pathological pain remain elusive, these models, along with the clinical disease mimicking inflammatory models, currently offer the best chance of bridging the knowledge gap.

1.3.4 Measurable Outcomes

All models require measurable outcomes from which researchers can infer pain. As mentioned above, spontaneous pain is clinically the most significant symptom of pathological pain; many neurogenic and inflammatory models monitor spontaneous behaviours, including flinching, biting, tail flick, blinking and altered body position/or movement (Gregory et al., 2013; Kemper et al., 1998; Laird et al., 2001a; Liu et al., 2013;

Sakurada et al., 1992). Pain sensitivities are monitored by eliciting responses to mechanical or thermal stimuli. Common tests for mechanical sensitivity include the von Frey test and paw withdrawal thresholds which apply a static force to the hindpaw (Gilchrist et al., 1996; Gregory et al., 2013; Otuki et al., 2005). Thermal tests include withdrawal from hot or cold surfaces and water, commonly used are the Hargreaves test (light source), hot/cold plate tests, tail withdrawal/flick tests and acetone application (Gregory et al., 2013; Honda et al., 2008; Ko et al., 1998; Negri et al., 2006; Yalcin et al., 2009). While these observations are relevant to clinical symptoms, the relevance is often limited, and some clinical symptoms are ignored altogether. For example, spontaneous behaviours mentioned above are induced by direct neuronal stimulation or inflammation, dissimilar to non-evoked spontaneous pain in clinical chronic pain patients (Backonja et al., 2004; Hansson, 2003). Similarly, static mechanical testing ignores clinical instances of allodynia induced by dynamic stimulation. Further, paraesthesia (abnormal sensations) and sensory loss are ignored in animal pain models (Hansson, 2003). Consideration of these less explored, yet clinically relevant symptoms may provide greater context to the relevance of currently utilised models.

1.4 Neuroimmune pain

1.4.1 Neuroimmune background

As described earlier, the nervous system contains numerous non-neural cell types including satellite glia, Schwann cells, astrocytes, microglia and oligodendrocytes, which are important for maintaining nervous system homeostasis (Greenhalgh et al., 2020; Huang et al., 2013; Stevens, 2003). Following injury or illness, astrocytes and microglia are considered the major responsive cell types of the CNS, and along with CNS infiltrating peripheral monocytes and T-cells are involved in identifying and removing pathogens and repairing associated damage (Ransohoff et al., 2012). This involves release of immune mediating cytokines, chemokines and neurotrophic factors which instigate changes in surrounding glia and neurons (Grace et al., 2014; Greenhalgh et al., 2020). This communication is referred to as neuroimmune signalling and ultimately leads to alteration in processing of nociceptive signals in both the PNS and CNS following illness or injury (Grace et al., 2014). The three major contributors are neurons (pre- and post-synaptic terminals), astrocytes and microglia which form a tetrapartite synapse, an important site for central neuroimmune communications (De Leo et al., 2006). Outside of the CNS, important neuroimmune communication occurs at peripheral nerve terminals influenced by peripheral immune responses and neuropeptide release (Baral et al., 2019). As such,

neuroimmune signalling is a bidirectional relationship between neural and non-neural elements to affect outcomes of nociceptive input (Baral et al., 2019; Cao et al., 2008).

1.4.2 Immunologically active glia

Glial cells are able to respond to illness and injury due to the plethora of pattern recognition receptors (PRRs) they express. PRRs recognise molecular epitopes released by damaged and stressed endogenous cells known as DAMPs (danger associated molecular patterns), as well as exogenous pathogens known as PAMPs (pathogen associated molecular patterns) (Takeuchi et al., 2010). Commonly expressed PRRs include toll-like receptors (TLRs) and NOD-like receptors (NLRs). Glia also express receptors which detect neuropeptides released by nociceptors and postsynaptic neurons including SP, glutamate, CGRP, ATP, fractalkine, prostaglandins (PGs) and nitric oxide (NO) (Cao et al., 2008).

Constitutively expressed membrane receptors are key to initiating microglial responses and include TLRs, purinergic receptors and fractalkine receptors (CX3CR1) (Cao et al., 2008). Microglia respond rapidly following stimulation, resulting in morphological changes (stratified to ameboid appearance), increased proliferation, altered migration and phagocytosis, and changes in gene expression. This includes changes in cell-surface molecules including, complement receptor 3 (CD3), major histocompatibility complex (MHC) class 1 and 2, PRRs and numerous cytokine and chemokine receptors (Hanisch, 2002; Olson et al., 2004). Further to changes in receptor expression, multiple soluble factors are released, including cytokines; interleukins (IL-1β, IL-6, IL-8, IL-10, IL-18 IL-2) and tumour necrosis factor α (TNFα), chemokines (incl. CCL2, CCL5, M-CSF), NO, ATP and PGs (Hanisch, 2002; Inoue, 2006; Li et al., 2003; Ransohoff et al., 2012).

Evidence suggests the molecular signals released following afferent damage is key to engaging microglial signalling, of which ATP plays a significant role. ATP is an important microglia "reactivator;" binding P2-type purinergic receptors (Basbaum et al., 2009). P2X4, P2X7 and P2Y are expressed on microglia and upregulated following activation. P2 receptors are cation channels, activation therefore increases intracellular calcium, promoting activation of p38 mitogen-activated protein kinase (MAPK) (Trang et al., 2012). p38 MAPK activity following P2X4 activation results in release of brain-derived

neurotrophic factor (BDNF), while IL-1β and cathepsin S are released following P2X7 activation (Clark et al., 2010; Clark et al., 2007; Trang et al., 2009).

CX3CR1, the fractalkine receptor is another cation channel upregulated in microglia, activation of which results in increased intracellular calcium and increased p38 MAPK (Chapman et al., 2000; Verge et al., 2004; Zhuang et al., 2007). CX3CR1 in the CNS is restricted to microglia and considered to have neuroprotective function in uninjured tissues, supporting neuronal and microglial survival in neurotoxic environments (Cardona et al., 2006). Cathepsin S, released by microglia following ATP stimulation, cleaves fractalkine from the presynaptic membrane, resulting in a positive feedback loop further enhancing microglia responses, providing a mechanism by which fractalkine signalling contributes to the pathological pain state. Indeed, cathepsin S inhibitor LHVS attenuates mechanical hypersensitivity associated with neuropathic pain in rats (Clark et al., 2007). Chemotactic cytokine receptor 2 (CCR2) is another receptor upregulated on microglia in inflammatory conditions, enhancing chemoattraction, and microglial activation (Abbadie et al., 2003; Zhang et al., 2018).

Multiple TLRs (1 - 9) are expressed at low levels on microglia in healthy tissues and upregulation occurs rapidly upon activation (Bsibsi et al., 2002; Konat et al., 2006; Lehnardt, 2010). TLR activation results in activation of transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB), leading to transcription of interleukin-1 (IL-1) family genes, producing pro forms of IL-1 and IL-18 (Chakraborty et al., 2010; Ransohoff et al., 2012). Key to the conversion or pro-IL-1β and pro-IL-18 into their active forms (IL-1β, IL-18) is caspase-1, which itself is activated by inflammasomes, large structures containing pattern recognition NLRs, adaptor proteins and effector caspases (Martinon et al., 2004). Extensively studied in microglia, inflammasome (NLRP3) activation is induced by numerous DAMPS and PAMPS including ATP, uric acid and bacterial protein and RNA (Halle et al., 2008; Petrilli et al., 2007; Ransohoff et al., 2012). Caspase independent cleavage of pro-IL-1β has also been reported in neuropathic pain following TNFα-induced microglial activation, by matrix metalloprotease-9 (MMP-9) (Kawasaki et al., 2008). Of particular interest due to their apparent role in pathological pain are TLR2 and TLR4. In models of nerve injury, knockout of both TLR4 and TLR2 produce significant reduction in cytokine output and

reduced glial morphological changes, highlighting their role in glial responses to injury (Kim et al., 2007; Tanga et al., 2005). TLR4 responds to exogenous ligands including gram negative bacterial cell wall component LPS, and endogenous ligands including heat shock proteins (HSPs), causing release of proinflammatory cytokines (interferon-γ (IFN γ), IL-1β, IL-6 and TNFα) and other immunomodulatory substances including inducible-nitric oxide synthase (iNOS) (Nicotra et al., 2012; Olson et al., 2004; Tanga et al., 2005). TLR4 activation also induces microglial neuroprotective effects including phagocytic repair (Glezer et al., 2006). TLR2 activation results in the same pro-inflammatory milieu induced by TLR4 although alternate danger signals activate the receptor, including gram positive bacterial cell wall components (Kawai et al., 2007b; Kim et al., 2007).

Astrocyte responses are delayed and persist compared to those of microglia in preclinical pain models. Changes in astrocyte activation often correlate with induction of altered behavioural sensitivity (Hashizume et al., 2000; Ren et al., 2008). Interestingly in contrast to astrocytes, microglial stimulation is not always required for persistent pain in animal models and human tissues, as observed in rat CINP and clinical HIV tissues (Robinson et al., 2014; Shi et al., 2012). In contrast to microglia, astrocytes constitutively express only few TLRs; in humans TLR 2 and -3 have been detected, while TLR2, -3, -4, and -9 have been reported in murine astrocytes (Bowman et al., 2003; Bsibsi et al., 2002; Gorina et al., 2009). Following stimulation of TLRs and NLRs astrocytes produce inflammatory cytokines IL-1β and IL-6, and chemokines CCL2, CXCL1, CXCL10 and CXCL12 (Ransohoff et al., 2012). Similar to microglia, cleavage of pro-IL-1β in astrocytes is mediated by MMP-2, however at later time points associated with its relatively late response (Kawasaki et al., 2008). This cleavage of pro-IL-1β is also observed in satellite glia with comparable timing (Kawasaki et al., 2008). In further contrast to microglia, expression of MHC in astrocytes is low, which brings into question their *in vivo* significance to antigen presentation. However there is strong evidence that astrocytes are heavily involved in T-cell activation (Tian et al., 2012). Along with microglia, astrocyte-induced chemokines recruit T-helper cells (Th1, Th2), promote recruitment of T regulatory cells (Treg) via cytokine transforming growth factor β (TGF- β) and CXCL1, while also releasing anti-inflammatory IL-10, capable of suppressing T cell and microglia activation (Meiron et al., 2008; Nair et al., 2008; Trajkovic et al., 2004).

Upregulation of gap junction proteins is observed in CINP, CCI and SCI pain models (Chen et al., 2014; Cronin et al., 2008; Robinson et al., 2015). Of particular importance is connexin 43 (CX43), upregulated in aforementioned pain models, inhibition of which results in reversal of mirror image allodynia, brief attenuation of ipsilateral thermal hyperalgesia and improved recovery times (Cronin et al., 2008; Spataro et al., 2004). These results suggest that astrocytic communication influences distant synapses, potentially through release of pro-inflammatory cytokines IL-1 and IL-6 (Spataro et al., 2004). Further, CX43 forms hemichannels following nerve injury, allowing release of numerous neuromodulator substances including ATP, glutamate and CXCL1, exacerbated by the concurrent upregulation of CX43 (Chen et al., 2014; Huang et al., 2012; Ji et al., 2019).

Glutamate transporters (GLT1, GLAST) constitutively expressed on astrocytes and important for maintaining glutamate homeostasis at the synapse, are downregulated in preclinical pain models. PSNL and CINP models report astrocytic downregulation of GLT1 and GLAST in rats (Robinson et al., 2015; Xin et al., 2009). While minocycline pre-treatment prevents decreases in glutamate transport in a CIPN model (Robinson et al., 2015). Interestingly, SC analysis shows an initial upregulation in GLAST and GLT-1 4 days after CCI, followed by downregulation at 14 days. PSNL initiates expression of GLT-1 and GLAST in microglia, potentially explaining rapid increases in SC glutamate transporters, while delayed downregulation is associated with later responding astrocytes (Sung et al., 2003).

It is clear that activation of typically immune receptors and associated signalling can alter the neuromodulatory elements surrounding neurons. These include changes to glutamate, cytokine, chemokine, ATP, NO, cathepsin S and PG levels. In addition to DAMPs and PAMPs, elements released by/from neurons, such as SP, glutamate, fraktaline and ATP also stimulate glia to induce further changes. It is now important to understand how these neuroimmune interactions alter nociceptive transmission, both centrally and in the periphery.

1.4.3 Neuroimmune interactions – peripheral sensitisation

Peripheral sensitisation results in increased excitability of nociceptors, causing hyperalgesia and allodynia within their receptive field (Grace et al., 2014). Excitability changes are a result of lowered activation thresholds, by a process known as sensitisation.

Sensitisation can result from numerous mechanisms, including; upregulation of, or altered ion channel kinetics (increased receptor activation for a given stimulus), and changes in thresholds for NaV opening (Campbell et al., 2006). Immune activation in the periphery, or release of immune modulating neurotransmitters and neuropeptides from nociceptor terminals contribute to peripheral sensitisation.

Peripheral inflammation, including neuronal injury results in secretion of monocyte-chemoattractant protein 1 (CCL2) and leukemia inhibitory factor (LIF), recruiting macrophages to the injury site, a key step in the process of Wallerian degeneration (Sugiura et al., 2000; Tofaris et al., 2002). Further recruitment of mast cells, basophils, fibroblasts, neutrophils, T- and B-cells and Schwann cells produces a proinflammatory milieu along with resident endothelial cells, neurons and keratinocytes (Basbaum et al., 2009; Woolf et al., 2007). Often concurrently, multiple nociceptor inputs result in action potential propagation along collateral axon branches to the periphery releasing neurotransmitters which contribute to the inflammatory milieu (Yam et al., 2018). This milieu contains a multitude of potential neural sensitisers including cytokines, chemokines, PG, nerve growth factor (NGF), SP, CGRP, bradykinin, NE, 5-HT, glial derived neurotrophic factor (GDNF), TNFα, activin, prokineticin, proteases, protons, NO and ATP (Basbaum et al., 2009; Woolf et al., 2007; Yam et al., 2018). Sensitising agents bind their respective receptors initiating intracellular signalling, of which important regulators include; protein kinase C (PKC), protein kinase A (PKA), phospholipase C (PLC), phosphoinositide 3-kinase (PI3K), ERK- and p38- MAPK (Cheng et al., 2008; Yam et al., 2018).

PKC, PKA and PI3K are implicated in phosphorylation of TRPV1 receptors, increasing their response to chemical stimuli (Varga et al., 2006; Zhang et al., 2005). Similarly, PKC activation following bradykinin stimulation sensitises TRPA1 channels to cold stimuli (Bautista et al., 2006). Mechanical sensitivity induced by NGF, which binds to receptor tyrosine kinase (TrkA) and neurotrophin receptor p75, is attenuated by inhibitors of PLC, ERK and PI3K (Malik-Hall et al., 2005). TNFα, acting on TNFR1 causes modulation of NaV channels via activation of p38 MAPK, facilitating channel opening (Jin et al., 2006). These studies also highlight the key effector channels in peripheral sensitisation, namely TRP and NaV channels. Of which TRPV1, TRPA1 and NaV-1.7, -1.8 and -1.9 contribute significantly to peripheral sensitisation (Amaya et al., 2006; Caterina et al., 2000; del

Camino et al., 2010; Kerr et al., 2001; Nassar et al., 2004; Varga et al., 2006). Membrane channel activity is also altered by lipid membrane components which can be altered by these intracellular signalling effectors. Hydrolysis of phosphatidylinositol 4,5-bisphospate (PIP_2) by bradykinin- and NGF-induced PLC activation releases TRPV1 from PIP_2 mediated inhibition (Chuang et al., 2001).

While the primary role of cytokines in peripheral sensitisation is the potentiation of the inflammatory response, there is evidence of direct effects. Nociceptors express a variety of classical immune receptors including those for TNFα and IL-1β (Opree et al., 2000). Direct sensitisation of nociceptors by TNFα and IL-1β has been reported. TNFα induces joint sensitivity in the rat knee joint via p38 MAPK, which is associated with increased excitability of excised DRG neurons (Richter et al., 2010). IL-1β reduces rat nociceptor thresholds to noxious heat, potentiating accumulation of intracellular calcium (Obreja et al., 2002). Both TNFα and IL-1β produce mechanical hypersensitivity following intraplantar injection in mice lasting up to 5 h, in the case of IL-1β however this was via downstream PG release (Cunha et al., 2005). Further, IL-1β is shown to increase expression of NGF, resulting in increased surface expression of TRPV1 and sensitisation of NaV channels in rat DRG neurons (Binshtok et al., 2008; Ebbinghaus et al., 2012; Kanaan et al., 1998; Lindholm et al., 1987).

Upregulation of ion channels TRPV1 and TRPA1 on injured nociceptors is associated with increased hot and cold sensitivity respectively in neuropathic pain models (Hudson et al., 2001; Katsura et al., 2006b). Likewise, rat and mouse models of inflammatory pain report upregulation of NaV1.7 and NaV1.8 in DRG neurons as well as NaV1.3 in the rat and NaV1.9 in mice (Black et al., 2004; Strickland et al., 2008). Further to ion channels, there is also upregulation of neurotransmitters, neuropeptides and cytokines (SP, CGRP, BDNF, TNFα and IL-1β) in nociceptors following inflammation and/or nociceptive activity (Cheng et al., 2008). Upregulation of CGRP, BDNF and P2X3 has also been reported in uninjured nociceptors following nerve ligation (Fukuoka et al., 2001; Fukuoka et al., 1998; Tsuzuki et al., 2001) Inflammation and nerve injury also result in both upregulation and downregulation of MORs respectively (Kohno et al., 2005; Puehler et al., 2004).

Neurotransmitters released in the periphery act at multiple targets to induce an inflammatory response and drive neurogenic inflammation. SP and CGRP both act at vasculature promoting neurogenic inflammation by inducing permeability, oedema and NO release (Baral et al., 2019). SP targets monocytes and macrophages inducing IL-1β, IL-6 and TNFα release (Cunin et al., 2011; Lotz et al., 1988). In contrast, CGRP also induces anti-inflammatory effects including release of IL-10. The overall impact of these contradictory mechanisms on nociceptive outcomes is yet to be elucidated (Baliu-Pique et al., 2014; Baral et al., 2019). SP also acts directly at mast cells resulting in degranulation, releasing numerous nociceptor effector substances including histamine, leukotrienes, TNFα, tryptases and PG (Baral et al., 2019; Lewin et al., 1994).

Peripheral nerve injury can induce increased firing of the injured nociceptor population or in the surrounding uninjured nociceptors away from their peripheral terminals, known as afterdepolarisation or ectopic discharge (Blumberg et al., 1984; Djouhri et al., 2006; Liu et al., 2002). Not classical nociceptive action potentials, this ectopic firing results from abnormal membrane excitability at the site of injury. Proposed mechanisms include TNFα released from Schwann cells, altered activity of potassium and calcium channels, hyperpolarisation-activated current and Ca^{2+}-activated chloride currents (Hilaire et al., 2005; Lee et al., 2005a). Like inflammation at peripheral terminals, the resulting sensitisation results in reduced activation thresholds due to altered membrane surface molecules and function, upregulation of neurotransmitters, and spontaneous firing.

1.4.4 Neuroimmune interactions – central sensitisation

Central sensitisation is the common mechanism responsible for multiple chronic pain states, associated with decreased activation thresholds in nociceptive projection neurons and increased responsiveness, resulting in a state of neuronal excitability within the spinal cord (Costigan et al., 2009; Grace et al., 2014). This change is mediated by afferents supplying the region, associated inflammatory responses and changes to descending input (Campbell et al., 2006). Central sensitisation can be broadly broken down into neural plasticity which is functional and protective, and neuroimmune components present in injury or illness, which can induce longer lasting changes leading to pathological pain states.

Neural inputs to the spinal cord can be considered homosynaptic (input from a single synapse) or heterosynaptic (input from multiple synapses) on the second order neuron (Latremoliere et al., 2009). Intense, repetitive, sustained high frequency homosynaptic input such as spontaneous firing following nerve injury results in "wind-up"; and long-term potentiation (LTP). Wind-up results in a prolonged response lasting tens of seconds, while LTP amplifies subsequent stimulations and can last for several hours (Campbell et al., 2006; Dickenson et al., 1987; Ji et al., 2003; Latremoliere et al., 2009). NMDA receptors which are usually inactive, become active after sustained glutamate, SP, CGRP and BDNF release from nociceptors alters the receptor pore. The resulting increased calcium influx boosts synaptic efficiency and consequently amplifies the signal to produce this LTP effect (Mayer et al., 1984). Increased calcium influx boosts synaptic efficiency by activating kinases (PKC, PKA, ERK, Ca^{2+}/Calmodulin-dependant protein kinase CaMKII) which induces changes in NMDA and AMPA activation kinetics and surface expression (Latremoliere et al., 2009). Long term synaptic changes can result from activation of transcription factors, upregulating expression of NK1 and TrkB (Latremoliere et al., 2009). Heterosynaptic input is responsible for interactions between large magnitude C-fibre inputs and low threshold input from mechanoreceptors (Aβ-fibres) (Campbell et al., 2006; Ji et al., 2003). Multiple C-fibre inputs alter the activity of the postsynaptic membrane as described above, augmenting the response to a secondary synapse. Therefore, heterosynaptic activity is responsible for secondary hyperalgesia and allodynia, whereas homosynaptic inputs can only increase responses to noxious input of the same synapse and contribute to primary hyperalgesia (Latremoliere et al., 2009). In pathological pain this functional plasticity is augmented by continuous neuroimmune interaction at the tetrapartite synapse.

The importance of neuroimmune interactions to generation of central sensitisation is highlighted by the attenuation of neuropathic pain by glial and inflammatory attenuator propentofylline (Raghavendra et al., 2003). As described above, glial activation following nerve injury and inflammation results in release of a proinflammatory milieu. TNFα, IL-1β and IL-6 are all implicated in spinal mediated nociceptive alteration correlating with nociceptive hypersensitivity in numerous injury and inflammatory-induced preclinical pain models, and chemically-induced pain in clinical models (Arruda et al., 2000; De Leo et al., 1997; Hutchinson et al., 2013; Peng et al., 2006; Raghavendra et al., 2004; Sweitzer et al.,

1999). Further highlighting their importance, antagonism of TNFα, IL-1β and IL-6 activity reverses pain hypersensitivity (Arruda et al., 2000; Milligan et al., 2003; Peng et al., 2006). It should be noted that centrally administered IL-6 produces both pro- and anti-nociceptive effects seemingly dependent on the nociceptive stimulation type, complicating our understanding of its role in nociception (Choi et al., 2003; Flatters et al., 2003). As mentioned above, p38 MAPK is believed to be a key integrator of multiple stimuli resulting in cytokine release. Inhibitor of p38 MAPK, CNI-1493, blocks the pro-inflammatory pain inducing cytokine responses and reverses thermal and mechanical hypersensitivities (Milligan et al., 2001a; Milligan et al., 2003). Decreased phosphorylation of p38 MAPK has been directly associated with decreased dorsal horn TNFα expression and mechanical hypersensitivity (Peng et al., 2006). Inflammatory associated increases in NGF also facilitate central nociceptive signalling by increasing NMDA receptor activity (Lewin et al., 1994).

As in the periphery, both glia and neurons express pro-inflammatory cytokine receptors (Vitkovic et al., 2000). As discussed above this direct activation causes further cytokine release and proliferation in glia. TNFα application to superficial lumbar spinal cord results in increased spontaneous firing in WDR neurons (Onda et al., 2002). TNFα induces a change in membrane conductance by altering calcium channel function, similar mechanisms have been proposed for changes in neuronal excitability by IL-6 and IL-1β (Qiu et al., 1998; van der Goot et al., 1999; Wilkinson et al., 1996). IL-1β also induces central cyclooxygenase-2 (COX-2), important for the production of PG in the SC (Samad et al., 2001). Centrally, PG facilitates presynaptic neuropeptide release, including SP and CGRP (Vasko, 1995). Microglial release of TNFα is associated with increased synaptic glutamate due to its effect on astrocytes. This mechanism involves PG release from astrocytes, which in an autocrine and paracrine fashion induces glutamate release from self and surrounding astrocytes. This signal is self-propagating leading to further increases in TNFα, PG and glutamate resulting in an excited synaptic environment (Bezzi et al., 2001). Astrocytes further amplify this feedback loop, releasing CXCL12 which binds to astrocyte and microglial CXCR4 receptors further increasing TNFα, and further increasing glutamate levels (Bezzi et al., 2001). Bradykinins further augment glutamatergic release from presynaptic terminals, which appears to depend on TRPA1 mediated calcium influx (Bautista et al., 2006; Kosugi et al., 2007; Wang et al., 2005). Bradykinins also alter

AMPA and NMDA receptors at postsynaptic terminals increasing spontaneous currents (Wang et al., 2005).

A number of factors released from both neuronal and non-neuronal sources can reduce inhibitory input in the dorsal horn. Downregulation of MORs following nerve injury results in decreased inhibition by endogenous opioids (Kohno et al., 2005). ATP-induced microglial BDNF release following nerve injury downregulates potassium chloride co-transporters (KCC2), altering the ionic gradient and altering GABA receptor function to induce depolarisation, thus reducing inhibitory and enhancing excitatory tone (Coull et al., 2005; Coull et al., 2003). Effects on glycinergic inhibition have been observed in an inflammatory pain model. PG, acting at the PGE2 receptor alters strychnine-sensitive glycine receptor (GlyR) function in a PKA-dependant mechanism. This results in the excitatory interneurons and secondary projection neurons which express GlyR becoming unresponsive to glycinergic input inducing both mechanical and thermal hypersensitivity (Harvey et al., 2004). Finally, injury-induced apoptosis due to glutamate toxicity in the dorsal horn results in the loss of GABAergic inhibitory interneurons (Scholz et al., 2005).

Outcomes of central sensitisation therefore depend on the relationship of multiple excitatory and inhibitory processes which are impacted by neuronal and classically immune input. Although neural elements of central sensitisation alone account for relatively short-term hypersensitivity, neuroimmune interaction can lead to long lasting pathological pain. Despite this knowledge, the key mechanisms for generating and maintaining an environment of central sensitisation remain elusive and continue to drive pain research.

1.5 Conclusion

Evidence presented here clearly highlights the importance of neuroimmune interaction in the alteration of nociceptive signal transmission. The consequences of these interactions can result in centrally and peripherally mediated alterations in pain sensitivity contributing to chronic pain. Preclinical pain models typically target either the neuronal mechanism, evidenced by numerous neurogenic pain models; direct immune stimulation, or unspecific activation of elements of neuroimmune signalling via nerve injury. The most relevant options are those models which aim to mimic clinical pathophysiology. One potential improvement in this space are models which independently, and therefore controllably

stimulate each of the elements involved in neuroimmune signalling. This will allow further mechanistic investigation with behavioural outcomes, as well as offer the possibility of investigating how timing and duration of each signalling modality independently affects long term nociceptive outcomes. Due to the unreliability of therapeutics to combat chronic pain; further research into the clinical importance of neuroimmune interactions is required. This thesis will now provide an investigation into a potentially clinically relevant neuroimmune interaction between TLR4 and TRPV1.

Chapter 2: Introducing toll-like receptor 4 and transient receptor potential cation channel subfamily V member 1

This chapter introduces the clinically relevant neuroimmune interaction between toll-like receptor 4 (TLR4) and transient receptor potential cation channel subfamily V member 1 (TRPV1). Each receptor is introduced individually in relation to their role in pain, before a discussion of their functional interaction. Along with the opening chapter, the information presented will highlight the importance of continued research into the mechanisms and relevance of the interaction and frame the aims of this thesis.

2.1 Toll-like receptor 4 (TLR4)

TLR4 is a transmembrane innate immune receptor responsible for initiating inflammatory signal cascades. TLR4 is expressed in multiple tissue types including; respiratory, urinary and digestive epithelia, endothelial cells, cardiac myocytes, peripheral and central myeloid cells (e.g. glia, macrophages) and peripheral and central neurons (Buchanan et al., 2010; Ferraz et al., 2011; Frantz et al., 1999; Ghosh et al., 2015; Helley et al., 2015; Kielian, 2006; Smolinska et al., 2008; Taylor et al., 2004). Activation can occur by interaction with a number of danger and pathogen associated molecular patterns (DAMPs/PAMPS), including heat shock proteins (HSPs), yeast cell wall components, protozoan glycolipids and viral envelope proteins (Lee et al., 2007; Ohara et al., 2013; Ransohoff et al., 2012). However, the most well-known and widely used agonist is the gram-negative bacterial cell wall component lipopolysaccharide (LPS) (Poltorak et al., 1998; Shimazu et al., 1999).

2.1.1 Structure

TLR4 consists of an extracellular region containing a leucine rich repeat domain and intracellular region containing a toll/interluekin-receptor-1 (TIR) domain (Buchanan et al., 2010; Hashimoto et al., 1988). An extracellular binding partner, myeloid differentiation factor 2 (MD2) is required for agonist binding (Shimazu et al., 1999). While co-receptor CD14 and extracellular LPS binding protein (LBP) act to chaperone LPS to the TLR4/MD2 complex; CD14 dramatically increases sensitivity to LPS (Delude et al., 1995; Janova et al., 2016; Zanoni et al., 2011).

2.1.2 Signal transduction

LPS binding induces dimerisation of the TLR4/MD2 complex, recruiting TIR domain containing adaptor proteins which interact with the TIR domain of TLR4. Two distinct

signalling pathways are involved in TLR4 signal transduction, each with separate TIR containing adaptor proteins. Myeloid differentiation primary response protein 88 (MyD88), and MyD88-adaptor protein (MAL) (also known as TIR domain-containing adaptor protein (TIRAP)) associate with TLR4 to initiate the MyD88 dependent pathway, known as the early phase response (Buchanan et al., 2010; Horng et al., 2002). While TIR domain-containing adapter inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) are recruited to induce the late response phase MyD88 independent pathway. Evidence from a microglial (BV2) cell line suggests initial increases in phosphatidylinositol 4,5-bisphosphate (PIP_2) following LPS promotes TIRAP translocation to the membrane (Nguyen et al., 2013). Similarly, another study concluded PIP_2 is key for facilitating MyD88 interaction with the TLR4 complex by binding and mediating TIRAP membrane translocation; MyD88 engineered to bind PIP_2 does not need TIRAP to activate the MyD88 dependent pathway (Kagan et al., 2006). Activation of the MyD88 dependent pathway occurs mainly at the plasma membrane, and TLR4 association with TIRAP/MyD88 induces translocation of transcription factors nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1). Transcription factor translocation occurs after a chain of kinase activations including, IL-1R-associated kinases (IRAKs), TNF receptor-associated factor 6 (TRAF6), transforming growth factor β-activated kinase 1 (TAK1), mitogen activate protein kinases (MAPKs) (JUN N-terminal kinase (JNK), p38, extracellular signal related kinases (ERKs)) and the IκB kinase (IKK) complex (Fig. 2.1). This pathway induces transcription of proinflammatory cytokines and chemokines including tumour necrosis factor α (TNFα) and CCL2 (Kawai et al., 2007a; Kielian, 2006; Molteni et al., 2016). In contrast, the MyD88 independent pathway occurs in the cytoplasm following TLR4/MD2 internalisation (Buchanan et al., 2010; Tanimura et al., 2008). This pathway leads to nuclear translocation of transcription factors interferon regulatory factor 3 (IRF3) and NFκB/AP-1 via TRIF/TRAM activation of TRAF3 and TRAF6 respectively (Fig. 2.1). IRF3 promotes interferon (IFN)-β synthesis and further production of type 1 IFNs which counteract the pro-inflammatory NFκB outcomes (Buchanan et al., 2010; Kielian, 2006). CD14 is important for modulating responses by inducing TNFβ mediated negative feedback and for TLR4 internalisation following LPS activation, suggesting it is important for induction of the MyD88 independent pathway (Zanoni et al., 2011).

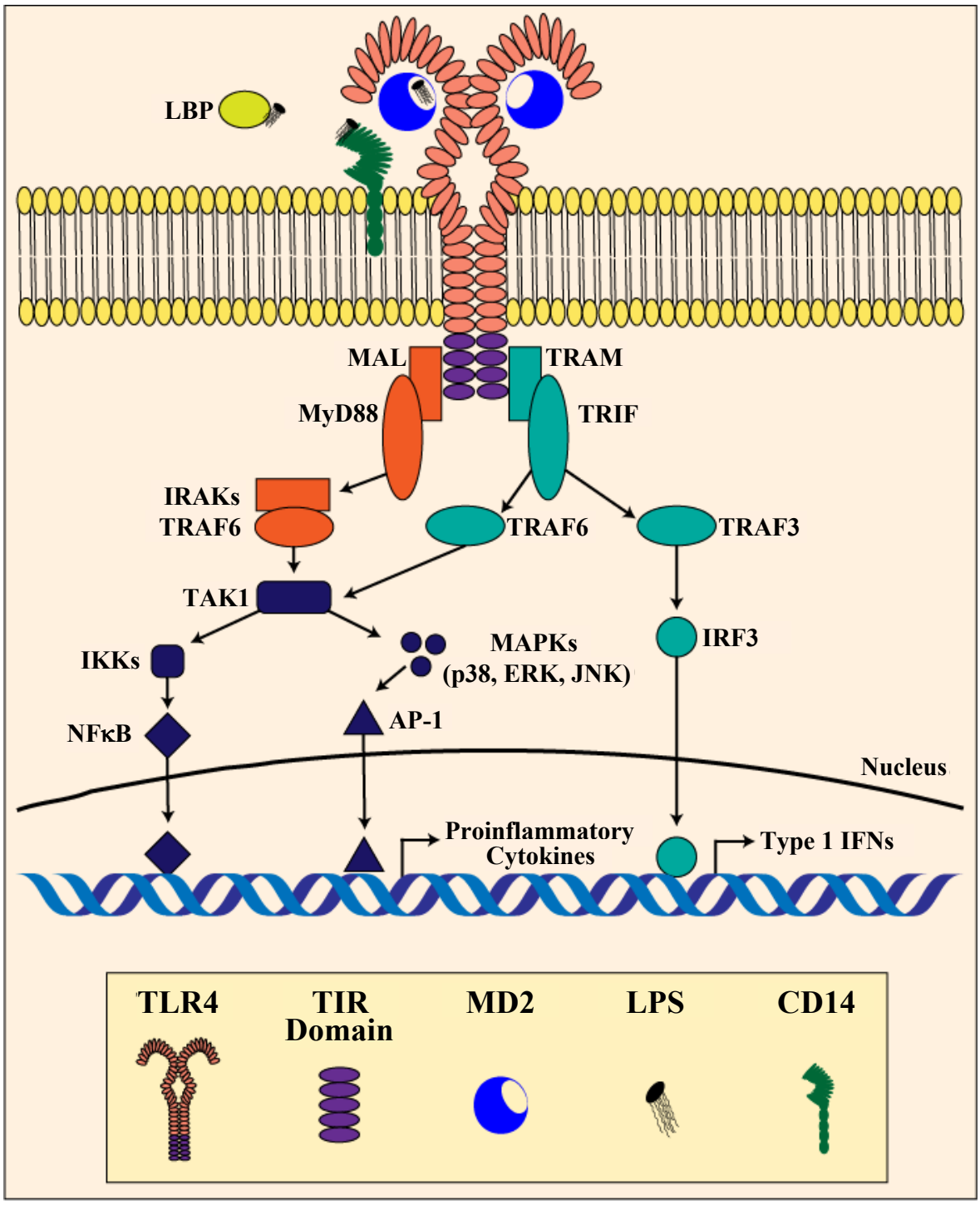


Figure 2.1: TLR4 Signalling Cascade Highlighting MyD88 Dependant and Independent Pathways. LPS binding involves LBP, CD14, MD-2 and TLR4. Orange and green proteins represent signalling cascades unique to MyD88 dependant and independent pathways respectively. Dark blue represents proteins common to both pathways resulting in nuclear translocation of AP-1 and NFκB.

In addition to the pathways described above, TLR4 activation induces a host of intracellular changes which influence the pro-inflammatory outcome. These include changes in intracellular calcium, expression of microRNAs (miRNAs) and activity of multiple kinases. LPS binding causes increases in cytosolic calcium in mouse dendritic cells and DRG neurons, from both intracellular stores and extracellular sources upon activation of calcium release activated calcium channels (CRAC) (Grobner et al., 2014). Despite this data some discussion persists around LPS being the true initiator of these calcium changes (Salter et al., 2009). Nevertheless, it is a phenomenon also observed in human bladder and mouse lung epithelial cells (Song et al., 2007; Tauseef et al., 2012). Multiple NFκB dependent miRNAs have been identified following TLR4 activation, including miR-145, miR-146 and miR-348. miRNA influence at multiple points of the NFκB signalling cascade negatively regulates the pro-inflammatory response (O'Neill et al., 2011; Taganov et al., 2006).

TLR4 mediated increases in protein kinase A and C (PKA, PKC), and calcium dependent increases in phospholipase C (PLC) and phosphoinositide 3-kinase (PI3K) following LPS have been reported (Cabral et al., 2015; Kim et al., 2015). PI3K is important for activation of the serine/threonine kinase, Akt, and production of IL-1β, TNFα and macrophage activating factor-2 (MAF-2) (Ojaniemi et al., 2003). While PLC-induced diacylglycerol (DAG) increase correlates with rapid NFκB translocation in mouse lung epithelial cells (Tauseef et al., 2012; Yamamoto et al., 1997). LPS results in calcium dependent activation of *Src* kinase associated with production of oxidants in a mouse macrophage (RAW264.7) cell line (Check et al., 2010). Activation of *Src*, as well as other members of the Src tyrosine kinase (STK) family, *Fyn* and *Yes,* are reported to last between 5 and 60 min following LPS administration in human lung microvascular epithelial cells, reversed by TLR4 small interfering (si)RNA-induced knockdown (Gong et al., 2008). Highlighting their role in TLR4 outcomes, antagonising STK activity inhibits output of TLR4 induced pro-inflammatory mediators in human and murine macrophages, including; TNFα, IL-8, IL-10, CXCL10 and inducible nitric oxide synthase (iNOS) (Lee et al., 2005b; Smolinska et al., 2008). In this case, cytokine and chemokine outcomes were independent of NFκB activation and ERK, JNK and p38 phosphorylation, although modulation of AP-1 was observed, suggesting an effect on the MyD88 independent pathway (Smolinska et al., 2008). Another STK member, *Lyn* kinase, is important for phosphorylation of the TIR

domain and endotoxin induced signalling (Medvedev et al., 2007). Altogether, this evidence suggests kinase activity is an important regulator of the TLR4 signalling system. It appears further mechanistic elucidation of kinase activation following TLR4 ligand binding is necessary due to the varied evidence of TLR4-induced intracellular calcium elevation.

2.1.3 TLR4 and pain

Change in pain sensitivity is associated with TLR4 activity through a number of mechanisms, including peripheral and central sensitisation, and direct nociceptor TLR4 activation. Neuronal subsets express TLR4, MD2 and CD14, which can be directly influenced by TLR4 agonists (Ochoa-Cortes et al., 2010; Tse et al., 2014). In mouse dorsal root ganglia (DRG) neurons, LPS increases both the generation of action potentials following electrical stimulation and the expression of IL-1β, TNFα and cyclooxygenase -2 (COX-2) in a MyD88 dependent fashion (Ochoa-Cortes et al., 2010; Tse et al., 2014). LPS also induces calcium influx in rat trigeminal (TG) and DRG neurons; the number of DRG neurons responding to LPS increases above 10 ng/mL (Diogenes et al., 2011; Li et al., 2015). In addition to LPS, HSP70 binding to TLR4 expressing TG neurons is a suggested mechanism of action for referred tongue pain during tooth pulp inflammation; while chemotherapy agent paclitaxel increases presynaptic glutamate release, an effect blocked by TLR4 antagonist LPS-RS in mice spinal cord (SC) slices (Ohara et al., 2013; Yan et al., 2015).

Indirect effects of TLR4 have been widely reported following systemic and peripheral LPS administration. Intravenous (i.v.) LPS (4 ng/kg) reduces pain thresholds to electrical, mechanical and cold stimulation in humans (de Goeij et al., 2013). While lower doses (0.4 ng/kg) produce mechanical visceral hypersensitivity (Benson et al., 2012). Similarly, 0.4 ng/kg i.v. produces peripheral mechanical hypersensitivity which interestingly is dependent on anatomical location, observed on the back and shoulder, and only observed at the calf or neck at higher doses (0.8 ng/kg) (Wegner et al., 2014; Wegner et al., 2015). This opens the interesting possibility of anatomically distinct nociceptive processes involving TLR4. Similarly, a mouse nerve injury model of orofacial pain produced widespread TLR4 dependent pain sensitivity at distant sites (hind paw) but not at the vibrissae pad, again suggesting region specific nociceptive mechanisms (Hu et al., 2017).

In the clinical examples above, peak pro-inflammatory cytokine levels (IL-6, IL-8, IL-10, TNFα) occurred between one and three hours post LPS, correlating with peak hyperalgesia at 3 h for all anatomical locations tested (Benson et al., 2015; Wegner et al., 2014). Peripheral application of between 30 and 300 ng of LPS induces mechanical hypersensitivity 3 h after intraplantar (i.pl.) injection into the mouse paw. This involves nociceptor sensitisation by MyD88 dependent increases in IL-1β, TNFα and CXCL1 (Calil et al., 2014). Further, two intraperitoneal (i.p.) (0.2 mg/kg) or intracerebroventricular (i.c.v.) (0.2 mg) doses of LPS produce a priming effect, whereby the second dose 24 h later produces increased mechanical and thermal hypersensitivities when compared to the initial dose (Cahill et al., 1998). Intrathecally (i.t.) applied LPS induces mechanical hypersensitivities correlated with astrocytic and microglial derived TNFα (Saito et al., 2010).

TLR4 has been implicated in the development and maintenance of multiple neuropathic pain models. I.t. delivery of TLR4 antagonists LPS-RS, (+)-naloxone and (+)-naltrexone reverse mechanical hypersensitivity 14 days following chronic constriction injury (CCI) for up to 3 h (Hutchinson et al., 2008). Likewise, systemic application of (+)-naloxone reverses mechanical hypersensitivity for up to 3 h. This attenuation was correlated with reduction of microglial marker CD11b, but not astrocyte marker GFAP, following sustained (4 day) delivery of (-)- or (+)-naloxone (Hutchinson et al., 2008). Another study correlated CCI-induced mechanical hypersensitivity with increased spinal cord and DRG TLR4 expression (Jurga et al., 2016). Interestingly 3 days of spinal cord stimulation attenuates CCI-induced TLR4 and NFκB expression, tissue IL-1β, IL-6 and TNFα, and associated mechanical hypersensitivity (Yuan et al., 2014). Use of peroxisome proliferator-activated receptor gamma (PPAR-γ) antagonist to reverse CCI hypersensitivity is also correlated with decreased spinal cord IL-1β, TNFα and TLR4 mRNA (Jia et al., 2016). Paclitaxel treatment causes chemotherapy-induced neuropathic pain (CINP) and is associated with TLR4 activity. TLR4 and MyD88 expression in DRG neurons are increased at 1- and 7-days , while TRIF is upregulated at 7 days; corresponding with the observed mechanical hypersensitivity beginning 7 days post paclitaxel administration (Li et al., 2014). Both LPS and MyD88 antagonists result in attenuation of paclitaxel-induced mechanical hypersensitivity; although interestingly LPS antagonism results in reduced DRG MyD88 but not TRIF, suggesting an alternative source. Interestingly, increased

TLR4 expression has been observed from days 1 to 21 following paclitaxel administration in typically late responding astrocytes, but not microglia (Li et al., 2014). Finally, TLR4 signalling is also implicated in joint inflammation associated hyperalgesia, diabetic neuropathy, opioid-induced hyperalgesia, postoperative pain, bone cancer pain, and migraine models (Aguado et al., 2018; Chen et al., 2017a; Guerrero et al., 2016; Lan et al., 2010; Su et al., 2018; Xing et al., 2018).

In addition to the activation of peripheral immune mechanisms, centrally activated glia play an important role in the action of TLR4 at the spinal cord level. Briefly mentioned above, upregulation of microglia and astrocyte TLR4 levels in paclitaxel CINP and CCI correlate with observed mechanical hypersensitivity (Bettoni et al., 2008; Li et al., 2014; Tanga et al., 2005). Further, paclitaxel-induced CINP reduces surface expression of glial transporters, glial glutamate transporter 1 (GLT1) and glutamate aspartate transporter (GLAST) on astrocytes, effects which are reversed by LPS-RS (Yan et al., 2015). Knockdown of TLR4 by i.t. siRNA administration reduced microglial activation and development of bone cancer pain (Lan et al., 2010). While a spinal nerve transection pain model shows that microglial and astrocyte activation markers are significantly reduced in TLR4 knockout animals compared to wildtype, correlating with attenuation of mechanical and thermal hypersensitivity (Tanga et al., 2005). Finally, in a skin and muscle incision pain model, TLR4 upregulation in astrocytes and microglia at 10 days, correlates with peak mechanical hypersensitivity (Sun et al., 2015b). It is evident that the action of TLR4, whether indirect by the action of pro-inflammatory signalling, or direct binding of neuronal TLR4, is influential to preclinical and clinical nociceptive outcomes.

2.2 Transient receptor potential cation channel subfamily V member 1 (TRPV1)

TRPV1 is a non-selective cation channel belonging to transient receptor potential (TRP) channel family and vanilloid subfamily (Zheng, 2013). The receptor was first classified in 1997 by Caterina and colleagues as vanilloid receptor 1 (VR1), a receptor for capsaicin, the pungent ingredient of chilli peppers, and resiniferatoxin (RTX), an ultrapotent capsaicin analogue from *Euphorbia* plants (Moroccan cactus) (Caterina et al., 1997; Szallasi et al., 1989). Initial cloning of the receptor also revealed it was activated by heat and modulated by protons (Caterina et al., 1997).

TRPV1 is found on numerous cell types including; keratinocytes, cardiomyocytes, oral and gastric epithelium, mast cells, thymocytes, T-cells, fibroblasts, pancreatic islet cells, hepatocytes, lung epithelium, urothelial cells, male germ cells, microglia and peripheral and central neurons (Akiba et al., 2004; Amantini et al., 2004; Bertin et al., 2014; Birder et al., 2001; Biro et al., 1998; Denda et al., 2001; Dvorakova et al., 2001; Jang et al., 2012; Kato et al., 2003; Kido et al., 2003; Lowin et al., 2015; Mezey et al., 2000; Mizrak et al., 2008; Wadachi et al., 2006). We will be focussing on TRPV1 effects related to pain and nociceptive processing. Compared to other tissue types mentioned, TRPV1 is abundant on primary afferent neurons as seen by expression in dorsal, trigeminal and nodose ganglia; where it is expressed on small diameter C-fibres and medium diameter Aδ-fibres responsible for detection and transduction of nociceptive stimuli (Brito et al., 2014; Jang et al., 2012; Kunert-Keil et al., 2006; Szallasi et al., 2007). Within the CNS, TRPV1 is expressed in the SC dorsal horn, hypothalamus, hippocampus and cortex (Mezey et al., 2000).

2.2.1 Structure

The TRPV1 channel is a homo-tetramer with an internal pore consisting of an external selectivity filter and a lower gate (Cao et al., 2013). Each monomer consists of an intracellular TRP domain at the C-terminal end, 6 transmembrane domains and an intracellular ankyrin repeat segment at the N-terminal end. A pore forming loop is present between transmembrane domains 5 and 6, forming part of the pore and influencing selectivity (Liao et al., 2013; Nilius et al., 2007). Channel opening threshold is altered by changes in phosphorylation state and the availability and/or activity of potentiating or negative regulators which will be discussed in detail below.

2.2.2 TRPV1 in pain literature

Activation of TRPV1 is widely used to study pain physiology and analgesic efficacy. In this respect TRPV1 provides an interesting juxtaposition, where activation results in spontaneous pain and extended hypersensitivity or analgesia, depending on dose and duration of stimulation (Holzer, 1991; Laird et al., 2001a; Menendez et al., 2004; Rashid et al., 2003a; Sakurada et al., 1992). The importance of TRPV1 in the generation of thermal hypersensitivity has been highlighted in multiple knockout models, while sensitivity to mechanical stimulation is also observed following channel activation (Caterina et al., 2000;

Cavanaugh et al., 2009; Davis et al., 2000; Ren et al., 1994). Further, centrally and peripherally acting TRPV1 antagonist AS1928370 attenuates spontaneous capsaicin-induced pain and mechanical hypersensitivity associated with spinal nerve ligation (SNL), highlighting the role of TRPV1 in physiological and pathological pain (Watabiki et al., 2011).

The importance of TRPV1 in pathological pain is highlighted in numerous preclinical pain models. TRPV1 is upregulated in CFA-induced inflammatory pain, opioid-induced hyperalgesia, dextran sulfate sodium-induced colitis, CFA-induced orofacial pain and spinal cord injury (SCI) (Amaya et al., 2003; Lapointe et al., 2015; Vardanyan et al., 2009; Watase et al., 2018; Wu et al., 2013). In contrast, total numbers of TRPV1 expressing neurons remain unchanged or decrease following CCI (Malek et al., 2014; Malek et al., 2015). Blockade of TRPV1 in CFA-induced orofacial pain leads to attenuation of head withdrawal thresholds to mechanical and thermal stimuli (Watase et al., 2018). Likewise, blocking TRPV1 in a model of orthodontic pain reduced expression and mouth wiping behaviour (Gao et al., 2016). While TRPV1 antagonists attenuate mechanical hypersensitivity in a paclitaxel CINP model (Li et al., 2015). Centrally, TRPV1 upregulation has been observed in mouse prelimbic and infralimbic structures following spinal nerve injury (SNI), with antagonism of TRPV1 at these sites resulting in attenuation of mechanical hypersensitivity (Giordano et al., 2012). While centrally administered TRPV1 antagonists block postsynaptic TRPV1 activity and attenuate mechanical allodynia, suggesting TRPV1 is an important element in the disinhibition of SC GABAergic inhibitory interneurons (Kim et al., 2012).

Clinically, TRPV1 activation by capsaicin is potentiated in patients with unilateral sciatica and rheumatoid arthritis, consistent with upregulation observed in preclinical studies (Shenker et al., 2008; Sumracki et al., 2012). TRPV1 upregulation is also observed in human tissues from irritable bowel syndrome patients (Akbar et al., 2008). There is abundant literature to suggest TRPV1 plays a significant role in both physiological and pathological pain. Characterising the key mechanisms with which TRPV1 contributes to pathological pain would represent an important step in pathological pain research.

2.2.3 Activation and agonists

In addition to capsaicin and RTX, direct activation is possible via natural exogenous agonists including; inhibitor cystine knot peptides (component of spider venom), piperine (component of black pepper), eugenol (component of clove oil), gingerols, shogaols, zingerone (components of ginger) and camphor (component of camphor trees) (Table 2.1) (Iwasaki et al., 2006; Liu et al., 1996; Siemens et al., 2006; Yang et al., 2003). Direct activation can occur by a number of endogenous ligands, including: polyamines (e.g. spermine) and lipid compounds comprising biogenic amines (including endocannabinoids anandamide (AEA) and *N*-arachidonoyl dopamine (NADA)), oxygenated eicosatetraenoic acids (e.g. oleoylethanolamide (OEA)) and lipoxygenase products (e.g. 12-(S)-hydroperoxyeicosatetraenoic acid (12-(S)-HPETE)) (Table 2.1) (Ahern, 2003; Ahern et al., 2006; Brito et al., 2014; Hwang et al., 2000; Toth et al., 2003; Vriens et al., 2009; Zygmunt et al., 1999). While not considered a direct activator, protons can sensitise the channel by binding directly, allowing the temperature threshold to drop below physiological temperatures at low enough pH (< 5.6) (Tominaga et al., 1998). Acidification produced by osteoclast activity in bone cancer models is associated with TRPV1 mediated pain sensitivity (Ghilardi et al., 2005). Similarly, basic conditions following ammonia application sensitise the channel, although through unknown mechanisms theorised to involve deprotonation of key residues (Dhaka et al., 2009). In lieu of direct agonist binding, TRPV1 activation can result from temperatures above 43°C, however this threshold can be reduced (sensitised) by proton binding and multiple intracellular pathways detailed below (Caterina et al., 1997).

Capsaicin is a widely utilised TRPV1 agonist and warrants further introduction. Consisting of an aromatic ring, amide bond and hydrophobic side chain, capsaicin binds an intermembrane region between transmembrane domain 3 and 4 (Elokely et al., 2016; Yang et al., 2015). Capsaicin elicits an initial burning sensation in mammals followed by a period of increased sensitivity, followed by a period of desensitisation resulting from depletion of neurotransmitters, calcium-induced cytotoxicity and calcium-induced receptor desensitisation (Caterina et al., 2001). As a result of its contradictory effects on nociception, capsaicin has a number of applications from self-defence (pepper spray) to analgesic agents (Szallasi et al., 1999; Szallasi et al., 2007). Currently, capsaicin is used to treat multiple pain conditions, including post herpetic neuralgia, diabetic neuropathy, arthritis and post-surgical pain (Baranidharan et al., 2013; Szallasi et al., 2007). Capsaicin-

induced pain models are utilised in both clinical and preclinical pain studies due to its ability to produce observable spontaneous pain behaviours and produce symptoms associated with both peripheral and central sensitisation (Hutchinson et al., 2013; Kinnman et al., 1995; Laird et al., 2001a). Measurable sensitisation effects include induction of primary and secondary hypersensitivities and visible neurogenic flare responses (Aykanat et al., 2012; Hutchinson et al., 2013; O'Neill et al., 2012; Tominaga et al., 1998; Willis, 2002).

Table 2.1: Naturally occurring TRPV1 agonists

Exogenous	
Camphor	(Xu et al., 2005)
Capsaicin	(Caterina et al., 1997)
Eugenol	(Yang et al., 2003)
Gingerol	(Dedov et al., 2002)
Inhibitor cystine knot (ICK) peptides	(Siemens et al., 2006)
Piperine	(McNamara et al., 2005)
Resiniferatoxin (RTX)	(Caterina et al., 1997)
Shogaol	(Iwasaki et al., 2006)
Zingerone	(Witte et al., 2002)
Endogenous	
5-(S)-hydroxyeicosatetraenoic acid (5-(S)-HETE)	(Hwang et al., 2000)
12-(S)-hydroperoxyeicosatetraenoic acid (12-(S)-HPETE)	(Hwang et al., 2000)
15-(S)-hydroperoxyeicosatetraenoic acid (15-(S)-HPETE)	(Hwang et al., 2000)
15-(S)-hydroxyeicosatetraenoic acid (15-(S)-HETE)	(Hwang et al., 2000)
Anandamide (AEA)	(Zygmunt et al., 1999)
Leukotriene B_4 (LTB_4)	(Hwang et al., 2000)
N-arachidonoyldopamine (NADA)	(Huang et al., 2002)
Oleoylethanolamide (OEA)	(Ahern, 2003)
Spermine	(Ahern et al., 2006)

2.2.4 Regulation

As mentioned above, the activity of TRPV1 is altered by multiple phosphorylation events. Below the key extracellular mediators and intracellular contributors to phosphorylation are discussed.

2.2.4.1 Kinases

Kinase activity induced by inflammatory mediators and/or calcium influx are a key contributing factor of TRPV1 activation and therefore nociceptive outcomes. PKC, PKA and Ca^{2+}-calmodulin dependant kinase II (CaMKII) phosphorylate key sites resulting in receptor activation and sensitisation. PKC and CaMKII mediate receptor function by phosphorylating key serine (S502, S800) and threonine (T704) residues (Bhave et al., 2003; Numazaki et al., 2002). Mutation of S800 alone results in attenuation of capsaicin-induced spontaneous pain and inflammatory muscle pain (Joseph et al., 2019). While S502 and T704 phosphorylation by CaMKII is required for capsaicin binding (Jung et al., 2004). PKA phosphorylates serine (S502, S116), and threonine (T117, T370) residues (Bhave et al., 2002; Mohapatra et al., 2003, 2005). Upregulation of PKA, PKC and CaMKII in TRPV1 positive cells is observed following CCI in rats (Malek et al., 2015). While downregulation of PKC and PKA and the subsequent effect on TRPV1 has been associated with the analgesic effect of electroacupuncture (Yang et al., 2017). Scaffolding protein A-kinase anchoring protein 150 (AKAP150) in rodents (human orthologue AKAP79) is crucial to the function of PKA and PKC (Faux et al., 1997; Jeske et al., 2008; Jeske et al., 2009; Zhang et al., 2008). Src kinase similarly acts to lower activation threshold by phosphorylation of tyrosine residue Y200 (Jin et al., 2004; Zhang et al., 2005). In addition to phosphorylation, kinase activity is associated with increased TRPV1 activity by mediating translocation of the receptor to the cell membrane. PKC is associated with increased TRPV1 surface expression by promoting SNARE-mediated exocytosis (Morenilla-Palao et al., 2004). While forskolin induced cyclic adenosine monophosphate (cAMP)-dependant PKA activity has been associated with rapid cell membrane insertion of TRPV1, although further mechanistic analysis of this pathway is required (Vetter et al., 2008).

Dephosphorylation of the aforementioned TRPV1 residues is associated with decreased TRPV1 activity known as receptor desensitisation. Short term, desensitisation can be categorised as either acute desensitisation, resulting in reduced response due to prolonged

agonist exposure; and tachyphylaxis, a reduction in response to repeated agonist applications (Vyklicky et al., 2008). Literature suggests these responses result from the balance between numerous phosphorylation and dephosphorylation events induced by calcium. Highly phosphorylated in its resting state; decreased phosphorylation of TRPV1 is observed following capsaicin application (Bhave et al., 2002). Highlighting the role of calcium-induced desensitisation, solutions replacing calcium with barium do not result in desensitisation of TRPV1 following capsaicin exposure. Interestingly, piperine agonism does result in desensitisation under these conditions, suggesting alternate mechanisms depending on agonist binding site (Gunthorpe et al., 2000; McNamara et al., 2005). Following from this, direct calcium application alone reduces capsaicin-induced current, the same effect is not seen following direct magnesium application (Rosenbaum et al., 2004). Calcium dependant messenger protein Calmodulin (CaM) binds two sites on the TRPV1 monomer, an N-terminal and a C-terminal region, both of which are important to channel desensitisation (Numazaki et al., 2003; Rosenbaum et al., 2004). In low intracellular calcium conditions, CaM in a calcium independent fashion binds at the C-terminal site, however ATP outcompetes CaM for the N-terminal ankyrin repeat domain (Lishko et al., 2007; Vyklicky et al., 2008). Due to increased intracellular calcium (such as following TRPV1 activation), CaM activity is altered, leading to binding both N and C-terminal sections, altering receptor configuration, resulting in desensitisation (Lishko et al., 2007; Vyklicky et al., 2008). Fluorescence resonance energy transfer (FRET) shows CaM also interferes with the association of AKAP150 and TRPV1. CaM mediated disruption AKAP150/TRPV1 interaction is associated with decreased capsaicin-induced calcium accumulation in Chinese hamster ovary cells (Chaudhury et al., 2011). Another regulator of desensitisation is the calcium and CaM dependent protein phosphatase 2b (calcineurin), which produces acute desensitisation and tachyphylaxis of TRPV1 (Jung et al., 2004; Mohapatra et al., 2005). Calcineurin desensitisation is hypothesised to depend on relative levels of calcineurin dephosphorylation and PKA and CaMKII phosphorylation events (Jung et al., 2004; Mohapatra et al., 2005). In addition to phosphorylation events, Fas-associated factor 1 (FAF1), co-expressed on primary afferents and associated by physical interaction, attenuates TRPV1 responses to acids, thermal and chemical stimuli (Kim et al., 2006). Longer lasting desensitisation results from receptor internalisation and degradation as observed following extended capsaicin application in TRPV1 expressing HEK293FT cells and DRG neurons. This action is mediated by calcium dependent PKA phosphorylation of S116 and a clathrin mediated endocytosis pathway (Sanz-Salvador et

al., 2012). The kinases mentioned above are activated by multiple inflammatory mediators which are discussed individually below.

2.2.4.2 Lipids

Lipid regulation of TRPV1 function is controversial due to the contrasting *in vitro* literature which centres around the role of PIP_2. Evidence suggesting that PIP_2 interactions are inhibitory, report calcium dependent activation of PLC and subsequent hydrolysis of PIP_2 into inositol triphosphate and DAG, releases TRPV1 from lipid mediated inhibition. This results in potentiation of TRPV1 responses to low pH, capsaicin and heat (Chuang et al., 2001; Prescott et al., 2003). In contrast, multiple studies in HEK393 cells and primary DRG neurons report that PIP_2 is required for channel sensitisation, and is an important component of recovery of channel function following desensitisation (Klein et al., 2008; Stein et al., 2006; Sun et al., 2015a; Ufret-Vincenty et al., 2015; Yao et al., 2009). This mechanism also involves PIP_2 hydrolysis by calcium dependent PLC action, resulting in the breakdown of an interaction between the proximal C-terminal region of TRPV1 and PIP_2. This PIP_2/TRPV1 separation prevents the desensitising binding action of calmodulin (CaM) to both C- and N-terminal domains (Kwon et al., 2007; Lishko et al., 2007). Similarly to the CaM mechanism discussed above, ATP is critical to re-sensitisation, responsible for PIP_2 resynthesis by phosphatidylinositol-4-kinase activity (Liu et al., 2005). One study reports both an inhibitory and sensitising effect of PIP_2 based on the concentration of capsaicin administered. In this case the authors suggest any inhibitory effect is likely to be indirect due failure to observe the inhibition in excised patches (Lukacs et al., 2007). Membrane protein phosphoinositide interacting regulator of TRP (Pirt) has been proposed as an important competent of PIP_2 regulation. Pirt is associated with both the C-terminus of TRPV1 and PIP_2, and is a critical component of PIP_2 mediated enhancement of heat- and capsaicin-induced currents in DRG neurons (Kim et al., 2008). Although the weight of evidence points to PIP_2 sensitisation of TRPV1, more study is required to understand the conditions in which multiple studies have reported an inhibitory effect.

2.2.4.3 Prostaglandins

Binding of prostaglandin E_2 (PGE_2) and I_2 (PI_2) to their respective receptor's EP and IP, reduce the temperature threshold of TRPV1 to below physiological temperature. This effect is PKC and PKA mediated depending on the receptor type activated (Moriyama et

al., 2005). Disruption of AKAP79/150 following EP or IP activation abolishes prostaglandin sensitisation effects on TRPV1, highlighting the importance of PKA/PKC activity (Zhang et al., 2008). TRPV1 sensitisation by PGE_2 is responsible for increased spontaneous pain in a model of chemotherapy-induced mucositis (Yamaguchi et al., 2016).

2.2.4.4 Nerve growth factor (NGF) and bradykinin

Highlighting their importance to nociceptive transmission, both bradykinin and NGF produce thermal hypersensitivity following intraplantar injection (Chuang et al., 2001). NGF activation results in potentiation of TRPV1 responses to capsaicin stimulation through multiple mechanisms. The major mechanism is activation of a PI3K/PKCδ/Src kinase mediated phosphorylation (Zhang et al., 2005). PI3K associates with TRPV1, forming a complex with TrkA (NGF receptor), which once activated by NGF results in PIP_2 phosphorylation to PIP_3, promoting TRPV1 trafficking to the cell membrane (Stein et al., 2006; Stratiievska et al., 2018). Another sensitisation pathway, common to both NGF and bradykinin activation involves PLC and PKCε activity, resulting in receptor phosphorylation (Cesare et al., 1999; Zhang et al., 2005). This same pathway is implicated in the PIP_2 hydrolysis scenarios mentioned above. Therefore, continued TrkA activation inhibits recovery of the desensitised channel (Liu et al., 2005). Finally, an NGF-induced PI3K/ERK pathway is also implicated in capsaicin-induced thermal sensitivity (Zhuang et al., 2004).

2.2.4.5 Serotonin and dopamine

Serotonin (5-HT) is implicated in the sensitisation of TRPV1 following visceral capsaicin administration by promoting CaMKII activity (Qin et al., 2010). This is corroborated by work in TG neurons reporting 5-HT increases capsaicin-induced calcium accumulation and CGRP release, attenuated by targeting peripheral 5-HT receptors (Loyd et al., 2011). In contrast, dopamine is associated with reduced TRPV1 current induced by capsaicin. Inward current is attenuated by inhibition of CaMKII, but not PKC or PKA (Chakraborty et al., 2015).

2.2.4.6 Opioids

Activation of μ- and δ-opioid receptors (MOR, DOR) have been linked with TRPV1 sensitisation via recruitment of scaffolding protein β-arrestin 2. Coimmunoprecipitation

reveals an interaction between TRPV1 and β-arrestin 2 in rat TG neurons, while siRNA knockdown potentiates and reduces desensitisation to capsaicin by modulating PKA phosphorylation (Por et al., 2012). Activation of MOP with DAMGO and morphine in TG neurons results in β-arrestin 2 recruitment and dissociation from TRPV1. This is associated with potentiated capsaicin responses and TRPV1 dependent thermal hypersensitivities following chronic DAMGO and morphine exposure (Rowan et al., 2014a; Vardanyan et al., 2009). Interestingly, DOP agonist SNC80 produced comparable results in rats but not mice, suggesting species specific effects (Rowan et al., 2014b). In contrast, short term morphine administration potentiates capsaicin-induced TRPV1 desensitisation (Endres-Becker et al., 2007; Shaqura et al., 2014). Centrally, TRPV1 and MOP interaction has been reported in the rostroventral medulla (RVM), simultaneous activation resulting in reduced ON-cell tone, inhibited by both TRPV1 and MOP antagonists (Maione et al., 2009).

2.2.4.7 Cytokines

Cytokines are responsible for activating kinase pathways leading to upregulation and sensitisation of TRPV1 attributed to increases in pain sensitivity. IL-6 induces upregulation of TRPV1, as well as activation of the Janus activated kinase (JAK)/PI3K signalling pathway and subsequent receptor sensitisation, associated with mechanical hypersensitivity in a bone cancer pain model (Fang et al., 2015). IL-1β is also associated with upregulation of TRPV1 in arthritis pain models, correlating with increased thermal hypersensitivity (Ebbinghaus et al., 2012). TRPV1 dependent thermal hypersensitivity resulting from increased IL-1β is associated with prostaglandin and PKC activity (Russell et al., 2009). Similarly, TNFα results in TRPV1 upregulation in cultured DRG neurons in an ERK dependent mechanism (Hensellek et al., 2007). A TNFα-induced upregulation of TRPV1 expression is found in CINP models, thermal and mechanical hypersensitivity are attenuated by both TRPV1 and TNFα inhibitors (Wang et al., 2018). Interestingly, blockade of TRPV1 in orthodontic pain models results in reduced IL-1β secretions in gingival fluid, highlighting the role of TRPV1 in peripheral immune activation (Gao et al., 2016).

2.2.4.8 Other

Activation of numerous other receptor types also influence TRPV1 thresholds. Purinergic P2Y receptor activation by ATP results in reduction of temperature threshold in both

HEK293FT cells and rat DRG neurons, dependent on PKC activation (Kress et al., 1999; Tominaga et al., 2001). Protease-activated receptor 2 (PAR2), co expressed with TRPV1 in rat DRG neurons also causes PKC dependent sensitivity changes. Increasing responses to protons, capsaicin and reducing temperature threshold upon stimulation in TRPV1/PAR2 co-expressing HEK293FT cells (Dai et al., 2004). Similarly, activation of prokineticin receptors (PKR1, PKR2), sensitises thermal hypersensitivity and potentiates capsaicin responses (Negri et al., 2006).

2.3 TLR4 / TRPV1 interaction

Potentiation of capsaicin responses following immune activation was discovered prior to the capsaicin receptor itself. Rat vagus nerve exposed to LPS for 5 h showed potentiated capsaicin-induced calcitonin gene related peptide (CGRP) content, correlating with increased capsaicin-induced CGRP release from excised trachea. Interestingly this effect was not observed following a 2 h LPS treatment and was blocked by IL-1β and COX inhibitors (Hua et al., 1996). The timing and effect of antagonising pro-inflammatory mediators suggests an indirect relationship of LPS on capsaicin effects. In a study investigating headache mechanism, systematic LPS decreased the incidence of a multitude of intracisternal capsaicin-induced immobilisation behaviours, which did not correlate with increased neuronal activation marker, c-Fos (Kemper et al., 1998). This data suggests the potentiated behavioural effects are not related to changes in c-Fos and that the output of capsaicin sensitive neurons was increased by LPS. As above, a 5 h LPS incubation was required to observe these changes, suggesting an indirect effect resulting from LPS stimulation (Kemper et al., 1998). Following discovery and characterisation of TLR4 and TRPV1, these studies lead to the exploration of a potential functional interaction between the two receptors. Functional TLR/TRP relationships have been proposed for other receptor types. TLR4 activation indirectly results in activation of transient receptor potential cation channel subfamily C member 6 (TRPC6) due to LPS-induced DAG production (Tauseef et al., 2012).

While the above examples suggest indirect, immune sensitisation and kinase activation events, the possibility of direct TRP and TLR interactions have previously been observed. Coimmunoprecipitation reveals an interaction between TLR7 and TRPA1, enhanced by

TLR7 and TRPA1 agonist miRNA lethal-7 (let-7b). Further, TLR7 and TRPA1 are colocalised in mouse DRG neurons (Park et al., 2014). This interaction induces functional consequences; let-7b activated TRPA1 mediated inward currents are enhanced by co-expression of TLR7 in HEK293FT cells. This potentiation is unaffected by blocking common intracellular mediators (PKA, PKC, PLC) and TRPA1 currents are reduced in TLR7 deficient DRG neurons (Park et al., 2014). Taken together, this data suggests a functional relationship between TLR7 and TRPA1, although further studies are required to ascertain a potential direct protein-protein interaction. Investigation of a TLR4/TRPV1 relationship *in vitro* and *in vivo* has attempted to clarify the nature of a potential interaction with mixed results.

2.3.1 Nociception - TLR4/TRPV1 in vitro

In vitro studies reveal expression of TLR4 in TLR4/TRPV1 co-expression systems is capable of modulating TRPV1 activity. Expression of TLR4 with TRPV1 in HEK293FT cells increases the amplitude of capsaicin responses and magnitude of calcium accumulation (Min et al., 2014). This mechanism is postulated to involve direct interactions between TRPV1 and the TIR domain of TLR4. An interaction which prevents internalisation and desensitisation of TRPV1 (Min et al., 2018). Contradicting this finding, evidence shows TRPV1 desensitisation to a second capsaicin stimulation in TLR4/TRPV1 co-expressed HEK293FT cells, a phenomenon not observed in cells expressing TRPV1 only (Li et al., 2015). It should be noted that this study introduced the whole TLR4 receptor complex, including MD2 and CD14, which were not co-expressed in the studies mentioned above. The same paper reported that short LPS (15 min, 10 ng/mL) exposure potentiated inward currents in HEK293FT cells co-expressing TLR4 and TRPV1, as well as the chemotherapy and TLR4 stimulating agent paclitaxel (Li et al., 2015). Therefore, in engineered overexpression systems, the presence of TLR4 potentiates TRPV1 responses.

Multiple studies investigating primary cultures have shown that TLR4 activation is able to alter the function of TRPV1. A 15 min LPS stimulation of rat trigeminal neurons produces potentiation of capsaicin evoked calcium accumulation and CGRP release attenuated by antagonising TLR4 (Diogenes et al., 2011; Ferraz et al., 2011). These and other studies reveal co-expression of TLR4 and TRPV1 in rat TG, DRG and human dental pulp and DRG neurons (Diogenes et al., 2011; Ferraz et al., 2011; Li et al., 2015; Wadachi et al., 2006; Wu et al., 2019). The short LPS incubation times of these and engineered co-

expression studies contrasts the previously discussed interaction in rat vagus nerves and suggests the possibility of a direct receptor interaction as postulated by studies in HEK293FT cells.

2.3.2 Pain – TLR4/TRPV1 in vivo

Preclinical pain models have given greater relevance to the *in vitro* results discussed above. The chemotherapy agent paclitaxel increases TRPV1 sensitisation in a TLR4 dependant manner in a rat CINP model, as well as excised human and rat DRG neurons (Li et al., 2015). Similar to the rat TG studies above, increased TRPV1 mediated calcium responses in the presence of paclitaxel were attenuated when TLR4 antagonist LPS-RS was co-administered in DRG culture. Paclitaxel not only induces nociceptor changes, but spinal changes; *ex vivo* studies show an increased rate of miniature excitatory postsynaptic currents (mEPSCs) to a second application of capsaicin following paclitaxel treatment, an effect blocked by LPS-RS. Although, no changes in spontaneous EPSCs (sEPSCs) were observed suggesting the paclitaxel effect was on pre-, not post-synaptic terminals (Li et al., 2015). In contrast, long term paclitaxel effects on postsynaptic TRPV1 responses were reported due to increased sEPSCs 7 days following paclitaxel treatment (Li et al., 2015). Comparable findings are observed in a 2,4,6-trinitrobenzine sulfate (TNBS)-induced colitis model. TLR4 knockout results in attenuated TRPV1 expression, consistent with the above theory that TLR4 prevents internalisation and thus desensitisation of TRPV1 (Wu et al., 2019). Upregulation of TLR4 in WT mice is associated with increased TRPV1 expression and visceral hypersensitivity. Excised DRG neurons also revealed that the inflammatory conditions predictably potentiated capsaicin-induced current, however, responses from TLR4 knockout animals were significantly attenuated in both TNBS and control animals, suggesting TLR4 expression alone is responsible for increasing capsaicin responses (Wu et al., 2019).

Evidence of functional and relevant TLR4/TRPV1 interaction in humans has been established in a clinical capsaicin model (Hutchinson et al., 2013). In this case, potentiation of mechanical allodynia and hyperalgesia was observed at three hours, but not two hours or directly following intravenous low dose (0.4 ng/kg) LPS. Interestingly this effect was anatomically variable, observed on the forearm but not the forehead. Further, the timing of potentiation correlated with peak levels of serum IL-6 (Hutchinson et al., 2013). Therefore, the mechanisms of action here appears to be one of inflammatory mediated receptor

sensation, rather than the rapid changes observed in primary DRG, TG and HEK293FT cultures.

2.3.3 Outside pain literature

Outside of pain literature, but within the sensory space, there have been increasing investigations regarding the role of TLR4/TRPV1 interactions in chronic itch. Increases in TLR4 expression are associated with increased histamine-induced itch. While histamine-induced calcium currents are potentiated by LPS in DRG neurons of animals with increased TLR4 expression (Ji et al., 2018). Similarly, histamine-induced calcium signals are reduced in TLR4-deficient neurons. These results suggest histamine mediated itch is altered by TLR4's impact on TRPV1 function (Min et al., 2014). Stroke models have also investigated the role of TLR4 and TRPV1. TLR2 and -4 are increased around the infarct, interestingly TRPV1 antagonism both reverses infarct volume and reduced TLR2 and -4 expression. This suggests the possibility that TRPV1 can modulate TLR4 expression (Hakimizadeh et al., 2017). TRPV1 modulation of TLR4 function has also been observed in macrophages and salivary gland epithelial cells where capsaicin application attenuates TLR4 mediated inflammatory responses (Park et al., 2004; Shin et al., 2013).

2.4 Conclusion

Neuroimmune interactions play an important role in pathogenesis of chronic pain and warrants further investigation to uncover novel therapeutic targets. Improving the relevance of preclinical models to screen novel targets would be an important step. TLR4 and TRPV1 represent receptor targets which are important in multiple pathological pain models and neuronal tissues. Further, multiple studies have highlighted a potential functional interaction whereby TLR4 mediates TRPV1 functional changes. The interaction involving TLR4 and TRPV1 offers a potential novel neuroimmune mechanism with clinical relevance in pathological pain states, the nature of this mechanism is the focus of current research. Further characterisation of the TLR4/TRPV1 interaction is required, including a greater understanding of mechanisms leading to direct and rapid receptor sensitisation. Rapid increases in receptor sensitivity are observed following LPS application, while the presence of TLR4 alone is also proposed to alter channel activity by direct protein-protein interaction. TLR4 activation by LPS results in increased levels of multiple kinases (PKC, PKA, PI3K, Src) as well as intracellular calcium, all of which are

associated with increased TRPV1 activity due to alteration in receptor phosphorylation and cell membrane expression. Potential direct and rapidly induced TLR4-dependent routes of TRPV1 sensitisation are presented in Figure 2.2 based on the literature of each receptor presented above. Investigation of both rapid, and proinflammatory driven sensitisation is required to understand the potential role in generation and/or maintenance of central sensitisation and pathological pain. The importance of each receptor to pathological pain states and central sensitisation suggests the interaction warrants further investigation in this context. Ultimately, novel mechanisms which influence central sensitisation are potential drug targets in efforts to improve chronic pain treatment outcomes.

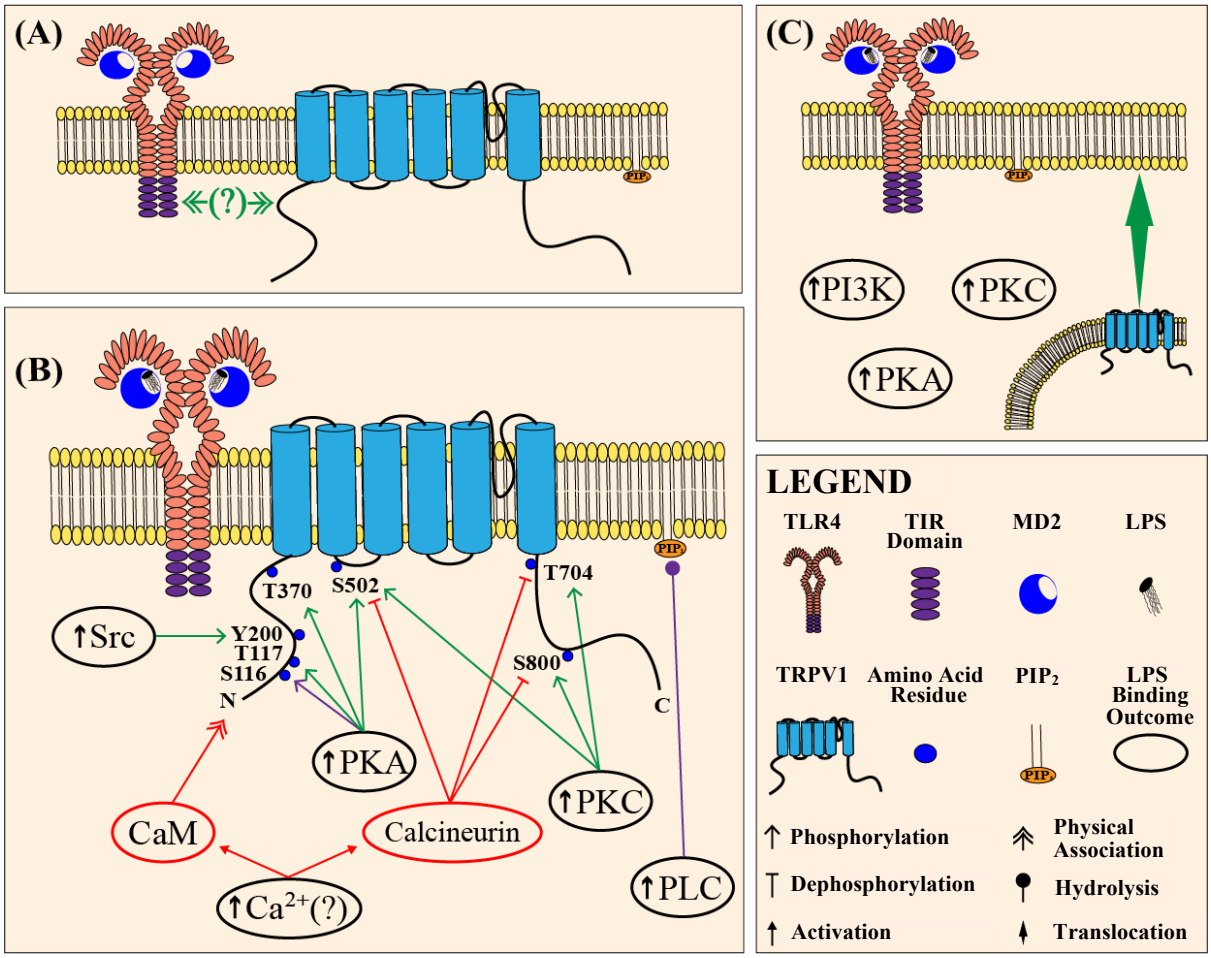


Figure 2.2: Proposed mechanisms of direct and rapid TLR4 induced TRPV1 sensitisation. (A) Direct interaction proposed between the TIR domain of TLR4 and TRPV1. (B) LPS-induced increase in enzymatic activity (Src, PKA, PKC, PLC) alters TRPV1 sensitisation. (C) LPS-induced increase in enzymatic activity (PKC, PKA, PI3K) results in increased trafficking of TRPV1 to the cell membrane. Green represents actions resulting in receptor sensitisation; red represents actions resulting in receptor desensitisation; purple represents actions where evidence exists of both sensitising and desensitising effects.

Thesis Aims and Hypotheses

The introductory chapters highlight that further research is required to ascertain the exact nature of a TLR4/TRPV1 functional interaction. It is clear that sensitisation of the TRPV1 channel occurs through multiple agents induced by inflammation. The timing of potentiated capsaicin responses in multiple *in vivo* studies correlates with the induction of pro-inflammatory responses, suggesting an indirect mechanism of receptor sensitisation. However, *in* vitro studies point to a much shorter LPS incubation altering TRPV1 function. Further, both *in vitro* and *in vivo* studies suggest the presence of TLR4 alone, in the absence of activation, alters TRPV1 function. The nature of this relationship could be any one of a number of candidates, from physical interaction, alteration of scaffolding proteins, the fast activation of multiple relevant kinases, or alteration of membrane lipid composition. Understanding this relationship will help improve our understanding of neuroimmune interactions important in the generation and maintenance of chronic pain states.

The aim of the first research chapter is to further investigate mechanisms surrounding the rapid induction of sensitisation *in vitro*. This involves creating a TLR4/TRPV1 co-expression system and assessing TRPV1 in the presence and absence of TLR4. Further, we aim to reproduce previously observed short term agonist-induced sensitisation in order to target multiple intracellular components which are potentially responsible. Based on the literature presented above, we hypothesise we will see a TLR4 dependent potentiation of capsaicin-induced intracellular calcium in TLR4/TRPV1 expressing HEK293FT cells. We then expect agonism of TLR4 with LPS will induce rapid (< 20 min) potentiation of calcium responses compared to vehicle treated TLR4/TRPV1 expressing cells. Further we hypothesise that antagonism of TLR4 signalling will inhibit potentiation, as will inhibition of TRPV1 acting protein kinases (PKA, PKC).

The second major aim relates to understanding the mechanisms underlying LPS-induced potentiation of capsaicin sensitivity in humans. A significant obstacle in the chronic pain field is the preclinical to clinical translation and relevance of preclinical pain models. One exciting possibility, given the clinical endotoxin-capsaicin model, is a unified preclinical, clinical model. Such a paradigm would offer the unique possibility of studying central cellular and molecular changes associated with both preclinical and clinical behavioural outcomes. We aim to reproduce the clinical endotoxin-capsaicin model in a preclinical

BALB/c mouse model. Following the creation of the model we aim to investigate if key mechanisms are central or peripheral by targeted TLR4 antagonism. Further we aim to characterise central glial activity and pro-inflammatory cytokine levels. We hypothesise that systemic endotoxin will potentiate mechanical hypersensitivity in a BALB/c mouse model, associated with an inflammatory response and glial activation.

In conjunction with this second aim, we will present a systematic review discussing the use of capsaicin in pain models. We hope such a review will help inform our decisions and interpretations of a BALB/c endotoxin-capsaicin model. Likewise, the review will assist future researchers and inform decisions to help improve the use and relevance of capsaicin in preclinical pain models.

Achieving these aims will further our understanding of potentially important neuroimmune interactions, and their role in pain sensitivity. Further, we hope to produce a novel neuroimmune pain model which targets both immune and neural elements separately to produce heightened pain sensitivity. This will create a unified clinical and preclinical model, a unique situation in pain literature. We hypothesis that TLR4 plays a critical role in TRPV1-induced pain and these investigations will improve understanding of the TLR4/TRPV1 interaction, it's importance to pain hypersensitivities, and relevance as a novel therapeutic target.

Chapter 3. Activation and antagonism of toll-like receptor 4 signalling potentiates TRPV1 mediated calcium accumulation in HEK293FT cells.

3.1 Abstract

Interaction between nociceptive signalling and the immune system is becoming increasingly relevant in the search for novel therapeutics to treat chronic pain. Two receptor types in particular have been repeatedly implicated in the generation and maintenance of chronic pain in preclinical models. These are the innate immune receptor, toll-like receptor 4 (TLR4), and the neuronal ion channel, transient receptor potential cation channel subfamily V member 1 (TRPV1). Recently, a functional relationship between these receptors has been reported in clinical and preclinical models. We utilise a dual overexpression model in HEK293FT cells in an attempt to understand the mechanism of this interaction. We report potentiated intracellular calcium and altered influx dynamics in cells expressing both TLR4 and TRPV1 compared to TRPV1 only, including increased rate of calcium accumulation ($p < 0.0001$). TLR4 antagonists LPS-RS, (+)-naloxone, 1J and TAK-242 do not affect calcium influx dynamic but do increase intracellular calcium accumulation. Likewise, short term (18 min) but not long term (4 h) stimulation of TLR4 with LPS does not affect calcium influx dynamic, but produces increased calcium accumulation in TLR4/TRPV1 expressing HEK293FT cells. TLR4 is able to alter the function of the TRPV1 receptor independent of TLR4 signalling events raising the possibility of a physical protein-protein interaction which alters TRPV1 mediated calcium influx.

3.2 Introduction

Chronic pain represents a significant worldwide health problem, affecting an estimated 20% of adults globally, with an estimated economic impact in 2010 of between $560 and 635 billion in the United States (Goldberg et al., 2011; Loeser, 2012). Changes in ion channel sensitivity are associated with increased neural excitability and symptoms of chronic pain (Gonzalez-Ramirez et al., 2017). Increasing evidence implicates transient receptor potential cation subfamily V member 1 (TRPV1) in mechanical and thermal hypersensitivities; as a result, research has focussed on improving understanding surrounding regulation of TRPV1 function (Caterina et al., 2000; Malek et al., 2015).

A neural receptor found centrally and peripherally, TRPV1 is activated by endogenous and exogenous ligands (e.g. anandamide, capsaicin) as well as environmental factors including temperature and pH (Brito et al., 2014; Caterina et al., 1997; Szallasi et al., 2007). Sensitisation and/or increased expression of TRPV1 leading to increased neuronal excitability occurs in the presence of numerous inflammatory mediators including interleukins (IL-1, IL-6), prostaglandins (PGs), bradykinins, adenosine triphosphate (ATP) and nerve growth factor (NGF) (Chuang et al., 2001; Fang et al., 2015; Hensellek et al., 2007; Moriyama et al., 2005; Tominaga et al., 2001; Zhang et al., 2005). Use of knockout models and expression studies reveal its importance in the maintenance of numerous pain phenotypes including morphine-induced thermal hyperalgesia, orofacial pain, orthodontic pain and complete Freund's adjuvant (CFA)-induced inflammatory pain (Amaya et al., 2003; Gao et al., 2016; Vardanyan et al., 2009; Watase et al., 2018).

The importance of neuroimmune interactions on neural sensitivity is becoming increasingly evident; and the role of innate immune toll-like receptors (TLRs) in particular, which are heavily linked to chronic pain (Lacagnina et al., 2017; Nicotra et al., 2012). TLR3, 4, 5 and 7 have all been implicated in pain via upregulation in pain models or presence on primary afferent nociceptors. (Chen et al., 2017b; Lacagnina et al., 2017; Qi et al., 2011; Shi et al., 2017). The most intensively studied is TLR4, well known for its detection of gram-negative bacterial cell wall component lipopolysaccharide (LPS). TLR4 antagonists LPS-RS and (+)-naloxone reverse neuropathic pain in rat models, while low dose LPS produces location-specific hypersensitivities in clinical trials (Hutchinson et al., 2008; Jurga et al., 2016; Lewis et al., 2012; Wegner et al., 2014). TLR4 has also been implicated in direct neural activation (Chiu et al., 2013). Unsurprisingly, due to anatomical proximity and their apparent roles in pain hypersensitivities, a functional relationship between TLR4 and TRPV1 has been proposed.

LPS potentiates capsaicin-induced neural responses in a number of primary cell and *ex vivo* models including rat vagus nerve, dorsal root and trigeminal ganglia (Diogenes et al., 2011; Ferraz et al., 2011; Hua et al., 1996). Hutchinson et al. revealed the clinical relevance of a TLR4/TRPV1 interaction, reporting low-dose endotoxin potentiates mechanical hypersensitivity following intradermal forearm application of capsaicin (Hutchinson et al., 2013). The timing of potentiation creates questions around the nature of

the TLR4/TRPV1 interaction. Clinical potentiation and *ex vivo* vagus nerve studies suggest an indirect relationship via increased production of proinflammatory mediators (Hua et al., 1996; Hutchinson et al., 2013). While primary cell and overexpression system studies suggest potentiation after a much shorter stimulation period (15 min), suggesting a more direct interaction (Diogenes et al., 2011; Ferraz et al., 2011; Li et al., 2015).

Despite the apparent clinical implications of a TLR4/TRPV1 interaction, the exact nature of this relationship is yet to be determined. This study aims to further define the functional relationship between TLR4 and TRPV1 by exploring changes in TRPV1 function in the presence of TLR4 alone, as well as during agonist and antagonist induced TLR4 signalling manipulations.

3.3 Methods

3.3.1 Materials

Human TRPV1 cloned into a PcDNA 3.1 D-V5/His6 TOPO mammalian expression vector was kindly donated by Dr. Christopher Reilly of the University of Utah, USA (Reilly et al., 2003). Gibco™ Hanks' Balanced Salt Solution (HBSS) – supplemented with calcium, magnesium and glucose; phenol red and sodium pyruvate free, purchased from ThermoFisher (NSW, AUS, cat#: 14025). 1M HEPES purchased from Sigma-Aldrich (NSW, AUS), diluted to 25 mM in HBSS. Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 purified by phenol extraction purchased from Sigma-Aldrich (Castle Hill, NSW). (+)-Naloxone and 1J were kindly provided by Dr Kenner Rice (Chemical Biological Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD), solubilised to 10 mM in 100% DMSO. LPS from *Rhodobacter sphaeroides* (LPS-RS) purchased from Invivogen (CA, USA), solubilised to 100 μg / mL in 1X PBS. CLI-095 (TAK-242) purchased from Invivogen (CA, USA), solubilised to 10 μM in 1X PBS. Capsazepine purchased from Sigma-Aldrich (NSW, AUS) and solubilised to 5 mM in 100% DMSO. Capsaicin purchased from Sigma-Aldrich (NSW, AUS), solubilised to 50 mM in 100% DMSO. Viafect™ purchased from Promega (WI, USA). Fluo-4AM purchased from Thermo Fisher (NSW, AUS), solubilised to 1 mM in 100% DMSO.

3.3.2 Cell culture

Human embryonic kidney 293-FT (HEK293FT) cells were purchased from Life Technologies (VIC, AUS), HEK293FT cells stably expressing TLR4 and accessory proteins (CD14 and MD2) (TLR4 cells) were purchased from InvivoGen (CA, USA). HEK293FT and TLR4 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, (10%) (w/v) fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin and 50 mg/mL streptomycin. TLR4 cell line was further supplemented with 100 μg/mL normocin, 50 μg/mL hygromycin, and 10 μg/mL blasticidin as per the manufacturer's protocol.

3.3.3 TRPV1 transient transfection

Cells were plated in 96 well clear bottom black cell culture plate (plate-based assay) or 8 well ibidi ibiTreat μ-Slide 8 well (live-cell assay) 24 h prior to transfection. All cell types were plated in basic media (antibiotic free DMEM supplemented with 4.5 g/L glucose, (10%) (w/v) fetal bovine serum (FBS), 2 mM L-glutamine) in a total volume of 200 μL. HEK293FT and TLR4 cell lines were plated at 25000 and 45000 cells/well respectively. Transfection was carried out 24 h after plating when cells were 60-80% confluent using ViaFect™ as per the manufacturer's protocol. ViaFect™ was used at a ratio of 6:1 (6 μL/1 μg DNA), 80 ng of DNA was added to each well. Cells were used for plate based or live cell calcium assay 24 h post transfection.

3.3.4 Fluo-4AM calcium assay

Supernatant was aspirated and 200 μL of HBSS added. 150 μL of supernatant was removed and replaced with 150 μL, repeated for a total of three washes – all remaining supernatant was removed during the final wash. 50 μL of 3 μM Fluo-4AM in HBSS + 25 mM HEPES was added to each well and left to incubate in the dark for 45 min at room temperature (RT). Fluo-4AM was removed and the cells were washed as above. Following the removal of the last wash step, 90 μL (96 well) or 150 μL (8 well) HBSS + 25 mM HEPES with/without TLR4 agonist/antagonist or TRPV1 antagonists were added to each well.

3.3.4.1 Plate reader

BMG FLUOstar OPTIMA was used for plate-based calcium assays and preheated to 37°C before all experiments. The plate was added immediately following the addition of 90 μL HBSS + 25 mM HEPES and read 18 min after removal of Fluo-4AM to allow appropriate de-esterification (timing optimised in pilot studies). Fluorescence was measured at 2 s intervals for 32 s (exc. 485, em. 520) per well with a maximum of 16 wells per run, 10 μL of capsaicin or vehicle was injected at 5.5 s.

3.3.4.2 Live cell

Olympus FV3000 Confocal and associated FV31S-SW viewer software (Ver2.3) was used to record live cell calcium flux. Slides were added to the preheated stage (37°C) following addition of 150 μL HBSS + 25 mM HEPES and allowed to sit for 18 min to allow appropriate de-esterification prior to the addition of capsaicin. Imaging was initiated, and 5 s later 150 μL of 1 μM capsaicin was administered via PE10 tubing attached to a 30G needle and 1 mL syringe. Total image time was 40 s per well. ImageJ software (Ver 2.0.0-rc-69/1.52p) was used to analyse fluorescence changes in responding cells.

3.3.5 Antagonist assays

Capsazepine (10 μM), (+)-naloxone (200 μM), and 1J (10 μM) were applied to cells after removal of the final wash. TAK-242 (1 μM) and LPS-RS (200 ng/mL) were applied 1 and 3 h respectively, prior to the first HBSS wash and re-applied during the 45 min Fluo-4AM incubation and following the final wash in HBSS + 25 mM HEPES to ensure total pre-treatment time of 2 and 4 h respectively.

3.3.6 Endotoxin – capsaicin study

An 18-min LPS pre-treatment; 10, 100 or 1000 ng/mL in HBSS + 25 mM HEPES was added after the final wash in HBSS + 25 mM HEPES. For the 4 h pre-treatment; 10, 100 or 1000 ng/mL LPS in basic media was added to cells 3 h prior to the first wash and re-applied during the 45 min Fluo-4AM incubation and after the final wash in HBSS + 25 mM HEPES.

3.3.7 Assessment of TLR4 function

Transfection of HEK293FT and TLR4 cell lines was performed as above. 24 h following transfection, supernatant was removed and cells treated with LPS in basic media (100 μL - 100 ng/mL) or vehicle (media only) for 4 h. 80 μL of supernatant was removed, and IL-8 content was measured using an ELISA kit (BD-Bioscience, Cat#555244, USA) according to the manufacturer's instructions. Due to the high concentrations of IL-8 in the supernatant, samples were diluted (1:25) in assay diluent so they did not exceed the detection limits (samples receiving LPS-RS were the exception (1:2)).

3.3.8 Analysis

GraphPad Prism (Ver 8.2.0) was used to analyse all data. To compare AUC, time to maximum intensity and linear regression slope between HEK293FT and TLR4 expressing cell lines, two-way ANOVA with Sidak's multiple comparisons test was performed. To compare intensity over time from plate reader experiments two-way ANOVA with Geisser-Greenhouse correction and Tukey's multiple comparison test was performed. All other AUC and ELISA data was analysed by one-way ANOVA with Tukey's multiple comparisons test. An unpaired two-tailed t-test was used to compare linear regression slope between populations following live cell experiments.

3.4 Results

3.4.1 TLR4 alters TRPV1 dependent capsaicin responses in HEK293FT cells

Capsaicin induces a sudden TRPV1 dependent increase in Fluo-4AM signal in cells transfected with TRPV1 (Fig. 3.1A, B). Post hoc analysis following two-way ANOVA revealed that TLR4 cells have significantly higher AUC at 50 ($p < 0.05$) and 0.5 μM ($p < 0.001$) compared to HEK293FT cells, no differences are observed at the lower concentrations tested. Time to maximum fluorescence intensity was significantly reduced for all concentrations tested (Fig. 3.1D), while the rate of intensity increase calculated by the slope from baseline to maximum was significantly higher in TLR4 expressing cells at 50 μM ($p < 0.0001$), 0.5 μM ($p < 0.0001$), and 50 pM ($p < 0.05$) but not 50 fM (Fig. 3.1E). ELISA results confirmed functionality of TLR4 in cells expressing TRPV1 (Fig. 3.1F). Post hoc analysis revealed LPS-RS significantly attenuated LPS-induced IL-8 responses in

both TRPV1 and empty vector control transfections ($p < 0.0001$) (Fig. 3.1F). Interestingly, the TRPV1 antagonist capsazepine also attenuated the IL-8 response in both TRPV1 ($p < 0.0001$) and empty vector controls ($p < 0.001$). No significant difference in IL-8 response between TRPV1 and empty vector transfected cells was observed ($p = 0.81$). Together, these results indicate an altered TRPV1 dependant capsaicin-induced calcium influx dynamic in the presence of functional TLR4.

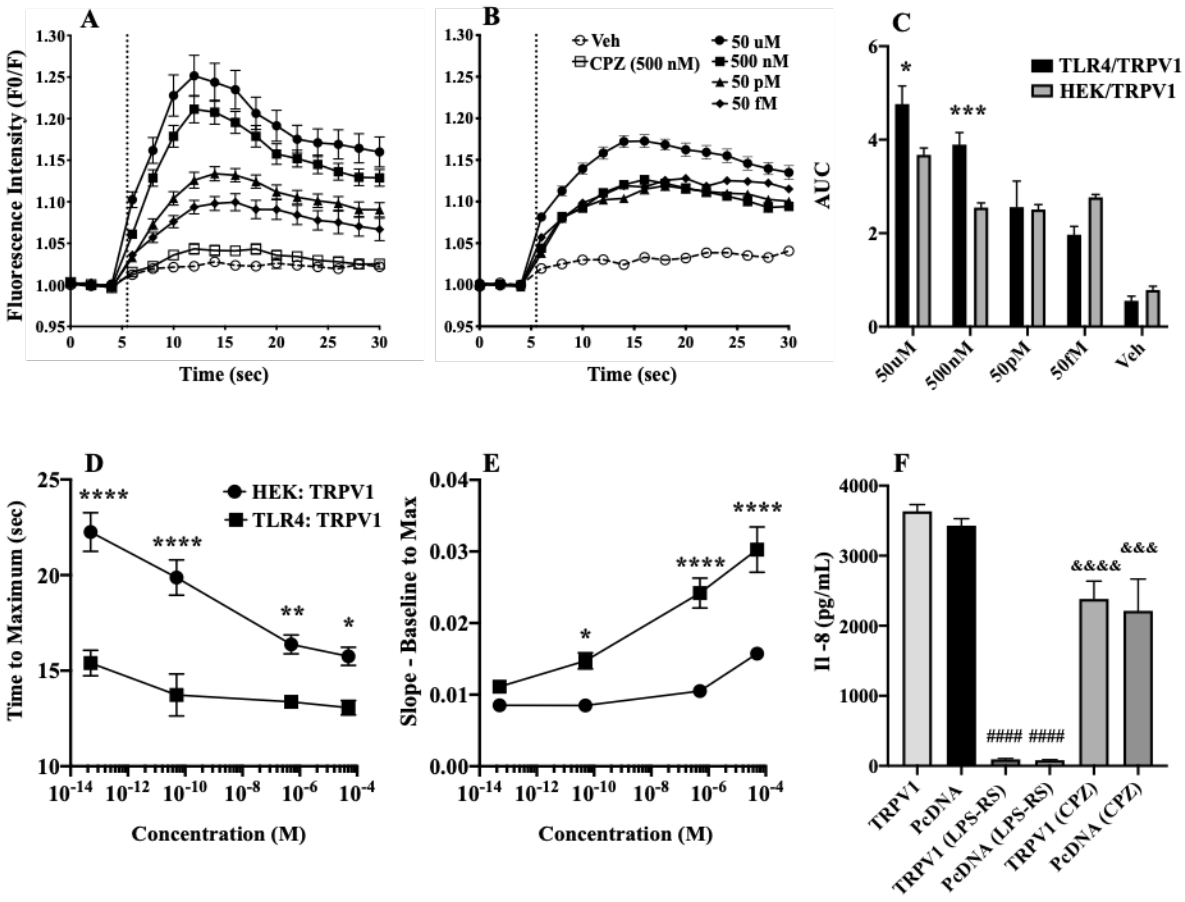


Figure 3.1: **TLR4 alters TRPV1 dependent capsaicin responses in HEK293FT cells.** Capsaicin dose response (50 μM, 0.5 μM, 50 pM, 50 fM) in TLR4/TRPV1 (A) and TRPV1 expressing cells (B) with AUC comparison (C). Comparison of time to peak fluorescence intensity (D) and rate of intensity increase (E). (F) TLR4 functionality confirmation in TRPV1 transfected cells by human IL-8 ELISA. n = 4 for calcium assay studies, n = 5 for ELISA results. Error bars represent SEM, dotted vertical line represents time of capsaicin injection. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$ for TLR4/TRPV1 vs TRPV1 expressing cells. #### = $p < 0.0001$ for LPR-RS + LPS vs LPS only cytokine response for respective transfections. &&& = $p < 0.001$, &&&& = $p < 0.0001$ for CPZ + LPS vs LPS only cytokine response for respective transfections.

3.4.2 Live cell calcium analysis reveals a population of fast responding TLR4/TRPV1 HEK293FT cells.

Live cell images reveal a rapid increase in calcium accumulation following capsaicin treatment in TRPV1 and TLR4/TRPV1 expressing cells. The rate of intensity increase varies within the population (Fig. 3.2). Linear regression of baseline to maximum intensity revealed a significant difference between the mean slope of TLR4/TRPV1 (5.3 ± 0.5) and TRPV1 (3.5 ± 0.3) cells ($p < 0.001$). Distribution of the two populations suggests a separate population with unique response characteristics may exist within the TLR4 responders (Fig. 3.2C).

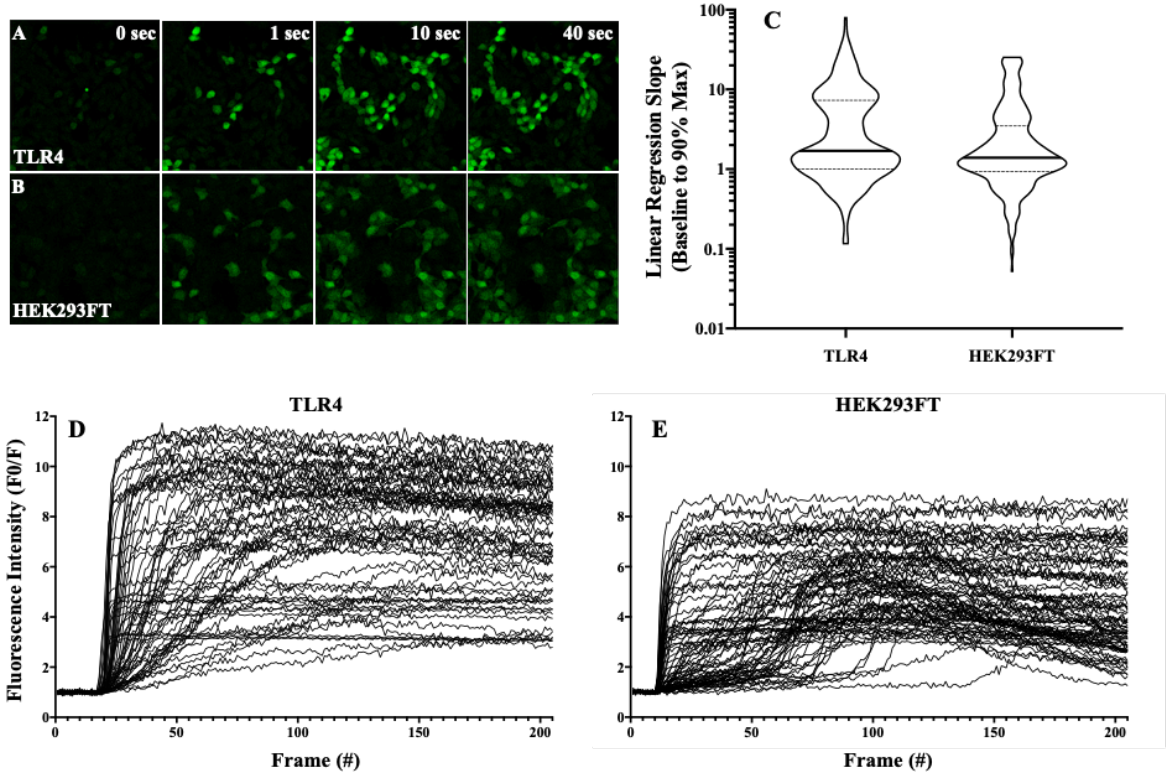


Figure 3.2: **Live cell analysis reveals a fast responding population within the TLR4/TRPV1 cell line**. Example recoding of TLR4 (A) and HEK293FT (B) cells transfected with TRPV1 at baseline, 1, 10 and 40 s following capsaicin administration (500 nM). (C) Violin plot comparing linear regression slope (baseline to max.) of the two populations of cells showing median, 1st and 3rd quartiles. Representative single cell fluorescent changes for TLR4/TRPV1 (D) and TRPV1 expressing HEK293FT cells (E). A, B, D and E represent one trial only. n = 5 recordings for violin plot (n = 326 & 356 individual capsaicin responders from TLR4/TRPV1 and TRPV1 populations respectively).

3.4.3 TLR4 antagonists potentiate capsaicin-induced calcium accumulation in TLR4/TRPV1 expressing HEK293FT cells.

TLR4 antagonists LPS-RS, (+)-naloxone, TAK-242 and 1J were administered to assess the potential action of TLR4 on TRPV1 function. Two-way ANOVA revealed an effect of time ($F_{(1.9, 28.9)} = 2393$, $p < 0.0001$) and interaction ($F_{(60, 225)} = 2.3$, $p < 0.0001$) but not treatment ($F_{(4, 15)} = 2.0$, $p = 0.15$) when capsaicin fluorescence intensity plots were analysed (Fig. 3.3A). Multiple comparisons revealed no significant differences at all time points for LPS-RS, TAK-242 and 1J compared to capsaicin only. (+)-Naloxone showed increased fluorescence intensity at 12 s post capsaicin but at no other time point (Fig. 3.3A). Comparison of AUC analysis by one-way ANOVA revealed that all TLR4 antagonists; LPS-RS ($p = 0.012$), TAK-242 ($p = 0.0017$), 1J ($p = 0.0025$) and (+)-naloxone ($p < 0.0001$) produced a significant increase compared to vehicle treated cells (Fig. 3.3B). Unlike all other antagonists, LPS-RS produced a significant intensity increase over vehicle cells in the absence of capsaicin from 14 s post addition of vehicle until the end of recording (Fig. 3.3A). This difference was observed in AUC analysis in LPS-RS treated cells ($p = 0.0011$), no statistical differences were observed between vehicle treated cells and all other antagonists tested; (+)-naloxone ($p = 0.873$), TAK-242 ($p = 1.0$), 1J ($p = 1.0$) (Fig. 3.3B). These results suggest TLR4 antagonist activity increases overall capsaicin-induced calcium accumulation without altering the influx dynamics.

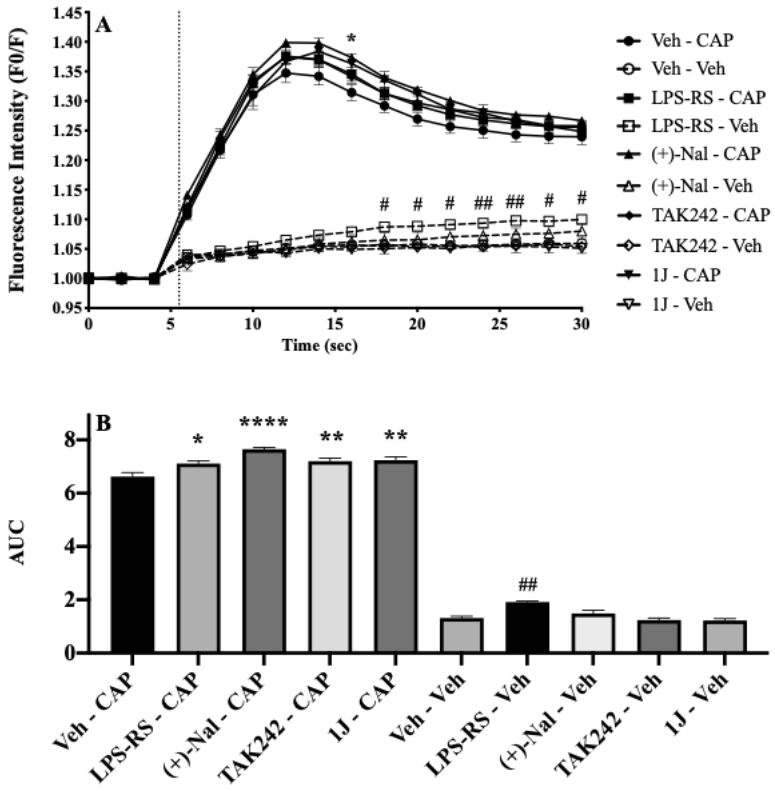


Figure 3.3: **TLR4 antagonists potentiate capsaicin-induced calcium accumulation in TLR4/TRPV1 expressing HEK293FT cells**. (A) Intensity changes induced by capsaicin (500 nM) in the presence of TLR4 antagonists LPS-RS (200 ng/mL), (+)-naloxone (200 μM), TAK-242 (10 μM) and 1J (10 μM). (B) AUC analysis of intensity plot in (A). n = 5 for capsaicin-vehicle and vehicle-vehicle treated cells, n = 4 for all other groups except cells administered 1J (n = 3). Error bars represent SEM, dotted vertical line represents time of capsaicin injection. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$ for (+)-naloxone + capsaicin (A) and antagonist + capsaicin (B) vs vehicle + capsaicin treated cells. # = $p < 0.05$, ## = $p < 0.01$for LPR-RS + vehicle vs vehicle + vehicle treated cells.

3.4.4 Endotoxin potentiates capsaicin-induced calcium accumulation following 18 min but not 4 h pre-treatment.

Representing the range of LPS doses previously found to potentiate TRPV1 responses; 10, 100 or 1000 ng/mL LPS was administered 18 min or 4 h prior to capsaicin (Ferraz et al., 2011; Li et al., 2015; Wu et al., 2019). After an 18 min pre-treatment a two-way ANOVA revealed significant effect of time ($F_{(1.9,23.2)} = 387$, $p < 0.0001$) but not treatment ($F_{(3,12)} = 1.0$, $p = 0.4$) or interaction ($F_{(45,180)} = 1.4$, $p = 0.05$) when capsaicin treated data was analysed (Fig. 3.4A). Two-way ANOVA of cells not treated with capsaicin reveals a significant effect of time ($F_{(2.0, 24.0)} = 119$, $p < 0.0001$), treatment ($F_{(3,12)} = 6.9$, $p = 0.006$) and interaction ($F_{(45,180)} = 5.9$, $p < 0.0001$) (Fig. 3.4A). Post hoc analysis revealed no time point differences between LPS-capsaicin and vehicle-capsaicin treated cells (Fig. 3.4A). Significant differences were observed for 1000 ng/mL LPS-vehicle compared to vehicle-vehicle treated cells from 20 s onwards (Fig. 3.4A). AUC analysis revealed a significant difference between 1000 ng/mL ($p < 0.01$) and 100 ng/mL ($p < 0.0001$) LPS-capsaicin and vehicle-capsaicin treated cells (Fig. 3.4C). Likewise, compared to vehicle-vehicle treated cells, AUC analysis revealed significant difference with 1000 ng/ml LPS-vehicle ($p < 0.001$) and 100 ng/ml LPS-vehicle ($p < 0.001$) groups (Fig. 3.4C). Following a 4 h pre-treatment two-way ANOVA revealed significant effect of time ($F_{(1.6, 19.7)} = 353$, $p < 0.0001$). However, no significant effect of treatment ($F_{(3,12)} = 0.2$, $p = 0.9$) or interaction ($F_{(45,180)} = 0.4$, $p = 1.0$) was found when capsaicin treated data was analysed (Fig. 3.4B). Two-way ANOVA of cells not treated with capsaicin reveals a significant effect of time ($F_{(2.1,25.3)} = 104$, $p < 0.0001$) and interaction ($F_{(45,180)} = 1.8$, $p = 0.004$) but not treatment ($F_{(3,12)} = 2.5$, $p = 0.1$). Post hoc analysis revealed no time point differences between vehicle-capsaicin and all LPS-capsaicin groups, or vehicle-vehicle and all LPS-vehicle groups (Fig. 3.4B). AUC analysis revealed a significant difference between 100 ng/mL LPS-vehicle and vehicle-vehicle treated cells ($p < 0.05$) (Fig. 3.4D). Despite this increase, there were no differences due to LPS treatment in capsaicin treated cells (Fig. 3.4D). Therefore, the shorter 18 min, but not 4h pre-treatment, resulted in potentiated capsaicin-induced calcium accumulation. However, LPS alone induced increased calcium accumulation at the same concentrations (100, 1000 ng/ml) following 18 min pre-treatment.

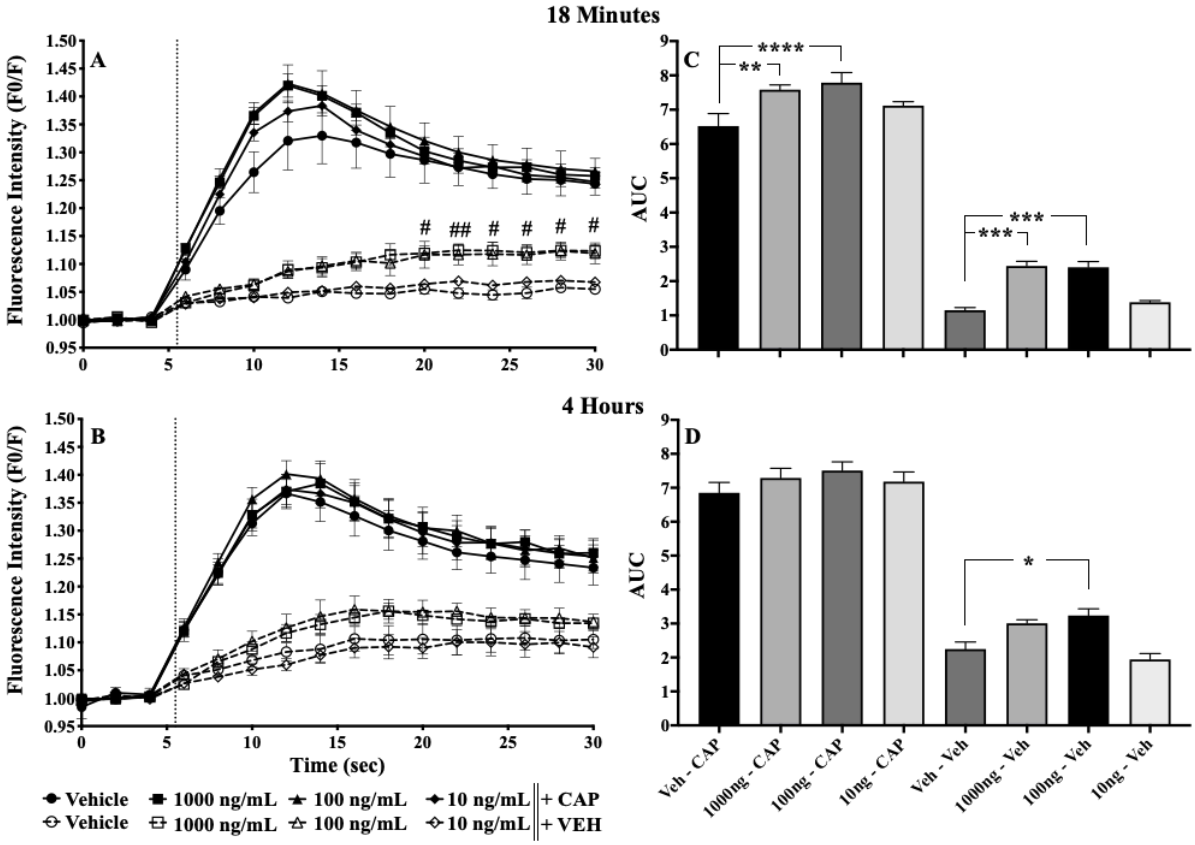


Figure 3.4: **Endotoxin potentiates capsaicin-induced calcium accumulation following 18 min but not 4 h pre-treatment in cells expressing TLR4 and TRPV1.** Intensity changes induced by capsaicin (500 nM) pre-treated with LPS for 18 min (A) or 4 h (B). AUC of intensity plot for 18 min (C) and 4 h (D) pre-treatment. n = 4 for all groups. Error bars represent SEM, dotted vertical line represents time of capsaicin injection. # = $p < 0.05$, ## = $p < 0.01$, for 1000 ng/mL - VEH vs VEH - VEH. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

3.5 Discussion

Neural sensitisation resulting in pain hypersensitivity leads to symptoms of chronic pain. TRPV1 plays an important role in nociception and hypersensitivity; it's relationship with immune receptor TLR4 is a potentially important neuroimmune interaction contributing to receptor sensitisation. Here we show that TLR4/MD2/CD14 (TLR4), co-expressed in HEK293FT cells with TRPV1, alters the dynamics of calcium influx following capsaicin exposure. The presence of TLR4 increased the rate of calcium accumulation when compared to un-transfected controls (Fig. 3.1). This agrees with similar studies in TLR4/TRPV1 expressing HEK293FT cells which showed increased calcium influx when compared to ionomycin control treatments (Min et al., 2018; Min et al., 2014). In contrast to these studies, we did not observe a sustained calcium difference for the duration of recording, although we used a different (non-ratiometric) calcium indicator and a 20-fold lower capsaicin concentration. It should also be noted that these studies do not mention co-expressing TLR4 associated CD14 and MD2. Primary cell patch clamp studies report similar findings in mouse DRG neurons; TLR4 knockout tissue shows decreased current density when compared to wild-type (WT) controls (Wu et al., 2019). Itch studies have postulated increased TRPV1 mediated histamine-induced itch is potentiated in older (75 day) compared to younger (45 day) mice as a result of increased DRG expression of TLR4 (Ji et al., 2018). Upregulation of TLR4 *in vivo* is also associated with increased capsaicin responses in models of inflammatory pain; paclitaxel-induced neuropathy and TNBS-induced colitis (Ji et al., 2018; Li et al., 2015; Wu et al., 2019). Our live cell analysis reveals a fast responding population of TLR4/TRPV1 cells, resulting in significantly higher calcium accumulation rate following capsaicin when compared to TRPV1 expressing cells (Fig. 3.2). These separate populations potentially explain the fluorescence ratio slope differences observed between TLR4/TRPV1 and TRPV1 expressing HEK293FT cells in a plate reader assay.

These unstimulated effects strengthen a proposed direct protein-protein interaction between TRPV1 and TLR4. The toll-interleukin receptor 1 (TIR) domain of TLR4 has been proposed as a potential site of interaction. TLR4 mutants without a TIR domain, expressed with TRPV1, results in decreased capsaicin responses compared to wild-type TLR4, whose responses resemble TRPV1 only expressing HEK293FT cells (Min et al., 2018). TIR domain potentiates TRPV1 activity by blocking activation-induced desensitisation (Min et al., 2018). It is unclear however if the interaction is direct; no

interaction between TIR domain containing proteins and TRP channels has been previously reported. While the authors identifying the TIR/TRPV1 interaction acknowledge unidentified intermediary proteins may be involved in the observed potentiation of TRPV1 activity (Min et al., 2018). One potential intermediary is cytoplasmic scaffolding protein A-kinase anchoring protein 79 (AKAP79) (rodent orthologue AKAP150), which is important to the sensitising effect of protein kinase A and C (PKA, PKC) on TRPV1 (Faux et al., 1997; Jeske et al., 2008; Jeske et al., 2009; Zhang et al., 2008). TLR-TRP interactions have been observed previously, co-immunoprecipitation revealed an interaction between TRPA1 and TLR7 which potentiates TRPA1 induced inward current, suggesting a physical interaction (Park et al., 2014). Further research is required to confirm a protein-protein interaction between TLR4 and TRPV1. Detection of unmodified protein interactions in tissues remains elusive due to the unreliability of available TLR4 antibodies (McCarthy et al., 2017). Advances in protein-protein interaction assays such as bioluminescence resonance energy transfer (BRET) have the potential to improve our understanding using overexpression models (Dimri et al., 2016).

TLR4 antagonists were applied to identify potential TLR4 signalling effects. Interestingly, at concentrations which inhibit LPS-induced IL-8 release in TLR4 cells, all antagonists showed increased calcium accumulation analysed by AUC when compared to vehicle treated cells (Fig. 3.3). (+)-Naloxone is the opioid inactive stereoisomer of opioid antagonist (-)-naloxone, and a TLR4 antagonist; its mechanism of action requires further investigation (Lewis et al., 2012). Like (+)-naloxone, (+)-naltrexone is an opioid inactive stereoisomer of an opioid antagonist, and TLR4 antagonist. Unsurprisingly its analogue IJ, modified for increased potency and decreased cytotoxicity, resulted in the same significant increase as (+)-naloxone (Fig. 3.3) (Selfridge et al., 2015). LPS-RS prevents many endotoxin sources from interacting with MD-2 (Gaikwad et al., 2015; Kutuzova et al., 2001). While TAK-242 inhibits the association of TLR4 with adaptor molecules toll/interleukin-1 receptor domain-containing adaptor protein (TIRAP) or toll/interleukin 1 receptor domain-containing adaptor protein inducing interferon-β-related adaptor molecule (TRAM) by binding a cysteine residue (C747) in the intracellular TIR domain (Matsunaga et al., 2011; Shirey et al., 2020). As interfering with this conserved region potentiates, rather than inhibits capsaicin-induced calcium accumulation, it is unlikely this represents

an important residue for a potential protein-protein interaction between TLR4 and TRPV1. Further, it suggests that TIRAP or TRAM don't facilitate an interaction. Taken together these results reveal blocking external and internal signalling components of the TLR4 signalling cascade causes an increase in calcium influx following capsaicin. This is in contrast to Diogenes et al. who did not report an antagonist effect on capsaicin response, either calcium accumulation or CGRP release from rat TG neurons (Diogenes et al., 2011).

To further elucidate mechanism, we tested the widely reported endotoxin-capsaicin paradigm where LPS potentiates TRPV1 dependent calcium responses. Studies using rat TG and mouse DRG report potentiated capsaicin responses (Ca^{+2} influx, CGRP release and current density) following 5 and 15 min LPS treatments (Diogenes et al., 2011; Ferraz et al., 2011; Wu et al., 2019). Importantly, antagonism of TLR4 reversed the effect in rat TG neurons and the potentiated capsaicin responses were not observed in DRG neurons from TLR4 knockout mice (Diogenes et al., 2011; Wu et al., 2019). Due to limitations with calcium detection by Fluo-4AM, we were unable to replicate this timing exactly but applied capsaicin after an 18 min LPS incubation. We observed potentiation at 1000 and 100 but not 10 ng/mL LPS (Fig. 3.4). In contrast, potentiation of inward current has been observed after 10 ng/ml LPS over a similar timeframe in TLR4/TRPV1 overexpressing HEK293FT cells; although the serotype used was not reported in this case (Li et al., 2015). In the same study, activation of TLR4 by chemotherapeutic agent paclitaxel over the same time period produced TRPV1 potentiation (Li et al., 2015). This effect is in contrast with primary cell studies which concluded 20 and 200 ng LPS did not potentiate a response, and between 2 and 200 μg/mL were required (Diogenes et al., 2011; Ferraz et al., 2011; Wu et al., 2019). This result is not unexpected given *in vitro* TLR4 overexpression models are likely to show greater sensitivity to LPS. Given the low dose of LPS (0.4 ng/kg) which produced potentiated capsaicin-induced pain behaviours in the clinical model, this data further strengthens the argument of immune mediator driven rather than direct receptor interaction causing potentiation in the clinical setting. This *in vitro* data further supports the hypothesis that early consequences of TLR4 signalling cause rapid sensitisation of TRPV1. Together with our antagonist data, we may also suggest that conformational changes induced by binding at TLR4 can affect TRPV1 function if a protein-protein interaction is present.

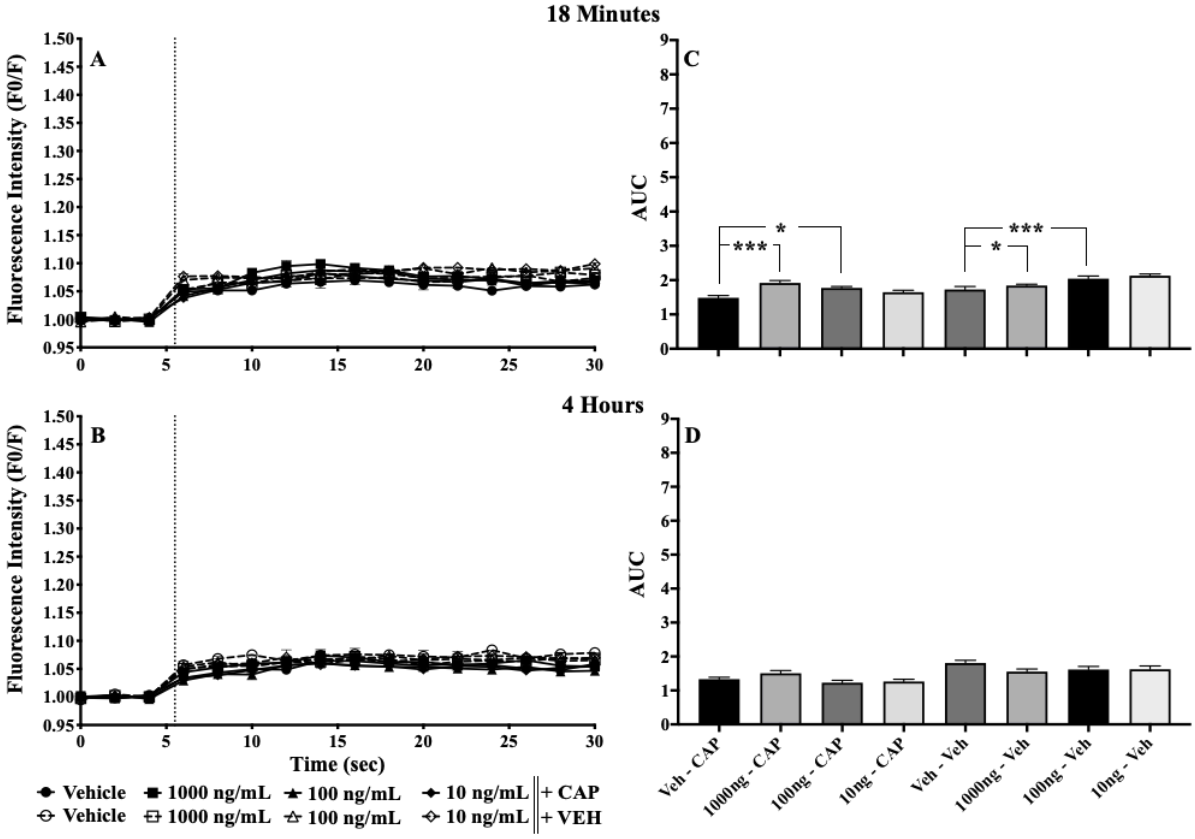
Potentiation of capsaicin responses caused by longer term LPS stimulation have been observed in rat *ex vivo* and behavioural studies (5 hr LPS treatment) as well as clinical studies (3.5 hr LPS) (Hua et al., 1996; Hutchinson et al., 2013; Kemper et al., 1998). Interestingly a clinical endotoxin-capsaicin study observed potentiation at 3.5 h but not at earlier time points (2 h) using a low dose of endotoxin (Hutchinson et al., 2013). In contrast animal studies required a much higher LPS concentration to observe a potentiated capsaicin response (Hua et al., 1996; Kemper et al., 1998). Nevertheless, in both cases the changes were associated with inflammatory cytokines. In humans, the timing of potentiation correlated with peak IL-6, while blockade of cyclooxygenase-2 (COX-2) and IL-1β activity reversed potentiation in *ex vivo* rat trachea (Hua et al., 1996; Hutchinson et al., 2013). Our *in vitro* studies were unable to replicate potentiation over a range of LPS concentrations despite producing a detectable inflammatory response (Fig. 3.1, 4). Despite the IL-8 increase we observed in our overexpression system, perhaps the gamut of inflammatory mediators known to sensitise TRPV1 were not present in our homogenous single cell culture system. While verifying the function of TLR4 in our TLR4/TRPV1 overexpression system, TRPV1 antagonist capsazepine reduced IL-8 output. TRPV1 antagonism with AMG9810 has been shown to decrease TLR2 and TLR4 expression in rat stroke models suggesting TLR4/TRPV1 relationship is not one-way, potentially explaining our result (Hakimizadeh et al., 2017). Like TLR4's impact on TRPV1, the potential impact of TRPV1 on TLR4 function requires further investigation.

LPS caused significant increases in calcium accumulation past 20 seconds in vehicle treated cells after an 18 min pre-treatment at 1000ng/mL, a result also observed following LPS-RS pre-treatment (Fig. 3.3, 4). While not significant, the same situation is observed after 4 h of LPS treatment (Fig. 3.4). Increase in baseline calcium levels, a phenomena previously observed in microglia could potentially explain enhanced neuronal excitability following LPS treatment (Hoffmann et al., 2003; Tzour et al., 2017). Multiple studies suggest LPS alone is capable of exciting nociceptive neurons, increasing action potential generation following long and short term exposure (Diogenes et al., 2011; Ochoa-Cortes et al., 2010; Tzour et al., 2017). Interestingly the same effects were not observed in cells not co-expressing TRPV1 (Sup. Fig. 3.1). Similar results have been observed in nodose and trigeminal ganglion neurons, where calcium influx following LPS treatment are independent of TLR4 expression and dependant on expression of ion channel TRPA1

(Meseguer et al., 2014). These increases potentially explain the increased calcium accumulation observed following capsaicin treatment. However, increased calcium accumulation in LPS-vehicle treated cells did not correlate with potentiated responses in LPS-capsaicin treated cells following 4 h pre-treatment. Further investigation is required to ascertain if these effects are mediated by TRPV1, TLR4 or both in combination.

In the present study we have shown cells expressing TLR4, MD-2 and CD14 have altered TRPV1 mediated calcium influx dynamic following stimulation with capsaicin. Antagonist investigation reveals the effect is unlikely to be connected to TLR4 signalling. We were able to observe a potentiated capsaicin induced calcium accumulation following short term administration of LPS at 1000 and 100 ng/mL but not following a longer-term administration as previously observed in primary cell lines. More investigation into a potential physical interaction between the receptors is required to fully understand this mechanism. Both a potential physical interaction and secondary effect of TLR4 signalling on TRPV1 function play a role in neuronal hypersensitivity, and further insight could improve our understanding of crucial elements underlying pain hypersensitivities.

3.6 Supplementary figures



Supplementary Figure 3.1: **Endotoxin effects on intracellular calcium in TLR4 expressing HEK293FT cells are observed following short, but not long term incubations.** Intensity changes induced by capsaicin (500 nM) pre-treated with LPS for 18 min (A) or 4 h (B). AUC of intensity plot for 18 min (C) and 4 h (D) pre-treatment. n = 4 for all groups. Error bars represent SEM, dotted vertical line represents time of capsaicin injection. * = $p < 0.05$, *** = $p < 0.001$.

The next section of this thesis will relate to our second aim and focus on the generation of a preclinical endotoxin-capsaicin model. The first chapter confirmed a functional relationship between the two targeted receptors. Taking into account chapter 3 and previous studies we will look to incorporate a second, shorter time point. We begin the development of an endotoxin-capsaicin model by delivering a literature review analysing the use of capsaicin in preclinical animal models utilising behavioural assessment. The review began as a means of understanding the best approach to back-translate the clinical model into a rodent model. As a consequence, we have been able to analyse the capsaicin literature, providing a valuable resource for future researchers utilising capsaicin-induced behavioural pain assessment.

Chapter 4. Review: The use of capsaicin as a pro-nociceptive stimulus in animal pain models utilising behavioural assessment

4.1 Abstract

Animal models of pain have been instrumental in advancing our understanding of pain mechanisms. However, translating this understanding into positive clinical outcomes has been lacking, with pain remaining a significant worldwide burden. As a result, researchers are having to re-evaluate their use of animal models in pain research, aiming to improve clinical relevance and improve therapeutic options. One commonly used model of pain is the application of capsaicin, the pungent ingredient of chilli peppers. Capsaicin directly activates neurons by activating transient receptor potential cation channel subfamily V member 1 (TRPV1), resulting in burning pain and sensitivity to numerous stimuli. This review analyses capsaicin use as a pro-nociceptive stimulus in pain literature, reporting on its current and historical use. We report species and sex used, routes of administration, associated behavioural testing, range of doses and duration of nociceptive hypersensitivity induced. We then discuss potential improvements on the use of capsaicin in the context of the broader pain literature with the aim of helping to inform the decisions of future research utilising capsaicin-induced pain in animal models.

4.2 Introduction

Capsaicin is an alkaloid recognisable as the main pungent ingredient of chilli peppers (Szallasi et al., 1999). It binds transient receptor potential cation channel subfamily V member 1 (TRPV1), a heat sensitive non-selective cation channel (Caterina et al., 1997; Szallasi et al., 2007). TRPV1 is found on multiple tissues types, including small to medium primary afferent neurons known as nociceptors, responsible for the transmission of noxious stimuli to produce the sensation of pain (Brito et al., 2014; Szallasi et al., 2007). TRPV1 is also activated by protons (low pH), resiniferatoxin, and various endogenous ligands including anandamide (Szallasi et al., 2007). Following activation of the receptor, cation influx results in action potential generation and nociceptive firing in the peripheral nervous system (PNS), ultimately leading to generation of pain sensation in higher brain regions (Brito et al., 2014; Sutherland, 2014). This makes capsaicin an excellent compound for the study of basic mechanistic questions around nociception both *in vitro* and *in vivo*.

In mammals, application of capsaicin leads to a sensation of burning, followed by a period of increased sensitivity (sensitisation), and finally a loss of sensation known as desensitisation/analgesia (Sawynok, 2005). Desensitisation is likely the result of one or multiple of the following factors; a depletion of neurotransmitters (e.g. substance P), calcium-induced receptor desensitisation or calcium-induced neuronal ablation (Caterina et al., 2001). As a result, capsaicin has multiple practical applications ranging from self-defence (pepper spray) to therapeutic agents targeting pain (Szallasi et al., 1999; Szallasi et al., 2007). Capsaicin has been used as an analgesic for over 100 years, currently a number of topical patches are available to treat chronic pain conditions including post herpetic neuralgia (PHN), diabetic neuropathy, arthritis and post-surgical pain (Baranidharan et al., 2013; Szallasi et al., 2007).

The dose, application method and age of the subject all contribute to the effects of capsaicin; single, low doses are able to produce nociceptor sensitisation without long term desensitising effects (Holzer, 1991). Sensitisation of TRPV1 produces nociceptor sensitivity to a number of mechanical and thermal (hot and cold) stimuli (Szallasi et al., 1999). In clinical studies this sensitisation is referred to as either hyperalgesia, an exaggerated painful response to noxious stimuli, or allodynia, a painful response to a previously non-noxious stimulus (Ji et al., 2003; Milligan et al., 2009). Capsaicin is able to cause sensitisation to areas surrounding the application site, known as secondary

hyperalgesia/allodynia as well as at further sites, known as referred hyperalgesia/allodynia (Kinnman et al., 1995; Laird et al., 2000; Willis, 2002). Mice lacking TRPV1 do not develop thermal hyperalgesia following capsaicin administration, highlighting the importance of the receptor to this sensitisation event (Caterina et al., 2000). The processes by which capsaicin is able to cause sensitisation to secondary areas of tissue are known as peripheral and central sensitisation (Carlton et al., 2001; Dai et al., 2002).

Peripheral sensitisation is a term given to multiple events on or within neurons of the PNS which result in lowered activation threshold and therefore increased chance of firing (Grace et al., 2014). Likewise, central sensitisation results in increased neuronal excitability, but refers to neurons within the central nervous system (CNS) (Woolf et al., 2000). These processes are of particular interest to the field of chronic pain, a disease state affecting 1 in 5 adults worldwide and costing billions of dollars annually (Goldberg et al., 2011; Loeser, 2012). Allodynia, hyperalgesia and spontaneous pain are major symptoms of many chronic pain types, as such, capsaicin is an interesting option for mechanistic investigations (Mogil, 2009). Further to symptomatic similarities, TRPV1 is upregulated in multiple preclinical animal models of chronic pain (Rashid et al., 2003a; Rashid et al., 2003b).

Capsaicin pain models have been described as potential surrogates for models of neuropathic pain due to the production of secondary hyperalgesia comparable to nerve ligation models, which are both attenuated by the same opioid combinations (Joshi et al., 2006). Beneficially, capsaicin models are able to reduce time and cost to the experimenter and reduce the amount of pain animals endure compared to nerve ligation models. However, the relevance of animal models which look to emulate human disease is being debated due to the poor translation of results for new therapeutic agents. Numerous examples exist of positive preclinical results not translating into successful therapeutic agents, including neurokinin-1 (NK1) receptor antagonists, sodium channel and gap junction blockers and microglial attenuators (Goadsby et al., 2009; Hill, 2000; Landry et al., 2012; Wallace et al., 2002).

Successful examples of translation from preclinical to clinical results exist but are less common than the failures. N-type voltage sensitive sodium channel blocker (ziconotide) showed promise in rat pain models, which translated into clinical trials and to Food and

Drug Administration (FDA) and European Medicines Agency (EMEA) approval. However, side effects limit use to individuals experiencing severe pain (Bowersox et al., 1996; Schmidtko et al., 2010). To a lesser extent aforementioned capsaicin patches are another example, however low compliance rates due to multiple painful applications limit their success (Baranidharan et al., 2013). Despite these positive outcomes of animal model use, there is an obvious need to improve use of animals in respect to finding improved clinical therapeutic options.

Behaviour assessed is a crucial element of preclinical models; one potential reason for the lack of success in preclinical translation is the use of irrelevant behavioural measures (Mogil, 2009). Researchers can observe both stimulus-evoked (elicited) or stimulus independent (spontaneous) responses which represent mechanical and thermal hyperalgesia/allodynia and spontaneous pain observed in pain patients, making these measures seemingly relevant (Vierck et al., 2008). Sufferers of chronic pain report sensitivity to thermal and mechanical sensitivities. However, the most prevalent symptom is spontaneous pain (Backonja et al., 2004). It is important that researchers consider models that mimic these phenotypes in order to measure relevant outcomes. Likewise, species selection, dose and location of stimulus application can affect the timing, intensity and location of pain (Gregory et al., 2013; Henze et al., 2010; Holzer, 1991). Therefore, multiple factors need to be considered when assessing the validity of a pain model to a particular condition.

Capsaicin is a valuable tool in which to study pain due to both the pain response it induces and the importance of TRPV1 to pain mechanisms. Given the relative efficacy of current therapeutics utilising capsaicin, TRPV1 also remains a viable target in pain medicine. To improve the clinical relevance of capsaicin research we must understand how capsaicin is currently used. This review will explore the use of preclinical capsaicin pain models and discuss the relevance of current approaches and potential improvements. We will focus on capsaicin use intended to provoke an observable nociceptive response, not intended for analgesia, desensitisation, denervation or treatment in alternative pain models.

4.3 Methods

4.3.1 Research strategy

The PRISMA statement was used to prepare the following systematic review. Literature searches of PubMed and Embase databases were used to identify all eligible studies. Search terms included "pain," "capsaicin", "behaviour" and related words. Papers used were published in English up until February 2018, these were the only limits of the search. The exact search string can be found in Table 4.1. Additional eligible studies were identified after manually searching the reference lists of included articles.

4.3.2 Systematic review publication selection criteria

To determine eligibility; titles, abstracts and/or full text were screened. Studies were eligible for inclusion if they were an original study which used a preclinical *in vivo* model to assess behaviour following application of capsaicin. Studies were excluded if they (i) did not include a vehicle control group in the behavioural assessment, (ii) used capsaicin for the purpose of denervation/desensitisation/analgesia; (iii) the behaviours observed were not related to pain or pain related behaviour (i.e. drinking).

4.3.3 Systematic review publication data extraction

The following was extracted from each included study: (i) study characteristics (first author, year of publication); (ii) animals used (species, sex, strain), (iii) route of capsaicin administration (including anatomical location); (iii) dose of capsaicin used, (iv) behavioural test used (including testing time and duration of observed sensitivity post capsaicin application), (v) purpose of study (hypothesis/stated aim) and (vi) the role of capsaicin in study design. Behavioural consequences of capsaicin administration were assessed.

Table 4.1. Search terms

Pubmed	
	capsaicin
AND	allodynia OR hyperalgesia OR assessment, pain[MeSH Terms] OR animal behavior[MeSH Terms]
AND	animal model[MeSH Terms] OR mice OR rat OR primate

Embase	
	capsaicin
AND	allodynia OR hyperalgesia
AND	behaviour OR pain assessment OR von Frey test OR Hargreaves OR licking OR bite
AND	animal model OR mouse OR rat OR primate

4.4 Results

4.4.1 Systematic review search outcomes

The screening process is described in Figure 4.1. The initial search yielded 540 results, 73 of which were duplicates and immediately discarded. Upon examination of abstracts a further 148 were excluded for not meeting entry criteria, including, studies that were not preclinical, reported no vehicle control in capsaicin experiments, used alternative TRPV1 agonists, included no behavioural assessment following capsaicin and were not primary research articles. The remaining 319 articles underwent full text review. 85 additional articles were identified based on references while a further 96 were excluded. The majority of exclusions were due to primary intentions of capsaicin either being denervation/desensitisation or as an analgesic intervention rather than to induce a pro-nociceptive response. Articles were also excluded for analysing non-pain behaviours (i.e. drinking), insufficient reporting and not being primary research articles. 309 articles remained and underwent further analysis.

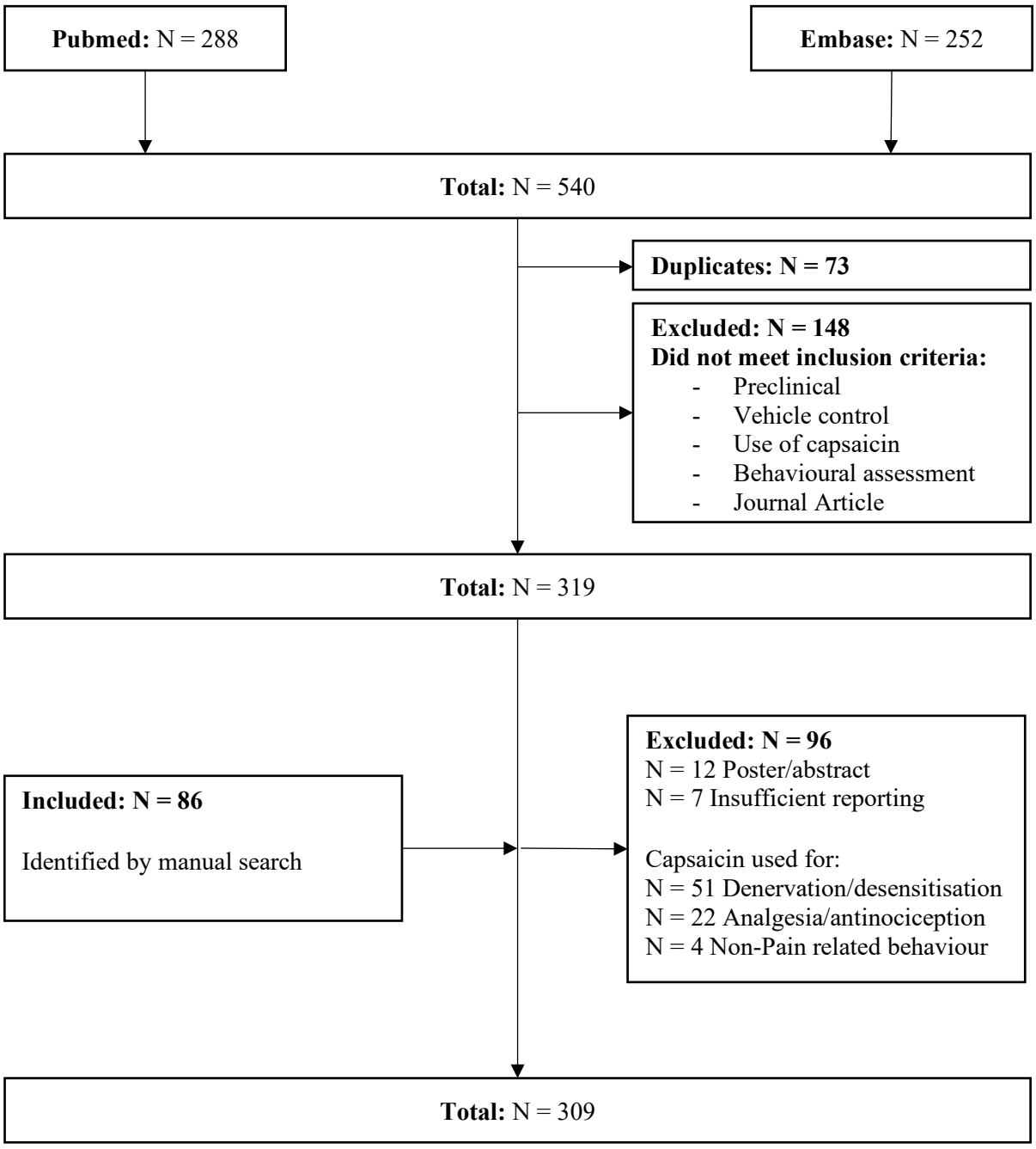


Figure 4.1. Flow diagram outlining study selection process.

4.4.2 Paper characteristics

Use of capsaicin in preclinical models to induce nociceptive behaviours increased in the latter half of the 1990's and has remained a constant research interest (Fig. 4.2A). Administration, observations and pain testing are primarily focused at peripheral sites (90.3%), the remaining minority explore visceral sites (9.7%). Rodent species are the most common animal model used, accounting for 93.2% of all identified studies, followed by primates of the genus *Macaca* (*mulatta* & *fuscata)* which account for 6.1% (Fig. 4.2D). Two studies in pigs were also identified. It should be noted the use of primates has reduced with only one incidence in the 5 years prior to this review, while porcine use is relatively recent, the first paper published in 2011 (Fig. 4.2B). Interestingly, there has been a shift in the last decade towards the use of mice compared to rats (Fig. 4.2B). Sex is unevenly distributed amongst studies using rodents with 18% and 10% of mouse and rat studies respectively including females. This discrepancy is not present in the smaller number of porcine and primate studies. The relative proportion of male and females used in rodent studies has remained constant for the past two decades (Fig. 4.2C).

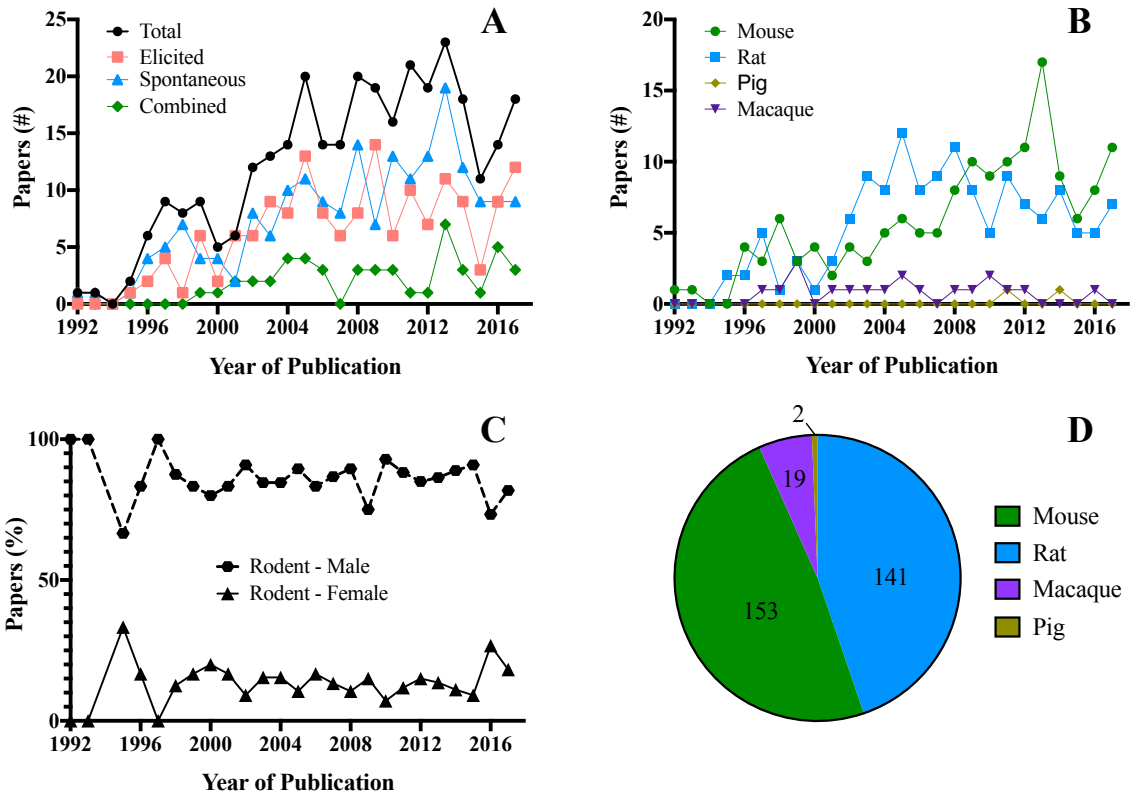


Figure 4.2. Study characteristics. Total number of papers by year of publication (A), behaviours assessed (A), species (B) and sex (C). (D) Species used by total number of papers.

4.4.3 Behaviour assessed

Following capsaicin administration, both the direct impact (spontaneous behaviour) and longer lasting sensitisation are assessed. 48% of studies assessed spontaneous behaviours only, 36% assessed elicited behaviours only and 16% assessed both (Fig. 4.3A).

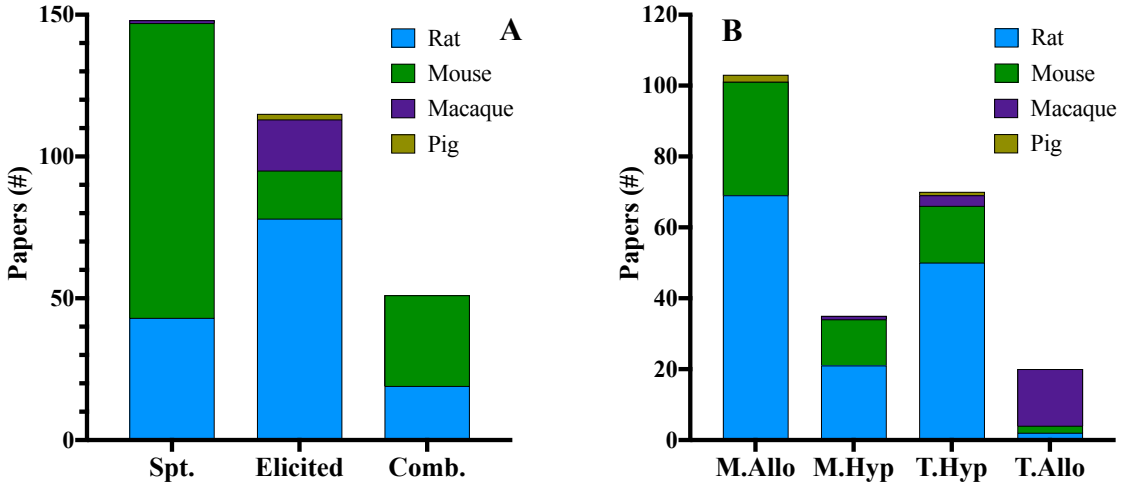


Figure 4.3. Study characteristics. Total number of papers by behaviours assessed (A), and type of sensitivity assessed (B).

4.4.3.1 Spontaneous pain

Mice are the most common species used to identify spontaneous behaviours following capsaicin administration (68.7%) followed by rats (30.8%) and primates (0.5%) (Fig. 4.3A). 32 different tests were used to quantify spontaneous behaviours (Table 4.2). Five techniques require subjective pain scale evaluation, the remaining quantify behaviours by number or duration of behaviours, or distance travelled. The most common behaviours assessed include movements of the hind paw including licking, flinching, biting, retracting, lifting, wiping and grooming (Table 4.2). Paw licking was the assessment first used in 1992 and remains the most common spontaneous measure following capsaicin (Fig. 4.4A). Assessment of spontaneous behaviours for visceral pain (squashing, retracting, licking, stretching) originated in 2000 and remain in use and unchanged to the present day (Fig. 4.4A). No new techniques for spontaneous behavioural assessment have been widely adopted in the last decade (Fig. 4.4A). Visceral pain behaviours hunching (2010) and abdominal contraction (2014), as well as peripheral pain behaviours, withdrawal latency

(2010) and flicking (2013) are the most recent to be introduced to the field; none of which have been published more than twice. It is clear that mice are favoured in studies assessing spontaneous behaviours, and that historical measures of spontaneous pain dominate modern capsaicin-pain research.

4.4.3.2 Elicited pain

Rats constitute the most utilized animal to study elicited pain following capsaicin (58.2%), followed by mice (29.7%), primates (10.9%) and pigs (1.2%). 20 different behavioural tests have been used to evaluate elicited pain responses (Table 4.2). Mechanical stimulation is used more often (61%) than thermal (hot or cold) (39%). Allodynia is more commonly reported after mechanical stimulation (75%) compared with hyperalgesia (25%). In contrast, hyperalgesia is more commonly reported following thermal challenge (77%) than allodynia (23%). Primates account for 84.2% of papers reporting thermal allodynia, and 84.2% of papers utilising primates investigate thermal allodynia via water immersion withdrawal latency. Commonly used techniques remain those first published before 2003 (Fig. 4.4B). The von Frey test was the first elicited pain test used following capsaicin (1995) and remains the most utilised, comprising 48.5% of all tests used and 43.6% of tests used over the past decade. Multiple von Frey techniques have been used, 'best of' techniques arose in the capsaicin literature in 1995 and account for 29.5% of von Frey analysis. Von Frey methods assessing mechanical threshold were introduced in 1997 and account for 64.3% of von Frey analysis. 'Up Down' threshold methods are frequently used in this analysis, Chaplan et al. 1994 'up down' method is frequently cited, a new method (SUDO) was introduced in 2014 and has been published three times in the capsaicin literature. Recently introduced von Frey analysis methods include withdrawal latency and pain score, which are found in 5 and 2 articles respectively. Air puff (2016), cold plate (2013), colorectal distension (2015), dynamic hot/cold plate (2009), thermal gradient response (2017), optogenetic stimulus positioning (2017), pinprick (2009), sticky tape (2013), mechanical threshold versus reward (2011), withdrawal latency to cold (2014) and grip force (2013) tests have been introduced in the last decade. These techniques have only been published once, with exception of the pinprick and mechanical stimulus versus reward tests (twice). Therefore, in contrast to spontaneous behaviours, rats are most often utilised to analyse elicited behaviours, and there is less reliance on rodents overall. However, like spontaneous behaviours, novel behavioural assessments have not been widely adopted and historical approaches remain the most commonly utilised. The

reviewer considers any threshold measurement as testing for allodynia, likewise the use of innocuous stimuli. As a result, allodynia was recorded for tests which met these criteria regardless of what the authors reported. Inconsistency of behavioural reporting is discussed in section 4.4.3.3.

Table 4.2: Behavioural tests used to classify responses to capsaicin administration in preclinical literature.

Spontaneous Behaviour	Incidence (#)	Elicited Test	Incidence (#)
Activity Score	4	**Acetone**	1
Abdominal Pain Score	1	**Air Puff**	1
Blinking	4	**Cotton Bud**	1
Biting	20	**Cold Plate**	1
Eyes Closed	2	**Colorectal Distension**	1
Contractions	2	**Dynamic Cold Plate**	1
Distance Travelled	2	**Dynamic Hot plate**	1
Escape Behaviour	2	**Hargreaves**	37
Eye Movement Score	2	**Hot Plate**	10
Exploring	2	**Movement (thermal stimuli)**	1
Flicking	1	**Position (optogenetics)**	1
Flinching	28	**Pin Prick**	2
Grooming	11	**Sticky Tape**	1
Guarding	5	**von Frey**	99
Hump-backed	1	**With. Lat. (radiant heat)**	5
Hunching	1	**With. Lat. (rad. heat vs rew.)**	3
Immobilisation	2	**With. Lat. (mechanical)**	2
Lifting	13	**With. Lat. (water immers.)**	27
Licking	146	**With. Thre. (mechanical)**	7
Locomotion Score	3	**With. Thre. (mech. vs rew.)**	2
Pain Scale	2		
Rubbing	10		
Retracting	17		
Rearing	1		
Rest	3		
Shaking	12		
Squinting	1		
Squashing	15		
Scratching	10		
With. Lat.	1		
Wiping	14		

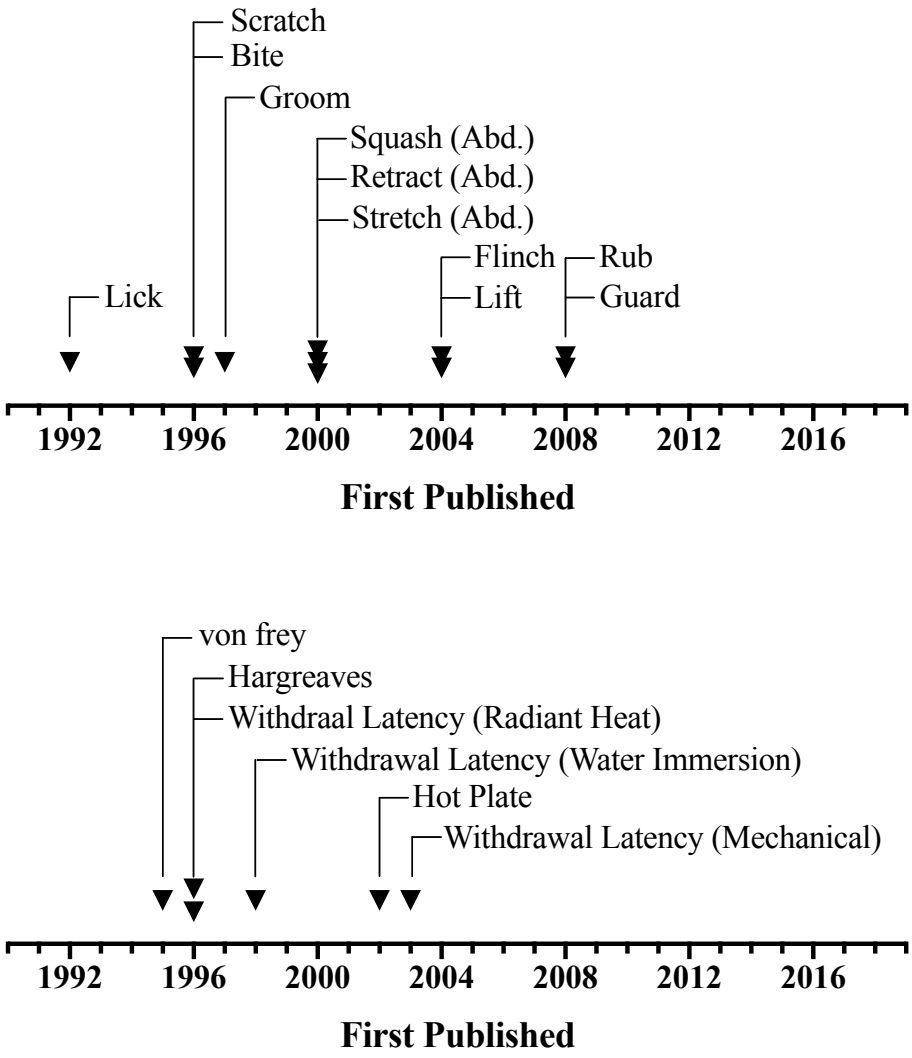


Figure 4.4. First reported use of the most commonly (>10 studies) recorded spontaneous behaviours (A) and elicited pain tests (B) following capsaicin in preclinical literature.

4.4.3.3 Reporting consistency

Reporting of allodynia or hyperalgesia was inconsistent across the articles reviewed. Mechanical threshold showed the greatest reporting inconsistency. Mechanical allodynia is reported in 47% of threshold testing with 53% reporting mechanical hyperalgesia. Interestingly, of the papers that reported hyperalgesia, 3 also referred to allodynia in the introduction and/or concluding remarks. Similarly, there are 3 articles which refer to thermal hyperalgesia and allodynia when only using innocuous heat stimuli. Reporting of von Frey data also displayed inconsistency. Twenty-three articles using a 'best of' von

Frey approach report hyperalgesia and allodynia when applying a noxious or innocuous stimulus respectively (innocuous stimulus for von Frey is considered a fibre that elicits lower than one response per trial). In contrast, nine articles referred to hyperalgesia due to increased response rate when testing both innocuous and noxious filaments, with one example using only noxious stimuli while reporting allodynia. It should be noted the last occurrence of such an inconsistency was 2005. Four articles use the terms allodynia and hyperalgesia interchangeably throughout the text, one of which only referring to a single stimulus (cotton bud wiping). Ten articles, 9 of which were published post 2010, did not classify elicited responses as either allodynia or hyperalgesia, but rather referred to 'hypersensitivity', 'sensitivity', 'withdrawal threshold' or 'reactivity.'

4.4.4 Capsaicin administration

4.4.4.1 Administration route and location

Route of administration is distinct for peripheral and visceral pain (Table 4.3). Intra-colonic administration is the most common method of delivery for visceral pain assessment accounting for 86.7 and 87% of elicited and spontaneous observations respectively, primarily used in mouse models, with three incidences in rats. The intra-colonic method is the only technique which is utilised to investigate referred (visceral) elicited pain using von Frey stimulation of the abdomen. One study utilised intraperitoneal administration to induce visceral pain, while two others inject the prostate to investigate prostate-specific pain, all of which observe spontaneous behaviours only, using unique pain scores (abdominal and eye movement scores respectively).

Peripheral application is more varied. The dominant method is intraplantar, used in 185 studies (Table 4.3). Intraplantar administration has been utilised in both rat and mouse models. Mice usage is dominant in spontaneous observations accounting for 77.2%, which differs from elicited studies, 31.8% of which use mice (Table 4.4). Additionally, hind paw administration is also achieved via subcutaneous (dorsal aspect) and topical routes, accounting for 32% and 15.8% of subcutaneous and topical studies respectively. Subcutaneous application is the second most prevalent peripheral administration route, accounting for 51 studies. In studies investigating elicited sensitivities, the tail is the most common location accounting for 59%, due to this being the dominant method of delivery in primates. Hind-paw and vibrissae pad are the most common subcutaneous administration sites for studies examining spontaneous behaviour. Topical application

accounts for 19 studies, all of which investigate elicited behaviours; with the cheek, tail and hind paw being used in multiple studies (Table 4.4). Intraocular administration is the only other method present in greater than 10 studies, used to observe spontaneous blinking almost exclusively in rats (one study in primates) (Table 4.3, 4). Despite a wide array of administration routes and sites it is evident that intraplantar and intra-colonic administration are overwhelmingly used for peripheral and visceral pain respectively.

Table 4.3: Frequency of various capsaicin administration routes in experiments observing spontaneous or elicited responses.

Peripheral Pain		
Administration (total papers)	**Elicited (%)**	**Spontaneous (%)**
Intra-Articular (2)	1.3	-
Intracranial (3)	0.7	1.1
Intra-Dental (2)	-	1.1
Intradermal (7)	2.0	3.9
Intramuscular (6)	4.0	1.1
Intraocular (11)	-	6.1
Intra-Oral (1)	-	0.6
Intraplantar (185)	60.7	68.3
Intra-Prostatic (2)	-	1.1
Intrathecal (6)	0.7	3.3
Subcutaneous (51)	18.0	14.4
Topical (19)	12.7	-
Visceral Pain		
Intra-Colonic (22)	86.7	87.0
Intraperitoneal (2)	13.3	4.3
Intra-Prostatic (2)	-	8.7

Table 4.4: Location of capsaicin administration by species and behaviour assessed. Three marks indicates species used > 50% of studies, two marks indicates species used < 50% of studies, one mark indicates only a single study uses that location in a particular species.

	Spontaneous				Elicited			
Location	**Rat**	**Mouse**	**Mac.**	**Pig**	**Rat**	**Mouse**	**Mac.**	**Pig**
Intra-Articular								
Ankle	-	-	-	-	✓✓✓	-	-	-
Intra-Colonic	✓✓	✓✓✓	-	-	✓✓	✓✓✓	-	-
Intracranial								
Cisterna Magna	-	-	-	-	✓✓✓	-	-	-
I.VL. PAG	-	-	-	-	✓	-	-	-
Intra-Dental								
Crown Cavity	✓✓✓	-	-	-	-	-	-	-
Intradermal								
Calf	✓	-	-	-	✓	-	-	-
Cheek	-	✓✓✓	-	-	-	-	-	-
Neck	-	✓	-	-	-	-	-	-
Tail	-	-	-	-	-	-	-	✓
Vibrissae Pad	✓	-	-	-	-	-	✓	-
Intramuscular								
Masseter	✓✓✓	-	-	-	✓✓✓	-	-	-
Plantar	-	-	-	-	✓	-	-	-
Intraocular								
Cornea	✓✓✓	-	✓	-	-	-	-	-
Intra-Oral								
Tongue	✓	-	-	-	-	-	-	-
Intraperitoneal	✓	-	-	-	✓	✓	-	-
Intraplantar	✓✓	✓✓✓	-	-	✓✓✓	✓✓	-	-
Intra-Prostatic	✓✓✓	-	-	-	-	-	-	-
Intrathecal	✓✓	✓✓	-	-	✓	-	-	-
Subcutaneous								
Cheek	-	✓✓✓	-	-	✓✓✓	-	-	-
Flank	-	-	-	-	-	✓	-	-
Hind Paw	✓✓	✓✓✓	-	-	✓✓✓	-	-	-
Lip	✓	✓	-	-	✓	-	-	-
Tail	-	-	-	-	✓✓	✓	✓✓✓	-
Upper Limb	-	✓	-	-	-	-	-	-
Vibrissae Pad	✓✓✓	✓	-	-	✓✓✓	✓	-	-
Topical								
Cheek	-	-	-	-	✓✓✓	✓	✓	-
Ear	-	-	-	-	✓	-	-	-
Flank	-	-	-	-	-	-	-	✓
Hind Paw	-	-	-	-	✓✓✓	✓✓	✓	-
Tail	-	-	-	-	-	-	✓✓✓	-

* Mac. = Macaque

4.4.4.2 Dose and sensitivity

Delivery method, species and associated anatomic location are an important contributor to dose. Administration to sensitive highly innervated tissues (e.g intrathecal, intraocular and intracranial) show lower doses most commonly in the nanogram range (Fig. 4.5). Larger tissues including viscera are able to tolerate larger doses, as seen in the case of intra-colonic, intraperitoneal, intramuscular and topical capsaicin application with doses commonly between 100 - 200 micrograms (Fig. 4.5). Well established doses in the capsaicin literature include intraplantar doses in both mice (1.6 μg) and rats (10 μg) having been used in 51 and 28 studies respectively (Table 4.5). This dose is also often used in subcutaneous administration of the dorsal hind paw in mice. Similarly, in Macaques, subcutaneous tail administration dose is well established with 10 studies – 63% - using 10 μg of capsaicin (Table 4.5). Reporting of topical doses is difficult to quantify as application of specific volumes is not often stated, instead 'applied liberally' is commonly used. Duration of reporting following capsaicin administration is highly variable. Well established timelines are intra-colonic capsaicin in mice and subcutaneous capsaicin in Macaques, at 20 and 15 min respectively (Table 4.5).

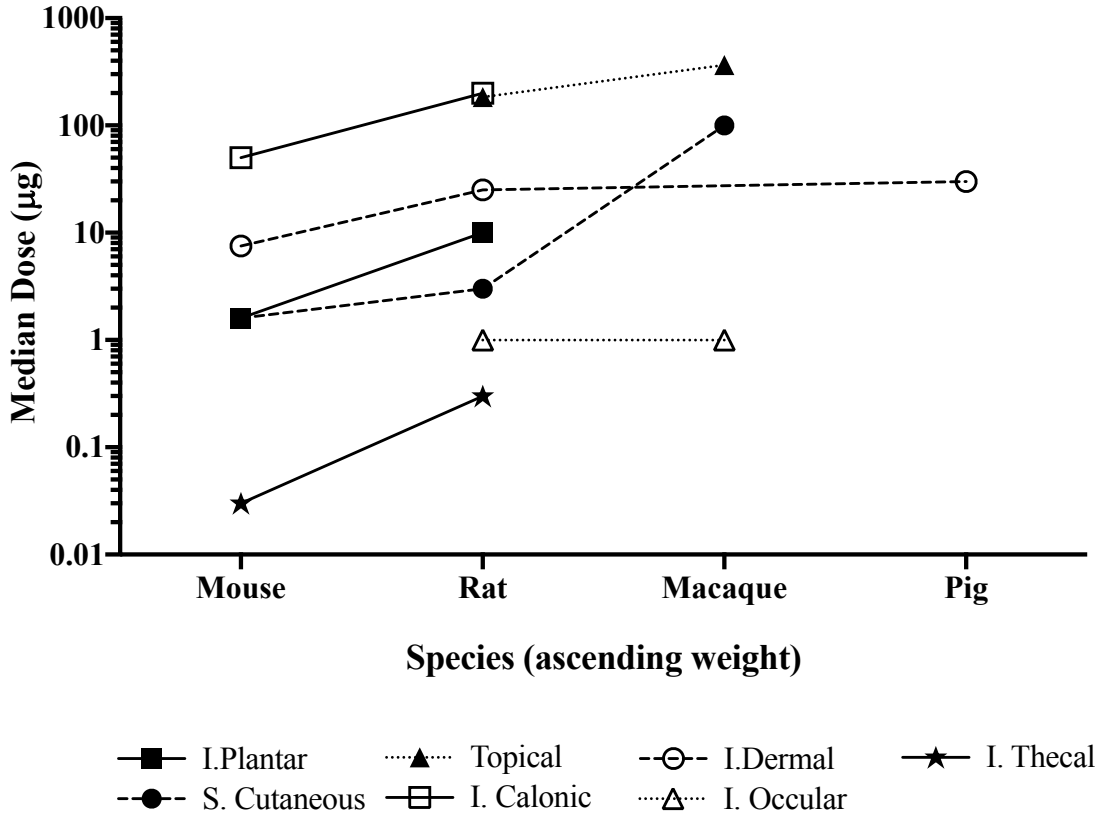
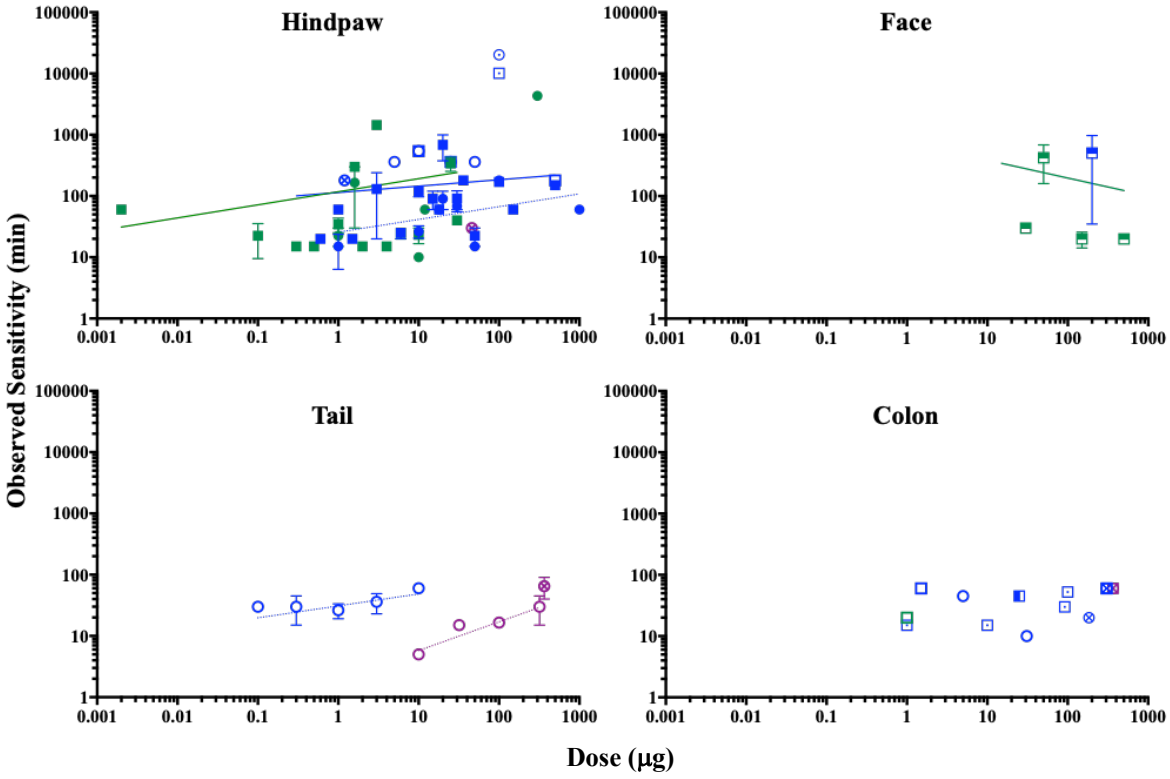


Figure 4.5. Median dose administered for route of administration across multiple species.

Table 4.5: Range of doses and median observation/testing times reported in each species.

Administration (incidence)	Dose (μg) - (incidence)			Median Observation/Test Duration (min) – (incidence)	
	Minimum	Median	Maximum	Spontaneous	Elicited
Rat					
Intra-Articular (2)	100 (2)	100 (2)	100 (2)	N/A	20220 (0)
Intracranial (6)	3.2 E-4 (1)	0.032 (1)	1.8 (1)	6.5 (0)	60 (1)
Intra-Colonic (3)	200 (3)	200 (3)	200 (3)	20 (2)	50 (1)
Intra-Dental (5)	3 (1)	100 (2)	150 (1)	60 (2)	N/A
Intradermal (1)	25 (1)	25 (1)	25 (1)	15 (1)	60 (1)
Intramuscular (8)	1 (1)	100 (5)	100 (5)	3.5 (0)	90 (3)
Intraocular (9)	0.001 (2)	1 (4)	4 (2)	5 (4)	N/A
Intra-Oral (6)	0.001 (1)	0.55 (0)	100 (1)	10 (1)	N/A
Intraperitoneal (2)	750 (1)	875 (0)	1000 (1)	30 (1)	60 (1)
Intraplantar (87)	0.3 (1)	10 (28)	1500 (1)	11 (0)	120 (12)
Intra-Prostatic (4)	0.3 (1)	27.5 (0)	305 (1)	30 (2)	N/A
Intrathecal (9)	0.003 (1)	0.3 (1)	6 (1)	5 (1)	N/A
Subcutaneous (37)	0.1 (1)	3 (5)	500 (2)	20 (2)	60 (3)
Topical (3)	1.2 (1)	184 (1)	305 (1)	N/A	30 (2)
Mouse					
Intra-Colonic (24)	7.5 (1)	50 (11)	500 (3)	20 (13)	20 (6)
Intradermal (10)	0.15 (1)	7.5 (0)	40 (1)	30 (2)	120 (1)
Intraperitoneal (2)	25 (1)	27.5 (0)	30 (1)	N/A	180 (1)
Intraplantar (124)	2 E-6 (1)	1.6 (52)	300 (1)	5 (66)	62.5 (0)
Intrathecal (5)	0.008 (1)	0.03 (1)	0.06 (2)	10 (1)	N/A
Subcutaneous (27)	1.56 E-3 (1)	1.6 (11)	50 (2)	5 (11)	105 (0)
Macaque					
Intraocular (1)	1 (1)	1 (1)	1 (1)	N/A	N/A
Subcutaneous (16)	10 (2)	100 (10)	320 (1)	N/A	15 (6)
Topical (7)	46 (1)	366 (4)	366 (4)	N/A	75 (0)
Pig					
Intradermal (1)	30 (1)	30 (1)	30 (1)	N/A	240 (1)

There is no strong correlation in the literature between increasing dose and duration of elicited sensitivity. Groups containing greater than 10 studies were analysed for dose - duration correlations (Fig. 4.6). Mechanical stimulation in mice and rats and thermal stimulation in rats following intraplantar capsaicin showed a positive correlation (0.21 (n = 29), 0.10 (n = 58) and 0.21 (n = 29) respectively) (Fig. 4.6). R squared values indicate a poor fit in each case, 0.05, 0.01 and 0.18 respectively (Fig. 4.6). A similar pattern is observed in subcutaneous tail administration in rats (n = 13) and Macaques (n = 14) with a positive slope of 0.20 and 0.47 respectively with R squared values of 0.10 and 0.40 respectively. Mice receiving intra-colonic capsaicin (n = 15) produced a weak correlation (R squared = 0.03) and negative slope -0.29 (Fig. 4.6). It is evident that dramatic increases in dose have little effect on duration of reported sensitivity. It should be noted that duration of sensitivity is often dependent on historically chosen timeframes and behavioural experiments are rarely carried out until complete reversal of hypersensitivity. For this reason, it is difficult to make concrete conclusions regarding a relationship between dose and duration of hypersensitivity and compare doses and responses across species. It is evident that researchers are using doses above those necessary to produce hypersensitivity duration suitable for their testing timeframes.



I.pl.: Rat - Mechanical
I.pl.: Rat - Thermal
S.c.: Rat - Mechanical
S.c.: Rat - Thermal
Top.: Rat - Mechanical
Top.: Rat - Thermal
I.m.: Rat - Mechanical
I.m.: Rat - Thermal
I.d.: Rat Mechanical
I.col.: Rat - Mechanical
I.pl.: Mouse - Mechanical
I.pl.:Mouse - Thermal
S.c.: Mouse - Mechanical
I.col.: Mouse - Mechanical
S.c.: Macaque - Thermal
Top.: Macaque - Mechanical
Top.: Macaque - Thermal

Figure 6. Correlation between dose and reported sensitivity due to elicited stimulation following capsaicin. Correlations included for conditions (route of administration + species + stimulus type) reported in ≥ 10 studies.

4.4.5 Sex differences

For the past two decades between 85 – 90 % of rodent studies published each year used male subjects only (Fig. 4.2C). Of all species 77% used male only subjects, 7% used female only and 12% used both; 4% of papers did not report sex. Of studies that investigated both sexes, 67% did not report sex independent results, 13% reported no sex specific effects on capsaicin-induced behaviours and 5% reported an effect of sex.

Of studies which analysed both sexes independently, six studies reported no sex difference including two using spontaneous and 6 using elicited (4 mechanical, 4 thermal) responses (Carey et al., 2016; Entrena et al., 2009; Hu et al., 2010; Lavand'homme et al., 1999; Neubert et al., 2008; Slivicki et al., 2016; White et al., 2014). Two studies show sex differences in capsaicin-induced sensitivities using both mechanical and thermal stimulation (Barrett et al., 2003; Nasir et al., 2016). Using capsaicin as a pain model to examine opioid effects, three studies report greater antinociceptive effects of mixed-opioids following capsaicin in males (Lomas et al., 2007; Lomas et al., 2008; Saloman et al., 2011). Despite the majority of papers reporting no sex effect on capsaicin-induced nociception in preclinical models, mixed results in a small sample size suggest more investigation is required. This outcome is not aided by low levels of female inclusion, and poor reporting of sex independent results in studies which include both sexes.

4.4.6 Capsaicin purpose

The majority of capsaicin use in preclinical literature aimed to investigate TRPV1 specific molecular pain mechanisms (49%). A high proportion of studies (39%) used capsaicin as a pain model for screening analgesic compounds. Development of new pain models using capsaicin accounted for 9% of all studies. Fewer studies were conducted to understand drug/treatment effect mechanism (2%) and discovery of novel TRPV1 antagonists (0.5%). One paper was identified as investigating a novel treatment mechanism - involving TRPV1 targeting adenovirus (Xiang et al., 2017).

4.5 Discussion

Since the discovery of its receptor, capsaicin has been consistently used as a direct pain activating agent eliciting an observable behavioural response (Caterina et al., 1997). Behavioural responses highlight changes influenced by multiple body systems, adding

relevance to *in vivo* and *in vitro* cellular and molecular results. Using capsaicin as a nociceptive stimulus is most commonly utilised to explore basic physiological questions, providing valuable evidence for the role of TRPV1 in pain signalling. Another significant utility for capsaicin is as a screening tool for novel therapeutic agents. The relevance of capsaicin as a model for drug screening requires further discussion, especially considering aforementioned translatability limitations of preclinical pain research.

4.5.1 Species assessed

Rodent models are overwhelmingly used in preclinical pro-nociceptive capsaicin studies involving animal behaviour (91%), in studies investigating spontaneous behaviours this rises to 99% (1 primate study being the exception). Considering the lack of successful clinical translation, and 40% of papers were investigating novel therapeutic options, it may be relevant to consider alternative models. Although infrequently, non-human primates and pigs offer ready tested options for thermal and mechanical elicited sensitivities respectively following capsaicin (Asad et al., 2016; Di Giminiani et al., 2014; Kupers et al., 1997). Larger animal species are part of the wider pain space, as well as pigs and non-human primates, dogs have been used to study acute (synovitis) and chronic inflammatory pain therapies (natural arthritis) (Brown et al., 2008; Hamilton et al., 2005; Henze et al., 2010). A sheep neuropathic pain model has also been developed, evidence the field is actively seeking to find potentially more clinically relevant models (Wilkes et al., 2012). Rodents absolutely have their place within pain research, offering excellent opportunities for mechanistic discoveries. However, novel alternatives should be investigated if screening for clinically relevant compounds is a study aim.

4.5.2 Pain type assessed

Two response types are reported following capsaicin; non-elicited (spontaneous) and elicited; the latter comprising allodynia and hyperalgesia from either mechanical or thermal stimuli. 64% of studies reported spontaneous data, while 52% reported sensitivity to elicited stimuli. In chronic pain; clinical studies report higher prevalence and intensity of non-elicited compared to elicited symptoms (Attal et al., 2008; Backonja et al., 2004). The prevalence of spontaneous behaviours in capsaicin literature is promising given what is being reported in the wider pain community. Between 2000 and 2004 only 10% of animal studies published in the journal ‘Pain’ report spontaneous behaviours, thus overlooking important mechanisms relevant to clinical pain (Mogil, 2009; Mogil et al., 2004). Further

to simply reporting on non-evoked behaviours, we must consider which are an appropriate representation of pain that may lead to improved clinical outcomes.

In the capsaicin literature 32 different spontaneous behavioural observations were reported. Licking was by far the most commonly reported, accounting for 43% of all tests, well above the next behaviour, flinching (8%) (Table 4.2). Unfortunately, despite the lack of clinical translation in pain literature in general, these historical methods remain the most commonly utilised behaviours to judge "pain" in capsaicin models (Fig. 4.4). Discovering and introducing different behavioural cues as reliable indicators of spontaneous pain into the capsaicin model are needed to improve its relevance in anti-nociceptive drug discovery. Facial grimace scores have more recently been used in models with similar duration (formalin test, intraplantar mustard oil) (Langford et al., 2010). This type of approach, observing responses not directly directed towards the application/injury site is rare in capsaicin literature. Two studies using a capsaicin-induced non-bacterial prostatitis model have used similar eye movement scores (Chuang et al., 2008; Chuang et al., 2007). The capsaicin-induced prostatitis model also reports on locomotion, another potential pain score based on automated analysis. As well as locomotion (type and distance) automated behavioural analysis can pick up on grooming and gustation behaviours that change based on pain state. Locomotion (activity) scores have been used in a single drug discovery model; reporting that novel compound AMG0347 decreases activity in the first 30 seconds following intrathecal capsaicin (Wu et al., 2008). This result helped confirm the compounds activity at TRPV1 but AMG0347 has not yet been used in further pain studies. Activity scores (movement, distance and rest time) have also been used in rat intraplantar capsaicin models exploring central pathways as well as central and peripheral kinase effects, however not in drug discovery studies (Fang et al., 2002; Palecek et al., 2002; Sun et al., 2007). Other non-evoked behaviours used in pain literature include burrowing, weight bearing and gait analysis, which offer potential alternatives for investigation in preclinical capsaicin models (Deuis et al., 2017).

Evoked responses in the capsaicin literature heavily lean on static mechanical allodynia and hyperalgesia following von Frey stimulation (49%). However, evoked hypersensitivities reported by pain sufferers occurs via both static and dynamic mechanical stimuli (i.e. clothes brushing against the skin) (Hansson, 2003; Ochoa et al., 1993). The dynamic cotton bud test has been used twice in the capsaicin literature, testing hind paw

responses following intraplantar capsaicin to examine the roles of tachykinin receptors (NK1) and cation-chloride co-transporters (NKCC1) (Laird et al., 2004; Laird et al., 2001b). Air puffs have also been utilised in a single model of orofacial pain (intradermal vibrissae application) to study the role of protein kinase C gamma (PKCγ) expressing interneurons in orofacial sensitivity (Peirs et al., 2016). Therefore, despite a much higher clinical prevalence (20-40%) only 2% of studies utilising capsaicin tested a dynamic stimulus (Hansson, 2003). Thermal sensitivities are tested in 39% of evoked behavioural studies following capsaicin (and 47% of drug screening studies using evoked responses). Although the symptom does present in clinical trials, sensitivity to heat is not reported as a common problem in daily life for neuropathic pain patients (Hansson, 2003; Maier et al., 2010; Staud et al., 2012). Interestingly, three assays tested cold hyperalgesia following capsaicin with mixed results. Dynamic cold plate following topical application of the hind paw revealed cold sensitivity based on number of jumps (Yalcin et al., 2009). However, standard cold plate tests revealed no capsaicin effect and cold probes show an increase in thresholds (Honda et al., 2014; Roberson et al., 2013).

Very few novel techniques have been introduced into the capsaicin animal model literature. Three studies introduced operant reward for measuring evoked responses, all of which were attempting to develop new models (Neubert et al., 2008; Neubert et al., 2006; Nolan et al., 2011; Rohrs et al., 2015). Movement of mice over a thermal gradient surface is a unique technique developed to study vasomotor symptoms using capsaicin, with capsaicin causing the animals to spend longer at cooler temperature compared to controls (Krull et al., 2017). It should also be noted that sensory loss, hypoalgesia, paraesthesia and dysesthesias are also symptoms of clinical pain that can be assessed using evoked behavioural tests and are not reported here (Hansson, 2003; Maier et al., 2010). Although these are rarely tested for in pain literature, they would have been screened out of our search with articles using capsaicin for desensitisation and/or analgesia (Mogil, 2009). Interestingly, operant and condition-placed preference behavioural analysis produces different results when compared to historically utilised evoked behavioural testing. This preclinical data correlates with clinical behavioural observations, suggesting these novel models should be investigated further (Clark, 2016; King et al., 2009).

Capsaicin is overwhelmingly used to elicit peripheral pain, with only 9.7% of studies exploring mechanisms of visceral pain. TRPV1 is implicated in numerous visceral pain models and importantly is implicated in clinical irritable bowel syndrome and functional dyspepsia cases (Akbar et al., 2008; Du et al., 2019; Hammer et al., 2008). The majority of studies included here explored visceral pain mechanisms and drug mechanisms associated with visceral pain, only 12% used a capsaicin model to assess novel drugs. Interestingly 92% of studies targeting visceral sites measure non-evoked behaviours involving abdominal movements, 100% of intra-colonic capsaicin models assess abdominal licking, stretching, squashing and retracting. A significant proportion (54%) of these studies report both evoked (colorectal distension, referred mechanical hyperalgesia) and non-evoked behaviours, while less than 1% report only evoked behaviours; in complete contrast to the peripheral capsaicin studies discussed above.

Lastly, it is prudent to note the inconsistencies in use of pain specific nomenclature within the studies analysed. In an attempt to make the animal models seem relevant, clinical symptoms of allodynia and hyperalgesia are used to describe behaviours in animal pain models. However, due to the inability of animals to self- report, it is difficult to define a painful versus non-painful stimulus, leading to researchers describing either allodynia or hyperalgesia employing the same behavioural methodology. We observed a growing number of publications using the terms; ‘hypersensitivity’, ‘sensitivity’ and ‘reactivity’ rather than allodynia and hyperalgesia. In either case, confusion arises from the use of multiple terms to describe the same behavioural phenomenon, and the next step is to agree upon a universally accepted nomenclature for these behaviours. The author suggests hypersensitivity is an appropriate term which would suit the studies using elicited behaviours in this review. While the terms allodynia and hyperalgesia absolutely have a place in clinical studies, it may be appropriate to find alternatives in preclinical literature to eliminate confusion caused by inconsistent reporting.

4.5.3 Dose and capsaicin administration

Both species and route of administration influence the dose used. Higher doses are typically used in larger animals to produce comparable sensitivities to those observed in smaller animals. Within the same species, higher doses are used for deeper administration (e.g. visceral, intramuscular) compared to superficial application, such as topical or intraplantar. An ethical consideration arising from this data is the use of high doses of

capsaicin to elicit pain. This data reveals no strong correlation between increasing dose and observed length of sensitivity (Fig. 4.6). The strongest correlations were following subcutaneous tail administration in primates ($R^2 = 0.47$). In this instance doses 3.5 - 10 times greater were used to elicit sensitivity lasting only an extra 15 min (Fig. 4.6). This is observed in many routes of application and it may be useful to consider when making decisions regarding refinement of methods. There were notable exceptions, two studies using intraplantar capsaicin in mice reported sensitivity at 24 and 72 h post administration, well beyond anything else for the comparable species/administration combination (Chen et al., 2009; La et al., 2017).

Larger doses of capsaicin are used to cause denervation and analgesia in adult animal studies. A single subcutaneous application can be between 10 and 1000 times greater than the median dose observed here, with multiple applications (Miranda et al., 2015; Saade et al., 2008). Topical application for desensitisation delivers comparable low doses to studies examined here, however multiple applications over multiple days are administered (Yamaoka et al., 2007). Likewise, intrathecal doses are comparable but administered for extended time periods, up to 24 h (Kamei et al., 2000; Mousseau et al., 1994).

4.5.4 Sex bias

A greater proportion of chronic pain patients are females and recent evidence suggests there may be mechanistic difference in the way sexes process pain (Fillingim et al., 2009; Mogil, 2012). Despite this, male subjects remain overwhelmingly the most utilised sex in preclinical pain research (Mogil et al., 2005). Capsaicin behavioural studies analysed here are typical of what is found in the wider pain community, with 80% of studies using male only subjects (Mogil et al., 2005). Of studies which did use both sexes, a large proportion (69%) did not report sex independent results or excluded females from behavioural experiments. Three papers stated sexes would be evenly distributed and analysed together, presumably other studies did the same. Of the small number of studies remaining which treated males and females separately there is no consensus on the sex effect of capsaicin-induced behaviours, which warrants further investigation and greater inclusion of female subjects in future studies. Similar contradictory evidence is also found in the clinical capsaicin literature (Frot et al., 2004; Jensen et al., 2006). Further emphasising the need for the use of both sexes was the increased antinociceptive ability of mixed-opioid analgesics in males, observed in multiple studies (Lomas et al., 2007; Lomas et al., 2008; Saloman et

al., 2011). The effect of sex on capsaicin sensitivity requires further clarification, this can be improved initially by researchers reporting sex independent results when both sexes are used. Further, attitudes need to shift in regards to variability imparted by oestrous, and researchers should become more open to including female subjects in their studies (Mogil et al., 2005).

4.5.5 Limitations

One of the main drawbacks of using a capsaicin model is the increasingly complicated nature of central sensitisation observed in chronic pain. The cross talk between immune modulating elements of the CNS (e.g. glia, infiltrating T-cells) and neurons, known as neuroimmune signalling, cannot be replicated in models which only use capsaicin (Grace et al., 2014). Despite producing comparable behavioural outcomes, capsaicin's relevance to chronic pain is potentially a little superficial. Further, the time frame suggests it does not cause prolonged changes from periphery to higher brain regions, again making the model different to the theorised mechanisms of chronic pain in humans (Vierck et al., 2008). Lastly, blinding of animal experiments is very difficult due to obvious flare response elicited by capsaicin, especially for commonly used subcutaneous and intraplantar administration. This is perhaps another reason to move away from user evoked behavioural assays towards operant or position place preference style assays where user subjectivity is less influential.

4.6 Conclusion

Currently, use of animal behaviour is crucial to pain research. It enables researchers to answer basic physiological questions, validate model relevance and preclinical molecular observations, and assess therapeutic options. No doubt behavioural observation following capsaicin administration will continue to be used for these purposes. This systematic review will aid in advising decisions on capsaicin dosing, behaviour assessed, sex selection and to improve and eliminate inaccurate reporting. These points will improve animal welfare, standardise confusing nomenclature and improve clinical relevance.

Following a review of capsaicin literature, we were ready to attempt a back-translation of the clinical endotoxin-capsaicin model. The first step is in line with our original aim, to replicate the conditions of the preclinical model. However, due to the results of chapter 3, and previous work in primary cells, we also plan to investigate a more directed LPS approach. This enables investigation into the relevance of short LPS activation influencing TRPV1 activity *in vivo*. Therefore, the rodent endotoxin-capsaicin model will be investigated in terms of both a systematic and localised LPS administration at previously examined short (< 20 min) and longer immune activating incubation periods.

Chapter 5. A peripherally administrated primed LPS response potentiates capsaicin-induced mechanical hypersensitivity in BALB/c mice.

5.1 Abstract

Interactions between immune and neural elements of the nervous system are important contributing mechanisms to chronic pain, which represents a significant global disease burden. Recently, a functional interaction between innate immune receptor, toll-like receptor 4 (TLR4) and neuronal ion channel, transient receptor potential cation channel subfamily V member 1 (TRPV1) has been discovered both *in vitro* and in clinical models. The clinical model reports that systematic endotoxin potentiates the effect of intradermal TRPV1 agonist, capsaicin. Potentiation of flare responses as well as mechanical allodynia and hyperalgesia are observed. Our aim is back-translate this clinical model into a rodent model and investigate the molecular and cellular mechanisms behind the response. We generated an intraplantar capsaicin pain model that allowed assessment of mechanical hypersensitivity to 90 min. Systemic (1 μg, 1 mg/kg) and local (100 ng) lipopolysaccharide (LPS) administration did not potentiate mechanical hypersensitivity at multiple time points. Interestingly we observed potentiation of capsaicin responses using two separate LPS doses administered into the hind-paw; an effect blocked by TLR4 antagonist (+)-naloxone. However, there appeared to be two populations of responders in this study, animals who showed LPS-induced potentiation and animals who showed no potentiation. Therefore, we were unable to replicate the clinical endotoxin-capsaicin model but did observe a population in which a locally primed endotoxin response results in potentiated capsaicin-induced mechanical hypersensitivity.

5.2 Introduction

Effective chronic pain treatment remains elusive, with the condition affecting quality of life for billions worldwide (Goldberg et al., 2011; Jacobs, 2005). Current options offer poor efficacy and negative side effects including tolerance, potential addiction, constipation and drowsiness, impacting on the ability to work, care and drive; necessitating the search for novel targets (Jacobs, 2005; Nightingale, 2012; Rogers et al., 2013; Whitten et al., 2005). Chronic pain refers to pain that extends past the resolution of injury, typically longer than three months, and may result from numerous common insults including nerve injury, cancer, chemotherapy, diabetes, surgery and infection (Milligan et al., 2009; Nightingale, 2012).

Enhanced neuronal excitability is the cause of several symptoms of chronic pain including hypersensitivity to a range of noxious (hyperalgesia) and non-noxious (allodynia) stimuli, as well as spontaneous pain (Backonja et al., 2004; Ji et al., 2003; Milligan et al., 2009). However, targeting neuronal excitability is the aim of currently underperforming pharmaceutical options including opioids, antidepressants and anticonvulsants (Whitten et al., 2005). In light of this evidence, the search for novel target mechanisms to treat chronic pain has switched away from its neuro-centric stance.

Understanding that both neural and non-neural elements communicate to create an environment of enhanced excitability has been an important advance in chronic pain science (Grace et al., 2014; Woolf et al., 2000). The peripheral and central nervous systems (PNS/CNS) contain multiple interacting cell-types including neurons, glia (astrocytes, microglia, oligodendrocytes, satellite glia) and peripheral immune cells. Their interactions are collectively referred to as neuroimmune signalling, important in altering neuronal excitability in both the PNS and CNS; processes known as peripheral and central sensitisation respectively (Grace et al., 2014; Woolf et al., 2000). T-cell knockout, blocking glial signalling cascades (e.g. sphingosine-1-phosphate receptor (S1PR1), toll-like receptors (TLRs), ERK pathway, Src kinase activity), and disrupting cytokine/chemokine binding (e.g. interleukin-1 receptor antagonists (IL-1Ra), colony stimulating factor 1 (CSF-1) have all attenuated generation or maintenance of pain sensitivity in animal models (Guan et al., 2016; Katsura et al., 2006a; Kobayashi et al., 2015; Lacagnina et al., 2017; Milligan et al., 2001b; Milligan et al., 2003). Targeting

neuroimmune interaction has the potential to revolutionise therapeutic outcomes for chronic pain.

Despite the importance of neuroimmune mechanisms observed in preclinical studies, there has been little clinical impact. Clinical trials which attempt to alter neuroimmune communication have not produced promising results. (Huggins et al., 2012; Landry et al., 2012). However, evidence shows pain sensitivity is altered by disease and inflammatory stimuli in humans (Hutchinson et al., 2013; Shenker et al., 2008; Sumracki et al., 2012; Wegner et al., 2014). Again, this suggests a lack of relevant mechanistic understanding; if neuroimmune targeting therapies are to be introduced, relevant clinical mechanisms must be understood. Development of quality clinical and preclinical models of pain which encompass both neuro- and immune- molecular mechanisms and aspects of somatosensory processing would be an important step forward.

The capsaicin pain model is currently utilised in both the clinical and preclinical literature (Kinnman et al., 1995). Capsaicin is the pungent component in chilli peppers and agonist of ion-channel, transient receptor potential cation channel subfamily V member 1 (TRPV1) (Caterina et al., 1997; Szallasi et al., 2007). It is commonly used due to its ability to directly activate pain signalling neurons (nociceptors) and produce symptoms of central sensitisation including allodynia, hyperalgesia and spontaneous pain (Aykanat et al., 2012; Simone et al., 1989; Tominaga et al., 1998; Wong et al., 2014). Capsaicin also causes sensitivity in surrounding (secondary) tissues following intradermal injection, indicative of threshold changes within the CNS, and further evidence of central sensitisation (Willis, 2002). For these reasons the capsaicin model has been likened to neuropathic pain models, despite solely targeting neural pain mechanisms (Joshi et al., 2006).

Pain produced by capsaicin is potentiated in individuals diagnosed with unilateral sciatica and rheumatoid arthritis, suggesting TRPV1 function is altered in the chronic pain environment (Shenker et al., 2008; Sumracki et al., 2012). Evidence of a relationship between the immune system and TRPV1 exists and potentially explains these clinical results. Activating the innate immune system with gram-negative cell wall component lipopolysaccharide (LPS), potentiates TRPV1 response in a the rat vagus nerve and dorsal root ganglia neurons (Diogenes et al., 2011; Ferraz et al., 2011; Hua et al., 1996). Along

with the well-established capsaicin pain model, these results open the possibility of a model involving direct manipulation of both immune and neural elements of pain.

The clinical relevance of this endotoxin/TRPV1 interaction was demonstrated by Hutchinson et al. (2013). A low dose of systemic LPS potentiated capsaicin-induced allodynia, hyperalgesia and flare when administered to the forearm (Hutchinson et al., 2013). Further, the potentiation occurred at 3.5 hrs post LPS administration and not at earlier time points, correlating with maximum serum IL-6 and suggesting an immune-induced sensitisation event. This endotoxin-capsaicin model is important evidence that neuroimmune interactions are relevant in clinical pain and offer the exciting potential of a unified clinical and preclinical model in which to study neuroimmune interaction.

It is not possible for further cellular and mechanistic investigation of the clinical endotoxin-capsaicin model, leaving a myriad of unanswered mechanistic questions. For example, it is not known if LPS is acting centrally by effecting glial activity, or on peripheral afferent fibres by direct activation or peripheral immune activity. LPS is an agonist of innate immune receptor toll-like receptor 4 (TLR4); with the potential to activate both peripheral nociceptors and glia in the CNS (Borges et al., 2012; Buchanan et al., 2010; Lin et al., 2015; Wadachi et al., 2006). Questions also surround the nature of a TLR4/TRPV1 relationship, if this potentiation is resulting from direct interaction, or indirect via TLR4 induced transcription outcomes as the serum IL-6 correlation suggests. Translating the clinical endotoxin-capsaicin model into a preclinical model will allow further elucidation of molecular and cellular mechanisms.

“Back-translation” of clinical results into preclinical animal models has been successfully demonstrated for numerous pain therapeutic agents including; non-steroidal anti-inflammatories (NSAIDs), opioids and those approved for chronic neuropathic pain (anticonvulsants, antidepressants and anaesthetics) (Berge, 2011; Whiteside et al., 2008). However, pharmacological comparison of responses in humans and animal models reveal differences in sensitivity to these compounds, hypothesised as differences in tissue penetration, metabolism or binding characteristics (Whiteside et al., 2008). This gives hope of potential back translation of the endotoxin-capsaicin model. However, particular care must be taken in drug delivery and dose in order to achieve a comparative systemic immune activation.

This study aims to 'back-translate' the clinical endotoxin-capsaicin model into a preclinical model, and to study the potentiation mechanism at a molecular and cellular level. We hypothesise that in BALB/c mice we can recreate the LPS potentiated capsaicin-induced hypersensitivity. In order to achieve this, we will look to replicate as many aspects of the clinical model as possible, including sex, relative doses, relative timings and behavioural outcomes in a mouse intraplantar capsaicin model. If successful, this raises the exciting possibility of a unified clinical and preclinical model of neuroimmune potentiated pain. A novel situation, with the potential to reveal key similarities and differences between the models we use to screen therapeutic options, and the clinical system we aim to treat.

5.3 Methods

5.3.1 Animals

Male adult BALB/c mice (6-12 weeks) (n = 6 - 10 per group) obtained from Laboratory Animal Services of the University of Adelaide (Adelaide, SA) were used in these experiments. Despite no previous usage in capsaicin-induced preclinical pain models, BALB/c mice were used due the characteristics of their immune response. Given the LPS challenge that accompanies capsaicin in this model, it was decided the Th2 dominant BALB/c strain would provide a more balanced immune response compared to the Th1 dominant, commonly used C57/Bl6 strain (Watanabe et al., 2004). All mice were group-housed in the Medical School Animal Facility of the University of Adelaide. Mice were allowed free access to food and water in a temperature-controlled environment (23 ± 3°C) maintained on a 12:12 h light/dark cycle (lights on at 0700). Animals were left to acclimatise to the facility for at least one week before handling began. One week of handling and acclimatisation to the experimental set-up was carried out prior to use. All experiments reported in this study were approved by the University of Adelaide Animal Ethics Committee (Ethics approval number M-227-13B) and complied with the Australian code for the care and use of animals for scientific purposes.

5.3.2 Materials

Capsaicin and (2-Hydroxypropyl)-β-cyclodextrin were purchased from Sigma-Aldrich (Castle Hill, NSW). (+)-Naltrexone was kindly provided by Dr Kenner Rice (Chemical

Biological Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD). Sterile saline (0.9%) was purchased from Baxter Australia (Old Toongabbie, NSW, Australia). Von Frey filaments were purchased from North Coast Medical Inc. (Gilroy, California).

(2-Hydroxypropyl)-β-cyclodextrin was diluted in 0.9% sterile saline to make a 38% solution. A stock capsaicin solution of 5 mg/mL was created using 38% (2-Hydroxypropyl)-β-cyclodextrin, protected from light, and stored at 4°C for a maximum of 1 month. On the morning of use, this was further diluted in (2-Hydroxypropyl)-β-cyclodextrin to the experimental concentration. (+)-Naltrexone was made up to the desired concentration in 0.9% sterile saline the week of experiment, and kept at 4°C.

5.3.3 Intraplantar drug (i.pl.) administration

Intraplantar administration was chosen for this model as the most commonly utilised technique in capsaicin literature which allows assessment of secondary mechanical hypersensitivity, while resembling the targeted peripheral administration of the clinical model (intradermal). All capsaicin and β-cyclodextrin administrations were conducted using the following technique. All injections were performed with a primed 25 µL Hamilton micro-syringe and 30G needle. To prime the micro-syringe, the plunger was removed, and 0.9% saline was injected into the barrel of the syringe until no air remained inside, the plunger was then reinserted. A 30G needle was attached to a 1 mL syringe containing 0.9% saline and saline was dispensed, filling the needle tip. The 30G needle tip was then carefully removed, filled with 0.9% saline and attached to the full micro-syringe; leaving no air inside either the micro-syringe or needle tip. The plunger was then pushed all the way down, and pulled up to the 5 µL mark, creating a small air bubble. The desired volume of solution could then be withdrawn and dispensed. Animals were gently restrained in a thin towel and 5 µL of the solution was injected at the distal end of the plantar surface, directly under the skin at the footpads (Fig. 5.1).

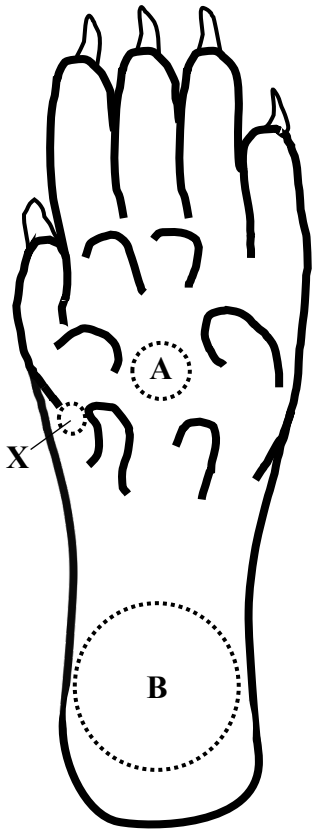


Figure 5.1: Intraplantar (i.pl.) drug administration. (X) represents the site of needle insertion, (A) represents the site of drug injection, (B) represents the site of von Frey mechanical stimulation.

5.3.4 Behavioural testing

A von Frey test modified from the Nicotra et al. (2014) method was performed on all animals. Immediately following all i.pl. drug administrations, animals were placed on a wire mesh shelf that allowed access to the plantar surface of the hindpaw and covered with an opaque cup. Von Frey filaments were touched to the proximal end of the plantar surface of the hindpaw to allow assessment of secondary hypersensitivity, as elicited in the clinical model (Fig. 1). Force was then applied until the filament bent and held for two seconds before being slowly removed for one second and reapplied. Animals were tested at 7, 45 and 90 min following i.pl. capsaicin administration, timings which would allow resolution of hypersensitivity based on review of preclinical capsaicin literature. All animals received 10 applications of each von Frey filament per time point, with a minimum of 8 min

between application of a different filament strength. Baseline testing was conducted 24 h prior to i.pl. capsaicin administration for all experiments.

5.3.5 Systemic endotoxin-capsaicin model

BALB/c mice were administered LPS (1 μg or 1 mg/kg) or the equivalent volume of saline by intraperitoneal (i.p.) injection. Capsaicin (0.8 μg/5 μL i.pl.) was administered 4 h following LPS. Animals were tested with filaments 1.65 (0.008 g), 2.44 (0.04 g) and 3.22 (0.16 g). Capsaicin dose for this and all subsequent experiments was based on data from pilot studies guided by review of literature utilising capsaicin to produce mechanical hypersensitivity.

5.3.6 Local administration endotoxin-capsaicin model

BALB/c mice were administered LPS (100 ng/5 μL i.pl.) (Calil et al., 2014) or equivalent volume saline. Capsaicin (0.8 μg/5 μL i.pl.) was administered 15 min or 4 h following LPS. Animals were tested with filaments 1.65 (0.008 g), 2.44 (0.04 g), 3.22 (0.16 g) and 3.84 (0.6g).

For experiments involving a primed LPS response, the first dose of LPS (100 ng/5 μL i.pl.) or saline was administered 7 days prior to the second dose, all other doses and administration timings remained the same. (+)-Naltrexone (60 mg/kg i.pl.) was administered 30 min prior to the first dose of LPS (Lewis et al., 2012).

5.3.7 Analysis

Data was analysed using GraphPad Prism v7.0a (GraphPad software, Inc. San Diego, CA). Two-way ANOVA with repeated measures and Tukey's multiple comparison test was used to compare treatment effect over time from all behavioural experiments. Sidak's multiple comparisons test was used to compare treatment groups at individual time points. P-values ≤ 0.05 were considered statistically significant.

5.4 Results

5.4.1 The human endotoxin-capsaicin model does not directly translate to BALB/c mice

Systemic LPS did not affect capsaicin-induced hypersensitivity in BALB/c mice. Capsaicin-induced hypersensitivity was observed across both LPS and saline groups as shown by increased von Frey responses (Fig. 5.2), however not consistently across all von Frey filaments tested. Post hoc analysis revealed that among LPS treated animals all filaments produced a significantly higher number of responses at 7 min post capsaicin compared to baseline (Fig. 5.2A. $p < 0.0001$, Fig. 5.2B. $p < 0.01$, Fig. 5.2C. $p < 0.05$). In the vehicle groups the 0.008 and 0.16g filaments produced a significant increase in the number of responses at 7 min compared to baseline (Fig. 5.2A. $p < 0.001$, Fig. 5.2C. $p < 0.05$), while there was no difference observed for the 0.04g filament. In all groups, hypersensitivity was resolved by 45 min, with no significant differences seen at 45 and 90 min post capsaicin when compared to baseline. Two-way ANOVA comparing the change in responses from baseline between LPS and vehicle groups revealed an effect of time following capsaicin administration for all three von Frey forces tested (Fig. 5.2A. $F_{(3,30)} = 19.82$, $p < 0.0001$, Fig. 5.2B. $F_{(3,30)} = 8.09$, $p < 0.001$, Fig. 5.2C. $F_{(3,30)} = 7.27$, $p < 0.001$), however no effect of treatment (Fig. 5.2A. $F_{(1,10)} = 0.78$, $p = 0.40$, Fig. 5.2B. $F_{(1,10)} = 0.07$, $p = 0.80$, Fig. 5.2C. $F_{(1,10)} = 0.018$, $p = 0.68$) and no interaction (Fig. 5.2A. $F_{(3,30)} = 0.28$, $p = 0.84$, Fig. 5.2B. $F_{(3,30)} = 0.75$, $p = 0.53$, Fig. 5.2C. $F_{(3,30)} = 0.19$, $p = 0.90$) (Fig. 5.2). Post hoc analysis revealed no significant difference between LPS and saline groups at any time point. These results are representative of multiple experiments using multiple LPS doses (1 μg, 1 mg/kg), capsaicin doses (1.6, 1.0, 0.8 μg/ 5 μL) and different time points (3, 4 h) (Ostberg et al., 2000) (Supp Fig. 5.1).

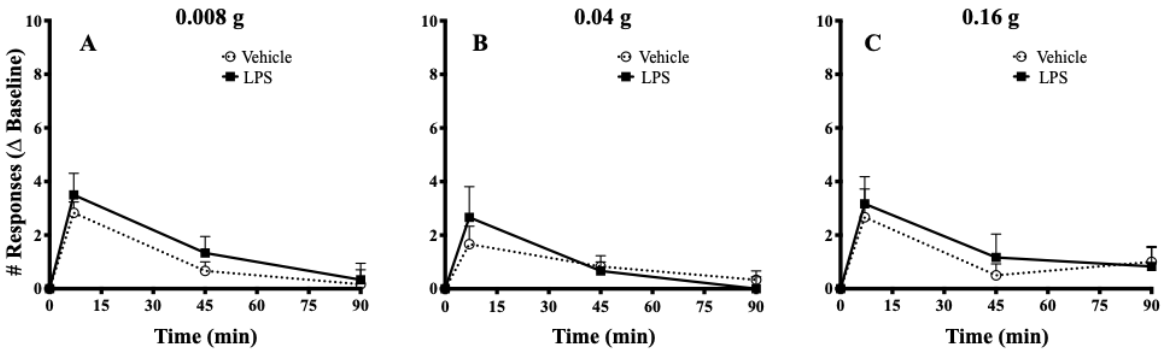


Figure 5.2: Systemic LPS does not potentiate capsaicin-induced hypersensitivity. Mice were administered LPS (1 mg/kg; i.p.) or saline 4 h prior to capsaicin (0.8 μg/5 μL; i.pl). Testing included 10 applications of von Frey filaments (0.008 (A), 0.04 (B), 0.16g (C)) and was performed at 7, 45 and 90 min following capsaicin administration. n = 6 for all groups, error bars represent SEM.

5.4.2 Local LPS administration does not consistently produce potentiated capsaicin-induced hypersensitivity at multiple time points

Applying LPS directly to the capsaicin injection site resulted in a large amount of variation in von Frey responses. Analysis revealed increased responses to the 0.6g filament in the LPS group for both 15 min and 4 h treatment groups (Fig. 5.3). However, the time at which potentiated hypersensitivity occurred was not consistent. A 15 min gap between LPS and capsaicin injection was chosen based on *in vitro* endotoxin-capsaicin studies (Diogenes et al., 2011). Two-way ANOVA revealed an effect of time for all filaments tested (Fig. 5.3A. $F_{(3,30)} = 7.79$, $p < 0.001$, Fig. 5.3B. $F_{(3,30)} = 4.3$, $p < 0.05$, Fig. 5.3C. $F_{(3,30)} = 7.41$, $p < 0.001$) but not treatment (Fig. 5.3A. $F_{(1,10)} = 0.95$, $p = 0.35$, Fig. 5.3B. $F_{(1,10)} = 0.42$, $p = 0.53$, Fig. 5.3C. $F_{(1,10)} = 4.50$, $p = 0.06$) or interaction (Fig. 5.3A. $F_{(3,30)} = 0.74$, $p = 0.54$, Fig. 5.3B. $F_{(3,30)} = 0.14$, $p = 0.93$, Fig. 5.3C. $F_{(3,30)} = 2.59$, $p = 0.07$) on von Frey responses when there was 15 min between LPS and capsaicin injections. Interestingly, post hoc analysis revealed that von Frey scores did not significantly deviate from baseline at 7 min post capsaicin (Fig. 5.3A-C), while at 45 min the LPS group had significantly higher von Frey responses to the 0.6g filament than the saline control ($p < 0.05$) which did not deviate from baseline (Fig. 5.3C). As with the systemic LPS administrations we considered 4 h between LPS and capsaicin; which corresponds to the timing of potentiation observed in the Human endotoxin-capsaicin model (Hutchinson et al., 2013). Two-way ANOVA revealed an effect of time for all filaments tested (Fig. 5.3D. $F_{(3,30)} = 22.63$, $p < 0.0001$, Fig. 5.3E. $F_{(3,30)} = 22.73$, $p < 0.0001$, Fig. 5.3F. $F_{(3,30)} = 7.41$, $p < 0.001$) and an effect of treatment for the 0.16g filament (Fig. 5.3E. $F_{(1,10)} = 6.337$, $p = 0.03$). No treatment effect was observed for the two other filaments tested (Fig. 5.3.D. $F_{(1,10)} = 1.61$, $p = 0.23$, Fig. 5.3F. $F_{(1,10)} = 1.96$, $p = 0.19$) and no interaction was observed for any filament (Fig. 5.3D. $F_{(3,30)} = 0.84$, $p = 0.48$, Fig.5.3E. $F_{(3,30)} = 1.20$, $p = 0.33$, Fig. 5.3F. $F_{(3,30)} = 2.90$, $p = 0.05$). Unlike for the 15 min time-point, capsaicin-induced hypersensitivity was observed for all filaments; with post hoc analysis revealing that von Frey responses significantly increased at 7 min compared to baseline for both saline ($p < 0.0001$ (Fig. 5.3D, F); $p < 0.001$ (Fig. 5.3E)) and capsaicin ($p < 0.0001$ (Fig. 5.3D-F)) groups. Post hoc analysis also revealed that for the 0.6g filament there was a significantly increased response in the LPS group at 90 min following capsaicin ($p < 0.05$) (Fig. 5.3F). A lower dose of LPS was also tested (50 ng/5 μL), interestingly again a treatment effect was observed ($F_{(1,9)} = 6.10$, $p = 0.04$) for the 0.16g filament, although this time in the 15min LPS group, no other group differences were observed (Supp. Fig. 5.2).

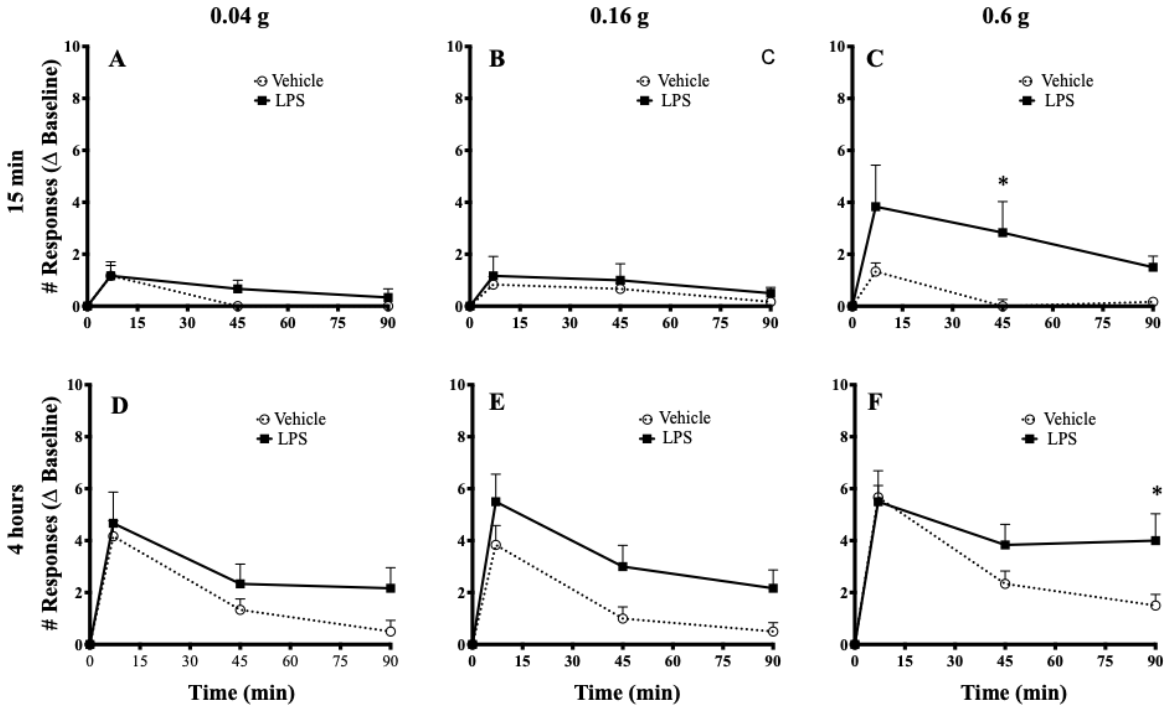


Figure 5.3. Local LPS (100 ng) administration does not consistently produce potentiated capsaicin hypersensitivity. BALB/c mice were administered LPS (100 ng / 5 µL; i.pl) 15 min or 4 h prior to capsaicin (0.8 µg/5 µL; i.pl.). Von Frey testing was carried out at 7, 45 and 90 min following capsaicin administration. Three von Fey filaments were used, 0.04 (A, D), 0.16g (B, E) and 0.6g (C, F). n = 6 for all groups. Error bars represent SEM. * $p < 0.05$.

5.4.3 A primed LPS response produces extended capsaicin-induced hypersensitivity, an effect reversed by (+)-naltrexone

In the next study, 2 doses of LPS (i.pl./i.pl.) separated by 7 days were administered prior to capsaicin; using the same von Frey stimulations as in the previous experiment. (+)-Naltrexone was used as a TLR4 antagonist and administered prior to the first LPS dose. Two-way ANOVA revealed a main effect of time (Fig. 5.4A. $F_{(3,93)} = 102.8$, $p < 0.0001$, Fig. 5.4B. $F_{(3,93)} = 119.4$, $p < 0.0001$, Fig. 5.4C. $F_{(3,93)} = 129.9$, $p < 0.0001$) across all three filaments. However, a treatment effect was only observed across the 0.04 (Fig. 5.4A. $F_{(2,31)} = 7.5$, $p = 0.002$) and 0.6g (Fig. 5.4C. $F_{(2,31)} = 7.0$, $p = 0.003$) filaments, with no treatment effect observed for the 0.016g (Fig. 5.4B. $F_{(2,31)} = 6.3$, $p = 0.05$) filament. An interaction effect was observed for all three filaments (Fig. 5.4A. $F_{(6,93)} = 3.1$, $p = 0.008$, Fig. 5.4B. $F_{(6,93)} = 3.5$, $p = 0.003$, Fig. 5.4C. $F_{(6,93)} = 3.4$, $p = 0.004$). Post hoc analysis revealed that LPS alone had no effect on von Frey scores (Fig. 5.4). For all filaments, extended hypersensitivity was observed; the LPS-capsaicin group had significantly increased von Frey responses when compared to the saline control at both 45 ($p < 0.01$) and 90 ($p < 0.001$) min. Despite significant differences between these groups, it should be noted that there appears to be two populations of responders. Between 6 and 8 out of 14 (depending on filament) animals tested produced a response that didn't significantly differ from the saline group, while the remaining animals showed potentiation and extended hypersensitivity (Fig. 5.4D-F). In fact, if the LPS primed group was split into two based on level of response, high (90 min score outside 2 standard deviations of vehicle control) and low, it is possible to view significant differences between the two (Fig. 5.4D-F). At 45 min a significant difference was observed at the 0.04g filament ($P < 0.01$) and at the 0.6g filament ($p < 0.05$). At 90 min all three filaments showed a significant difference between high and low groups ($p < 0.01$). The high responding group was significantly higher than vehicle for all filaments at 45 and 90 min ($p < 0.01$) and at the 7 min time point for the 0.04g filament ($p < 0.05$) (Fig. 5.4D-F). The group receiving (+)-naltrexone did not differ from the saline group at any point. Significant differences between (+)-naltrexone-LPS and LPS only groups were only observed at the 90min time point for 0.04g ($p < 0.05$) and 0.6g ($p < 0.01$) filaments (Fig. 5.4A-C).

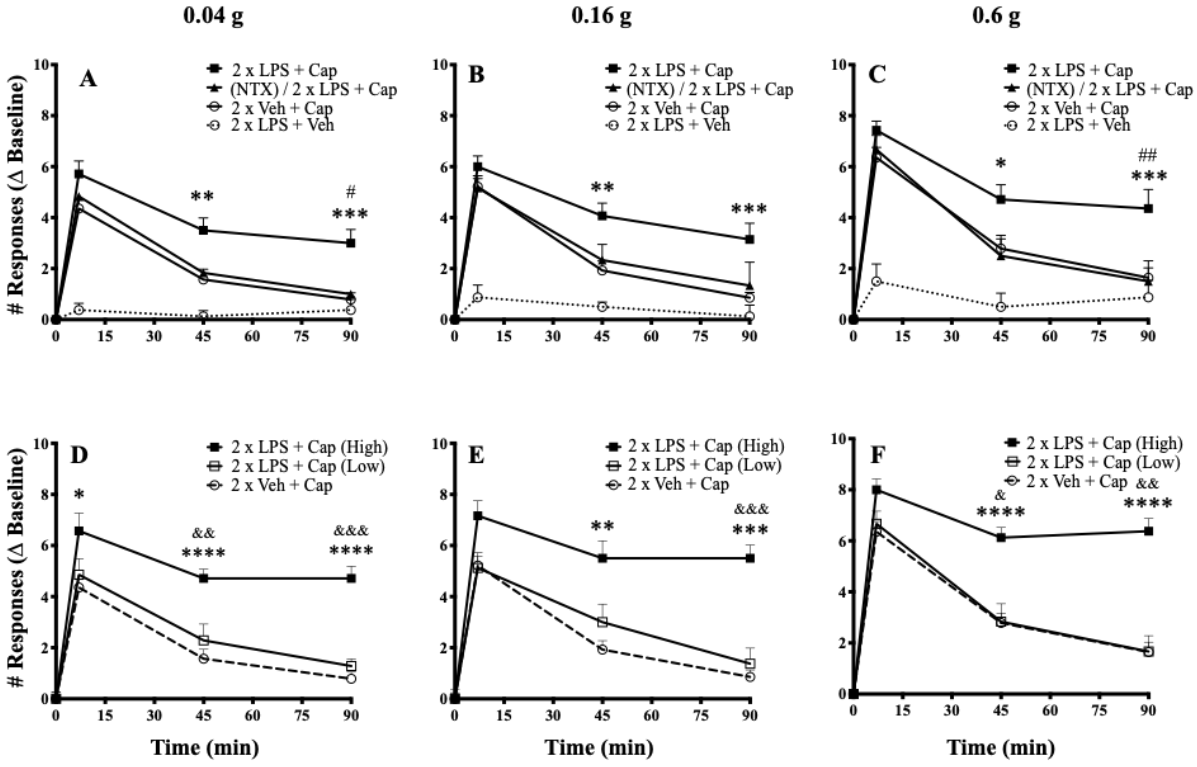


Figure 5.4. Two LPS doses prolongs capsaicin-induced hypersensitivity, an effect reversed by (+)-naltrexone. BALB/c mice were administered two doses of LPS (100 ng / 5 µL; i.pl.) 7 days apart. 4 h after the final dose animals received capsaicin (0.8 µg/5 µL: i.pl). Von Frey testing was carried out at 7, 45 and 90 min following capsaicin administration. Three von Frey filaments were used, 0.04 (A, D), 0.16g (B, E) and 0.6g (C, F). D, E and F represent the 2 x LPS + capsaicin group split into high and low responders compared with the 2 x veh + capsaicin control group. n = 6 for (+)-naltrexone group, n = 14 for all other groups receiving capsaicin, n = 8 for LPS-vehicle group. For graphs (D, E, F) which split 2 x LPS + capsaicin group, n = 7 (D), 6 (E) and 8 (F) for high responding group and 7 (D), 8 (E) and 6 (F) for the low responding group. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, $p < 0.0001$ 2 x LPS + capsaicin and 2 x LPS + capsaicin (high responders) vs 2 x saline + capsaicin; # $p < 0.05$; ## $p < 0.01$ NTX + 2 x LPS + capsaicin vs 2 x LPS + capsaicin; & $p < 0.05$; && $p < 0.01$, &&& $p < 0.001$ for 2 x LPS + capsaicin (high responders) vs 2 x LPS + capsaicin (low responders).

5.5 Discussion

The aim of this study was to back-translate the clinical endotoxin-capsaicin model into BALB/c mice. Not only would this enable investigation of mechanisms underlying the clinical endotoxin-capsaicin results but create a unified clinical/preclinical model; a unique drug screening paradigm. Further to these, it may also shed light on the nature of the relationship between TLR4 and TRPV1. We failed to replicate the mechanical hypersensitivity observed in the clinical endotoxin-capsaicin model using both systemic and targeted peripheral LPS application. LPS priming did cause an observed potentiated hypersensitivity, which was reversed by TLR4 antagonist (+)-naltrexone. However, the variability of this response suggests further investigation is required.

We produced a reproducible capsaicin-induced hypersensitivity in BALB/c mice using a best of 10 von Frey approach (Fig. 5.2), applying 0.8 μg of capsaicin per paw. To our knowledge this is the first time this model has been established in BALB/c mice. Hypersensitivity to von Frey stimuli is observed at 7 min and is resolved by 45 min; a timeframe which correlates well with clinical observations following intradermal capsaicin (Aykanat et al., 2012; Liu et al., 1998). Importantly the magnitude of hypersensitivity following 0.8 μg of capsaicin never exceeds 80% of the maximum response rate and drops rapidly, which allows room for potentiation of magnitude and duration to be observed.

Systemic LPS was unable to potentiate capsaicin-induced hypersensitivity at 1 μg or 1 mg/kg, across all filament strengths tested (Fig. 5.2, Sup Fig. 5.1). Multiple filaments were tested for numerous reasons, the most practical being minimising the chance of reaching an effect ceiling. Secondly, in the case potentiation occurs in only a particular subset of nociceptors. TRPV1 is found on both small and medium C and Aδ primary afferents, which have been shown to be activated by different stimuli and stimulus force (Hsieh et al., 2015; Szallasi et al., 2007). In the event of potentiation, using multiple forces may have revealed if a particular neuronal subset was responsible for the change. Finally, the lowest fibre we used elicited less than one response from 10 at baseline, considered completely innocuous (data not shown). The highest filament we used elicited an average of 2 responses from 10 and was considered noxious (data not shown). This gave the study the possibility of distinguishing if the observed hypersensitivity may be a result of an allodynic

(innocuous filament) or hyperalgesia (noxious filament) mechanism in the event that potentiation occurred.

Careful consideration was given to the timing of LPS as this was an important factor in clinically observed potentiation; peak plasma cytokine (IL-6) levels correlated with potentiated allodynia in that case (Hutchinson et al., 2013). Inflammatory mediators including TNFα, bradykinin, NGF, IL-1β, IL-6 are implicated in TRPV1 sensitisation and upregulation; suggesting pro-inflammatory conditions induced by TLR4 signalling may be responsible for the observed potentiation (Chuang et al., 2001; Fang et al., 2015; Hensellek et al., 2007; Zhang et al., 2005). Therefore, capsaicin potentiation was tested at observed peak cytokine following LPS administration in BALB/c mice (4 h) (Ostberg et al., 2000). Interestingly, recent evidence reports differences in cytokine response due to variation in LPS administration. With the exception of IL-10, cytokine levels were delayed and variable after i.p. administration compared to i.v.. Again, this suggests the use of an extended time period between endotoxin and capsaicin, compared to that observed in the clinical model (Somann et al., 2019). Timing is a factor that could be explored further, clinical studies which administered the same (0.4 ng/kg) and higher (0.8 & 2 ng/kg) LPS doses report peak pro-inflammatory cytokines (IL-6, TNFα, IL-8) at 2 h, earlier than reported by Hutchinson et al. during the clinical endotoxin-capsaicin study (Benson et al., 2012; de Goeij et al., 2013; Hutchinson et al., 2013; Wegner et al., 2014). Therefore, any future studies might consider a longer timepoint of 5-7 h, past peak plasma cytokine resulting from i.p. administration. While i.v. administration should also be considered if the nature of a systemic immune response is altered by administration location.

LPS potentiated capsaicin responses have also been observed in trigeminal nociceptors over much shorter time frames. LPS stimulation of rat trigeminal ganglion neurons for 15 min causes a potentiation of capsaicin-induced increase in intracellular calcium and CGRP release, which is attenuated by TLR4 antagonist LPS-RS (Diogenes et al., 2011; Ferraz et al., 2011). We attempted to stimulate peripheral neurons directly based on this direct and fast acting potentiation. TLR4/TRPV1 co-localisation has been observed in peripheral tissues including dental pulp, TG and DRG of humans, rats and mice respectively (Ferraz et al., 2011; Min et al., 2014; Wadachi et al., 2006). Reports of direct activation of afferent nociceptors by LPS alone, further supports evidence that TLR4 may be directly involved in

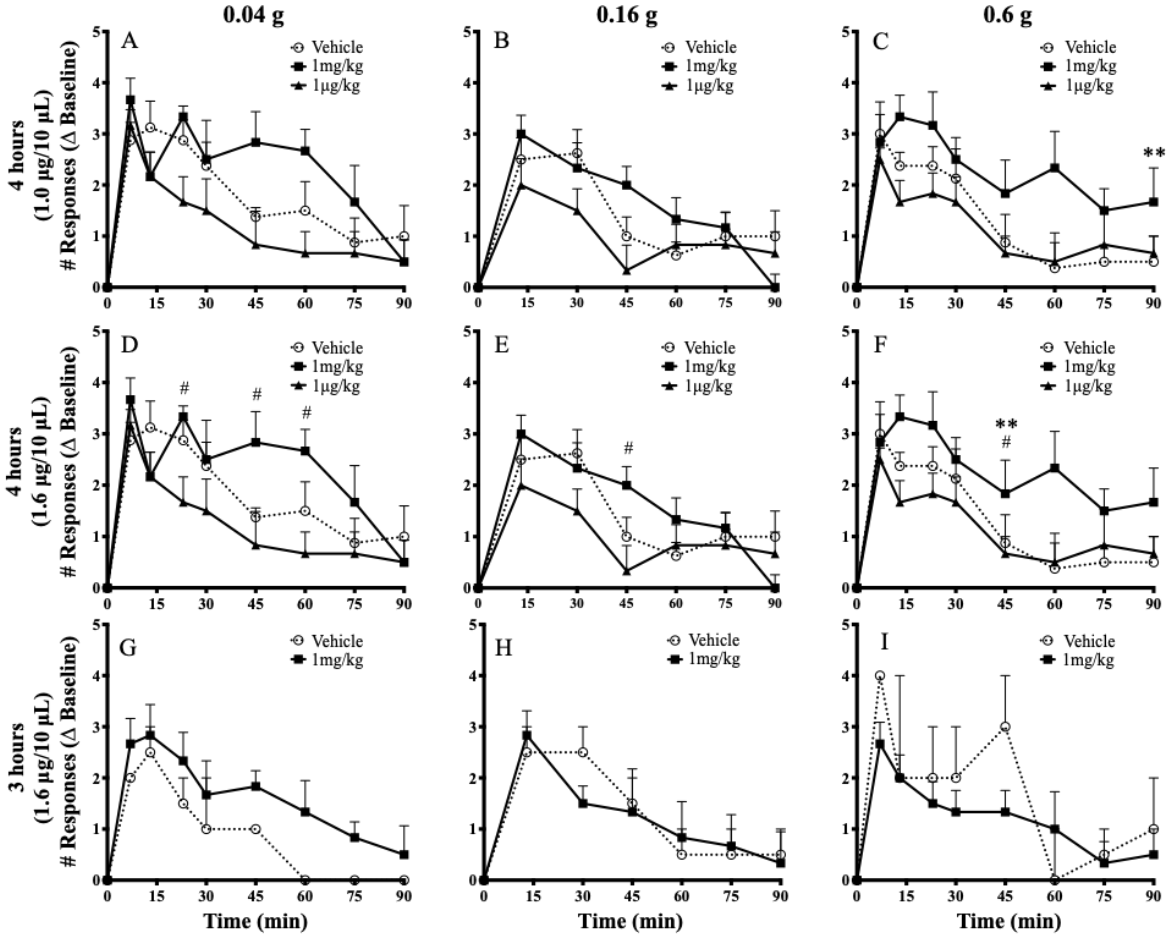
nociceptive responses (Li et al., 2015; Meseguer et al., 2014). Administration of local LPS did not potentiate capsaicin-induced hypersensitivity at either the 15 min or 4 h time points. In contrast with earlier studies, we observed no sensitivity to LPS alone at 100 ng/paw at the tested time points (15 – 105; 240 – 330 min) (data not shown) (Calil et al., 2014). Interestingly the addition of an intraplantar injection 15 min prior to intraplantar capsaicin-administration eradicated capsaicin-induced hypersensitivity (Fig. 5.3A-C). Further investigation is warranted to understand which systems attenuate capsaicin hypersensitivity following injection stressor in the hind paw.

Local priming of the LPS response produced a statistically different hypersensitivity in LPS primed and control groups. It has been observed that LPS priming has significant impact on pain sensitivity and immune cell activation when administered centrally (Cahill et al., 1998; Clark et al., 2010). Our model produced an all or nothing response, with animals either displaying an extended hypersensitivity to 90 min, or no change from vehicle control, resulting in a large amount of variability. An extra group of 8 animals was used after post hoc power analysis, which did lead to a statistically significant effect. However, this did not resolve the obvious variability. Rather than considering this an unreliable model, we suggest that differences in mechanisms between these two populations (high and low responders) would be of interest. Just as the mechanisms between a pain patient and a patient without pain if they had been given the same stimulus would be of interest – why did one develop the symptoms and the other did not? Interestingly the potentiation displayed a markedly different profile to the magnitude shift seen in the clinical model, with potentiated hypersensitivity not observed immediately following capsaicin administration. Taken together with the single peripheral application data above, it appears within BALB/c mice, for any given batch and serotype of LPS the response behavioural outcomes are highly variable. Genetic variability is a candidate for observed differences given evidence of significant variation in inbred strains including BALB/c's (Pritchard et al., 2006). For greater relevance it would be useful to determine how local LPS administration, and priming, elicits molecular and cellular changes in central and peripheral tissues. If analysis of immune cell infiltration or reactivation and/or locally induced inflammatory mediators also display significant intrasubject variability it is reasonable that they contribute to the variable behavioural outcomes.

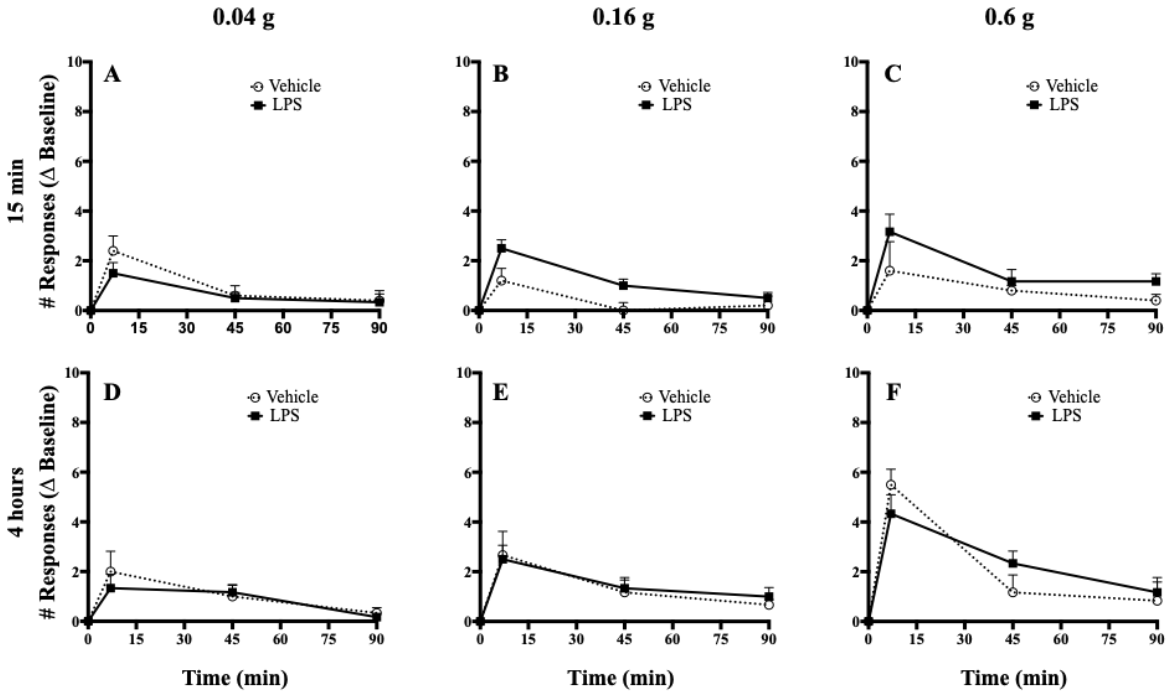
Although capsaicin-induced hypersensitivity is overwhelmingly tested using rodent hind-paw models, sensitisation by LPS in clinical models is region specific. In the clinical endotoxin-capsaicin model, allodynia and hyperalgesia were observed on the forearm, and not on the forehead, interesting considering preclinical endotoxin-capsaicin models have utilised trigeminal nociceptors (Hutchinson et al., 2013). Wegner et al. reported low-dose systemic LPS sensitisation in man, reporting lower back sensitisation while the calf and shoulder remain unaffected (Wegner et al., 2014). These results in particular introduce the possibility that some anatomical regions may involve different pain mechanisms, however anything from altered peripheral sub-populations to varied higher order CNS processing could account for such variances. Subcutaneous, intramuscular in intradermal capsaicin administration over the back or calf is perhaps an option in rodent models if further investigation was to occur, although new testing paradigms would need to be designed to assess mechanical sensitivity at these locations.

Poor preclinical to clinical translation of novel therapeutic agents has rightly brought into question the usefulness of current preclinical models in pain research (Hill, 2000; Huggins et al., 2012; Landry et al., 2012). We attempted to bridge the clinical to preclinical gap by creating a unified pain model involving both immunological and immune elements of pain signalling. Unfortunately, this study was unable to replicate the behavioural results seen in the clinical endotoxin-capsaicin model. Therefore, we were unable to shed any further light on the mechanism that underlies LPS-induced capsaicin potentiation observed in the clinical setting. We did observe a potentially interesting paradigm whereby a primed LPS response potentiates capsaicin hypersensitivity in a subset of our subjects, which warrants further investigation.

5.6 Supplementary figures



Supplementary Figure 1. Systemic LPS does not potentiate capsaicin-induced allodynia. Mice were administered LPS (1 µg/kg or 1 mg/kg; I.P.) or vehicle (0.9% sterile saline) 3- (G-I) or 4- (A-F) h prior to capsaicin (1.0 µg or 1.6 µg/10 µL; i.pl). Testing included 5 applications of a 0.008 (A, D, G), 0.04 (B,E,H) or 0.16g (C,F,I) von Frey filament. Testing was performed at 7, 13, 23, 30, 45, 60, 75 and 90 min following capsaicin administration, the 7 and 23 min timepoint were skipped for the 0.04g filament. n = 6 for all LPS groups except in the group receiving 1 mg/kg LPS and 1.0 µg/10 µL capsaicin (A - C) where n = 12; for vehicle groups n = 18 (A-C), 8 (D-F) and 2 (H-I). * $p < 0.05$ vehicle vs 1 mg/kg LPS; # $p < 0.05$, ## $p < 0.01$ 1 mg/kg LPS vs 1 µg/kg LPS, error bars represent SEM.



Supplementary Figure 2. Local administration of LPS (50 ng) does not potentiate capsaicin-induced allodynia. Mice were administered LPS (50 ng/5 µL; i.pl) or vehicle (0.9% sterile saline) 15 min (A-C) or 4 h (D-F) prior to capsaicin (0.4 µg/5 µL; i.pl). Testing included 10 applications of a 0.04 (A, D), 0.16 (B, E) or 0.6g (C,F) von Frey filament. Testing was performed at 7, 45 and 90 min following capsaicin administration. n = 6 for all groups, error bars represent SEM.

Chapter 5 revealed that intraplantar (i.pl.) administration of LPS 15 min prior to capsaicin completely attenuated capsaicin-induced mechanical hypersensitivity. This effect limits our ability to understand the *in vivo* relevance of short LPS stimulation on TRPV1 activity. We therefore aim to uncover mechanisms behind this response, as well as any potential effects on capsaicin responses following hindpaw administration. We hypothesised the role of endogenous opioids are key to this response, and we therefore investigate the role of opioid antagonists at various time points in relation to i.pl. administration of capsaicin. Further we look to examine if this peripheral stimulation elicits central changes.

Chapter 6. Naltrexone non-stereoselectively reverses intraplantar injection-induced analgesia and extends capsaicin-induced mechanical hypersensitivity.

6.1 Abstract

Capsaicin-induced pain models are used to investigate nociceptive physiology and screen for novel analgesic agents. Capsaicin activates neuronal ion channel, transient receptor potential cation channel subfamily V member 1 (TRPV1) to produce localised burning pain and extended hypersensitivity resulting from peripheral and central sensitisation. A common method of inducing pain in preclinical models is an injection into the ventral hindpaw of rodents. Our aim is to understand the effects of intraplantar (i.pl.) administration on the nociceptive outcome of capsaicin-induced pain. We found that i.pl. administration of saline (5 µL) 15 min before capsaicin challenge attenuates mechanical hypersensitivity in BALB/c mice. This effect was blocked by classical and non-classical opioid antagonists (-)- and (+)-naltrexone, restoring capsaicin-induced mechanical hypersensitivity to 90 min ($p < 0.05$). Both (-)- and (+)-naltrexone had no effect on capsaicin-induced mechanical hypersensitivity when administered pre-capsaicin. Interestingly, when administered following induction of hypersensitivity they significantly potentiated and extended capsaicin-induced mechanical hypersensitivity up to 135 min ($p < 0.0001$). If hypersensitivity recovered, naltrexone had no effect, even if administered prior to 135 min post capsaicin. Immunohistochemical analysis of the spinal cord revealed that extended hypersensitivity was not a result of increased neural activity measured by c-Fos activation at the spinal cord level. Therefore, i.pl. administration influences nociceptive outcomes of capsaicin administration by classical and non-classical opioid mechanisms. Behavioural effects of antagonising these mechanisms are dependent on nociceptive environment at the time of administration.

6.2 Introduction

Capsaicin is commonly utilised in pain research for its ability to produce observable, spontaneous behavioural responses and extended hypersensitivity (O'Neill et al., 2012). Capsaicin, the pungent ingredient of chilli peppers, is an agonist of transient receptor potential cation subfamily V, member 1 (TRPV1), a non-selective cation channel found on multiple tissue types including C and Aδ-fibre primary afferent neurons (Brito et al., 2014; Caterina et al., 1997; Szallasi et al., 2007). In addition to capsaicin, TRPV1 channel opening is induced by endogenous ligands and environmental factors (e.g. temperature, pH) resulting in activation of nociceptors, primary afferent neurons involved in transmission of noxious stimuli (Brito et al., 2014; Sutherland, 2014). Controlled, direct activation of nociceptive circuits by capsaicin makes it ideal for models investigating pain physiology; it is also frequently used to assess novel analgesic efficacy (Fialho et al., 2017; Hutchinson et al., 2013; Kang et al., 2010; Ren et al., 1994).

Preclinical pain models allow investigation of molecular and cellular mechanisms that are not possible in clinical models, while providing multi-system input that *in vitro* experiments lack. Use of capsaicin in preclinical animal models dates back multiple decades, utilising multiple animal species and routes of administration (Kinnman et al., 1995; Kupers et al., 1997; Laird et al., 2000). In addition to producing spontaneous pain behaviours, a number of common behavioural tests can be performed to establish hypersensitivity to mechanical and thermal stimuli following capsaicin administration. Mechanical application of force to the hind paw offers the ability to assess sensitivity at a secondary site, away from the application of noxious stimulation (Kinnman et al., 1995; Tamaddonfard et al., 2017). This gives researchers using capsaicin the ability to discriminate between peripheral and central origins of pain sensitivity, known respectively as peripheral and central sensitisation (von Hehn et al., 2012).

Peripheral and central sensitisation are separate mechanisms which produce unique outcomes, it is therefore important to distinguish between them to fully understand the effect of treatment in pain models. Both mechanisms result in exaggerated responses to noxious stimuli (hyperalgesia) and painful responses to innocuous stimuli (allodynia) (von Hehn et al., 2012). However, peripheral sensitisation involves changes to structure and

function of primary afferents; as such these symptoms are located at the site of damage/insult (von Hehn et al., 2012). Central sensitisation involves changes within the central nervous system (CNS), resulting in increased excitability and decreased inhibitory tone (Grace et al., 2014). The 'over-excited' environment within the CNS results in crosstalk between usually non-nociceptive sensory neurons and pain transmitting secondary afferents. As a result, innocuous stimuli can cause pain sensations (allodynia). Secondly, signals from primary afferents at sites away from the injury are amplified upon entering the 'over-excited' CNS; causing secondary hyperalgesia and allodynia (von Hehn et al., 2012). Importantly, capsaicin models are able to produce both primary and secondary hypersensitivities enabling researchers to analyse separate pain mechanisms.

Our group utilised a clinical intradermal capsaicin pain model in an attempt to investigate a relationship between capsaicin-induced nociception and systemic endotoxin (lipopolysaccharide (LPS)) (Hutchinson et al., 2013). This model used multiple behavioural measures, including spontaneous pain, mechanical allodynia, punctate mechanical hyperalgesia and flare, in order to elucidate either a central or peripheral effect. Endotoxin potentiated capsaicin-induced hyperalgesia, allodynia and flare, suggesting both central and peripheral actions, which required further investigation (Hutchinson et al., 2013). It is necessary to recreate the observed endotoxin potentiated capsaicin response in a preclinical model in order to ask detailed molecular and cellular questions within the CNS.

In our preclinical capsaicin model, studies revealed an attenuation of capsaicin-induced hypersensitivity due to pre-treatment injection timing, highlighting the complexities present in even this basic pain model. Of particular concern are the effects of endogenous opioid responses following a subcutaneous application of capsaicin to the hindpaw, an effect known as stress-induced analgesia (SIA) (Butler et al., 2009). Common endogenous opioid classes include endorphins, enkephalins, dynorphins and endomorphins which can be released following stressful stimuli, acting at classical opioid receptors (Feng et al., 2012; Labuz et al., 2016; Stein et al., 2003). Classical opioid receptors include μ-, κ- and δ-opioid receptors (MOR, KOR, DOR) (Ahlbeck, 2011; Feng et al., 2012). Upon activation, alteration of ion channel function attenuates production and release of neurotransmitters, subsequently reducing neuronal activity (Feng et al., 2012; Stein et al.,

2003). Further, the relatively non-selective opioid antagonist naloxone blocks opioid mediated SIA in multiple rodent models, including electroacupuncture, intraperitoneal hypertonic saline administration and foot shock (Hemingway et al., 1987; Watkins et al., 1982; Wright et al., 1985). There is evidence that endogenous opioid peptides also attenuate mechanical hypersensitivity in a mouse neuropathic pain model (Labuz et al., 2016). The potential of an endogenous opioid response in our preclinical capsaicin pain model complicates the endotoxin-capsaicin effect we are aiming to replicate. Therefore, it is necessary to investigate the potential role of opioid receptor activation following intraplantar (i.pl.) capsaicin administration.

Classical opioid responses are not the only means by which stress-induced analgesia occurs. Non-classical opioid effects refer to morphine-like analgesia which do not engage classical opioid receptors (McDonald et al., 2013). Glutamate, γ-aminobutyric acid (GABA), endocannabinoids and monoamines are examples of endogenous non-classical opioid compounds, due to their role in descending inhibitory facilitation (Butler et al., 2009). Synthetic, clinically approved compounds include Tapentadol, which inhibits norepinephrine re-uptake and MOR activation, however retains efficacy in MOR knockout models (Langford et al., 2016) (Kogel et al., 2011). Likewise, stereoisomers of classical opioid antagonists (+)-naltrexone and (+)-naloxone are able to attenuate neuropathic pain in rodents, despite lack of affinity to classical receptors (Hutchinson et al., 2008; Lewis et al., 2012; Valentino et al., 1983). Nociceptin/orphanin GQ (N/OFQ) receptor (NOP) is an identified non-classical opioid receptor. NOP is structurally homologous to classical opioid receptors but shows low affinity towards their agonists and is therefore recognised as a non-classical opioid receptor (Mollereau et al., 1994; Toll et al., 2016).

This study aimed to investigate the effects of i.pl. administration on capsaicin-induced mechanical hypersensitivity. I.pl. administration of saline (5 µL) 15 min before capsaicin challenge attenuates mechanical hypersensitivity in BALB/c mice. Both (+)-and (-)-naltrexone reversed the attenuated capsaicin response, while immunohistochemical examination showed no effect on c-Fos expression in the spinal cord. Both naltrexone stereoisomers were able to extend hypersensitivity, however only when administered post-capsaicin. The results suggest a local endogenous non-classical opioid response alters

neuronal function, the effects of which are dependent on the current activity levels of nociceptive circuitry.

6.3. Methods

6.3.1 Animals

Male adult BALB/c mice (6-14 weeks) (n = 6-10 per group) obtained from Laboratory Animal Services of the University of Adelaide (Adelaide, SA) were used in these experiments. All mice were group-housed in the Medical School Animal Facility of the University of Adelaide. Mice were allowed free access to food and water in a temperature-controlled environment (23 ± 3°C) maintained on a 12:12 h light/dark cycle (lights on at 0700h). Animals were left to acclimatise to the facility for at least one week before handling began. One week of handling and acclimatisation to the experimental set-up was carried out prior to use. All experiments reported in this study were approved by the University of Adelaide Animal Ethics Committee (Ethics approval number M-227-13B) and complied with the Australian code for the care and use of animals for scientific purposes.

6.3.2 Materials

Capsaicin in 38% (2-hydroxy-propyl)-β-cyclodextrin was kindly donated by the PARC clinical research group of the University of Adelaide (Adelaide, SA). (-)-Naltrexone was kindly provided by Dr Andrew Somogyi (University of Adelaide, School of Medicine, Adelaide, SA). (+)-Naltrexone was kindly donated by Dr Kenner Rice (Chemical Biological Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD). (2-Hydroxypropyl)-β-cyclodextrin was purchased from Sigma-Aldrich (Castle Hill, NSW). Sterile saline (0.9%) was purchased from Baxter Australia (Old Toongabbie, NSW, Australia). Von Frey filaments were purchased from North Coast Medical Inc. (Gilroy, California). PBS (10X) (137 mM sodium chloride, 2.7 mM potassium chloride, 1.5mM dihydrogen potassium orthophosphate, 8 mM disodium hydrogen orthophosphate (purchased from Chem Supply, South Australia, Australia)) was made and diluted in on-site Milli-Q filtered H_2O. Rabbit @ c-Fos antibody (K-25: sc-253) was purchased from Santa Cruz Biotechnology, Texas, USA. Donkey @ Rabbit secondary antibody and 3,3'-

Diaminobenzidine (DAB) substrate were purchased from Vector Laboratories (California, USA). Pierce™ streptavidin peroxidase-conjugated antibodies were purchased from ThermoFisher Scientific (North Ryde, NSW).

(2-Hydroxypropyl)-β-cyclodextrin was diluted in 0.9% sterile saline to make a 38% solution. A stock capsaicin solution of 5 mg/mL was created using 38% (2-Hydroxypropyl)-β-cyclodextrin, protected from light and stored at 4°C for a maximum of 1 month. On the morning of use, this was further diluted in (2-Hydroxypropyl)-β-cyclodextrin to the experimental concentration. Both (-)- and (+)-naltrexone were made up to the desired concentration in 0.9% sterile saline the week of the experiment and kept at 4°C.

6.3.3 Intraplantar drug administration

All capsaicin and β-cyclodextrin administrations were conducted using the following technique. All injections were performed with a primed 25 μL Hamilton micro-syringe and 30G needle. To prime the micro-syringe, the plunger was removed, and 0.9% saline was injected into the barrel of the syringe until no air remained inside, the plunger was then reinserted. A 30G needle was attached to a 1 mL syringe containing 0.9% saline and saline was dispensed, filling the needle tip. The 30G needle tip was then carefully removed, filled with 0.9% saline and attached to the full micro-syringe; leaving no air inside either the micro-syringe or needle tip. The plunger was then pushed all the way down, and pulled up to the 5 μL mark, creating a small air bubble. The desired volume of solution could then be withdrawn and dispensed. Animals were gently restrained in a thin towel and 5 μL of the solution was injected at the distal end of the plantar surface, directly under the skin at the footpads (Fig. 6.1).

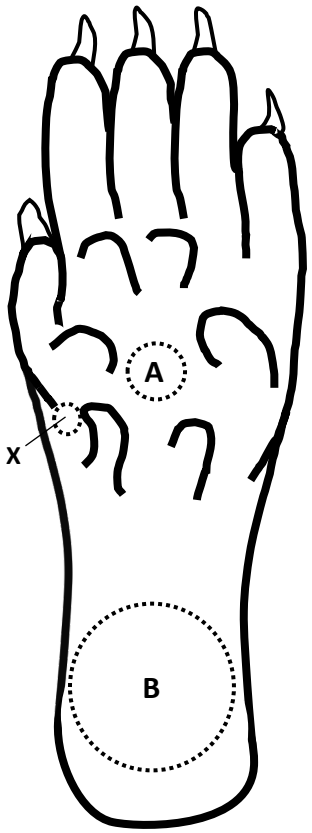


Figure 6.1: Intraplantar (i.pl.) drug administration. (X) represents the site of needle insertion, (A) represents the site of drug injection, (B) represents the site of von Frey mechanical stimulation.

6.3.4 Behavioural testing

A von Frey test modified from the Nicotra et al. (2012) method was performed on all animals. Immediately following i.pl. drug administration, animals were placed on a wire mesh shelf that allowed access to the plantar surface of the hindpaw and covered with an opaque cup. Von Frey filaments were touched to the proximal end of the plantar surface of the hindpaw to allow assessment of secondary hypersensitivity, as elicited in the clinical model (Fig. 6.1). Force was then applied until the filament bent and held for two seconds before being slowly removed for one second and reapplied. Animals were tested up to 180 min following i.pl. drug administration. All animals received 10 applications of each von Frey filament per time point, with a minimum of 8 min between application of a different

filament strength. Baseline testing was conducted 24 h prior to i.pl. capsaicin administration for all experiments.

6.3.5 Naltrexone studies

6.3.5.1 Intermediate saline

BALB/c mice were administered (-)-naltrexone (6 mg/kg) or equivalent volume saline by intraperitoneal (i.p.) injection 15 min pre i.pl injection of saline. 15 min later animals were administered capsaicin (0.4 µg/5 µL; i.pl.) or the equivalent volume of β-cyclodextrin (Bazzo et al., 2013). Animals were tested with filaments 2.44 (0.04 g), 3.22 (0.16 g) and 3.84 (0.6g) at 7, 45, 90 and 135 min following capsaicin administration.

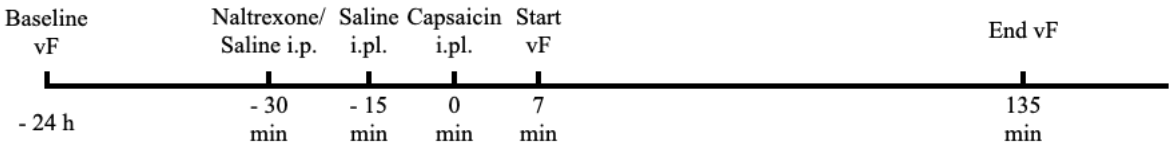


Figure 6.2: Intermediate saline effect. Time course of drug administration and behavioural testing investigating pre-capsaicin i.pl. injection stress effect.

6.3.5.2 Naltrexone pre-capsaicin

BALB/c mice were administered (-)- or (+)-naltrexone (6 mg/kg) or equivalent volume saline (i.p.) 30 min prior to capsaicin (0.4 µg/5 µL; i.pl.) or the equivalent volume of β-cyclodextrin (Bazzo et al., 2013). Animals were tested with filaments 2.44 (0.04 g), 3.22 (0.16 g) and 3.84 (0.6g) at 7, 45, 90 and 135 min following capsaicin administration.

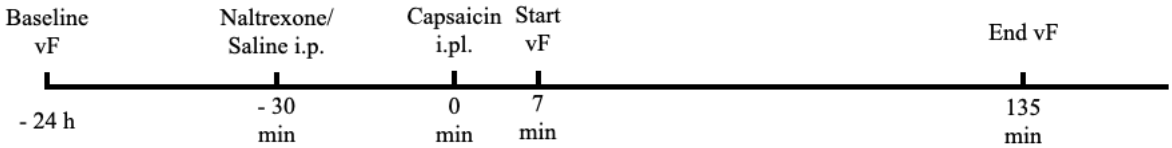


Figure 6.3: Pre-naltrexone effect on capsaicin-induced hypersensitivity. Time course of drug administration and behavioural testing investigating pre-capsaicin naltrexone.

6.3.5.3 Naltrexone post-capsaicin

BALB/c mice were administered (-)- or (+)-naltrexone (6 mg/kg) 30 min post capsaicin (0.4 µg/5 µL; i.pl.) or (-)-naltrexone (6 mg/kg) 110 min post capsaicin (0.4 µg/5 µL; i.pl.) (Bazzo et al., 2013). Control animals were administered equivalent volume saline (i.pl.) at the same time points. Animals were tested with filaments 2.44 (0.04 g), 3.22 (0.16 g) and 3.84 (0.6g) at 7, 45, 90 and 135 min following capsaicin administration. Animals administered (-)-naltrexone at 110 min, were also tested at 180 min following capsaicin.

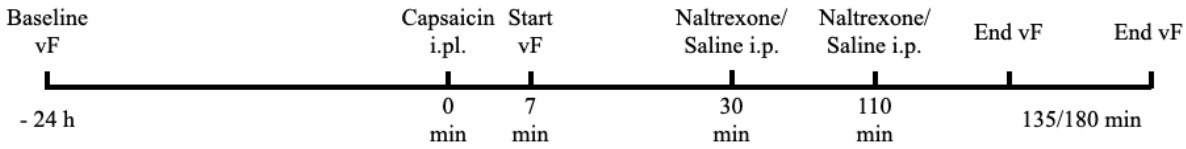


Figure 6.4: Post-naltrexone effect on capsaicin-induced hypersensitivity. Time course of drug administration and behavioural testing investigating post-capsaicin naltrexone.

6.3.6 Tissue collection

Animals were administered (-)-naltrexone (6 mg/kg) 30 min following capsaicin (0.4 µg/5 µL; i.pl.). 2 h following capsaicin, animals were sacrificed by i.p. sodium pentobarbitone (300 ng/ kg), immediately followed by transcardial perfusion with 4% paraformaldehyde (PFA) and flushed with sterile PBS (pH 7.4). The spinal column and brain were dissected and post fixed in 4% PFA for 48 h. Tissue was then washed twice for 30 min in sterile PBS (pH 7.4); the lumbar spinal cord (SC) was dissected out. Tissue was then stored in 70% ethanol before overnight processing in a Sakura Tissue Tek VIP Junior Vacuum Infiltration Processor (Olympus, Notting Hill, Australia). Tissue was then embedded in paraffin using a Sakura Tissue Tek TEC 5 Embedding Centre (Olympus, Notting Hill, Australia). Tissue was cut into 10 µm slices using a Leica RM2125 RT microtome (Leica Microsystems, Mt Waverly, Australia) and mounted on Superfrost microscope slides.

6.3.7 Immunohistochemistry

Slides were placed in an oven at 37°C for 15 min, then into a Dako CoverStainer (Santa Clara, United States) for de-waxing. Slides were removed, washed in water and placed in methanol containing 3% H_20_2 for 30 min. Slides were washed twice in PBS for 5 min. Heat-induced epitope antigen retrieval was performed in 10 mM sodium citrate (pH 6).

Slides in sodium citrate were brought to the boil and kept at 95°C for 10 min. Slides were allowed to cool to room temperature (RT) before two, 5 min washes in 1X PBS. Slides were dried and 3.3% normal horse serum (NHS) applied to each section for 30 min in a humidifying chamber at RT. NHS was removed and c-Fos antibody (1:3000) was added to each section. Slides were left in a humidifying chamber overnight at RT. Slides were washed twice in 1X PBS for 5 min and biotin conjugated Donkey @ Rabbit (1:250) diluted in 1X PBS was applied for 30 min at RT. Slides were washed twice for 5 min in 1X PBS and Pierce™ streptavidin peroxidase-conjugated antibodies (ThermoFisher Scientific) (1:1000) were applied for 60 min at RT. Slides were then washed in 1X PBS twice for 5 min before DAB was applied for 7 min. DAB was removed and slides washed in running water for 10 min before haematoxylin counterstaining and cover slipping in a Dako CoverStainer (Santa Clara, United States). Slides were allowed to dry overnight and imaged using a NanoZoomer (Hamamatsu C10730-12 2.0RS, Shimokanzo, Japan). Staining in dorsal horn lamina I - III was analysed for c-Fos positive cells and area stained using Image J (1.52t, Maryland, USA).

6.3.8 Analysis

Data was analysed using GraphPad Prism v7.0a (GraphPad software, Inc. San Diego, CA). Two-way ANOVA with repeated measures and Tukey's multiple comparison test was used to compare treatment effect over time for all behavioural experiments with the following exception. Sidak's multiple comparisons test was used to compare treatment groups at individual time points for (-)-naltrexone administered at 110 min post capsaicin and to compare individual time points to baseline for all experiments. One-way ANOVA with Tukey's multiple comparison test was used to compare treatment effect in each of the spinal cord sections analysed. P-values ≤ 0.05 were considered statistically significant.

6.4 Results

6.4.1 I.pl. saline attenuates i.pl. capsaicin-induced mechanical hypersensitivity when administered 15 min prior, an effect reversed by (-)-naltrexone

An i.pl. injection of capsaicin produces rapid hypersensitivity in BALB/c mice. This response was not observed when saline (i.pl.) is administered just 15 min before capsaicin

(Fig. 6.5). No values significantly differed from baseline at the lowest filament; a significant difference was observed for the 0.16g ($p < 0.01$) and 0.6g ($p < 0.05$) filaments at 7 min only (Fig. 6.5). The magnitude of these differences was much lower than observed in previous studies. (-)-Naltrexone (6 mg/kg) administered 30 min before capsaicin, reversed this effect. Two-way ANOVA revealed an effect of time (Fig. 6.5A. $F_{(2.5,52)} = 36.72$, $p < 0.0001$, Fig. 6.5B. $F_{(2.6,55)} = 50.81$, $p < 0.0001$, Fig. 6.5C. $F_{(3.3,70)} = 66.96$, $p < 0.0001$) and treatment (Fig. 6.5A. $F_{(2,21)} = 15.31$, $p < 0.0001$, Fig. 6.5B. $F_{(2,21)} = 19.98$, $p < 0.0001$, Fig. 6.5C. $F_{(2,21)} = 17.57$, $p < 0.0001$), as well as an interaction effect (Fig. 6.5A. $F_{(8,84)} = 11.98$, $p < 0.0001$, Fig. 6.5B. $F_{(8,84)} = 16.93$, $p < 0.0001$, Fig. 6.5C. $F_{(8,84)} = 16.14$, $p < 0.0001$), on von Frey responses for all filaments tested. Post hoc analysis revealed capsaicin-induced hypersensitivity was significantly greater in the (-)-naltrexone pre-treated group versus the saline pre-treated for all filaments at 7 ($p < 0.01$) and 45 min ($p < 0.05$) (Fig. 6.5). Interestingly for the heaviest filaments an extension in hypersensitivity was observed until 90 min ($p < 0.05$). Von Frey responses were also significantly different for the (-)-naltrexone-capsaicin group compared to the (-)-naltrexone-vehicle control at 7 ($p < 0.01$) and 45 min ($p < 0.05$) for all filaments tested (Fig. 6.5). Like the saline-capsaicin group, an effect was observed for the (-)-naltrexone-vehicle group compared to baseline at 7 min for the heaviest filament ($p < 0.05$). At all other points no significant difference from baseline were observed. Interestingly, (-)-naltrexone alone appeared to cause extended hypersensitivity at higher doses (60 mg/kg) (Supp. Fig. 6.1)

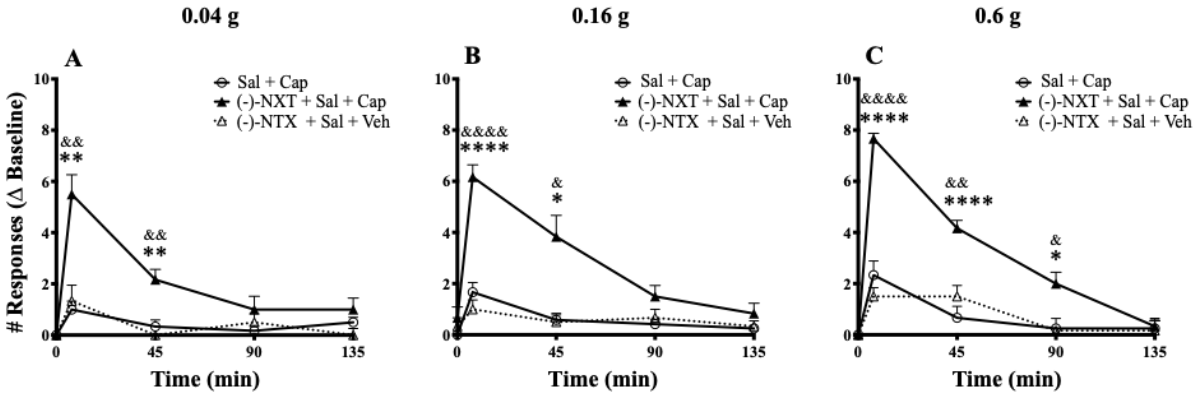


Figure 6.5. I.pl. saline 15 min pre-capsaicin attenuates mechanical hypersensitivity; an affect is reversed by (-)-naltrexone. Mice were administered 0.9% saline (i.pl.) 15 min prior to capsaicin (0.4 μg/5 μL; i.pl). (-)-Naltrexone (6 mg/kg; i.p.) or saline (0.9%; i.p.) were administered 15 min prior to saline (0.9 %; i.pl.), followed by either capsaicin (0.4 μg/5 μL; i.pl) or vehicle (5 μL; i.pl). Testing included 10 applications of von Frey filaments (0.04 (A), 0.16 (B), 0.6g (C)) and was performed at 7, 45, 90 and 135 min following capsaicin administration. n = 12 for saline + capsaicin, n = 6 for all other groups. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ (-)-naltrexone + saline + capsaicin vs saline + capsaicin; & $p < 0.05$, && $p < 0.01$, &&&& $p < 0.0001$ (-)-naltrexone + saline + capsaicin vs (-)-naltrexone + saline + vehicle. Error bars represent SEM.

6.4.2 Neither (+)- or (-)-naltrexone affect capsaicin-induced mechanical hypersensitivity when administered pre-capsaicin.

To examine the effects of naltrexone on capsaicin-induced hypersensitivity the 'intermediate' i.pl. saline injection was removed from the protocol; leaving a single i.p. administration of (+)- or (-)-naltrexone 30 min prior to i.pl. capsaicin. Two-way ANOVA revealed an effect of time (Fig. 6.6A. $F_{(3,63)} = 40.24$, $p < 0.0001$, Fig. 6.6B. $F_{(2.6,55)} = 38.64$, $p < 0.0001$, Fig. 6.6C. $F_{(3.2,66)} = 66.32$, $p < 0.0001$) on von Frey reposes for all filaments tested. A treatment effect was also observed for the 0.04 and 0.16 g filaments (Fig. 6.6A. $F_{(2,21)} = 5.13$, $p = 0.0153$, Fig. 6.6B. $F_{(2,21)} = 4.49$, $p = 0.0205$) but not for the 0.6g filament (Fig. 6.6C. $F_{(2,21)} = 1.603$, $p = 0.2249$). No interaction effect was observed for all filaments (Fig. 6.6A. $F_{(8,84)} = 1.395$, $p = 0.211$, Fig. 6.6B. $F_{(8,84)} = 1.232$, $p = 0.2909$, Fig. 6.6C. $F_{(8,84)} = 1.310$, $p = 0.2497$). Post hoc analyses revealed there was no significant difference between the (-)-naltrexone group and saline control at any time point tested (Fig. 6.6). Interestingly when (+)-naltrexone was administered hypersensitivity was observed at 135 min ($p < 0.05$) for the intermediate filament (0.16g) (Fig. 6.6).

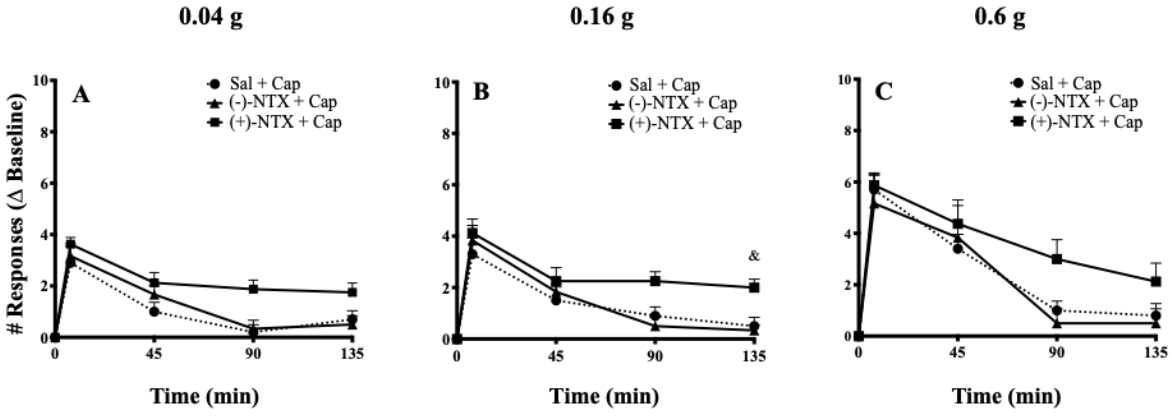


Figure 6.6. Neither (-)- or (+)-naltrexone effect i.pl. capsaicin-induced hypersensitivity. (+)-Naltrexone, (-)-naltrexone (6 mg/kg; i.p.) or saline were administered 30 min prior to capsaicin (0.4 μg/ 5 μL; i.pl.). Von Frey testing was conducted 7, 45, 90 and 135 min post capsaicin administration. n = 10 for saline group, n = 8 for (+)-naltrexone group, n = 6 for (-)-naltrexone group. & $p < 0.01$ (+)-naltrexone vs (-)-naltrexone. Error bars represent SEM.

6.4.3 Both (+) and (-)-naltrexone administered post capsaicin extend hypersensitivity.

Following the observed (+)-naltrexone effect (Fig. 6.6) and extended hypersensitivity observed at high (-)-naltrexone doses (Supp. Fig. 6.1); (+)- and (-)-naltrexone were administered 30 min post-capsaicin. We observed a significant effect of time (Fig. 6.7A. $F_{(3.3,70)} = 53.15$, $p < 0.0001$, Fig. 6.7B. $F_{(2.8,58)} = 74.28$, $p < 0.0001$, Fig. 6.7C. $F_{(3.0,62)} = 149.8$, $p < 0.0001$) and treatment ((Fig. 6.7A. $F_{(2,21)} = 6.988$, $p = 0.0047$, Fig. 6.7B. $F_{(2,21)} = 22.63$, $p < 0.0001$, Fig. 6.7C. $F_{(2,21)} = 42.89$, $p < 0.0001$) on von Frey responses for all filament strengths. An interactive effect was also observed for all filaments (Fig. 6.7A. $F_{(8,84)} = 3.17$, $p = 0.0034$, Fig. 6.7B. $F_{(8,84)} = 4.99$, $p < 0.0001$, Fig. 6.7C. $F_{(8,84)} = 13.43$, $p < 0.0001$). Of interest were the group differences at 45 min onwards (as naltrexone was introduced at 30 min). Post hoc analysis revealed that 15 min following naltrexone administration (+)-naltrexone produced significantly higher scores for the 0.04g ($p < 0.05$), 0.16g ($p < 0.0001$) and 0.6g ($p < 0.0001$) filaments compared to saline control animals. In contrast, (-)-naltrexone was significantly different than the saline group at 45 min using only the heaviest filament (0.6g) ($p < 0.0001$). Both (+)- and (-)-naltrexone produced significantly higher von Frey scores at 90 and 135 min post capsaicin (Fig. 6.7). There was no significant difference between the (+)- and (-)- naltrexone groups at any time point (Fig. 6.7). In the absence of capsaicin, (+) - and (-)-naltrexone had no effect on von Frey responses; no significant differences were observed from baseline at all time points (data not shown).

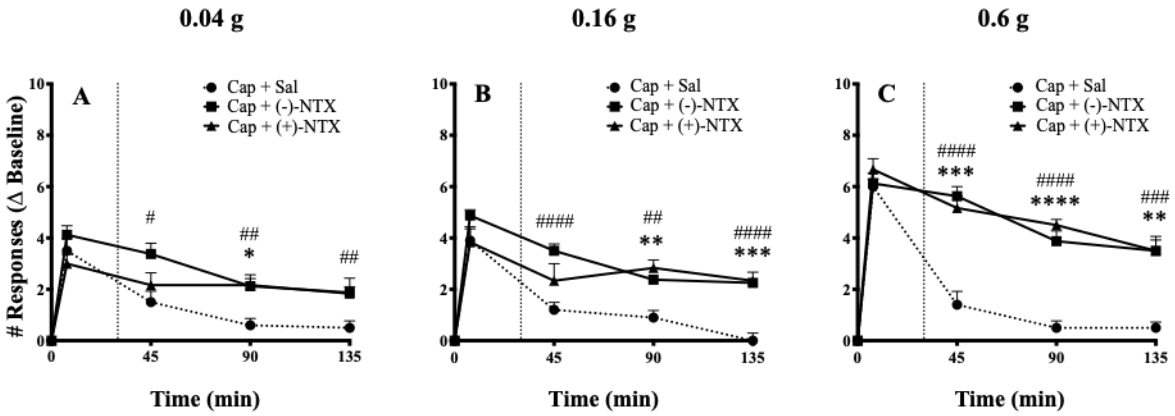


Figure 6.7. (+)- and (-)-naltrexone extended hypersensitivity when administered 30 min following i.pl. capsaicin. (+)-Naltrexone, (-)-naltrexone (6 mg/kg; i.p.) or saline were administered 30 min (dotted line) following capsaicin (0.4 μg/ 5 μL; i.pl.). Von Frey testing was conducted 7, 45, 90 and 135 min post-capsaicin. n = 10 for saline group, n = 8 for (+)-naltrexone group, n = 6 for (-)-naltrexone group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (-)-naltrexone vs saline; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, #### $p < 0.0001$ (+)-naltrexone vs saline. Error bars represent SEM.

6.4.4 Administration of (-)-naltrexone at later time points does not restore mechanical hypersensitivity following capsaicin.

Following the extended hypersensitivity observed above we asked whether addition of naltrexone at later time points would reverse a return to baseline. Using (-)-naltrexone we observed no difference compared to control when administered 110 min following capsaicin (Fig. 6.8). Two-way ANOVA revealed an effect of time (Fig. 6.8A. $F_{(2.9,29)}$ = 19.18, $p < 0.0001$, Fig. 6.8B. $F_{(2.8,28)} = 21.68$, $p < 0.0001$, Fig. 6.8C. $F_{(2.9,29)} = 31.51$, $p < 0.0001$), however, no treatment (Fig. 6.8A. $F_{(1,10)} = 0.62$, $p = 0.4508$, Fig. 6.8B. $F_{(1,10)}$ = 1.74, $p = 0.2163$, Fig. 6.8C. $F_{(1,10)} = 0.08$, $p = 0.7861$) or interaction effect (Fig. 6.8A. $F_{(5,50)} = 0.14$, p -= 0.98, Fig. 6.8B. $F_{(5,50)} = 0.91$, $p = 0.4862$, Fig. 6.8C. $F_{(5,50)} = 1.89$, $p = 0.1137$), on von Frey responses for all filaments tested. Post hoc analysis revealed no difference between groups at any time point. For the lightest filament (0.04g) there was no time point significantly greater than baseline past (-)-naltrexone administration. For the 0.16g filament, the (-)-naltrexone group only was significantly higher than baseline at 135 min ($p < 0.05$), neither group was statistically different than baseline at 180 min. Similarly, (-)-naltrexone group only was significantly higher than baseline for the 0.6g filament at 180 min ($p < 0.01$), neither group differed from baseline at 135 min (Fig. 6.8). Reflecting these results, we observe no effect when (-)-naltrexone is delivered at 155 min post capsaicin; no group differences and no change from baseline post (-)-naltrexone administration (Supp. Fig. 6.2).

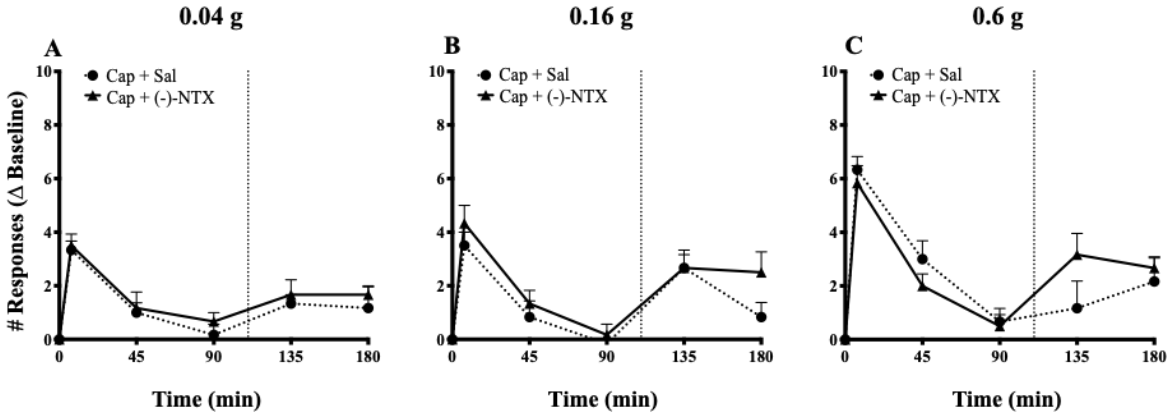


Figure 6.8. Administration of (-)-naltrexone 110 min following i.pl. capsaicin does not restore mechanical hypersensitivity. (-)-Naltrexone (6 mg/kg; i.p.) or saline were administered 110 min (dotted line) following capsaicin (0.4 μg/ 5 μL; i.pl.). Von Frey testing was conducted 7, 45, 90, 135 and 180 min post-capsaicin. n = 6 for all groups. Error bars represent SEM.

6.4.5 Spinal cord c-Fos is unaltered by application of (-)-naltrexone applied 30 min following capsaicin administration.

Neuronal activation marker c-Fos was investigated 120 min post capsaicin and 90 min post (-)-naltrexone, a time point where we observed significantly higher sensitivity compared to controls (Fig. 6.7). No changes were observed, in either the contralateral or ipsilateral dorsal horn of the lumbar spinal cord (Fig. 6.9). One-way ANOVA revealed no effect of treatment on cell count or area stained for all ipsilateral and contralateral spinal cord sections (Supp. Tab. 6.1). Post hoc analysis revealed no differences between treatments at all cord segments.

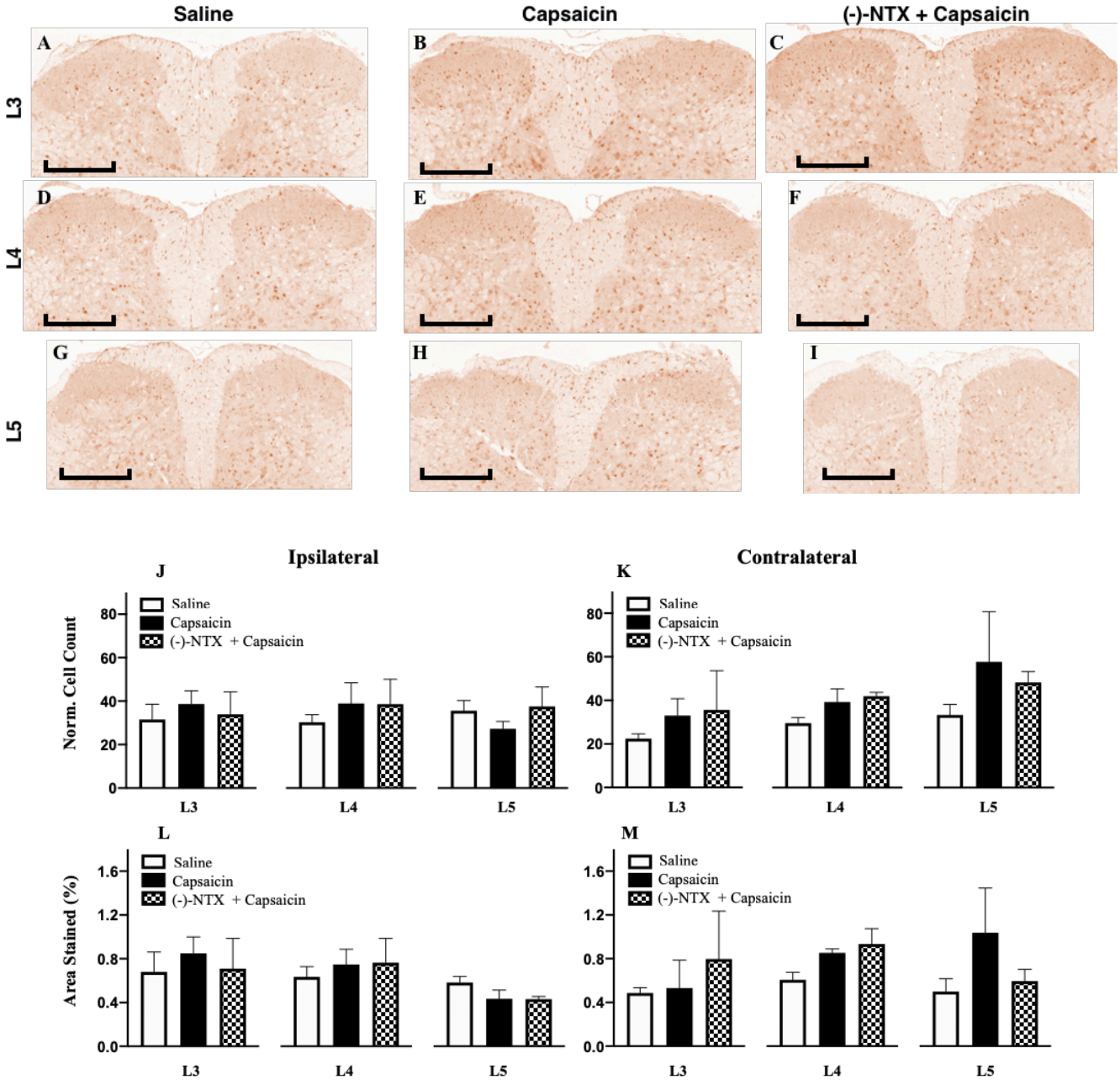


Figure 6.9. Administration of (-)-naltrexone 30 min post capsaicin does not alter c-Fos expression in the lumbar spinal cord lamina 1-3. C-Fos staining in the lumbar spinal cord 90 min following (-)-naltrexone (6 mg / kg; i.p.) and 120 min following capsaicin (0.4 µg/ 5 µL; i.pl.). Representative images at L3 (A, B, C), L4 (D, E, F) and L5 (G, H, I) of saline (A, D, G), capsaicin (B, E, H) and (-)-naltrexone-capsaicin (C, F, I) groups. Scale bar represents 250 µm. J and K represent the number of c-Fos positive cells in the ipsilateral and contralateral dorsal horn respectively. L and M represent the area stained by c-Fos antibody as a percentage of ipsilateral and contralateral dorsal horn lamina 1-3 respectively. n = 2, error bars represent SEM.

6.5 Discussion

The aim of this study was to investigate the effect of i.pl. administration on capsaicin-induced mechanical hypersensitivity. This would enable improved interpretation of i.pl. capsaicin behavioural data by gaining a more rounded multi-system understanding. We observed injection-induced attenuation of capsaicin hypersensitivity, which was reversed by both opioid receptor antagonist (-)-naltrexone and its opioid inactive stereoisomer (+)-naltrexone. Further to this, we were able to extend capsaicin-induced hypersensitivity when naltrexone was administered during a period of behavioural sensitivity post-capsaicin. Analysis of CNS c-Fos activation revealed no increase in c-Fos positive cells in the spinal cord dorsal horn that would account for the behavioural observations. These results suggest i.pl. drug administration recruits both endogenous opioid and non-classical opioid systems, and the consequences of these systems depends upon the current level of activity within the nociceptive pathway.

We were able to demonstrate that an i.pl. injection stressor 15 min before capsaicin administration attenuated the magnitude of hypersensitivity in BALB/c mice. To the best of our knowledge, the effect of an i.pl. injection stressor on nociceptive hypersensitivity has not been reported. Injection-induced stress effects in rodent models are well documented. I.p. saline administration in rats increases blood and tissue (ventral hippocampus) corticosterone, and decreases blood nitric oxide (Freiman et al., 2016). These findings suggest a single injection was able to activate the hypothalamic-pituitary-adrenal (HPA) axis which is involved in stress responses (Freiman et al., 2016). Further, corticosterone is elevated 30 min following intraperitoneal injection in male BALB/c mice, again suggesting involvement of the HPA axis (Drude et al., 2011). Rats and mice have also been used to show the effects of restraint handling during injection, with increased restraint resulting in increased blood cortisone; an area of concern for i.pl. administration which requires significant animal restraint (Meijer et al., 2006; Stuart et al., 2015). While there is no doubt that handling and injection cause stress responses in animal models, the mechanisms behind the analgesia reported here requires further investigation.

Stress-induced analgesia (SIA) is a well-documented phenomena with multiple potential mechanistic drivers including opioid, endocannabinoid and monoaminergic systems acting at all levels of pain circuitry (Butler et al., 2009). Injection stressors are not used to study SIA, making comparisons to this work difficult. I.p. injection of hypertonic saline causes

reduced tail withdrawal latency to radiant heat in rats, however in this case it appears the effect is due to the hypertonic nature of saline rather than the injection itself (Wright et al., 1985). Opioid antagonist naloxone did not reverse this effect but enhanced analgesia, again suggesting the mechanism is dissimilar to what we observe here (Wright et al., 1985). Electric foot shocks are a commonly used stressor in both clinical and preclinical pain models, and perhaps the most relevant method to the i.pl. effect we observe here. Both mouse and rat studies have used intrathecal naloxone to block the analgesic effects of foot shock (Chesher et al., 1977; Hemingway et al., 1987; Watkins et al., 1982). One study reported location-dependent opioid actions, with reversal of analgesia observed in forepaw, but not hindpaw (Watkins et al., 1982). This is in contrast to our study which found hindpaw analgesia was resolved by both classical and non-classical opioid antagonism. An interesting finding here is that naloxone could not reverse analgesia past onset; we did not administer naloxone between saline injection and capsaicin to test this in our model (Watkins et al., 1982). Such an experiment could be conducted in future to determine if our analgesia is similarly spinally mediated. Acupuncture-induced analgesia is another, less commonly utilised stressor with potentially similar impact to our model. In rabbits, naloxone reverses tail withdrawal analgesia induced by electroacupuncture (Zakusov et al., 1981). While SIA is a possible cause of the observed analgesia further characterisation of opioid vs non-classical opioid mechanism and peripheral or central action needs to be determined.

Both classical and non-classical opioid antagonists administered prior to the injection stressor, reversed analgesia in this study. Interestingly, not only was the analgesia reversed but hypersensitivity was observed at 90 min following stimulation with the highest filament. I.pl. capsaicin-induced hypersensitivity has never lasted 90 min in this, or any of our previous experiments utilising i.pl. capsaicin at higher doses. Evidence from the wider pain community for similar and higher capsaicin doses also shows resolution of hypersensitivity prior to 90 min using a von Frey test (Abdelhamid et al., 2013; Carey et al., 2016; Laird et al., 2001b). This suggests an effect of (+)- and (-)-naloxone in not only reversing attenuation but extending allodynia, an effect observed both with and without the intermediary i.pl. saline injection.

To further investigate, we administered naltrexone at later time points to observe their effect on the duration of capsaicin-induced hypersensitivity. Naloxone administered 30

min post capsaicin caused extensions of hypersensitivity for the duration of the testing period (135 min), significantly longer than that observed due to pre-capsaicin naltrexone. These results mimic those observed in a mouse complete Freund's adjuvant (CFA) pain model, which administered (-)-naltrexone post i.pl. administration (Corder et al., 2013). An inflammatory pain model, CFA produces mechanical hypersensitivity lasting 10 days, however (-)-naltrexone was infused throughout this entire resolution phase. Mechanical hypersensitivity remained elevated until the infusion was ended. The authors postulate a MOR mediated inhibition of pain is blocked by (-)-naltrexone (Corder et al., 2013). In contrast to our study however, the authors were able to reinstate mechanical and thermal hypersensitivity when (-)-naloxone was administered (i.p.) after initial resolution. They were also able to show that peripherally restricted naltrexone (naltrexone methobromide) had no effect, suggesting a central site of action (Corder et al., 2013). The authors propose enhanced pronociceptive synaptic strength via an N-methyl-D-aspartate (NMDA) mediated adenyl cyclase activation. While this is in contrast to our result which shows no reinstatement of hypersensitivity (Fig. 6.8), it agrees with multiple clinical and preclinical studies. In mice, surgical incision and remifentanil administration-induced mechanical hypersensitivity resolves by day 20. At day 21 mechanical hypersensitivity was reinstated by (-)-naloxone, but not (+)-naloxone or peripherally acting naloxone methiodide. Again, the authors identified the effect was centrally mediated, however through KOR, with involvement of NMDA receptors (NMDAR) (Campillo et al., 2011). Similarly, primary and secondary mechanical hypersensitivity was reinstated in a concentration dependent manner by (-)-naloxone in mice and humans following mild heat shock (Pereira et al., 2015). NMDARs are also implicated in non-nociceptive stress-induced latent pain sensitisation in rats (Le Roy et al., 2011). Latent sensitisation is a theory which postulates central sensitisation outlasts signs of hyperalgesia which can be reversed by antagonism of central opioid actions (Pereira et al., 2015). While much evidence exists for this process, our results only partially corroborate an opioid-induced latent central sensitisation due to the nature of classical and non-classical opioid extension of hypersensitivity.

While limited, there are studies which indicate a possible mechanism by which opioid action can increase intracellular calcium and neurotransmitter/neurotrophic factor release (Samways et al., 2006). Enkephalin, noradrenaline and adenosine are released from guinea-pig myenteric plexus, rat spinal cord and SH-SY5 cells respectively following opioid exposure (Cahill et al., 1993; McDonald et al., 1996; Xu et al., 1992). However, in

each case concomitant stimulation of a secondary receptor (muscarinic receptor, N-type voltage-operated calcium channels and G_i/G_o-coupled neuropeptide Y receptors respectively) was required. Similarly, in mouse dorsal root ganglion neurons capsaicin-induced calcium is able to induce a rapid increase (30 s - 10 min) of surface DOR. Upon activation, DOR's are able to release internal calcium stores leading to sustained elevated calcium (Bao et al., 2003). We suggest this mechanism warrants further investigation based on the context of this behavioural data. The rapid increase in surface DOR following capsaicin perhaps accounts for the observed potentiated responses only in the period immediately following capsaicin administration. Further evidence of naloxone-induced potentiation of hypersensitivity can be found in freezing behaviour induced by electric shock in rats (Fanselow et al., 1979). The authors suggest by blocking endogenous analgesic action, the shocks become more of an aversive stimulus, resulting in amplified and extended shock-induced behaviours (Fanselow et al., 1979). While we do not observe amplified hypersensitivity, we do observe an extended response. Interestingly the authors also note that timing is crucial to this effect. Naltrexone must be present before the shock, with no effect observed if naltrexone is administered post shock, in contrast to what we see here (Fanselow et al., 1979). The possibility of opioid and non-opioid mediated increases in intracellular calcium after TRPV1 activation offer an interesting alternative explanation to what we observe and warrants further study.

Despite the mechanisms, the central and/or peripheral origins of the naloxone effect need to be determined. Future studies should consider the use of peripheral or centrally restricted antagonists, such as those used in the studies discussed above. We used multiple filament strengths in this study in order to attempt to behaviourally distinguish mechanism. The lowest fibre we used elicited less than one response from 10 at baseline, considered completely innocuous (data not shown). The highest filament we used elicited an average of 2 responses from 10 and was considered noxious (data not shown). This gave the study the possibility of distinguishing between an allodynic (innocuous filament) and hyperalgesic (noxious filament) mechanism. Secondly, stimulating away from the site of injury (Fig. 6.1) narrowed focus on changes induced by central mechanisms. Including an array of behavioural tests in this protocol would have been beneficial. Firstly, to monitor spontaneous behaviours which are clinically relevant and secondly a wider range of elicited behaviours (Backonja et al., 2004). Mechanical sensitivities at the site of administration would have allowed for peripheral sensitisation effects but may require

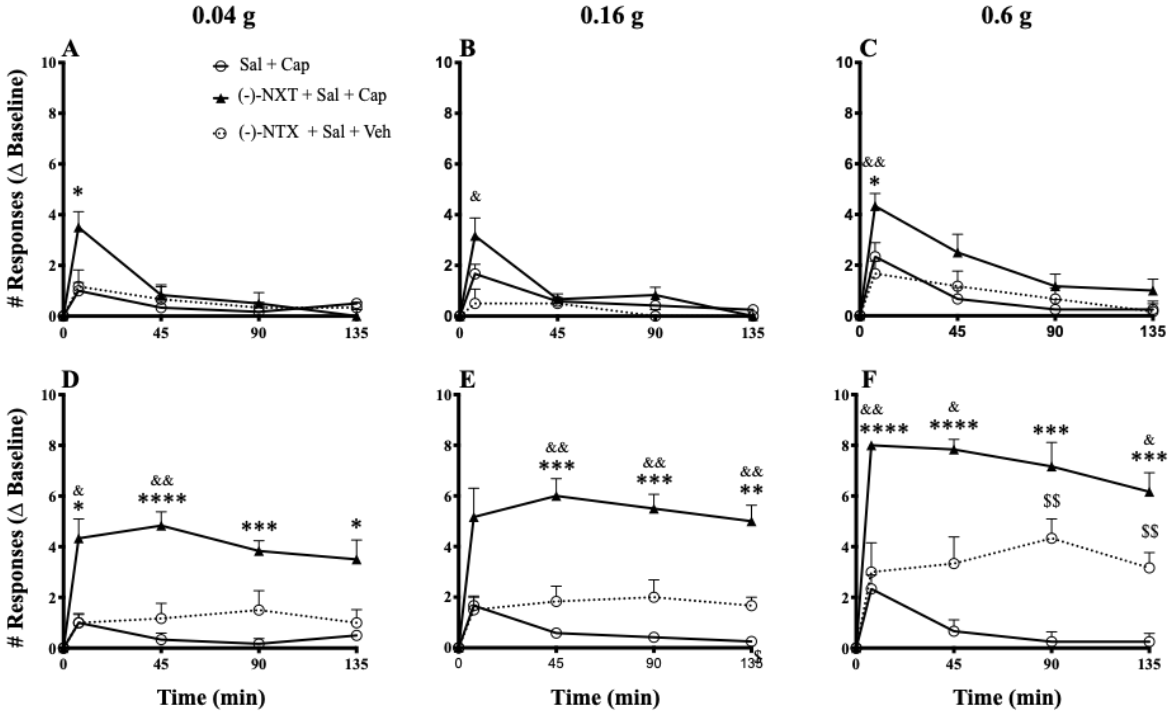
administration at separate sites that allow easier access to the injection site than the footpads of a mouse. For example, cheek or calf administration which have been demonstrated previously (Akiyama et al., 2014; Shimada et al., 2008). Secondly, introducing thermal testing, which would provide useful comparisons to previous studies in which opioid antagonism has varying effects on mechanical and thermal stimuli (Labuz et al., 2016).

We investigated neuronal activation (c-Fos) states in the dorsal horn of the spinal cord. Pilot studies were unable to identify differences between groups, suggesting that increased neuronal activation is not responsible for the observed behavioural differences. Multiple studies report increased c-Fos in superficial lamina at comparable doses between 30 min and 1.5 h post capsaicin (Hossaini et al., 2014; Hossaini et al., 2011; Zou et al., 2001). Despite the difference in timing, one would expect to see increased c-Fos in capsaicin treated animals. The induction of phosphorylated extracellular signal-regulated kinase (pERK) may also be worth considering as an alternative. Associated with noxious stimulation, pERK is produced rapidly in dorsal horn neurons correlating closely with induction of hypersensitivity compared to c-Fos, which is induced 30 - 60 min following stimulation (Gao et al., 2009). Further, pERK was used in the previously mentioned study by Corder et al. (2013). They use pERK to highlight the effect of naltrexone in reinstating mechanical hypersensitivity. Their protocol included anaesthetising mice and applying light mechanical stimulation of the hindpaw (cotton bud wiping) prior to sacrifice and histological processing (Corder et al., 2013). We did not mechanically stimulate the paw in our protocol, which should be considered in future immunohistochemical studies investigating the effect of naltrexone potentiation of mechanical hypersensitivity.

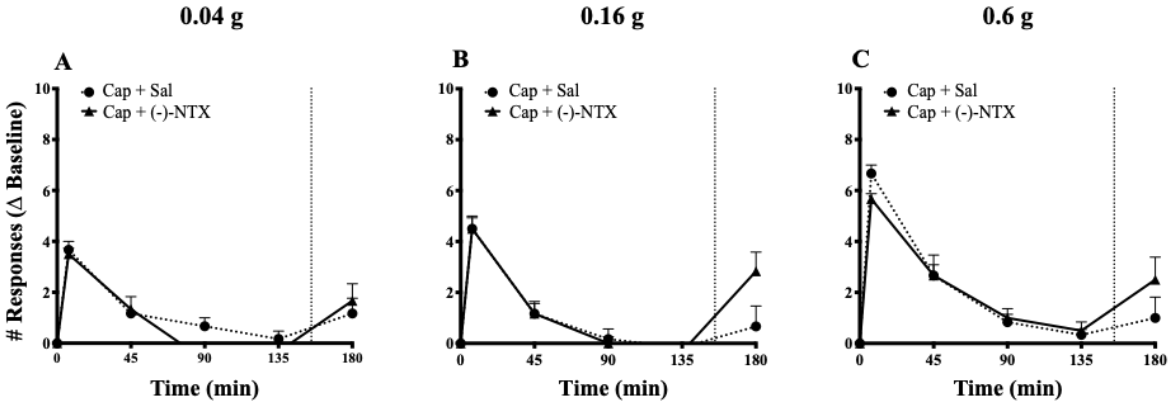
Understanding the multisystem effects elicited by drug administration is important when interpreting data from subsequent behavioural testing. Here we show that i.pl. administration can affect capsaicin-induced hypersensitivity. Further to this we show that blocking both opioid and non-classical opioid pathways can reverse this effect and extend hypersensitivity following capsaicin depending on the timing of administration. The production of extended hypersensitivity appears to be dependent on the environment of the nociceptive pathway at the time of antagonism but is not due to increased neuronal activity at the level of the spinal cord. Despite the need for further elucidation of mechanism, these

results improve understanding of the effects of i.pl. drug administration on nociceptive outcomes.

6.6 Supplementary figures



Supplementary Figure 6.1: (-)-Naltrexone reverses i.pl. saline attenuated capsaicin-induced mechanical hypersensitivity, which is extended at high doses. Mice were administered 0.9% saline (i.pl.) 15 min prior to capsaicin (0.8 µg/5 µL; i.pl). (-)-Naltrexone (0.6 mg/kg (A, B, C); 60 mg/kg (D,E, F); i.p.) or saline (0.9%; i.p.) was administered 15 min prior to saline (0.9 %; i.pl.), followed by either capsaicin (0.8 µg/5 µL; i.pl) or vehicle (5 µL; i.pl). Testing included 10 applications of von Frey filaments (0.04 (A, D), 0.16 (B, E), 0.6g (C, F)) and was performed at 7, 45, 90 and 135 min following capsaicin administration. n = 12 for saline + capsaicin, n = 6 for all other groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (-)-naltrexone + saline + capsaicin vs saline + capsaicin; & $p < 0.05$, && $p < 0.01$, &&&& $p < 0.0001$ (-)-naltrexone + saline + capsaicin vs (-)-naltrexone + saline + vehicle; \$ $p < 0.05$, \$\$ $p < 0.01$ (-)-naltrexone + saline + vehicle vs saline + capsaicin. Error bars represent SEM.



Supplementary Figure 6.2. (-)-Naltrexone does not restore capsaicin-induced mechanical hypersensitivity when administered 155 min following i.pl. capsaicin. (-)-Naltrexone (6 mg/kg; i.p.) or saline were administered 155 min (dotted line) following capsaicin (0.8 µg/ 5 µL; i.pl.). Von Frey testing included 10 applications of von Frey filaments (0.04 (A), 0.16 (B), 0.6g (C)) at 7, 45, 90, 135 and 180 min post-capsaicin. n = 6 for all groups. Error bars represent SEM.

Supplementary Table 6.1. One-way ANOVA results reveal no effect of treatment on cell count or area stained by c-Fos antibody (n = 2) (Fig. 6.9).

	Normalised Cell Count			Area (%)		
	L3	L4	L5	L3	L4	L5
Ipsilateral	$F_{(2, 3)}$ = 0.2032 P=0.8265	$F_{(2, 3)}$ = 0.3071 P=0.7562	$F_{(2, 4)}$ = 0.4837 P=0.6484	$F_{(2, 3)}$ = 0.1834 P=0.8411	$F_{(2, 3)}$ = 0.1908 P=0.8356	$F_{(2, 4)}$ = 2.926 P=0.1648
Contralateral	$F_{(2, 3)}$ = 0.3803 P=0.7125	$F_{(2, 3)}$ = 2.729 P=0.2113	$F_{(2, 3)}$ = 0.7842 P=0.5321	$F_{(2, 3)}$ = 0.3253 P=0.7449	$F_{(2, 3)}$ = 3.439 P=0.1674	$F_{(2, 3)}$ = 1.288 P=0.3946

Chapter 7. Discussion

The first aim of this thesis was to investigate the specifics of a functionally relevant interaction between toll-like receptor 4 (TLR4) and transient receptor potential cation channel subfamily V member 1 (TRPV1). Secondly, we aimed to utilise this interaction and create a novel preclinical pain model which directly engages both the immune and neuronal elements of neuroimmune signalling. This would create a unified preclinical and clinical pain model and enable further molecular and cellular investigation of mechanisms underlying endotoxin potentiated capsaicin-induced allodynia, hyperalgesia and flare in humans. We were able to successfully demonstrate TLR4 altered TRPV1 function in a HEK293FT cell line co-expressing both TLR4 and TRPV1. In this system we observed endotoxin potentiated increases in calcium accumulation following capsaicin but no altered influx dynamic. Interestingly we also observed TLR4 antagonists resulted in increased intracellular calcium accumulation. We were unable to replicate potentiated capsaicin responses observed in the clinical endotoxin-capsaicin study in a preclinical mouse model. Although we do report a potentiated mechanical hypersensitivity following primed locally applied LPS in a population of BALB/c mice. Complicating our efforts in creating a preclinical model was an apparent endogenous analgesic response following intraplantar capsaicin administration. We were able to identify this was the result of classical and non-classical opioid analgesic mechanisms, blocked by the addition of both (-)- and (+)-naltrexone. Therefore, we confirm the existence of a TLR4/TRPV1 functional interaction, both dependent and independent of TLR4 signalling, with relevant *in vivo* consequences.

We were able to confirm that the presence of TLR4 is sufficient to alter TRPV1 function in a co-expression system. This result agrees with multiple studies which show alteration of TRPV1 mediated current and calcium accumulation when co-expressed with TLR4 (Min et al., 2018; Min et al., 2014). Live cell analysis suggests a population of TLR4/TRPV1 co-expressing cells exhibit a rapid response, but what would cause this in our system is yet to be identified. Due to the unreliability of TLR4 antibodies we were unable to identify the relative expression of TLR4 protein, which may have provided more insight into these responses (McCarthy et al., 2017). In future, using conjugated LPS may offer an alternative while *in situ* TLR4 protein identification remains elusive (Diogenes et al., 2011). In the absence of any intentional added stimulus these results suggest either a physical interaction or some level of basal signalling. A physical interaction has been

proposed between TRPV1 and the TIR domain of TLR4, which when removed reverses potentiated TRPV1 mediated calcium responses (Min et al., 2018). To investigate basal signalling effects we applied multiple antagonists which inhibit various aspects of the TLR4 complex including the MD2/endotoxin interaction (1J, (+)-Naloxone, LPS-RS) and interaction of TLR4 with its intracellular TIR adaptor proteins (TAK-242) (Gaikwad et al., 2015; Matsunaga et al., 2011; Selfridge et al., 2015). These antagonists had no effect on the calcium response curve of TLR4/TRPV1 co-expressed HEK293FT cells, and surprisingly led to increased calcium accumulation following capsaicin, contrasting previous work in primary DRG neurons which shows no effect of LPS-RS on intracellular calcium (Diogenes et al., 2011). Based on these results, it is unlikely basal signalling is the cause of our altered capsaicin-induced calcium responses, further implicating physical interaction. Future studies will need to establish a physical protein-protein interaction to confirm this hypothesis; resonance energy transfer techniques such as bioluminescence or fluorescence resonance energy transfer (BRET or FRET) offer interesting avenues for this live cell work given the unreliability of TLR4 antibodies (Dimri et al., 2016).

Relating to our second aim, we hypothesised the potentiation of TRPV1 responses following activation of TLR4 by LPS *in vitro*. Activation with LPS did not alter TRPV1 mediated calcium influx dynamics, although it did induce increased calcium accumulation at high doses (100, 1000 ng/mL) following 18 min but not 4 h LPS incubation. It should be noted that cells not exposed to capsaicin expressing both TLR4 and TRPV1 showed increased calcium accumulation following 18 min LPS stimulation at 100 and 1000 ng/mL which may account for the difference observed in capsaicin stimulated cells. This response to LPS is also seen in rat DRG neurons, where the effect is blocked by TLR4 antagonism, suggesting the influx is TLR4 mediated; although LPS has no effect on calcitonin gene-related peptide (CGRP) release (Diogenes et al., 2011; Li et al., 2015). Likewise, we recorded increases in intracellular calcium accumulation in cells only expressing TLR4. Together, these results suggest that TLR4 mediated intracellular calcium increases do not influence neuropeptide release, but offer an alternative mechanism to rapid alteration in TRPV1 sensitivity.

Studies in both primary and HEK293FT cells agree that short LPS stimulation (< 1 min to 15 min) potentiates TRPV1 responses measured using alternate assays, including inward current and CGRP release (Diogenes et al., 2011; Li et al., 2015; Wu et al., 2019).

HEK293FT cells are used in overexpression studies due to their active signal transduction pathways; importantly they express the intracellular components important for the sensitisation of TRPV1, including AKAP79, PKC, PKA, PI3K and PLC (Atwood et al., 2011; Gardner et al., 2006; Song et al., 2014). Apart from ligand-induced physical changes, these scaffolding proteins and kinases represent a plausible route of rapid, calcium-induced sensitisation. Our conditions were almost identical to the HEK293FT overexpression system used previously which reported LPS-induced potentiation of capsaicin-induced inward currents (Li et al., 2015). Future studies which explore mechanism would be wise to include patch clamp data to ascertain if the small, rapid LPS-induced intracellular calcium changes translate to altered TRPV1 channel conductance.

In order to achieve our second aim and back-translate the clinical endotoxin-capsaicin model, an exhaustive literature review of preclinical pro-nociceptive capsaicin models was undertaken. It is evident that there are areas in need of refinement in this space; in particular in relation to capsaicin dose, reporting nomenclature and the use and reporting of data from both sexes. One such improvement is recognising which doses are necessary to produce duration of hypersensitivity required for the behavioural assessment. Rarely do researchers test animals for more than 3 h post capsaicin application, yet doses can vary hundreds of micrograms within the same species and application site. This is particularly evident in intra-colonic mouse studies, where a dose of 50 μg is established, yet to 500 μg is still used despite testing at the same 20minute time point (Gonzalez-Cano et al., 2017; Hockley et al., 2017). Likewise, in rat intraplantar injection studies 10 μg is established, while recently doses above 1 μg were administered for testing at the same or shorter time points (Huang et al., 2016; Leblanc et al., 2014). This is not true of all applications; 1.6 μg is commonly used at the mouse hindpaw with no evidence that significantly higher doses are used. We incorporated this dose in our pilot studies, eventually choosing to use 0.8 μg as it produced similar outcomes. We further reduced this to 0.4 μg in the later animal studies, finding it as effective as the higher doses. We suggest many doses used in capsaicin literature can be significantly refined. Another area in need of refinement is the use of language around subject responses. Throughout this manuscript we use the term ‘hypersensitivity’ in reference to reduced mechanical and thermal thresholds. It became clear in our literature search that terms allodynia and hyperalgesia - commonly used in clinical trials due to the ability of subjects to self-report pain – are used interchangeably. It

is recommended to reduce confusion that researchers agree on a consensus term to refer to reduced sensory thresholds in pain literature. Finally, only 10% of studies include female subjects, presumably due to the added variables introduced by oestrous. However, this removes a significant research interest and is an important step forward in future experimental design. In addition, poor reporting of sex-independent results should be reviewed in order to clarify persistent questions around sex-related difference in pain. In order to back-translate the clinical model, an attempt was made to replicate as close as practicable the clinical conditions, leading to the initial use of male mice. Further investigations into the potentiated responses following a primed LPS response should investigate the phenomenon in female mice. We were therefore able to use the literature review to refine or procedures and reporting, while future steps should aim investigate primed LPS-induced capsaicin potentiation in female mice.

We were unable to replicate potentiated capsaicin-induced mechanical hypersensitivity in BALB/c mice. As stated above, we attempted to replicate conditions present in the clinical model including; sex, stimulus intensity, route of administration and behavioural outcome; however, adaptions were necessary for a rodent model. These included relative capsaicin and LPS doses as well as LPS timing. Systemic LPS timing was investigated at both 3 and 4 h to firstly replicate the clinical study, and then at a later timepoint to reflect the comparatively delayed peak cytokine response in BALB/c mice (Ostberg et al., 2000). Capsaicin doses were optimised to generate a submaximal response to mechanical stimulation, allowing for detection of potentiation. LPS doses 1 μg/kg and 1 mg/kg reflect both low and moderate immune inducing stimulus in BALB/c mice, reflecting low levels of serum cytokine increase in the clinical model (Kadafi et al., 2019; Meneses et al., 2018; Ostberg et al., 2000). However, none of the variables tested produced potentiated mechanical hypersensitivity.

We chose to utilise a common method of measuring mechanical hypersensitivity in mice following application of capsaicin to the hindpaw. The clinical model reports potentiated mechanical allodynia, hyperalgesia and flare. Therefore, we adopted a von Frey approach using a range of filaments to replicate both innocuous and noxious mechanical stimulation. This would allow assessment of both allodynia and hyperalgesia in a rodent model. Adopting a best of 10 von Frey approach limited the number of fibres we could test due to

the rapid onset and transient nature of capsaicin-induced mechanical hypersensitivity; however, this offered significantly decreased variability over a best of 5 approach. Determining a threshold using either the Chaplan or the modified SUDO up-down methods rather than a best of 10 approach may be advantageous in future work using manual von Frey filaments across a short time frame. While they won't allow inferences regarding allodynic or hyperalgesic mechanism, they offer fewer applications per animal per time point (Bonin et al., 2014; Chaplan et al., 1994). Ideally, electronic von Frey would be used to further minimise filament applications. It should be noted that the clinical endotoxin-capsaicin model observed a larger potentiation to mechanical allodynia compared to hyperalgesia (Hutchinson et al., 2013). Hyperalgesia was measured using von Frey filaments, a static mechanical stimulus; while capsaicin-induced allodynia was measured using a foam brush, representing dynamic mechanical stimulation (Hutchinson et al., 2013). Dynamic and mechanical stimuli can be differentiated by use of analgesics which block only either static or dynamic stimulation and can be produced independently by different nerve injuries (Field et al., 1999). These results suggest that each stimulation type recruits different nociceptor subsets, raising the possibility that LPS potentiation may not occur in those activated by punctate mechanical stimulation. Dynamic stimulation has been reported in capsaicin literature with use of cotton bud wiping, an alternative to consider in future studies (Laird et al., 2004; Laird et al., 2001b).

In addition, we needed to modify the anatomical region tested when compared to the clinical endotoxin-capsaicin model. The hindpaw is an overwhelmingly popular choice to assess capsaicin-induced pain, however multiple studies have shown TLR4-dependent pain hypersensitivity is anatomically variable (Hu et al., 2017; Wegner et al., 2014; Wegner et al., 2015). In the clinical endotoxin-capsaicin model, the forearm produced potentiated sensitivity, however this is not an ideal location for administration of mechanical stimulation in rodent models. Alternatives already present in the capsaicin literature include intradermal application at the calf, vibrissae pad and nape (Akiyama et al., 2014; Peirs et al., 2016; Shimada et al., 2008). Based on clinical data suggesting LPS-induced mechanical sensitivity presents in back muscles, the back offers another potential alternative for intradermal capsaicin administration in mice (Wegner et al., 2014). A disadvantage of our intraplantar model is the endogenous analgesia induced by injection and its subsequent effect on hindpaw sensitivity. This becomes particularly relevant if these mechanisms impact any potential effects of LPS.

We identified that injection stress attenuated capsaicin responses in BALB/c mice within 15 min. Both classical and non-classic opioid antagonists (-)- and (+)-naltrexone reversed this response when administered pre-capsaicin. When administered post-capsaicin however, (-)- and (+)-naltrexone potentiated and extended capsaicin-induced mechanical hypersensitivity. Therefore, the timing of this effect was critical to this potentiation, and suggests it is not a result of nociceptor potentiation, but an attenuation of an endogenous analgesic response. Potential mechanisms must be rapid given the short time period between injection and altered capsaicin response and includes stress-induced analgesia resulting from intraplantar application of capsaicin, a phenomenon observed in rodent hindpaw following electric shock and acupuncture (Wright et al., 1985; Zakusov et al., 1981). However, more investigation is required to narrow down mechanism, importantly, identifying if the effects are peripherally or centrally mediated would enable a more targeted mechanistic investigation.

Although we weren't able to observe potentiated capsaicin-induced hypersensitivity following systemic LPS, potentiated responses were observed following locally applied LPS. Due to the effects of LPS on DRG neurons described above we attempted to cause potentiation by targeting peripheral neurons directly (Diogenes et al., 2011; Ferraz et al., 2011; Li et al., 2015; Wadachi et al., 2006). This was only possible at later time points due to the previously discussed analgesic action of intraplantar drug administration. We were unable to produce significant potentiation of capsaicin-induced mechanical hypersensitivity following a single LPS dose, with exception of a single time point (90 min) using the strongest filament tested (0.6 g). Based on evidence in mice that primed LPS responses produce thermal and mechanical hypersensitivities and single LPS doses can lead to long lasting (> 2 weeks) but slow inducing thermal hyperalgesia, we tested a primed LPS response with two identical doses separated by 7 days (Abu-Ghefreh et al., 2010; Cahill et al., 1998). We found that primed responses produced potentiated capsaicin-induced mechanical hypersensitivity; an effect completely attenuated by TLR4 antagonist (+)-naltrexone. This data displayed considerable variability, with apparent high and low responding populations within the primed LPS capsaicin group. Separating this group reveals responses to mechanical stimulation were significantly higher in 50, 42 and 58% of animals stimulated with 0.16, 0.04 and 0.6g filaments respectively; with the remaining animals not statistically different from vehicle treatment. This potentiation is comparable

to that reported in the clinical endotoxin-capsaicin study, with enhanced mechanical sensitivity over multiple time points. It should also be noted that primed LPS alone did not result in changes to mechanical hypersensitivity in this case. We propose that mechanistic differences between the two populations are of interest, shedding light on why only a portion of animals developed hypersensitivity may highlight important mechanisms. This is an important question in clinical pain, in which some people develop chronic, dysfunctional pain and others do not despite being exposed to the same stimuli, for example chemotherapy or surgery (Borsook et al., 2018). In this case both central and peripheral blockade of TLR4 would emphasise areas for more detailed investigation. Further regional analysis of cellular activation states, gene activation and inflammatory mediators may highlight potential biological factors responsible for biasing hypersensitivity over unaffected nociception.

The evidence presented here reinforces that innate immune receptors and pathogen associate molecular pattern (PAMP) activity are important elements of immune driven nociceptive sensitisation. Typically, innate immune responses are associated with inflammatory pain; nociceptive sensitisation to promote repair of damaged tissue. However, we show TLR4 influences nociception in the absence of activation, and LPS alters nociceptive processing at doses which do not induce inflammatory hypersensitivity. This suggests an alternate role in pain signalling. One suggestion is the direct activation or sensitisation of nociceptors by PAMPs as a danger signal similar to the effect of noxious chemical, thermal and mechanical stimuli. Direct activation of nociceptors by pathogens has been reported, with *Staphylococcus aureus* inducing pain hypersensitivity correlated with bacterial load. Interestingly, nociceptor activation by *Staphylococcus aureus* is associated with local nociceptor-induced immune suppression (Chiu et al., 2013). LPS induces TLR4 and TRPA1 dependent calcium influx in mouse DRG and nodose ganglion neurons respectively (Diogenes et al., 2011; Meseguer et al., 2014). In addition to TLR4, TLR3, -5, -7 and -9 are expressed on small and medium diameter nociceptors (Liu et al., 2012; Liu et al., 2010; Wadachi et al., 2006; Xu et al., 2015). Activation of TLR3 and TLR7 by their respective ligands results in inward currents in mouse DRG neurons (Liu et al., 2012; Liu et al., 2010). While activation of TLR3, -7 and -9 result in prostaglandin (PGE_2) release and upregulation of pro-inflammatory cytokines in murine DRGs (Qi et al., 2011). Activation of TLR5 by flagellin allows entry of membrane impermeable compound QX-314 in medium diameter DRG neurons, further evidence of direct pathogen

recognition by nociceptors via innate immune receptors (Xu et al., 2015). Therefore, further to the role in generation and maintenance of chronic pain following illness or injury, we suggest TLRs allow pathogen detection, sensitisation of neuronal ion channels and subsequent recruitment of local immune responses. Contradictory reports on the nature of nociceptive driven immune responses suggest further research is required. It is likely responses are pathogen specific, with suppression of immune reposes by some a host avoidance strategy, in other cases enhanced pro-inflammatory responses may represent an immune priming event.

We were unable to successfully back-translate the clinical endotoxin-capsaicin model, although there are a number of variables outlined above which can be investigated further. We were however able to show in a similar model involving targeted LPS application that LPS indeed induces *in vivo* changes in TRPV1 function; requiring further mechanistic investigation. There is clear evidence in the literature, supported here, that TLR4 enhances TRPV1 action *in vitro*, although the mechanisms underlying rapid receptor sensitisation need to be elucidated. Understanding if these rapid changes are relevant *in vivo* is also necessary, as evidence here and in the wider literature suggest TLR4 mediated potentiation of TRPV1 *in vivo* occurs over longer timeframes, consistent with inflammatory sensitisation events. Given the importance of both TLR4 and TRPV1 to pathological pain models, and the clinical endotoxin-capsaicin proof of concept, the implications of a functional physical receptor interaction offers an interesting, novel, and specific therapeutic target. Additionally, a unique unified clinical/preclinical model offers interesting possibilities for drug screening and investigation into the relevance of murine models in drug screening.

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