Investigation into the Mechanisms of Hyperalgesic Priming

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Abstract

Chronic pain is a major clinical problem that affects approximately 40% of the UK population. Understanding the mechanisms that underpin the development and maintenance of chronic pain permits more focused research and identification of potential targets for new therapies.

Hyperalgesic priming models the transition from acute to chronic pain. Subcutaneous inflammogens applied 72hrs apart induce a long-term hyperalgesia to mechanical stimulation that outlasts the resolution of the systemic swelling. The aim of this thesis was to investigate the underlying mechanisms of this phenomenon.

Two versions of hyperalgesic priming were initially assessed; carrageenan-induced and TNFα induced. The rats pre-treated with carrageenan (primed) (1%, 5µl) showed significantly reduced paw withdrawal thresholds post-intraplantar injection of PGE_2 (1µg, 5µl), these were maintained up to 7 days post-injection compared to saline pre-treated (unprimed). Rats pre-treated with TNFα (100ng, 5µl) also showed maintained reductions in PWTs post-PGE_2, this however was not significantly lower than saline unprimed rats.

To investigate changes in immune cell populations within the peripheral injury site, skin samples collected from carrageenan-primed rats after PGE_2 injection showed increased numbers of macrophages compared to saline-saline controls. Ionized calcium-binding adaptor protein (IBA1) immunostaining of the spinal cord showed that there were increased numbers of microglia in the dorsal horn in unprimed rats compared to carrageenan-primed rats. There were alterations in the presence of plasma lipid mediators of inflammation determined using mass spectroscopy in primed compared to unprimed rats. Unprimed rats having a higher level of anti-inflammatory mediators including PGE_2 EA and 18-HEPE suggests an alteration in production of inflammatory mediators due to hyperalgesic priming.

To evaluate the involvement of sub-sets of primary afferent neurones in priming, sensory nerve blockade using the local anaesthetic QX-314 and capsaicin or QX-314

and flagellin were administered before or after intraplantar injection of PGE_2 to selectively block Aβ and C nerve fibres. The injection of QX-314 + capsaicin or QX-314 + flagellin inhibited the PGE_2 induced reduction in PWTs however was unable to prevent the establishment of chronic pain. The injection of QX-314 + capsaicin in rats with established chronic pain inhibited the reduction in PWTs however the effect was only seen to last up to 4h post-injection. QX-314 + flagellin partially inhibited the reduction in PWTs up to 2h. These data showed that hyperalgesic priming is mediated by the activation of both C-fibres and partially by Aβ fibres.

To assess input changes in CNS processing of primary afferent fibres spinal electrophysiological recordings of wide dynamic range (WDR) neurones in lamina V were performed. These data showed a 300% increase in firing in response to low threshold mechanical stimulation after the intraplantar injection of PGE_2. There were no differences observed in WDR firing between primed and unprimed rats post-PGE_2.

Finally the role of the resolvin resolution pathway was investigated in preventing and reversing hyperalgesic priming. Aspirin-triggered resolvin D1 precursor 17-R-HDoHE has been shown to reduce inflammatory pain. 17-R-HDoHE (300ng, 300µl) was systemically administered before or after PGE_2 injection in carrageenan-primed rats. It was shown that administration of 17-R-HDoHE prior to PGE_2 significantly ($p<0.01$) inhibited the sustained reduction in PWTs in primed rats. However systemic administration of 17-R-HDoHE after PGE_2 in primed rats had no effect on PWTs. These data show an alteration in the responsiveness of primed rats to the activation of resolvin mediated resolution. Changes in microRNA (miRNA) has been linked to alterations in gene expression in different pain states. To investigate miRNA changes in chronic pain. The L4, L5 and L6 DRGs were collected. There were measured increases in miR-100 and miR-125b during carrageenan induced acute pain and hyperalgesic priming induced chronic pain post-PGE_2. Pre-treatment with 17-R-HDoHE was shown in inhibit the up-regulation of miR-100 and miR-125b in DRGs of primed rats. This demonstrated that alterations in the regulation of genes during acute pain may also influence changes during chronic pain.

This thesis demonstrates the potential involvement of several altered cellular processes that contribute to the induction and maintenance of long-term hyperalgesia by hyperalgesic priming. I observed decreases in numbers of microglia within the dorsal horn of primed rats. Hyperalgesic priming induced chronic pain requires activity within of both C and Aβ fibre primary afferent populations of sensory neurons. The firing rate of WDR neurones to mechanical stimulation was shown to be increased post-PGE_2 intraplantar injection however the increase was not maintained in primed rats. Levels of miR-100 and miR-125b were found to be increased during periods of acute and chronic pain and were found to be inhibited by resolvin treatment. Finally resolvin pre-treatment inhibited the generation of hyperalgesic priming induced chronic pain, but to reverse established pain.

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Contents

Chapter 1

General Introduction

1.1 An Introduction to Pain

Pain is a crucial evolutionary system developed for the purpose of protection. It notifies us when we are in danger of causing damage or when damage is occurring. Pain is made up of two components, the unpleasant sensation and the emotional component associated with the injury. Emotional responses are difficult to characterise so nociception is measured which is the actual detection of painful stimuli, which is controlled by the nervous connections between the damaged tissue and the central nervous system.

IASP define pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." *International Association for the Study of Pain (1979)*. There are two major pain sensations that are studied; **hyperalgesia** is an increased response to a stimulus that normally causes pain (a previously painful stimulus now perceived as more painful) and **allodynia** which is pain from a stimulus that does not normally cause pain (a previously innocuous stimulus now perceived as painful).

1.1.1 Nociception

The sensation of nociception is made up of several components that work together to notify the body of damage. Information from the periphery is converted into action potentials by specialised sensory nerve fibres called nociceptors. Action potentials are transduced along primary afferent fibres where they enter the dorsal horn of the spinal cord. The peripheral information is integrated via the activity of several cell types and passes up specialised ascending pathways to the brain. The incoming signals can then be modulated and then signals can be returned to the spinal cord through descending control pathways leading to amplification or inhibition of nociceptive information.

1.1.2 Primary Afferent Fibres

1.1.2.1 Aβ Fibres

Sensory nerve fibres are primarily categorised by their diameter, conduction velocity and function. There are three main classification of sensory nerve fibres Aβ, Aδ and C fibres (Millan, 1999). Aβ fibres are large diameter (>10µm) and fast conducting (30-100 m/sec) with their function mainly involved in the sensation of innocuous mechanical stimulation (Djouhri *et al.*, 1998) and so not directly involved in normal nociception. However Aβ fibres have been shown to have involvement in the mediation of allodynia after nerve injury via the upregulation of neuropeptide Y within the larger diameter sensory fibres (Campbell *et al.*, 1988), (Ossipov *et al.*, 2002).

1.1.2.2 Aδ Fibres

Aδ fibres have a smaller diameter axon (2-6µm) and are more thinly myelinated than the Aβ fibres this gives them a slower conduction velocity (12-30 m/sec). The Aδ fibres are responsible for the localised pain occurring rapidly after a noxious event and can be split into two groups known as type I and type II A-fibres. The type I fibres are polymodal therefore responding to mechanical, chemical and thermal stimuli (Burgess *et al.*, 1967). However they are more sensitive to mechanical stimuli, and type I fibres have a very high threshold to short duration heat stimuli, requiring >53°C to elicit activation (Caterina *et al.*, 1999) although long duration thermal stimuli will elicit a response at a lower temperature. The type II fibres are mechanically insensitive, but have a lower threshold to heat from both short and long duration stimulation (Treede *et al.*, 1998).

1.1.2.3 C Fibres

C-fibres are the smallest in diameter (0.4-1.2µm) of the sensory nerves with the slowest conduction speed (0.5-2 m/sec). A large proportion of the small diameter fibres are nociceptors, with around 10% of C-fibres also able to transmit non-noxious information (Snider *et al.*, 1998). The C-fibres are defined as polymodal as they are able to detect multiple noxious inputs such as mechanical, thermal and chemical (Meyer *et al.*, 2005). There are a group of C-fibres that are termed silent nociceptors (Schmidt *et al.*, 1995), which unresponsive during normal conditions, and are suspected to place a role in central sensitisation and other chronic pain states (Dubin *et al.*, 2010).

From early stages of development C-fibres fall into two subtypes, peptidergic and the non-peptidergic fibres. The C-fibres are formed from the neural crest and express tropomyosin receptor kinase A (TrkA) the receptor for nerve growth factor (NGF) due to the proneural transcription factor neurogenin 1 (Ma *et al.*, 1998). About half of the TrkA positive neurones will start to express the atypical tyrosine kinase receptor Ret instead of TrkA at between embryonic day 15 and postnatal day 7. Ret is the receptor for glial cell derived neurotrophic factor (GDNF). The Ret expressing C-fibres become non-peptidergic neurones and have a high binding affinity for isolectin B4 (Molliver *et al.*, 1997) due to the expression of α-D-galactose on their membranes (Silverman *et al.*, 1990) and so are known as the $IB4^+$ C-fibres. The peptidergic fibres produce several neuropeptides including calcitonin gene-related protein (CGRP) as well as substance P and neurokinin A. These fibres innervate deeper skin and visceral tissue (Bennett *et al.*, 1996), (Perry *et al.*, 1998). The non-peptidergic fibres mainly innervate skin and have a high expression of $P2X_3$ receptors that detect ATP a product usually released from damaged cells (Plenderleith *et al.*, 1993).

FIBRE	DIAMETER	CONDUCTION VELOCITY	MYELINATION
Aβ	10-15µm	30-100m/s	Thickly myelinated
Aδ	2-6µm	12-30m/s	Thinly myelinated
C	>1µm	0.5-2m/s	Unmyelinated

Table 1.1: Summary of sensory nerve fibres characteristics

1.1.3 Dorsal Horn of the Spinal Cord

The sensory fibres project from the periphery to the spinal cord where they enter the dorsal horn and synapse with second order neurones. The spinal cord is divided into distinct regions known as lamina originally described in the cat (Rexed, 1954; Rexed, 1952). This layering has also been identified in rat spinal cord (Molander *et al.*, 1995). There are ten distinct laminae within the spinal cord (denoted I-X), the dorsal horn consists of I-VI and is the main processing centre for sensory information. Different sensory fibre types terminate in different lamina. The Aβ fibres which are non-noxious synapse within lamina III-V (Brown *et al.*, 1981) as well as forming synapses within the more superficial lamina II (Woodbury *et al.*, 2008). The Aδ fibres along with the peptidergic C-fibres terminate in laminae of I and II (Light *et al.*, 1979). The non-peptidergic C-fibres synapse solely within lamina II (Lorenzo *et al.*, 2008).

These primary afferents enter the dorsal horn and terminate upon multi-synaptic complexes call glomeruli. They act as a crossroads allowing for the integration of information from different primary afferent inputs along with interneurone as well as supra-spinal modulatory inputs. These glomeruli are predominantly within lamina II where they are mainly innervated by Aδ and non-peptidergic fibres with post-synaptic connections to multiple axodendritic synapses allowing for the information to be communicated to the rest of the dorsal horn (Rethelyi *et al.*, 1982) (see review (Todd, 2010a).

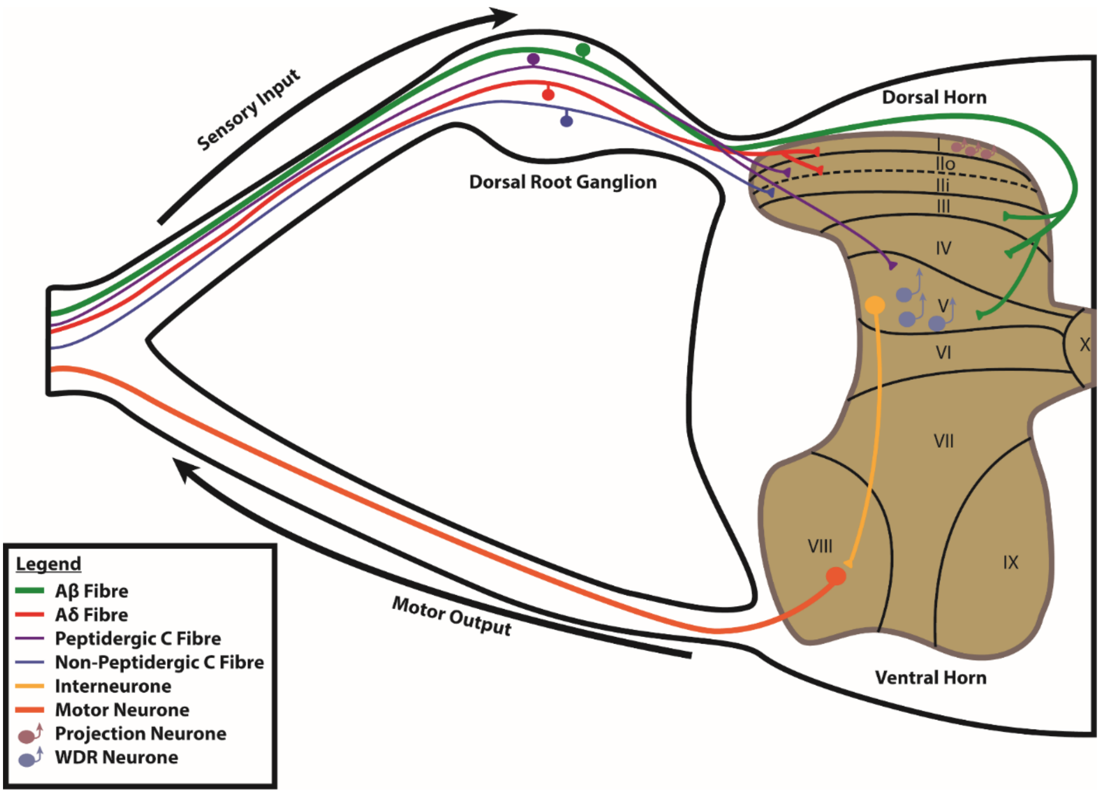


Figure 1.1 The spinal termination regions of Aβ, Aδ & C fibres in the dorsal horn of the spinal. Incoming noxious stimuli trigger the reflex arc causing a withdrawal from the noxious stimulus. Information is integrated by specialised neurones within lamina I and V where it is then transmitted to the brain via ascending tracts. Lamina V contains dense populations of wide dynamic range neurones (WDRs) that integrate signals from multiple sensory nerve types (based on Basbaum et al. 2009).

1.1.3.1 Cells of the Dorsal Horn

The dorsal horn consists of a multitude of different cell types. They have been characterised by their morphology, neurochemistry and electrophysiological responses. One of the major neuronal characterisation methods is to assess their response to primary afferent input. These can be divided into non-nociceptive, nociceptive-specific and wide dynamic range cells (WDRs) (Dubner *et al.*, 1983; Mense, 1993). The non-nociceptive cells are predominantly localised in laminae II-IV and receive their input from the innocuous signalling via Aβ fibres. The nociceptive-specific cells are located within laminae I and the outer layer of lamina II and V-VI receive input from Aδ and C fibre afferents (Mense, 1993). The wide dynamic range neurones are innervated by each of sensory neurones giving them graded responses (increasing in firing rate to more intense pain signals) (Mendell, 1966). They are present within lamina IV-VI however they can also be found within lamina I and IIo (Morgan, 1998). Wide dynamic range neurones are important to nociceptive transmission as they integrate the multiple inputs from cutaneous, visceral and muscular regions particularly those within lamina V that project to the brain via the spinothalamic tract (Dubner *et al.*, 1989; Mense, 1993).

1.1.3.2 Interneurones

A high proportion of neurones within laminae I-III have axons that remain within the spinal cord and terminate locally hence they are termed interneurones (Todd, 2010a). They were original split into two subtypes based on their morphology, islet cells and stalk cells (Gobel, 1978). They were later more clearly defined by function into islet, vertical, radial and central cells (Grudt *et al.*, 2002). These cells can be either excitatory or inhibitory using glutamate or GABA as their major neurotransmitters respectively.

The islet and central cells are both inhibitory GABAergic neurones, however excitatory populations of central cells have also been described (Yasaka *et al.*, 2010). The cell bodies of islet cells are located within lamina II forming dendroaxonic (dendrite to axon synapse) connections with primary afferent fibres, while also communicating with other excitatory interneurones via dendrodendritic (dendrite to dendrite) and axodendritic (axon to dendrite) connections (Gobel *et al.*, 1980). The central cells show similar connectivity with their cell bodies located throughout lamina II. Both islet and central cells receive input from C-fibres (Lu *et al.*, 2003) and it has been shown that a loss of inhibitory signalling through these interneurones can contribute to the generation of central sensitisation (Sivilotti *et al.*, 1994; Zheng *et al.*, 2010).

The vertical and radial cells are excitatory but there are subpopulations of inhibitory vertical cells. Both cells have complex dendritic trees, radial cells project dendrites in all directions whereas the vertical cells show ventral projections (Yasaka *et al.*, 2010) and receive inputs from Aδ and C fibres and serve to transmit nociceptive information throughout the dorsal horn (Lu *et al.*, 2005b). This system only classifies the interneurones on the basis of their morphology. However it is not that straightforward as during morphological studies of these interneurones it was found there was a population of approximately 30% which were unclassified, along with varied excitatory and inhibitory function within central cells (Todd, 2010a).

1.1.3.3 Glia

The cells of the central nervous system do not consist of solely neurones, in actuality there are a far greater number of glial cells compared to neurones (Herculano-Houzel, 2014). There are several types of glia including oligodendrocytes and ependymal cells. However it is the microglia and astrocytes that have been implicated in the modulation of pain processing.

Microglia

Microglia are macrophage-like cells and act as the immune defence of the central nervous system (Pessac *et al.*, 2001). They are produced from bone-marrow derived monocytes and are distributed heterogeneously within the central nervous system. Microglia exist in one of two main morphologies; a ramified form that actively senses their environment and an active amoeboid form during injury or inflammation (Eriksson *et al.*, 1993; Ji *et al.*, 2013). After microglia undergo the morphological change they show an up-regulation of microglial markers such as major histocompatibility complex II (MHC II) which is found on antigen presenting cells, and expression of ionized calcium-bind adapter molecule 1 (IBA1). Microglia have proven roles in neuropathic pain development and acute inflammatory pain (Inoue *et al.*, 2009). Studies have also shown that after peripheral nerve injury microglia will rapidly proliferate with clear increases visible at 2 days post-injury (Cherry *et al.*, 2014). Microglia release several cytokines including IL-6, IL-1β and TNFα (Ferrari *et al.*, 1997; Suzuki *et al.*, 2004). In the central nervous system TNFα is mainly produced by microglia and plays a part in the generation of central sensitisation and peripheral sensitisation (Andrade *et al.*, 2011).

ATP is released within the CNS from nociceptors and endothelial cells upon injury as well as local release from astrocytes (Davalos *et al.*, 2005). ATP is one of the main modulators of microglial cells through its activation of P2X and P2Y receptors that are widely expressed on microglia. $P2X_7$ is a nonselective cation channel that permits the influx of both Na^+ and Ca^{2+} as well as the efflux of K^+. It is widely expressed on several immune cells including macrophages (Steinberg *et al.*, 1987) and microglia (Visentin *et al.*, 1999), contributing to TNFα and IL-1β release. ATP binding to $P2X_7$ leads to microglial activation, blockade of $P2X_7$ leads to a reduction in the switch to the microglial amoeboid form (Monif *et al.*, 2009). It has also been shown that activation of $P2X_7$ leads to the release of ATP from microglia themselves causing autocrine activation (Pellegatti *et al.*, 2005). Microglia are also regulated by the metabotropic P2Y receptors: $P2Y_2$, $P2Y_6$ & $P2Y_{12}$ which are involved in the control of phagocytosis and chemotaxis (Hidetoshi *et al.*, 2012). It was demonstrated that microglia would move towards sites of ATP, which was antagonised by the administration of an antagonist for $P2Y_{12}$ (Irino *et al.*, 2008).

Microglia are important in the regulation of neuronal activity and control of sensitisation, so are important to study in different pain states. They have been implicated in the maintenance of chronic pain in cases such as spinal cord injury (Hains *et al.*, 2006) as well as in inflammatory models such as arthritis through their increased activation state, as studies have shown that the prevention or reversal of microglia activation can alleviate the associated pain within these models (Nieto *et al.*, 2016; Yang *et al.*, 2012).

There is currently a lot of debate over the polarisation of activated microglia into M1 and M2 subtypes. The M1 microglia are termed 'classically activated' they are pro-inflammatory causing the release of TNFα, IL-1β and reactive oxygen species (Block *et al.*, 2007). The M2 microglia follow an 'alternative activation' or 'acquired deactivation.' Alternatively activated microglia are triggered by IL-4 or IL-13 promoting tissue repair and extracellular matrix reconstruction (Colton, 2009). The acquired deactivation is caused by the uptake of apoptotic cells as well as exposure to IL-10 and transforming growth factor β (TGFβ) (Colton *et al.*, 2010; Sawada *et al.*, 1999).

However this is not a fully accepted view on microglia, discussions have stated that the categorisation of microglia as this early stage 'hinders rather than aids research progress' (Ransohoff, 2016). It is the view of some scientists that by applying solely two categories to microglia based off macrophages, which themselves have multiple variations (Langerhans cells, monocyte-derived, osteoclasts etc.) that it is an oversimplification of the microglial family (Ransohoff, 2016). It will therefore be important to work into the identification of individual microglia subtypes. These may themselves possess varying functions that will allow for the better understanding of different neuronal disorders caused by microglia dysfunction.

Astrocytes

Astrocytes are the regulatory cells of the CNS and work to support and regulate neuronal function. They regulate blood flow to the CNS and are able to modulate the diameter of blood vessels allowing the exchange of glucose and other nutrients (Koehler *et al.*, 2009). Astrocytes contain stores of glycogen so that neurones have a steady and maintained source of glucose including during situations of hypoglycaemia (Brown *et al.*, 2007). The astrocyte processes surround neuronal synapses and function in maintaining homeostasis via control over pH, ion release and transmitter presence within the extra-synaptic space. They able to do this using an array of transporters such as Na^+/H^+ exchangers, and bicarbonate and monocarboxylic acid transporters (Obara *et al.*, 2008). The release of gliotransmitters such as glutamate, GABA and purines (adenosine and ATP) can act to modulate synaptic function this gave rise to the hypothesis of a tripartite synapse suggesting that astrocytes play a direct role in synaptic transmission (Araque *et al.*, 1999; Perea *et al.*, 2009).

Altered astrocyte activity has been linked to control of both acute and chronic inflammatory pain. The subcutaneous injection of formalin increases number of GFAP (glial fibrillary acidic protein a structural protein expressed on astrocytes (Garrison *et al.*, 1994)) positive astrocytes within the spinal dorsal horn (Qin *et al.*, 2006). After an incision in the hindpaw of rats there is an increase in GFAP expression in laminae I-II in the dorsal horn that peaks at 5 days dissipating by day 7 post injury (Romero-Sandoval *et al.*, 2008). The increase in GFAP expression within the dorsal horn also occurs in models of chronic pain such as the injection of CFA (Shimizu *et al.*, 2009) and after spinal nerve injury (Tanga *et al.*, 2006). Increased astrocyte activity is linked to the local release of glutamate as a gliotransmitters promoting NMDA activity aiding in the generation of central sensitisation (Bardoni *et al.*, 2010) (see **1.3**).

1.1.4 Neurotransmitters of Pain

1.1.4.1 Glutamate

Glutamate is the primary excitatory transmitter of the central nervous system. Primary afferent fibres that form monosynaptic terminals are excitatory with all nociceptive afferents releasing glutamate (Broman *et al.*, 1993). Glutamate activates both ionotropic and metabotropic receptors allowing for quick transmission and slower activated changes in neurones respectively.

There are four main types of glutamate receptor **AMPA** (R, S-α-amino-3-hydroxy-5-methylisoxazole-4-propionate, **NMDA** (N-methyl-D-aspartate) and **kainate** receptors are all ionotropic the final group is the **metabotropic glutamate** receptors. The ionotropic receptors allow the movement of Na^+ and K^+ with the NMDA receptors also allowing Ca^{2+} passage (Hollmann *et al.*, 1994). All primary afferents and many of the interneurones are glutamatergic (Todd, 2010b) meaning that the roles of glutamate receptors are crucial to the transmission of pain.

1.1.4.2 Glutamate: AMPA Receptors

AMPA receptors are formed from four homologous subunits GluR1-R4. It has been observed using immunohistochemistry and in situ hybridization that the receptor subunits are expressed throughout the dorsal horn, with different subunit combinations being more prevalent in different lamina of the dorsal horn (Garry *et al.*, 2003). GluR1 is expressed mainly within the superficial laminae with reduced expression in deeper laminae. GluR2 is predominantly localised in lamina II with greater prevalence in lamina II inner compared to lamina II outer (Nagy *et al.*, 2004a; Polgar *et al.*, 2008). In the deeper laminae of III and IV dendrites and NK1 positive projection neurones express GluR2, R3 and R4 but not GluR1 (Todd *et al.*, 2009). The presence of GluR2 reduces Ca^{2+} influx, there are however Ca^{2+} permeable AMPA receptors in the post-natal dorsal horn within laminae I and II. It has been shown that activation of Ca^{2+} AMPA receptors within the dorsal horn can promote AMPA synaptic transmission (Engelman *et al.*, 1999). The AMPA receptors themselves are responsible for fast signalling in both acute and chronic pain states (Dickenson *et al.*, 1997; Seagrove *et al.*, 2004) as well as playing an important role in central sensitisation **1.3**.

1.1.4.3 Glutamate: NMDA Receptor

The NMDA receptor is crucial to changes in spinal plasticity and synaptic transmission. NMDA receptors possess several unique properties; they have a high permeability for Na^{+} and K^{+} as well as movement of Ca^{2+}. They require dual stimulation with both glutamate and glycine (a co-agonist that aids in efficient activation). Finally under normal resting membrane potentials the NMDA pore is blocked with a Mg^{2+} meaning that it can only open upon both agonist binding and depolarisation (Petrenko *et al.*, 2003).

NMDA receptors are heterotetrameric channels that are composed of NR1, NR2A-D and NR3A & NR3B. NR1 and NR2 are crucial to the function of the NMDA receptor and hold the binding sites for glycine and glutamate respectively. The presence of different NR2 subunits changes the activity and sensitivity of the channel to agonist and antagonists, as well as changing its rate of desensitisation (Hirai *et al.*, 1996; Laube *et al.*, 1997; Mori *et al.*, 1995). The main observed difference between subunits is that channels containing NR2A or NR2B have a high conductance when open and are very sensitive to Mg^{2+} block. NR2C and NR2D containing channels have lower conductance when open and a lower sensitivity to Mg^{2+} block (Petrenko *et al.*, 2003).

NMDA receptors are located mainly upon post-synaptic neuronal cells within the dorsal horn (Nagy *et al.*, 2004b). When activated they produced a long lasting depolarisation accompanied by increased excitability. The role of NMDA receptors are pivotal in central sensitisation (Ji *et al.*, 2003) and wind up (Herrero *et al.*, 2000). NMDA receptors have also been described as being located pre-synaptically on non-peptidergic C fibres behaving as autoreceptors (Liu *et al.*, 1994).

1.1.4.3 Glutamate: Kainate Receptors

Kainate receptors have a modulatory role in nociceptive transmission. They are present on pre-synaptic terminals particularly on non-peptidergic C fibres (Lucifora *et al.*, 2006), their activation leads to a decrease in glutamate release acting as a negative feedback loop. They have also been detected on post-synaptic terminals where they play a role in acute nociception as well as modulation of GABAergic transmission and substance P activity (Hegarty *et al.*, 2007; Lu *et al.*, 2005a).

1.1.4.4 Glutamate: Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) are divided into groups I, II and III (Endoh, 2004) based on their receptor structure and function (Ohashi *et al.*, 2002). Group I consists of mGluR1 and mGluR5 which are G_q coupled and located post-synaptically (Shigemoto *et al.*, 1997). They cause an increase in Na^+ and K^+ currents (Chu *et al.*, 2000) and also play a regulatory role on Ca^{2+} currents having both an excitatory and inhibitory effect on voltage dependent calcium channels (Endoh, 2004). Group I mGluRs are also able to promote NMDA receptor function (Skeberdis *et al.*, 2001). Group II and III are located pre-synaptically (Shigemoto *et al.*, 1997) and can be found on C-fibre terminals (Jia *et al.*, 1999). The group II and III mGluRs are coupled to $G_{i/o}$ leading to a reduction in NMDA activity via inhibition of adenylate cyclase (Ambrosini *et al.*, 1995).

1.1.4.5 GABA

The major inhibitory neurotransmitter is γ-aminobutyric acid (GABA). GABA originates from GABAergic interneurones as well as from descending control pathways synapsing within lamina II (Kato *et al.*, 2006). It is synthesised from glutamate via the enzyme glutamate decarboxylase (GAD). There are two main receptors for GABA; the ionotropic $GABA_A$ receptor and metabotropic $GABA_B$ receptors.

The $GABA_A$ receptor is a pentameric ion channel consisting of several different subunits (Miller *et al.*, 2014) it permits the movement of Cl^- ions leading to the hyperpolarisation of neurones reducing their ability to fire action potentials (Johnston, 1996). The receptors are located throughout the dorsal horn, however different there exist receptors with varying subunit compositions depending on their location. The β3 and γ2 subunits are expressed throughout the spinal cord however the α subunits are differentially expressed in different lamina. It has been shown that α2 and α3 and present in all dorsal horn laminae, but α1 and α5 are exclusive the laminae III-VI being absent from laminae I-II (Bohlhalter *et al.*, 1996).

The $GABA_B$ receptor performs a modulatory role on spinal excitability. They can be located pre-synaptically where they can mediate the release of GABA or other neurotransmitters by the inhibition of N-type or P/Q type Ca^{2+} channels (Amico *et al.*, 1995; Menon-Johansson *et al.*, 1993). The activation of the $GABA_B$ receptors can also be coupled to rectifying K^+ channels leading to the production of a slow inhibitory postsynaptic current. The K^+ efflux leads to the hyperpolarisation of neurones preventing the propagation of action potentials (Luscher *et al.*, 1997).

Alterations in GABA signalling has been implicated in the generation and maintenance of chronic pain, increased $GABA_A$ receptor trafficking to the synapse has been shown to be involved in the transition from acute to chronic pain (Price *et al.*, 2015). The use of $GABA_A$ agonists had been beneficial in reducing neuropathic injured by nerve injury (Braz *et al.*, 2012) and inflammatory pain (Kim *et al.*, 2016) this is due to the inhibitory role of GABA in reducing spinal excitability.

1.1.4.6 Glycine

Glycine is another inhibitory neurotransmitter of the central nervous system in addition to its role as a co-transmitter of the NMDA receptors. The glycine receptor is located mainly within the upper superficial laminae of the dorsal horn (Harvey *et* *al.*, 2009) however they have been found in lamina III and some deeper laminae (Todd, 1990). Approximately half of GABAergic dorsal horn neurones also express glycine receptors. Glycine receptors are pentameric like the $GABA_A$ receptor (Miyazawa *et al.*, 2003). Glycine receptors expressing the α3 subunit are found in the outer laminae of the dorsal horn and it has been shown that α3 knockout reduces central sensitisation however animals still show normal sensitivity to neuropathic and visceral pain (Hösl *et al.*, 2006) as well as in rheumatoid and osteoarthritic pain (Harvey *et al.*, 2009). It has also been shown that PGE_2 mediated activation of PKA leads to phosphorylation of the α3 subunit of glycine receptors inhibiting its activity (Harvey *et al.*, 2004).

1.1.4.7 Noradrenaline

Noradrenaline (NA) originates from the locus coeruleus (A6) , A5 and A7 nuclei of the pons forming part of the descending pathways from the brain which project to the dorsal horn (Satoh *et al.*, 1982). Many of these connections as previously stated work through volume transmission (Zoli *et al.*, 1999). NA acts on GPCRs divided into two subtypes the α_1 adrenoceptor coupled to G_q (activating phospholipase C) and the α_2 adrenoceptor coupled to G_i (inhibiting adenylate cyclase activity) (Summers *et al.*, 1993). NA inhibits the release of neurotransmitters from C-fibres (Lu *et al.*, 2007). NA can also cause hyperpolarisation of interneurones within lamina II via the activation of α_2 receptors, and induce depolarisation within lamina I via α_1 activation (Grudt *et al.*, 1995; North *et al.*, 1984). The antinociceptive effect of NA is linked to its action on α_2 adrenoceptors as intrathecal administration of α_2 agonists produce reductions in pain in animals including primates (Takano *et al.*, 1992; Yaksh *et al.*, 1981). There is also evidence of epidural administration of α_2 agonists alleviating pain in humans (Eisenach *et al.*, 1995). There are also noradrenergic connections peripherally from the sympathetic nervous system however these have little effect on pain modulation. It has been shown that peripheral administration of NA into the skin does not itself induce pain, but does cause thermal hyperalgesia (Fuchs *et al.*, 2001).

1.1.4.8 5-Hydroxytryptamine

5-hydroxytryptamine (5-HT) is another of the monoamine neurotransmitters along with NA. It has wide and varied functions due to the large number of 5-HT receptors (seven families identified) that are coupled to GPCRs with the exception of the 5-HT_3 receptor which is ionotropic. 5-HT can be pro-nociceptive and antinociceptive, causing inhibition of Aδ and C fibres as well as the hyperpolarisation of excitatory interneurones (Lu *et al.*, 2007) and increasing pain sensitivity under normal conditions (Rahman *et al.*, 2011; Suzuki *et al.*, 2002) and in different pain states (Wei *et al.*, 2010).

The action of 5-HT is able to have these varied effects due to the variety of the receptor subtypes. There is evidence that the antinociceptive effects of 5-HT may be linked to action of the 5-HT_{1A} (Jeong *et al.*, 2012; Lu *et al.*, 2007) and 5-HT_{2C} receptors (Obata *et al.*, 2004). The 5-HT_{1A} receptor is coupled to G_i which inhibits adenylate cyclase, activation of 5-HT_{1A} has been shown to increase K^+ influx leading to neuronal hyperpolarisation. The 5-HT_{2C} receptor acts through a G_q coupled mechanism and is able to modulate serotonergic activity via the signalling of GABAergic neurones upstream leading to a negative feedback control mechanism (Spoida *et al.*, 2014). The pro-nociceptive role of 5-HT is linked to its action on 5-HT_3 (Rahman *et al.*, 2011; Suzuki *et al.*, 2002), activation of 5-HT_3 leads to the influx of cations causing the excitation of dorsal horn neurones (Hoyer *et al.*, 2002).

1.1.4.9 Neurokinins

These are pro-nociceptive transmitters that include substance P. They are generated within the cell body of primary afferent fibres where they are transported to central and peripheral terminals. They are usually released centrally in conjunction with other transmitters as part of a multi-transmitter signalling network (Juranek *et al.*, 1997). In the dorsal horn substance P is co-released with glutamate leading to the generation of fast ionotropic mediated excitatory post-synaptic potentials (EPSPs) while the activation of NK1 (GPCR for substance P) leads to the generation of a slower

EPSP (De Felipe *et al.*, 1998). The use of substance P antagonists were looked at in clinical trials however they failed to exhibit any efficacy to a variety of pain states. This is thought to be due substance P being more important in some pain states than others. They have however been considered as potential adjuvants for analgesic treatment (Hill, 2000).

1.1.4.10 Other Peptides

There are many other peptides that lead to the activation or modulation of nociceptors. Growth factors such as nerve growth factor (NGF) and glial cell-line derived neurotrophic factor (GDNF) have been shown to play a role in pain sensation and during chronic pain. NGF has been shown to be expressed in high levels within damaged tissues and is able to facilitate pain transmission within nociceptors. It binds to the TrkA receptors which are expressed on nociceptive neurones (Fang *et al.*, 2005) where it co-localises with TRPV1 (Moran *et al.*, 2004). Activation of TrkA leads to the sensitisation of TRPV1 via the activation of PLC (Chuang *et al.*, 2001). NGF has been implicated in a number of chronic conditions such as arthritis (Halliday *et al.*, 1998) and cancer induced pain (Ye *et al.*, 2011).

GDNF is a small protein molecule that promotes the survival of several types of neurone. Promoting dopaminergic neurone survival and differentiation as well as preventing apoptosis in motor neurones (Lin *et al.*, 1993). Intramuscular injection of GDNF has been shown to induce hyperalgesia lasting up to 3 weeks (Alvarez *et al.*, 2012a). However GDNF has also been demonstrated to be analgesic preventing and reversing pain developed during neuropathic pain (Boucher *et al.*, 2000).

Calcitonin gene-related peptide (CGRP) that is released both peripherally and centrally having varied effects in each region. Centrally CGRP has different effects depending on its location, within the ventral horn it is synthesised in motor neurones and may help in the regeneration of nervous tissue. In the dorsal horn CGRP is synthesised in the cell bodies of DRGs where its release at central terminals promoting excitability via the potentiation of responses to substance P (Biella *et al.*, 1991; Chen *et al.*, 2010).

1.1.5 Ascending Pathways

1.1.5.1 Projection Neurones

Within the dorsal horn of the spinal cord noxious inputs are processed and modulated by excitatory and inhibitory neurones that relay the information to the brain. The information from the primary afferent fibres enters the dorsal horn and is relayed to the brain and supra-spinal regions by projection neurones localised within lamina I, they are also present in laminae III-VI (Todd, 2010b). Many of the projection neurones within lamina I receive monosynaptic connections from primary afferent fibres which go on to form part of the ascending pathway (Ruscheweyh *et al.*, 2004). These projections neurones transmit information to several different brain regions identified by retrograde tracing techniques. These include the parabrachial area, periaqueductal gray (PAG), thalamus and the caudal ventrolateral medulla (Todd, 2010b).

1.1.5.2 Spinothalamic Tract

The spinothalamic tract is involved in the transmission of pain, temperature and the sensation of itch (Craig, 2003; Craig *et al.*, 2001). The spinothalamic tract conveys information from the dorsal horn (mainly laminae I and V) up to the thalamus. In the thalamus projections terminate within six distinct regions: the posterior portion of the ventral medial nucleus, ventral posterior nuclei, ventral lateral nucleus, central lateral nucleus, parafascicular nucleus and the ventral caudal portion of the medial dorsal nucleus. With much of the innervation from the dorsal horn terminating within the ventral posterior nuclei (Craig, 2003).

In the thalamus the most clearly studied of the spinothalamic neurones innervate the ventral posteromedial (VPM) and ventral posterolateral nuclei (VPL) which form part of the ventral posterior nuclei (Craig *et al.*, 2001). Neurones from the VPL connect to the somatosensory cortex and aids in sensory discrimination i.e. pain location and intensity (Basbaum *et al.*, 2009). The VPM also projects to the somatosensory cortex as well as the insular cortex playing a role in the discrimination and the motivational aspects of pain (Gauriau *et al.*, 2004). Input from lamina V and VII project to the ventral lateral nucleus which relays information to the somatosensory and sensorimotor cortices (Craig, 2008). These variation in termination locations shows that the inputs from the spinal cord are each processed differently forming the overall sensation of pain.

1.1.5.3 Spinoparabrachial Tract

This ascending pathway transmits information to the parabrachial area located in the midbrain. Input from the spinal cord originates from lamina I with a slightly weaker contribution for laminae IV-VI (Chamberlin *et al.*, 1992). Many of these projection neurones within lamina I are NK1 positive and can be nociceptive cells with dendritic trees or wide dynamic range neurones (Todd *et al.*, 2000). Lamina III-VI projection neurones are also wide dynamic range neurones that express NK1 (Naim *et al.*, 1997). The information travels from the dorsal horn to the parabrachial area. The parabrachial area outputs to the amygdala and ventromedial hypothalamus, associated with the aversive reaction to pain and emotional and autonomic responses. The parabrachial area also projects to the thalamus joining with the spinothalamic tract (Bernard *et al.*, 1993). There are also several connections from this tract that link to the periaqueductal gray (PAG) and the rostroventromedial medulla (RVM), which then project back to the spinal cord (**1.1.6**) (Basbaum *et al.*, 2009; Gauriau *et al.*, 2002).

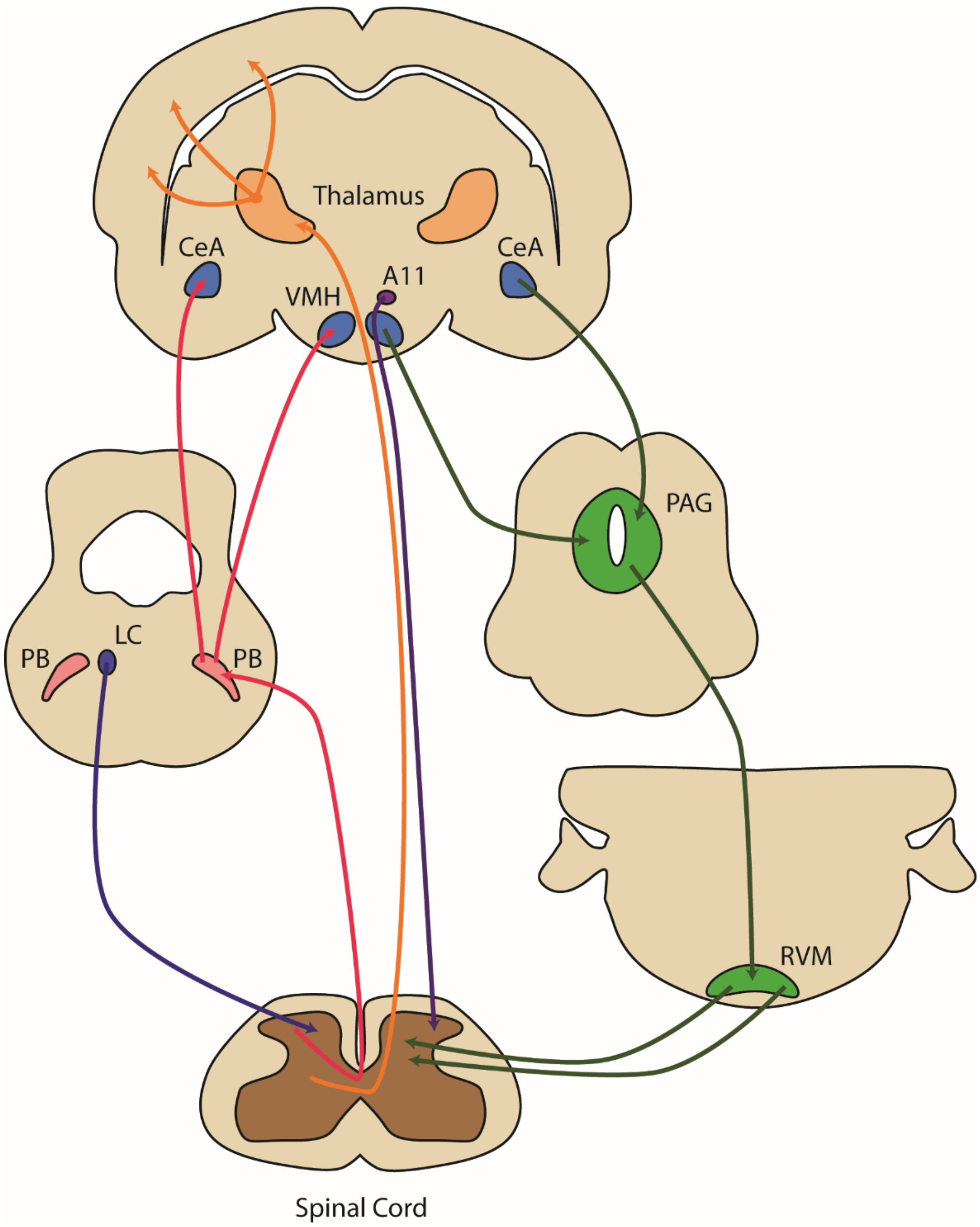


Figure 1.2 Ascending and descending pain pathways. Information from the dorsal horn travels up two major ascending tracts the Spinoparabrachial (red) transmits information to the ventromedial hypothalamus (VMH) and the central nucleus of the amygdala (CeA) via the parabrachial area (PB). The Spinothalamic (orange) takes information to the thalamus which then connects to several regions of the cortex.
There are several descending pathways from the periaqueductal grey (PAG) to the rostroventral medial medulla (RVM) to modulate the dorsal horn excitability (green). There are also descending noradrenergic (blue) and dopaminergic (purple) innervation from the locus coeruleus (LC) and the A11 nucleus of the hypothalamus respectively (based on Bradl et al. 2014, Koblinger et al. 2014).

1.1.5.4 Spinoreticular Tract

The spinoreticular tract originates within lamina 5 as well as connections located with laminae 1, 7 and 8, from here projections travel up the spinal cord to several regions within the hindbrain. The hindbrain structures that are innervated by the spinoreticular tract include the raphe magnus and median raphe nucleus. Information is then sent to the thalamus and onto the cerebral cortex promoting the behavioural and emotional aspects of pain (Millan, 1999; Sengul *et al.*, 2012).

1.1.5.5 Spinomesencephalic Tract

The spinomesencephalic tract consists of several projection pathways from the spinal cord to locations throughout the midbrain. There are 3 main pathways that make up the spinomesencephalic tract these include the spinotectal, spinoannular and parts of the spinoparabrachial tracts. Projections originate throughout the spinal cord from laminae I, III, IV, V, VII and X and terminate within the thalamus and medullary reticular formation. These pathways are integrated into the emotional component of pain (Menetrey *et al.*, 1982; Yezierski, 2007).

1.1.6 Descending Controls

Sensitivity to pain can be modulated by the activation of descending control pathways from the brain. The main regions that control spinal excitability are the PAG and the RVM located in the midbrain and brainstem respectively (Basbaum *et al.*, 1978; Basbaum *et al.*, 1984). The PAG and RVM modulate nociceptive sensitivity by integrating the information from the spinal cord and several brain regions including the medial prefrontal cortex, limbic system, hypothalamus and nucleus accumbens. This integrated signalling allows for the generation of the modulatory controls upon the dorsal horn.

1.1.6.1 Periaqueductal Gray

The PAG was the first brain region that was demonstrated to activate endogenous inhibitory modulation of pain responses (Basbaum *et al.*, 1978). The PAG received primary afferent input from projections neurones in the dorsal horn as well as from forebrain structures. The information is integrated allowing the PAG to make appropriate regulatory modulation. There were many studies that showed that the administration of opioids to, or electrical stimulation of the PAG caused a large inhibitory effect on pain responses in both animals (Reynolds, 1969; Tsou *et al.*, 1964) and humans. Studies showed that the inhibitory effect could be reversed via the opioid antagonist naloxone (Hosobuchi *et al.*, 1977; Lewis *et al.*, 1977). The PAG is now a well-established region for descending opioid mediated inhibition. The PAG has reciprocal connections with the RVM (Willis, 1985). Excitation of the PAG by glutamate administration caused increased firing within cells of the nucleus raphe magnus (NRM), which correlated with nocifensive behaviour in the rat (Behbehani *et al.*, 1979). The PAG then projections down to the RVM, but not directly to the dorsal horn.

1.1.6.2 Rostroventromedial Medulla

The RVM receives inputs from several brain regions including the PAG, thalamus, parabrachial area and locus coeruleus and is the final integration point for descending modulation. The RVM is able to both inhibit and facilitate spinal excitability this is carried out by two major cell types, the on cell have increased activity in response to noxious stimulation, the off cell however have shown an immediate halt to firing prior to pain induced reflex (Fields *et al.*, 1985; Fields *et al.*, 1991). Opioid administration is able to inhibit on cells as well as causing the excitation of off cells leading to potential analgesia (Fields *et al.*, 2005; Heinricher *et al.*, 2009). The activity within the RVM increases in response to injury or inflammation showing increased on cell activity (Gardell *et al.*, 2003; Zhang *et al.*, 2009). These project down to the dorsal horn of the spinal cord modulating its sensitivity.

1.1.6.3 Neurochemistry of Descending Control Pathways

The modulation of spinal excitability is a vast and complex area of research, this section will briefly cover the descending pathways connected to the modulation of nociception. There are three main descending pathways that modulate spinal excitability; serotonergic neuronal connections originating within the medulla raphe nucleus (Bowker *et al.*, 1983; Miletic *et al.*, 1984), inhibitory GABAergic connections originating from the RVM (Antal *et al.*, 1996) and noradrenergic connections from the locus coeruleus (Satoh *et al.*, 1982; Westlund *et al.*, 1983). These pathways work together to modulate how the dorsal horn processes incoming pain signals from the periphery.

These pathways enter and terminate throughout the dorsal horn, the GABAergic connections mainly terminating within lamina II with interneurones (Kato *et al.*, 2006) this leads to an inhibition in the nociceptive information within the spinal dorsal horn (Antal *et al.*, 1996; Millan, 2002). The serotonergic and noradrenergic pathways have a more indirect modulatory role and communicate via local release into the cellular environment allowing neurotransmitter to spread further from the synaptic cleft. This is known as volume transmission and allows the diffusion of transmitter to act on several different cells (Rajaofetra *et al.*, 1992; Zoli *et al.*, 1999). This modulation of nociceptive information is crucial to the creation of the overall sensation of pain.

1.2 Peripheral Sensitisation

1.2.1 Inflammation

When tissue is injured the process of inflammation is initiated, and this acts to protect the damaged site and remove any bacteria as well as damaged tissues. This acts as a form of homeostasis, returning the tissue to a normal state. There are several key processes to inflammation. Firstly vasodilation, this leads to increased blood flow to the damaged region causing the typical redness and increased heat. Injury to a tissue leads to damaged cells and the disruption to blood flow causing the release of vasoactive compounds including prostaglandins, histamine and thrombin which go on to mediate the inflammatory response (Gillitzer *et al.*, 2001; Kim *et al.*, 1998).

The infiltration of immune cells causes the stereotypical oedema (swelling) that is seen during an inflammatory reaction. Immune cells such as macrophages and neutrophils also produce further inflammatory mediators (discussed in **1.2.2**) Capillaries and other blood vessels within the injured site will constrict in response to damage but the vasoactive substances lead to vasodilation and increased vascular permeability of other non-damaged blood vessels (MacKay *et al.*, 2003).

The released inflammatory mediators have a multitude of effects to promote the activation of the immune system as well as the sensitisation and direct activation of nociceptive terminals transmitting pain signals to the central nervous system from the periphery (major receptors discussed in **1.2.3**). Histamine is produced by mast cells which degranulate during an inflammatory response releasing histamine into the surrounding extracellular space. Histamine binds to H1 and H2 GPCRs present on blood vessels within the damaged region causing vasodilation. Histamine does not directly cause pain by does act to promote nociceptor sensitisation. Prostaglandins are produced from arachidonic acid via the action of the enzyme cyclooxygenase (COX) (further discussed in Chapter 6) cause an increase in vascular permeability by binding to EP receptors present on blood vessels promoting the expression of I-CAM1 (a structural protein expressed on blood vessel walls that bind to monocytes and macrophages), allowing for the infiltration of monocytes and macrophages into the injured tissue to remove cell debris and any foreign invaders such as bacteria. (Bryant, 2007; Maroon *et al.*, 2010). Other inflammatory mediators such as bradykinin which is produced in the blood and binds to receptors that promote the phosphorylation of receptors present on nociceptive terminals such as TRPV1 (Chuang *et al.*, 2001; Huang *et al.*, 2006).

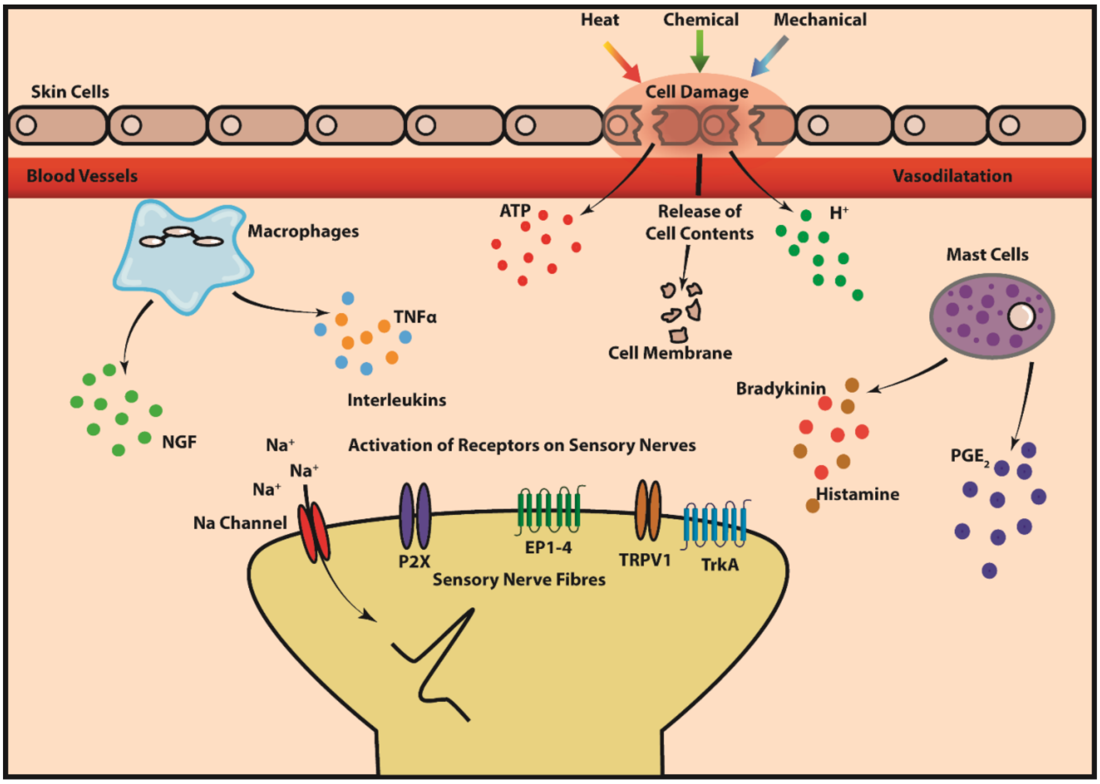


Figure 1.3 Summary of peripheral sensitisation. When a tissue is damaged by heat, chemical or mechanical stimulation, it causes the activation of the immune system promoting macrophages infiltration and mast cell degranulation. Release of inflammatory mediators causes the activation and sensitisation of receptors present on nociceptive terminals. Triggering the generation of action potentials via the opening of sodium channels transmitting the information to the dorsal horn of the spinal cord. ATP – adenosine triphosphate, NGF – nerve growth factor, TNFα – tumour necrosis factor alpha, PGE_2 – prostaglandin E_2 (based on Basbaum et al. 2009).

1.2.2 Immune Cells

1.2.2.1 Neutrophils

Neutrophils are the first member of the leukocyte family to enter damaged tissue, and this occurs within the first hour after injury. They respond to inflammatory mediators and act to clear away debris and bacteria (Kumar *et al.*, 2010). Neutrophils are able to carry out phagocytosis by binding to adhesion molecules in damaged tissue or invading bacteria engulfing and destroying targets through the action of intracellular enzymes such as lysozymes (Rausch *et al.*, 1975; Welsh *et al.*, 1971). Neutrophils themselves signal to other immune cells such as macrophages and mast cells via the release of chemokines and other inflammatory mediators (TNFα, IL1β, CCL3, CCL4 etc.) (Soehnlein *et al.*, 2009). Upon clearing away damaged tissue and bacteria the neutrophil undergoes apoptosis and are subsequently phagocytised by macrophages (Akgul *et al.*, 2001).

1.2.2.2 Macrophages

Within the body there is a source of tissue resident macrophages that await bacterial invasion or tissue damage. During inflammation they are stimulated into producing inflammatory mediators, and monocytes are also recruited from the circulation (Murray *et al.*, 2011). The infiltrating monocytes are able to differentiate into two different classes of macrophage, the M1 classical macrophage which is inflammatory or the M2 alternatively activated anti-inflammatory macrophage (Italiani *et al.*, 2014).

The M1 macrophages are regulated by several different chemotactic factors and cytokines including TNFα, IL1β and CCL2-6 (Beuscher *et al.*, 1990; Wetzler *et al.*, 2000). These can be released from keratinocytes as well as the tissue resident macrophages. Activation of toll-like receptors present on monocytes leads to the activation of the NF-κB and STAT-1 pathways causing their differentiation into the

M1 subtype (Menzies *et al.*, 2010; Sica *et al.*, 2012). These pro-inflammatory macrophages produce more inflammatory mediators like TNFα, IL6 and PGE_2 (Kuroda *et al.*, 2003; Laskin *et al.*, 1995) as well as further chemokines. If production of these mediators continues it can lead to the generation of chronic inflammation (Murray *et al.*, 2011; Porcheray *et al.*, 2005). Once the tissue debris or bacteria have been removed tissue resolution can take place through the action of the M2 macrophages.

The M2 subtype act to reduce inflammation via the production of anti-inflammatory mediators such as IL10 and CCL17, 18 & 22. IL10 in particular actively reduces the production of TNFα, IL1β and IL12 (Aste-Amezaga *et al.*, 1998; Opp *et al.*, 1995). It has also been shown that IL4 and IL13 stimulation of macrophages causes activation of STAT-3/6 leading to differentiation to the M2 subtype (Bhattacharjee *et al.*, 2013).

Macrophages are crucial to the whole inflammatory process it has been shown that tissue will heal without the need of neutrophils (Dovi *et al.*, 2003). However the absence of macrophages leads to incomplete tissue repair potentially causing chronic inflammation (Bryant, 2007).

1.2.3 Nociceptor Sensitisation

The peripheral terminal of the nociceptor is located in the tissue that it innervates and when damage occurs, receptors on its surface are activated. This leads to nerve depolarisation via the activation of Na^+ channels present on nerve terminals allowing the movement of Na^+ ions causing the generation and firing of action potentials which are propagated towards the spinal cord. The peripheral terminal expresses a wide variety of receptors that each has a specific function that allows the nociceptor to sense different types of noxious stimuli. During inflammation there is a release of a large number of inflammatory mediators including prostaglandins, growth factors (NGF & GDNF), chemokines, cytokines as well as ATP and H^+ ions (Woolf *et al.*, 2007). These form the 'inflammatory soup' which can activate receptors on nociceptors sensitising them, and leading to allodynia and hyperalgesia. These sensitizers bind to receptors present on the nociceptive terminal and lead to the activation of several downstream pathways including PKA (Varga *et al.*, 2006), PKC (Hucho *et al.*, 2005) and ERK (Mizushima *et al.*, 2007) for example. One of the most prominently studied channels is the transient receptor vanilloid 1 (TRPV1) many of these inflammatory mediators can modulate the activity of TRPV1. These receptor families are very well studied and too complex to be covered in this thesis, therefore a brief overview of each family and their involvement in nociceptive will be discussed.

<u>1.2.3.1 Transient Receptor Vanilloid 1</u>

TRPV1 is a tetrameric cation channel allowing the movement of both Na^+ and Ca^{2+} ions through its pore. It was originally discovered using capsaicin, a component of the chilli pepper, which is an agonist at the receptor and produces a burning sensation (Caterina *et al.*, 1997). It was due to the burning feeling produced by capsaicin that led to TRPV1 being proposed as a detector of noxious pain due to thermal stimuli. TRPV1 in fact has a thermal threshold of 43°C; and temperatures greater than this will evoke an activation of the receptor (Caterina *et al.*, 1999). TRPV1 like the nociceptor itself is polymodal and can be activated by more than one stimulus, it has already been said that capsaicin acts as an agonist for the receptor, there are other endogenous molecules that also can activate or sensitise the TRPV1 receptor; these include the endocannabinoids e.g. anandamide (Ross, 2003) and inflammatory mediators like bradykinin (Katanosaka *et al.*, 2008). When tissue is damaged there is a release of intracellular contents including H^+ ions. This leads to a decrease in the extracellular pH. It has been shown that a reduction in pH levels to <6 leads to the TRPV1 channel opening at room temperature (Tominaga *et al.*, 1998). Also the activation of PKA and PKC phosphorylate serine sites (S502 and S800) (Lee *et al.*, 2012) on the TRPV1 receptor, reducing its threshold to stimuli meaning that previously non-noxious stimuli can activate this channel (Mandadi *et al.*, 2006).

1.2.3.2 Sodium Channels

Sodium channels are crucial to the generation and propagation of actions potentials. There have been a series of knockout studies that looked into the involvement of a variety of voltage gated sodium channels including Na(v) 1.7, 1.8 and 1.9. These channels are expressed selectively on nociceptive peripheral nociceptors.

The knockout of Na(v) 1.7 lead to animals with increased mechanical and thermal pain thresholds. It was however shown that the absence of Na(v)1.7 during development lead to death of the animal (Nassar *et al.*, 2004). Although animals lacking the Na(v)1.7 and 1.8 channels still display neuropathic pain demonstrating their role in inflammatory but not neuropathic pain generation (Nassar *et al.*, 2005). There are also several cases of human mutation in Na^+ channels leading to a reduction or complete loss in pain sensation. The mutation of the Na^+ channel gene SCN9A which encodes for Na(v) 1.7 has been shown to have several different effects on how individuals experience pain. A missense gain in function mutation can cause erythromelalgia (a vascular disorder leading to periodic blockages in blood flow causing burning pain and redness). There is also gain of function mutations that cause the rare condition paroxysmal extreme pain disorder expressed as pain within the mandibular, ocular and rectal regions of the body. The presence of a nonsense mutation in Na(v) 1.7 can results in a loss of sodium channel function and channelopathy associated insensitivity to pain where sufferers cannot feel physical pain (Drenth *et al.*, 2007).

The knockout of Na(v)1.8 lead to a prevention of NGF induced thermal hyperalgesia, however failed to prevent PGE_2 induced hypersensitivity as well as neuropathic pain, still being able to be established (Kerr *et al.*, 2001). Na(v)1.9 is also thought to be involved in the generation of peripheral sensitisation. Its knockout in Na(v)1.9$^{-/-}$ mice lead to a reduction in the hypersensitivity generated by the administration of PGE_2, bradykinin, IL1β and capsaicin as well as agonists for P_2X_3 and P_2Y (Amaya *et al.*, 2006).

Pro-inflammatory mediators such as TNFα have also demonstrated the capacity to modulate the activity of sodium channels on primary afferent fibres. The administration of TNFα leads to increased currents from tetrodotoxin resistant sodium channels (Na(v)1.7, 1.8 & 1.9) via activation of the TNF receptor 1 and through action of p38 mitogen-activated protein kinase (Jin *et al.*, 2006).

1.2.3.3 Acid Sensing Ion Channels

There are several other channels that also contribute to the generation of peripheral sensitisation. H^+ ions can also be detected by the aptly named acid sensing ion channels (ASICs) which are also expressed by the peripheral terminals of nociceptors (Olson *et al.*, 1998). The ASICs are formed of three subunits and can form as either homomers or heteromers; they open in response to high [H^+] and allow the flow of monovalent cations (Yermolaieva *et al.*, 2004), however it can allow the movement of Ca^{2+} although it doesn't play a major physiological role (Samways *et al.*, 2009). Their function i.e. pH sensitivity and desensitisation rates can be modulated by the release of inflammatory mediators and signalling molecules such as arachidonic acid and nitrous oxide (NO) (Sluka *et al.*, 2009). The ASICs are expressed on primary afferent fibres, specifically the peptidergic C-fibres. It has been shown using immunohistochemistry that they are co-expressed with substance P and CGRP (Olson *et al.*, 1998).

1.2.4 Tissue Resolution

Peripheral sensitisation under normal conditions is not a permanent change once the damage has been repaired or the painful stimulus removed the tissue returns to a resting state. It was originally thought that inflammation ended due to the breakdown of inflammatory mediators meaning the response just died out (Serhan *et al.*, 2007). However maintained inflammation can become chronic causing long lasting damage (Bryant, 2007). Tissue resolution consists of several processes that act to remove inflammatory cells, and antagonise the release and action of inflammatory mediators. This thesis will provide a brief overview of the mechanisms by which tissue resolution occurs.

A major method of immune cell removal is through the process of apoptosis. Apoptosis is the process of controlled cell death acting to destroy expired cells. Apoptosis occurs via two main pathways, the intrinsic pathway is self-mediated cell death caused by the release of proteins from the mitochondria during times of cell stress (Kroemer *et al.*, 2007). Apoptosis of inflammatory cells occurs via an extrinsic pathway, a mechanism that involves alterations in the cell surface markers on immune cells such as neutrophils allowing recognition by macrophages to engulf and clear them away (Savill *et al.*, 1989). Apoptosis within immune cells can be induced by the free radical NO, which is produced during inflammation. NO acts to inhibit the NF-κB pathway reducing the production of survival proteins allowing for the activation caspases causing DNA fragmentation and altered expression of surface markers targeting them for phagocytosis leading to cell death (Maskrey *et al.*, 2011).

This signal change is induced by the action of chemical mediators that aid in resolution. These are bioactive lipid mediators consisting of resolvins, protectins and maresins are derived from arachidonic acid (AA) and promote resolution after injury. The resolvins are divided to two main groups the D series and E series that act to reduce the infiltration of immune cells such as neutrophils, they have shown potential in the reduction of inflammatory pain (further discussed in **5.1**). Protectins and maresins also reduce neutrophil infiltration and promote phagocytosis by macrophages helping remove debris and immune cells undergoing apoptosis (Serhan, 2010; Serhan *et al.*, 2009). This allows for the tissue to return to resting condition once an inflammatory stimulus has ended.

1.3 Central Sensitisation

Previous studies have shown that following a sustained high frequency noxious input into the spinal cord there are changes in the expression and sensitivity of receptors in the dorsal horn of the spinal cord which result in a long lasting sensitization of spinal neurones (synaptic plasticity) to incoming noxious and innocuous inputs. These events are known as central sensitisation, which results in the expansion of the receptive fields of spinal neurones to areas outside of the original site of injury (known as secondary hyperalgesia). Unlike peripheral sensitisation, central sensitisation does not seem to resolve after an injury has recovered (Woolf, 1983) and is linked to the manifestation of chronic pain conditions. Indeed, the heightened sensitivity to painful stimuli remains long after the tissue injury has repaired (Woolf, 1983). It is important to realise that central sensitisation is not a simple change to a synapse, but is made up of the culmination of several cellular processes each of which act to increase the ease of activation of the sensory pathway.

1.3.1 Generation of Central Sensitisation

The development of central sensitisation takes time; however it is calcium that plays a major part in the initiation and maintenance of the sensitised state (Luo *et al.*, 2008). When primary afferent C-fibres reach the spinal cord they enter the dorsal horn and synapse with second order neurones in the spinal cord. The nociceptors release glutamate and other transmitters which bind to receptors present on the post synaptic site depolarising the post-synaptic neurones (De Biasi *et al.*, 1988).

There are several glutamate receptors located on post synaptic terminals, AMPA receptors are ionotropic channels selective towards Na^+ ions, and depending on the subunit composition they can also allow Ca^{2+} ions through their channel as well (Polgar *et al.*, 2008). When a nociceptor is repeatedly stimulated, or generates longer and larger depolarisations particularly during inflammation or a nerve injury, there is an excessive release of glutamate (Inquimbert *et al.*, 2012). This excessive release causes more AMPA receptors to be activated therefore a large depolarisation occurs in the second order neurones. There is also a change in the subunit make up of AMPA receptors in favour of non-selective subunit GluR1 (Larsson *et al.*, 2008). Substance P is co-released with glutamate from peptidergic C-fibres, which works to produce slow depolarisations that maintain the change in membrane potential of the post-synaptic terminal (Afrah *et al.*, 2002).

Due to injury there is continuous stimulation of primary afferent fibres so there is a large concentration of glutamate released from synaptic terminals (Inquimbert *et al.*, 2012). The concentration is so great that the reuptake transporters and enzymes that metabolise the glutamate are unable to remove all the glutamate in the synaptic cleft. This means that the excess glutamate can reach the out-lying metabotropic glutamate receptors (mGluRs) (Azkue *et al.*, 2000). This leads to maintained depolarisation causing the removal of the Mg^{2+} ion block in NMDA receptors (Mayer *et al.*, 1984). Both Na^{+} and Ca^{2+} ions can pass through the pore within the NMDA receptor leading to further depolarisation.

The mGluRs activate PKC and PLC pathways through the release of Ca^{2+} from intracellular stores located in the endoplasmic reticulum (Fagni *et al.*, 2000). The increased concentration of Ca^{2+} activates a multitude of intracellular pathways including ERK which goes on to increase the sensitivity of AMPA and NMDA receptors by phosphorylation through PKA and PKC activation (Slack *et al.*, 2004) additionally there is an increase in the number of glutamate receptors expressed at the post-synaptic terminal (Galan *et al.*, 2004). ERK will also phosphorylate the Kv4.2 channels reducing K^{+} flow therefore increasing membrane excitability(Hu *et al.*, 2006).

PKC is activated downstream of the NMDA, NK_1, and mGluRs, and can also play a role in increasing the membrane excitability; it does this by reducing the GABAergic and glycinergic inhibitory neurones that act on the second order terminals (Lin *et al.*, 1996).

This is the same mechanism as long term potentiation (LTP), which is involved in learning and memory, however there are some important differences that occur only in central sensitisation (Ji *et al.*, 2003).

1.3.2 Types of Sensitisation

There are two forms of potentiation that happen in sensitised neurones homosynaptic potentiation and heterosynaptic potentiation, the latter never occurs with long term potentiation (LTP). Homosynaptic potentiation is a use-dependent facilitation of a single synapse caused by the activation of said synapse. Windup is a form of homosynaptic potentiation where a train of low frequency stimuli cause elicited action potentials to get larger after each subsequent stimulus (Mendell *et al.*, 1965). The repeated stimulation of the primary afferent causes long sustained depolarisation due to release of substance P and CGRP causing temporal summation of the slow synaptic potentials (Afrah *et al.*, 2002). The long depolarisation leads to the removal of the Mg^{2+} block in the NMDA receptors enhancing the sensitivity of the synapse. Heterosynaptic potentiation is specific to central sensitisation this is where repeated stimulus of one primary afferent causes an increase in the activity of its synapse (homosynaptic) this then leads to an increase in the activity of other surrounding synapses (heterosynaptic). This means that uninjured areas will exhibit allodynia and hyperalgesia and it will seem as though the pain has spread from its origin to neighbouring tissues; secondary hyperalgesia (LaMotte *et al.*, 1992). The method by which heterosynaptic potentiation occurs are unknown, however there are two possible mechanisms that could be involved. The first is the mGluRs, which are crucial to the development of central sensitisation as they are linked to large Ca^{2+} release from the endoplasmic reticulum. The other is nitrous oxide which diffuses rapidly to surrounding tissue when released and its downstream pathways could be key to the change in the sensitivity of synapses in surrounding regions (Moreno-Lopez *et al.*, 2006).

Central sensitisation is formed through a combination of changes to synaptic function, a decrease in the threshold for activation of glutamatergic receptors, translocation of receptors from internals stores to the membrane increasing their expression, and a reduction of inhibitory pathways. All these changes work to increase the sensitivity of the sensory nervous system to non-noxious and noxious stimuli.

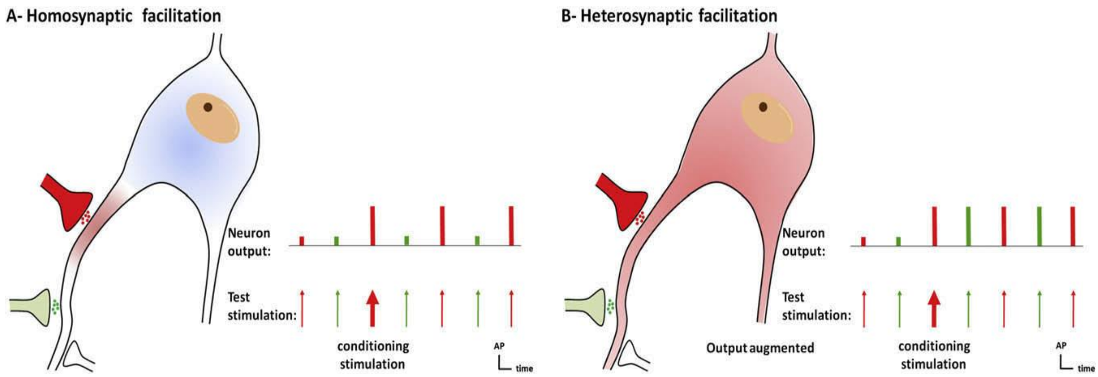


Figure 1.4 Types of sensitisation: **A:** Homosynaptic facilitation leads to the strengthening of a single synaptic connection via the increase in the expression of glutamatergic receptors on the post-synaptic site in a mechanism that is similar to long-term potentiation. **B:** Heterosynaptic facilitation is specific to central sensitisation, this causes a single synapse to activate its connected post-synaptic site and adjacent terminals. This leads to the spread of pain that is present in many chronic pain cases (Latremoliere et al. 2009).

1.3.3 Wind Up

One of the major examples of increased central excitability in the field of pain is wind up. This is a short lasting effect due to repeated peripheral input to the spinal cord leading to enhanced wide dynamic range neurone discharge caused by the application of a train of >10 electrical stimuli at between 0.5-5Hz (Mendell, 1966). This effect is reversible and non-pathological, and it can last from seconds to minutes leading to temporary hyperalgesia. The mechanism behind this effect is similar to the generation of long-term potentiation and involves the activation of NMDA receptors and increased Ca^{2+} (Thompson *et al.*, 1990), it has been shown that the blockade of NMDA receptors leads to a reduction in central excitability (Woolf *et al.*, 1991). The activation of NMDA receptors requires the summation of both fast and slow EPSP involving the action of glutamate and peptidergic transmission respectively (Murase *et al.*, 1986; Sivilotti *et al.*, 1993).

1.4 Hyperalgesic Priming

1.4.1 Animal Models of Pain

Pain is an important health problem and so a crucial area of study. Setting up and studying animal models of different pain states allows for better understanding of its mechanisms and potential discovery of therapeutics. There are two major types of pain; inflammatory and neuropathic. Neuropathic pain is caused by an injury or damage to a nerve. This can be studied in animals using models of chemical injury such as diabetic neuropathy (Byrne *et al.*, 2015) or surgically using chronic nerve constriction (Austin *et al.*, 2012). Inflammatory pain can be modelled in animal via the injection of inflammatory chemicals such as Complete Freund's Adjuvant (CFA) a solution of inactivated mycobateria (Ren *et al.*, 1999). These models directly induce chronic pain however because many chronic pain states start with acute inflammation (MacIntyre *et al.*, 1995; Melhorn, 1998) it is important to study how acute pain can become chronic.

Hyperalgesic priming is a relativity recent chronic pain model first being demonstrated by Jon Levine, University of California, San Francisco (Aley *et al.*, 2000). It models the transition from acute to chronic pain. This model can be useful in understanding the cellular and mechanistic changes that occur during the transition from acute short lasting pain to chronic long lasting pain.

1.4.2 Initiating Hyperalgesic Priming

Hyperalgesic priming involves the application of two acute inflammatory stimuli that lead to the generation of a long term sustained pain response. Hyperalgesic priming involves changes in the responsiveness of the animal to mechanical stimulation. It is characterised by a long term reduction in paw withdrawal threshold following the second stimulus. It was demonstrated that the administration of carrageenan (an extract from seaweed that induces an inflammatory reaction via the activation of toll-like receptor 4 (Bhattacharyya *et al.*, 2008)) led to the priming of nociceptors. When PGE_2, 5-HT or CGS-21680 (A_2 adenosine receptor agonist) were then administered into the same site it caused long term allodynia that lasted longer than normal (Aley *et al.*, 2000).

This effect requires a priming period to occur, if the secondary stimulus is administered prior to the 72 hour time-point after the initial injection then long term reductions in mechanical withdrawal thresholds don't occur (Bogen *et al.*, 2012). It was demonstrated that priming could be initiated via the use of NGF and GDNF as the initial stimulus prior to the administration of PGE_2 (Joseph *et al.*, 2010). This would suggest that the activation of either peptidergic or non-peptidergic C-fibres can lead to hyperalgesic priming from taking place. However it has been shown that for priming to occur it requires the activation of IB_4^+ neurones. The administration of IB_4-saporin intrathecally caused the destruction of all IB_4 expressing neurones. The loss of IB_4^+ neurones prevented priming from occurring this was demonstrated in both plantar skin (Joseph *et al.*, 2010) and gastrocnemius muscle (Alvarez *et al.*, 2012a; Alvarez *et al.*, 2012b).

Interestingly priming cannot be induced in female rats, administration of oestrogen into male rats also prevents the induction of hyperalgesic priming. Performing a gonadectomy on female rats also allows for the induction of hyperalgesic priming (Joseph *et al.*, 2003). However it has been shown that using activators that target downstream pathways can lead to the induction of hyperalgesic priming in females (see 1.4.3). Therefore this model is not a suitable pre-clinical model however is very useful for investigating the transition from acute to chronic pain.

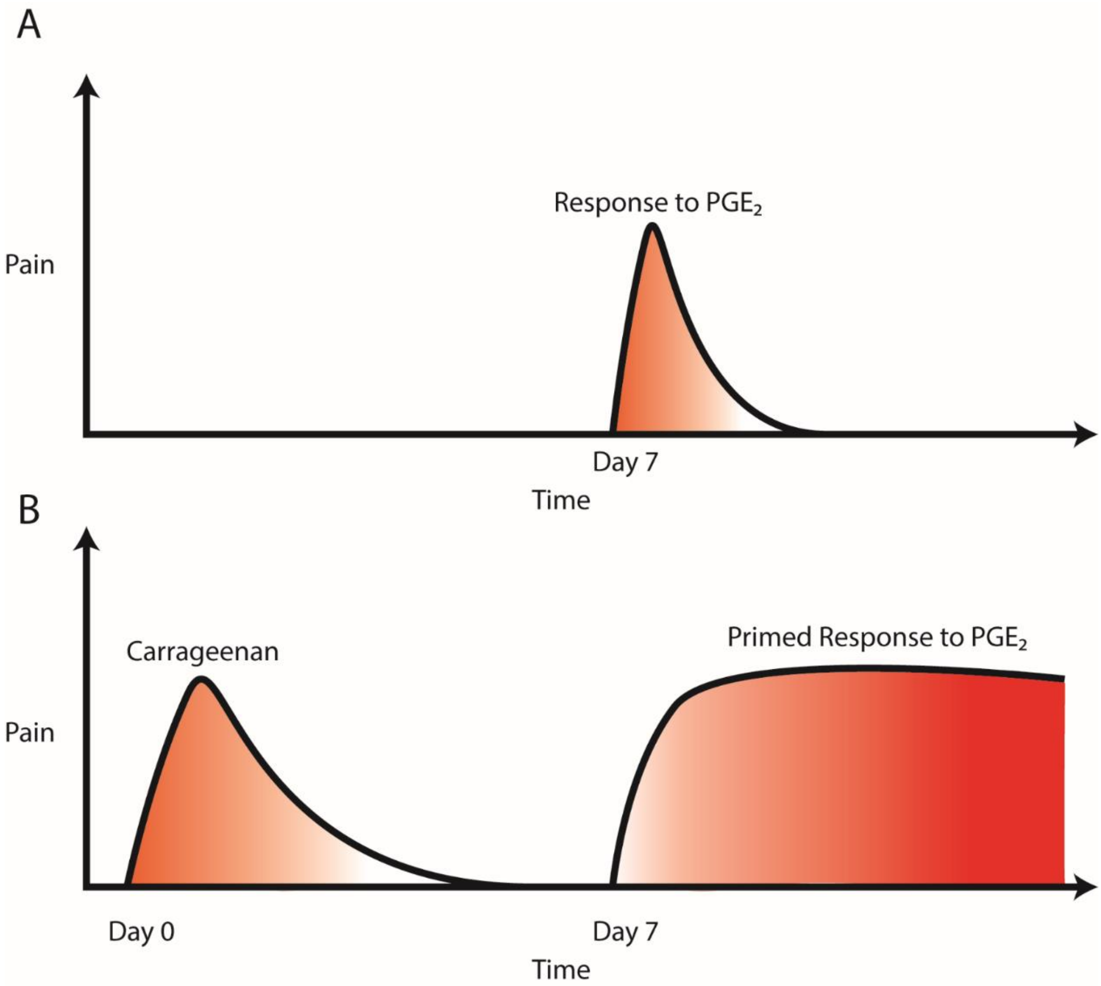


Figure 1.5 Hyperalgesic priming; the administration of PGE_2 alone causes a short reversible acute pain. By administering carrageenan one week prior causes PGE_2 to generate a long lasting chronic state (based on Reichling et al. 2009).

1.4.3 Underlying Mechanisms

The cause of the extended PGE_2 hyperalgesic response has been linked to a switch in its signalling pathway. Under normal conditions PGE_2 signals via a PKA dependent pathway however it has been shown that after priming has been initiated the PGE_2 response becomes biphasic. There is the normal acute phase (30mins-1 hour post injection) and then the long term chronic phase (>4 hours post injection) this is due to a switch from a PKA mediated response during the acute phase to a PKCε mediated chronic phase (Aley *et al.*, 2000). Priming was then shown to be initiated via the administration of PKCε activator (ΨεRACK) (Aley *et al.*, 2000; Ferrari *et al.*, 2013b) and blocked via the intrathecal injection of PKCε antisense oligodeoxynucleotides (Dina *et al.*, 2008).

The requirement for the 72 hour period between the first and second injections was suggested to be due to protein translational changes that were occurring due to the initial priming agent. This was investigated using rapamycin and cordycepin. Rapamycin is an mTOR inhibitor, which is responsible for cell growth, cell proliferation and importantly protein synthesis (Ballou *et al.*, 2008). Intraplantar treatment with rapamycin on days 1-3 post intraplantar carrageenan prevented the enhanced PGE_2 response caused by hyperalgesic priming. Cordycepin is an inhibitor of polyadenylation leading to a reduction in poly(A) tails on mRNAs. It has been shown to have a selective bias for inflammatory mRNA (Wong *et al.*, 2010). Administration of cordycepin has also shown to prevent the generation of hyperalgesic priming when injected after carrageenan 7 days prior to PGE_2. The preventative effect of cordycepin however was shown to be temporary if given 20 days prior to carrageenan it failed to prevent the PGE_2 hyperalgesia (Ferrari *et al.*, 2013a).

Studies of the downstream pathways that lead to hyperalgesic priming. It was shown that the intrathecal administration of antisense oligodeoxynucleotides against calmodulin kinase (CAM kinase) could prevent priming from being initiated. The use of αCAMKII (activator of CAM kinase) could itself induce hyperalgesia and be used as an initiator of priming demonstrated by causing PGE_2 hyperalgesia. αCAMKII was also able to induce hyperalgesic priming in female rats demonstrating that its effect was downstream of the inhibitory role of oestrogen (Ferrari *et al.*, 2013b). This lead to a theorised mechanism to the generation of hyperalgesic priming at the peripheral level.

There have been some recent investigations into the central changes involved in hyperalgesic priming focussing on alterations in dopaminergic and GABAergic signalling. A model using IL-6 in lieu of carrageenan was able to produce hyperalgesic priming and administration of 5, 7-DHT in conjunction with desipramine selectively ablates 5-HT neurones this was shown to block IL-6 induced pain and subsequently hyperalgesic priming (Kim *et al.*, 2015). However if the neurones were ablated post IL-6 administration it failed to prevent the generation of a primed state meaning that PGE_2 hyperalgesia could still occur. The ablation of noradrenergic innervation via DβH-saporin did not affect priming initiation. However the administration of 6-hydroxydopamine (6-OHDA) caused loss of dopaminergic connections from the A11 nucleus of the hypothalamus prevented hyperalgesic priming from occurring, even when administered before or after IL-6. It was then observed that the administration of D1/D5 antagonists could inhibit the PGE_2 response at 3 hours suggesting a dopaminergic mediated response (Kim *et al.*, 2015).

GABA activation has also been demonstrated to inhibit IL-6 induced pain via the use of muscimol ($GABA_A$ agonist) and midazolam (benzodiazepine). However the administration of these GABAergic modulators fail to block IL-6 induced hyperalgesic priming. $GABA_A$ antagonists were able to inhibit the PGE_2 response at 3 hours post injection. This suggested that there was an increase in GABA activity, which was due to an increase in GABA receptor trafficking via neurolignin 2. The disruption of neurolignin 2 by neurolide 2 partially inhibited PGE_2 hyperalgesia. Therefore the maintenance of the primed state maybe be due to changes within GABAergic signalling (Kim *et al.*, 2016).

Hyperalgesic priming is a model that shows potential for the better understanding of clinical chronic pain. By studying the transition from acute to chronic pain it may allow for the development of preventative measures as well as therapeutics for chronic pain.

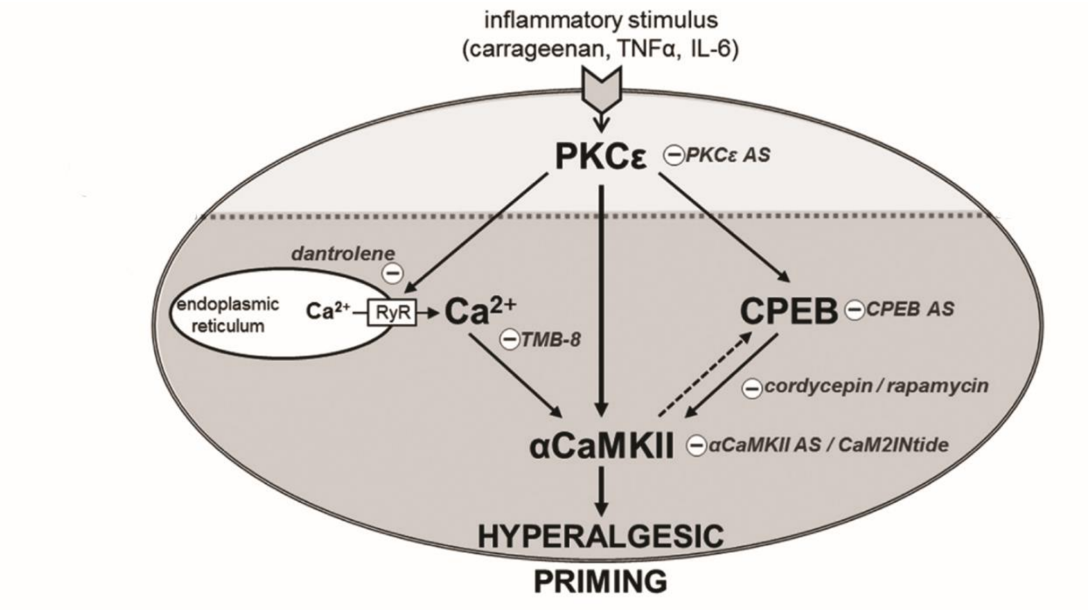


Figure 1.6 Proposed peripheral mechanism for the generation of hyperalgesic priming. Activation of PKCε leads to the activation of CPEB and Ca^{2+} release causing the activation of αCaMKII generating a primed state. (adapted from Ferrari et al. 2013)

1.5 Aims

Development of new animal models to study and evaluate chronic pain is of high importance. The aim of this thesis is to establish and evaluate the model of hyperalgesic priming by the assessment of different versions of the model. Then to investigate the cellular changes that occur during the chronic phase of the model so to determine the underlying changes that have occurred. This will be done by the use of immunohistochemistry to visualise immune cell populations within the affected region in the skin and changes in non-neuronal cells within the spinal cord (Chapter 3). The contribution of different peripheral sensory neurones to the development of hyperalgesic priming, will be investigated using selective blockade techniques. The changes in spinal excitability during the transition from acute to chronic pain will also be assessed using single unit recordings in the spinal cord of wide dynamic range neurones (WDR) (Chapter 4). Finally the role of resolution during hyperalgesic priming will be assessed using pro-resolving lipid mediators as interventions in the development of hyperalgesic priming as well as reversal of the chronic pain state.

Chapter 2

Materials and Methods

2.1 Animals

All procedures adhered to IASP and ARRIVE guidelines, were ethically approved and licenced by the UK Home Office. Experiments were all conducted upon male Sprague Dawley rats obtained from Charles River (Margate, UK). Animals were ordered in at a weight range of 179-199g so that they would be at a range of 200-250g upon commencing studies. During experiments animals were given free access to both food and water for the duration of the study and habituated to experimental equipment, and handled by the experimenter prior to experiments starting. Behavioural measurements were made between 0800 and 1700. The experimenter was blinded to the treatment of each animal.

2.2 Behavioural Tests

2.2.1 Weight Bearing Asymmetry

Weight-bearing asymmetry measures the distribution of mass the animal places on its hind limbs. During periods of pain in one hind limb the animal will place less weight on it to accommodate. For measurement of changes in weight distribution due to injury animals were tested using an incapacitance tester (Linton Instrumentation, Norfolk, UK). Animals were placed in a Perspex box with each foot placed over a force transducer. When taking measurements the incapacitance tester would measure the average weight each paw applied to their respective sensor over a 3 second period. Recordings were repeated three times and the average calculated for each paw.

2.2.2 Mechanical Paw Withdrawal Threshold

To measure the development of mechanical hyperalgesia animals were placed in a Perspex box with a mesh floor (Medical Engineering, University of Nottingham) where von Frey monofilaments were applied to the plantar surface of the hind paw exerting a bending force in grams (1, 2, 4, 6, 8, 10, 15 and 26g). Von Frey monofilaments were applied sequentially starting from 6g in increasing order to the plantar surface of both paws for three seconds until a withdrawal reflex was observed. This was defined as a rapid withdrawal of the paw from the stimulus, which was occasionally accompanied by slight shaking or licking of the paw. (Forces applied did not exceed 26g in any study). Each stimulus was applied for 3 seconds to the injured region of the paw, if no reflex was observed the next incremental filament was applied. Once a withdrawal reflex was observed, the next lower magnitude von Frey monofilament was used to test the paw to confirm no presence of a response (Calcutt *et al.*, 1997). The lowest weight of monofilament used which elicited a reflex was taken to be the paw withdrawal threshold.

2.3 Induction of the Model of Hyperalgesic Priming

2.3.1 Solution Preparation

2.3.1.1 Carrageenan

λ-Carrageenan (Sigma Aldrich, UK) was immersed in 0.9% saline and left in a water bath at 50°C for 24 hours to dissolve in the saline. The solution was then inverted several times within the tube to fully distribute the carrageenan throughout. Carrageenan when mixed in solution forms a thick emulsion. A concentration of 1% carrageenan was made for use in the induction of hyperalgesic priming. This concentration of carrageenan had been previously used in the generation of the hyperalgesic priming model (Aley *et al.*, 2000).

2.3.1.2 TNFα

TNFα (Sigma Aldrich) was dissolved in (0.9%) saline to make a stock solution of 100µg/ml. This was then diluted to a working concentration of 20µg/ml for injections. TNFα had also been used in several studies to induce hyperalgesic priming (Bogen *et al.*, 2012).

2.3.1.3 PGE_2

PGE_2 (Cayman Chemicals, Cambridge Bioscience, UK) was dissolved in a solution of 99% of 0.9% saline and 1% ethanol to produce a stock solution of 1mg/ml. This was diluted to a working concentration of 0.2mg/ml. This was used to confirm that hyperalgesic priming had occurred.

2.3.2 Anaesthesia

Intraplantar administration of compounds was performed on animals while under brief isoflurane (Abbot, Kent, UK) anaesthesia. The anaesthetic system consisted of a Perspex induction box that received a delivery of 3% Isoflurane via an anaesthetic vaporiser, in a nitrous oxide and oxygen gas (BOC gases, UK) mixture in a ratio of 33:67 pressure controlled by 0-4 bar multistage gas ARC multistage regulators. Flow rate was maintained by Platon glass variable flow meters connected to a small animal nose cone through a non-rebreathable tubing system. An active extraction system (Fluo Vac) was used to remove exhaled gasses via an aldasorber (Harvard Apparatus).

2.3.3 Intraplantar Administration of Substances

Animals were placed within an induction box and anaesthetised as detailed in **2.3.2**, upon the animal losing its righting reflex it was transferred to a small animal nose cone in prone position on a heating blanket. The animal's paws were positioned with the plantar surface facing up. The pedal reflex was tested to confirm the animal was areflexic. The plantar surface was disinfected with chlorhexidine (Hydrex) and a 28 gauge 0.3ml insulin syringe was used to administer a small 5µl bolus of noxious compound (carrageenan or, TNFα or, PGE_2) into the plantar skin into the intradermal space. The needle was then twisted 180° to aid in skin closure and then immediately removed. Animal was then allowed to recover in a separate cage. Once the self-righting had returned the animal was transferred back to its home cage.

2.4 Spinal Electrophysiology

2.4.1 Electrode Production

2.4.1.1 Electrode Etching

Tungsten wire electrodes were constructed and used to record from spinal dorsal horn neurones. These electrodes were manufactured in our laboratory using methods adapted from (Bullock *et al.*, 1988).

Lengths of tungsten wire (Harvard Apparatus, Kent, UK) of approximately 4cm were taped around a brass chuck (with 2.5cm projecting beyond then end); this was then attached to the arm of the etching unit (Medical Engineering, University of Nottingham, UK) and acted as the positive electrode. The chuck was then lowered into a bath solution containing KNO_2 (13.22M) with a carbon electrode also submerged in the solution. A current of 250mA is passed through the electrodes as the chuck is rotated in the solution at 5rpm, this leads to the tungsten electrodes becoming tapered to a point as the electrolysis removes tungsten from the ends of the wires. The electrodes are etched until the current drops to 200mA (Figure 2.1A).

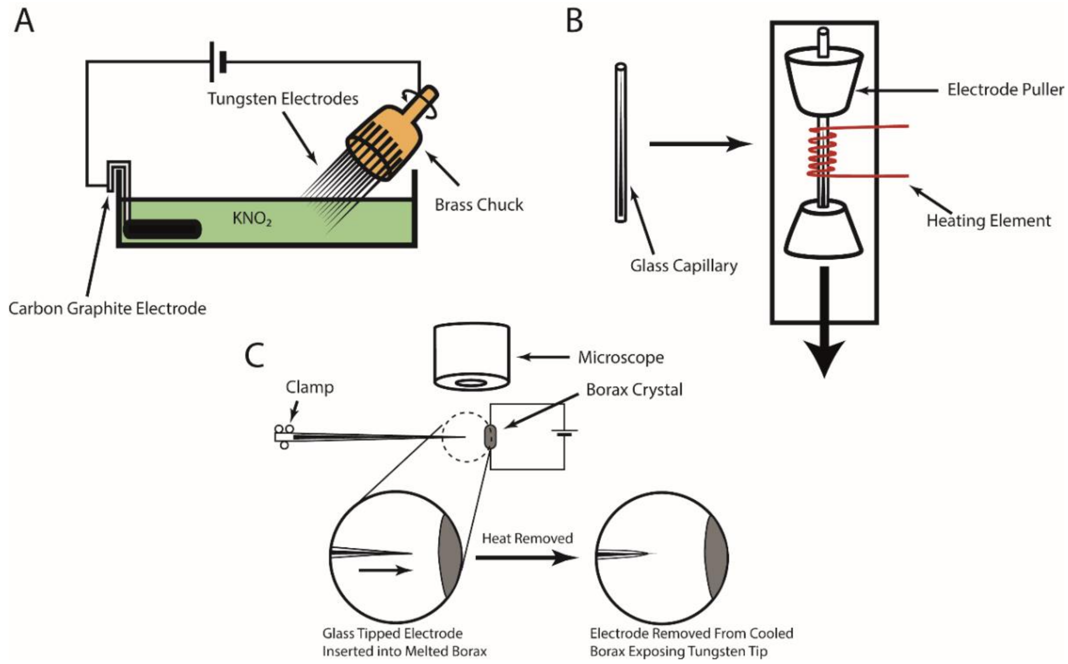


Figure 2.1: Electrode production apparatus. **A** Etching station tungsten electrodes are attached to a brass barrel and then rotated in a solution of KNO_2 while a current is applied. **B** Etched electrodes are placed into a glass capillary then heated in an electrode puller to coat them in glass. **C** Glass is removed to expose the tip of the tungsten electrode using a heated borax crystal under a microscope.

2.4.1.2 Glass Coating Electrodes

The etched electrodes need to be coated in glass to insulate the exposed metal. A glass capillary tube is stoppered at one end with a small amount of plasticine™; an etched piece of tungsten wire is dropped into the capillary blunt end first. The capillary tube is placed in an electrode pulling unit (Medical Engineering, University of Nottingham, UK) and clamped in place. The glass capillary is then heated to melt the glass, with the electrode pulled down by gravity coating the tungsten in glass (Figure 2.1B).

2.4.1.3 Removing the Glass Tip

To expose the tip of the tungsten, the coated electrode was held in a clamp under a light microscope. A bead of borax surrounding a cauterising heating element was melted and the end of the electrode was inserted into the melted borax. The heat the element was turned off allowing the borax to cool causing it to retract from the electrode tip removing the glass and exposing the tungsten (Figure 2.1C).

2.4.2 Electrophysiology Equipment

2.4.2.1 Anaesthetic

The anaesthetic system used was set up as laid out in **2.3.2** with anaesthetic being delivered by a tube connected to a tracheal cannula. The excess gasses were removed by passive absorption via an aldasorber. For surgery animals are placed in an induction box and anaesthetised with 3% isoflurane, during surgery the anaesthetic is reduced to 2% and maintained at 1.5% for recording.

2.4.2.2 Electrophysiology Rig

Spinal WDR activity was recorded using the glass-coated tungsten electrode (see **2.4.1**) with the signal amplified and recorded via a Neurolog system. The tungsten electrode was attached to a Neurolog headstage (NL100AK) and grounded to the animal and frame via a crocodile clips. The pre-amplifier (NL104) uses differential recording to record spinal nerve activity while removing the animal related background with the amplification set to 2K and then amplified through an AC-DC Amp (NL106). The signal passed through low and high-pass filters (NL125) set to a bandwidth of 0.8-1.5kHz to remove background and mains supply noise leaving only the neuronal signal.

For accurate measuring of neuronal activity the signal was sent through a spike trigger module (NL201) this allowed for a specific window height to be set so that only action potentials of sufficient amplitude were counted. Signal to noise ratio was typically 4:1.

Signals were visualised through a two channel (Tektronix TDS210) oscilloscope displaying both the raw spinal activity and an event count showing when an action potential was counted.

2.4.2.2 Electrophysiology Recording

Spinal neuronal input was converted to a digital signal using a CED1401 microprocessor and sampling conducted using Spike 2.0 version 6.05 (Cambridge Electronic Design, Cambridge, UK). This set up allowed for recording of the WDR neurone activity in real time. The activity induced by mechanical and electrical stimulation was then plotted on a histogram in Spike 2.0 (Kelly *et al.*, 2015; Woodhams *et al.*, 2012).

2.4.2.3 Surgery

Animals were anaesthetised as detailed in **2.3.3** the animal was then placed in the supine position with a nose cone. The skin above the throat was removed with scissors and the trachea exposed by blunt dissection of the surrounding muscle. The trachea was slightly raised by the insertion of a pair of sharp forceps below and then tilted upwards to allow for two ligatures (2.0 suture silk) approximately 1cm apart to be tied loosely around the trachea. An incision was made between the tracheal cartilage rings and a cannula (Portex nylon tubing internal diameter 1.50mm, external diameter 2.10mm) of 5-6cm in length was inserted approximately 5mm down into the trachea. The ligatures were tied with a reef knot blocking the upper trachea and securing the cannula in place. The cannula was then connected back to the anaesthetic system.

The animal was transferred to a stereotaxic frame (University of Nottingham, Medical Engineering) and the head stabilised with ear bars. A homeothermic heating blanket and rectal probe (Harvard Apparatus) was used to maintain the temperature of the animal at 37±1°C. The spinal column was exposed by an incision that was made down the midline of the animal above the spinal column; two small incisions were then made on either side of the spinal column above the rib cage. A spinal clamp was used to elevate the spinal column and to secure it in place, while the muscle covering the spinal processes was removed using a pair of fine tipped rongeurs. The spinal processes above the region of L4-7 were removed and the tip of the rongeurs inserted between the vertebrae and the bone above L4-5 removed thus exposing the spinal cord. Care was taken to make sure that the spinal cord was not damaged during the bone removal. A second clamp was secured caudally to the laminectomy this was then adjusted to make sure the column was straight and level, as well as making sure there was no movement due to respiration. In most cases the dura is removed as the vertebral bone is taken away. If the dura is still present (the spinal cord appears dull) it was removed using fine forceps, this allows a flow of CSF to keep the spinal cord moist. In most cases saline is applied to the surface of the spinal cord to ensure that is doesn't dry out. Upon completion of the laminectomy the isoflurane anaesthetic is reduced and maintained for the duration of the experiment at 1.5% (set up shown in Figure 2.2).

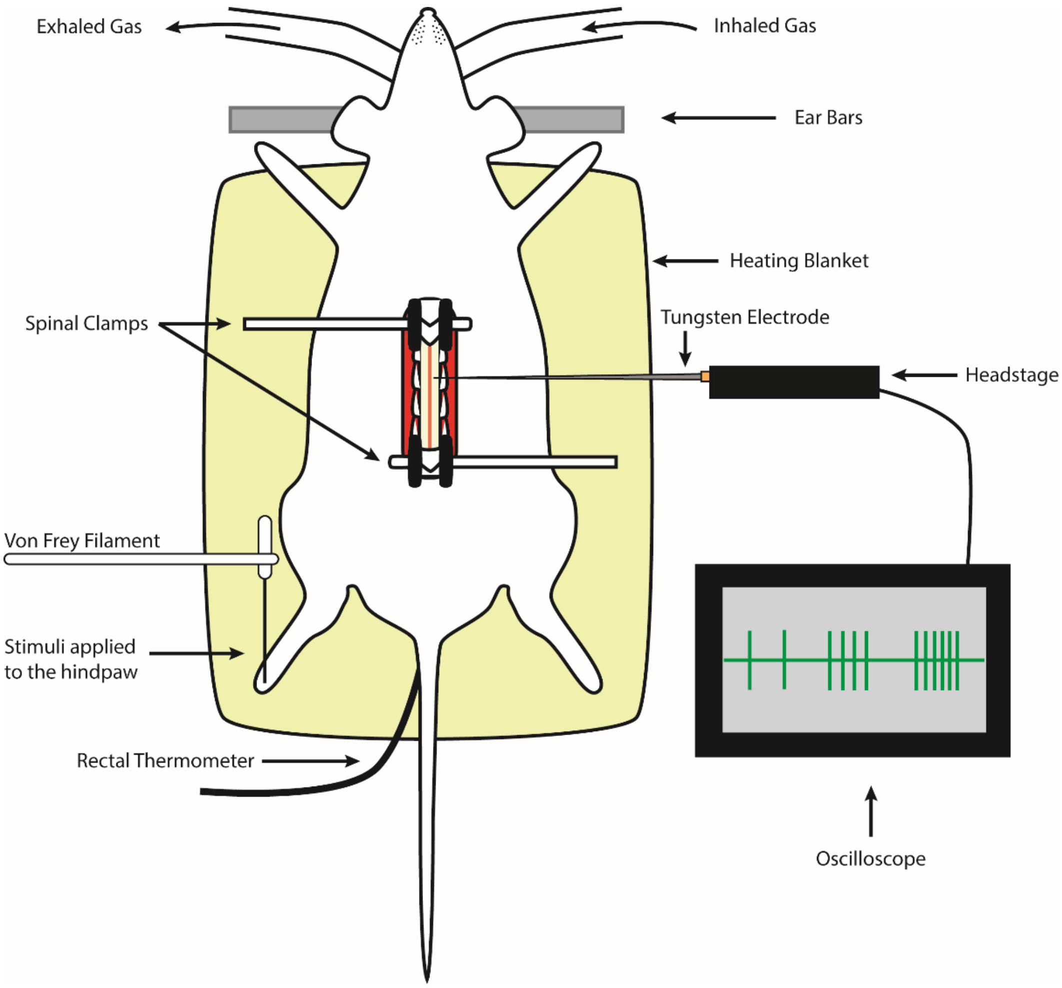


Figure 2.2: Spinal electrophysiology set up, rat is secured in a stereotaxic frame with ear bars. The spinal column held straight with spinal clamps with the spinal cord exposed. Recordings are made with a glass coated tungsten electrode connected to a Neurolog headstage and visualised on an oscilloscope. Data collected using Spike 2.0.

2.4.2.4 Locating and Stimulating WDR Neurones

The tungsten electrode is lowered manually using a micromanipulator until it touches the surface of the spinal cord (shown as a slight distortion in the membrane surface). The electrode is then slowly inserted into the spinal cord using a SCAT-01 microdrive (Digitimer); this allows the electrode to be moved in 10µm increments. The location of the WDR neurones was between 500-1000µm in depth from the surface of the spinal cord corresponding to lamina V, as the electrode was lowered the hind paw was stimulated by systematic tapping of the hind paw until a WDR neurone was located.

The criteria for the selection of the cell were that the cell had a graded response to mechanical stimulation from 8-26g (increased firing rate with higher weight von Frey), as well as an Aβ, Aδ and C fibre input measured through electrical stimulation. Electrical stimulation was produced using an NL800A stimulator box which delivered controlled stimuli to the hindpaw of the animal via 26 gauge pins inserted below the skin. The generated stimulus was controlled by a period generator (NL304) set to produce a 2ms pulse with a digital width module (NL401) controlling the delay between each stimulus set to 2s. A pulse generator (NL510) controlled the intensity of the current applied to the hindpaw with a counter module (NL603) gating the signal upon completion of the stimulus.

The C fibre threshold was measured by delivering an initial pulse of 0.5mA then increasing in increments of 0.1mA until the recordings included Aβ, Aδ and C fibre input, determined by the firing latency post-stimulus (0-20ms, 20-90ms, 90-300ms respectively). Once the threshold is found a train of sixteen stimuli are applied to the hindpaw at 3 times the C fibre threshold (figure 2.3).

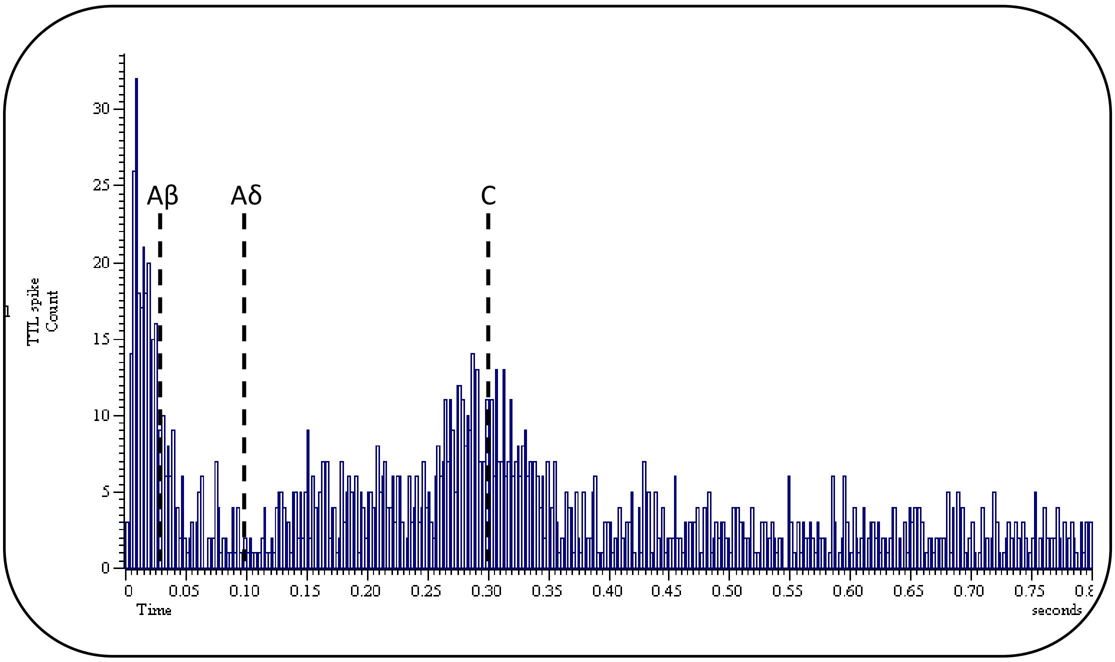


Figure 2.3: Representative histogram showing total neuronal firing post electrical stimulus as displayed in Spike 2.0. Trace is divided into distinct regions (indicated by dashed lines) that relate to the difference sensory nerve latencies. Aβ, Aδ and C fibres (0-20ms, 20-90ms, 90-300ms respectively). Anything post-C fibre latency was counted as post-discharge.

Once the cell had been electrically characterised von Frey filaments were applied to the receptive field of the cell on the hindpaw in ascending order from 8-26g. Each of the mechanical stimuli was applied for 10s with a gap of 10s between each von Frey with the neuronal activity visualised as shown in Figure 2.4. The mechanical stimulation trains were given every 20mins.

The size of the receptive field was measured by the application of 8g and the 26g stimuli to several locations on the plantar surface of the hindpaw. If the stimulus produced a response from the recorded cell it was deemed to be within the receptive field of the cell. This allowed for a map of the field to be drawn. Receptive field measurements were made at 10 and 15min intervals after the mechanical stimulus train. The stimulus protocol was repeated until the responses were in 10% of each other for each von Frey; once baselines were stable the experimental protocol was started (detailed in Chapter 4).

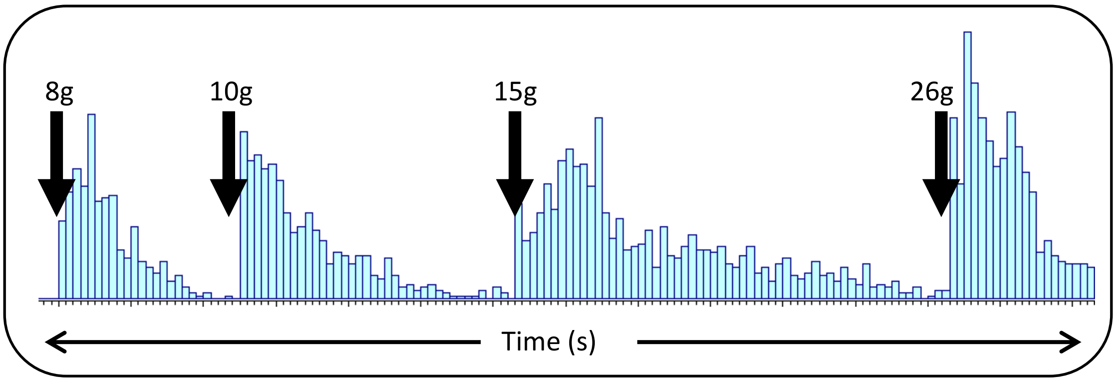


Figure 2.4: Representative histogram of WDR nerve activity as displayed in Spike 2.0 in response to mechanical stimulation of 8g, 10g, 15g and 26g von Frey application (application of stimulus indicated by arrows).

2.5 Immunohistochemistry

2.5.1 Tissue Collection for Analysis

2.5.1.1 Reagents

For the collection of fixed tissue: 4% paraformaldehyde (Sigma Aldrich, Kent, UK), sucrose azide (30% sucrose, 0.02% sodium azide) and saline (0.9% sodium chloride) were used.

2.5.1.2 Perfusion Fixation

Animals were humanely euthanized with an i.p overdose with sodium pentobarbitone. The pedal and corneal reflexes were tested to determine whether animal was deeply anaesthetised. The animal was placed in the supine position on a downflow unit (AFOS, Hull) a transverse incision was made above the sternum at the base of the ribcage. The diaphragm was exposed and incisions were made up and through on each side of the ribcage, the diaphragm was cut exposing the thoracic cavity. The sternum was clamped using a hemostat and the ribcage lifted over the head of the animal allowing for access to the heart. A bevelled pipette tip connected a perfusion pump was used to puncture the left ventricle the right atrium was then cut to allow for the circulatory system to be completely cleared through with 0.9% saline at 4°C (approximately 200ml of saline per animal). Once the blood had been cleared from the animal 4% paraformaldehyde was passed through to fix the animal tissue. Complete fixation was confirmed by the full rigidity of the animal. Spinal cord tissue was carefully removed via the use of fine nosed rongeurs and the plantar skin was removed from the hindpaw. The tissue was left to post-fix in 4% paraformaldehyde overnight and then transferred to sucrose azide for long term storage at 4°C.

2.5.2 Tissue Preparation for Immunohistochemistry

2.5.2.1 Spinal Cord Preparation

For use in immunohistochemistry the L5 region of the spinal cord was isolated. A scalpel blade was used to make an incision into the contralateral ventral horn so that the sides of the spinal slice could be identified under the microscope. The spinal cord was then mounted on a freezing microtome (LEICA, 2010R) and sliced at a thickness of 40µm. The slices were then stored free floating in sucrose azide at 4°C or washed in PBS for immediate use in immunohistochemical staining.

2.5.2.2 Spinal Cord Immunostaining

Spinal immunostaining was conducted on free floating slices. The tissue was allowed free movement in the solution during the staining protocol. The advantage of this is that both sides of the tissue are introduced to the antibody solution increasing total penetrance. The tissue placed into test tubes where it was washed in PBS for a duration of 10mins. This was repeated 3 times to ensure as much sucrose azide had been removed from the tissue, as formation of sucrose crystals would impair visualisation of the tissue. The tissue first went through a blocking phase using a 5-10% species specific serum, 0.1-0.3% triton in PBS for up to 2 hours, this aims to reduce the potential for non-specific binding of the secondary antibody. The species serum used was determined by the species the secondary antibody was raised in (i.e goat raised antibody, goat serum). The primary antibody against the targeted antigen was made up in blocking solution, the previous block was removed and the primary antibody solution was applied to the tissue. This was incubated for at minimum 2 hours up to 72 hours to allow for sufficient binding of antibody to antigen. During the incubation period the tissue was stored at either room temperature (25°C) or in a cold room (4°C) this was dependent on the antibody. The primary antibody was removed and the tissue washed in PBS for 10min incubations repeated 3 times. The secondary antibody was diluted in TTBS and recognises the main chain of the primary antibody. The secondary conjugated to a fluorescent tag therefore exposure to light was kept to a minimum as photo-bleaching can be a concern. Once washes were complete the secondary antibody was applied to the tissue and incubated for 2 hours at room temperature (25°C) in the dark. The tissue was again washed in PBS three times and then in ddH_2O, the spinal cord slices were mounted on gelatinised slides and left to dry in the absence of light. Fluromount was then applied to the slices to prevent fluorescent quenching, a coverslip was then applied over the tissue to protect it.

2.5.2.3 Plantar Skin Preparation

The injection site of the skin was isolated and then also sliced on a freezing microtome at 20µm. The skin was stored in sucrose azide or washed in PBS and then mounted onto gelatinised slides. Prior to staining the mounted skins slices were freeze thawed up to three times to reduce chances of the tissue lifting off the slides during the staining process.

2.5.2.4 Plantar Skin Immunostaining

The immunohistochemistry protocol was the same as detailed in **2.5.2.2**. The skin tissue was already on slides as the tissue can be relatively fragile so free floating staining could lead to damage. During the staining protocol the slides were placed in a humidity chamber this allowed the tissue to remain the dark and prevent the tissue from drying out during the staining process.

2.5.2 Immunohistochemistry Image Acquisition and Analysis

The stained tissue was imaged on a Leica fluorescence microscope (DMIRE2). The secondary antibody applied during the immunostaining protocol is conjugated to a fluorescent tag (FITC; green, TRITC; red or DAPI; blue). The microscope uses various wavelengths of light to stimulate the appropriate fluorophore generating a fluorescent signal that can be imaged. Images were captured using Volocity (Perkin-Elmer) software to control a Hamamatsu Orca C4642-95 camera. The spinal cord slices were imaged at 20x magnification with images of the dorsal horn collected for further analysis. The skin tissue was imaged using a 40x oil medium lens with 4-5 regions selected along the tissue slice to confirm any changes observed are consistent. Images were collected using the imaging program Volocity. Acquired images were exported as .TIFF files and opened in ImageJ for quantification.

2.6 Statistics

Time-courses of weight bearing asymmetry and von Frey paw withdrawal threshold studies as well as electrophysiology time-courses were analysed with two-way ANOVA with Bonferroni post hoc test. Immunohistochemistry comparisons were made using student t tests or for multiple groups a one-way ANOVA with Sidak post-hoc test. Group sizes were based off previously published work using the model of hyperalgesic priming (Aley *et al.*, 2000; Bogen *et al.*, 2012). All values are given as mean ± standard error of the mean. For determination of statistical significance a $p < 0.05$ was take to be significantly different. Data was tested for normal distribution prior to statistical tests normalisation confirmed using Shapiro-Wilks normality test.

Chapter 3

Establishing a Model of Hyperalgesic Priming and Investigating Cellular Changes

3.1 Introduction

3.1.1 Model of Hyperalgesic Priming

Hyperalgesic priming has been proposed to model the transition from acute to chronic pain using two consecutive acute inflammatory stimuli. The first stimulus causes the priming of the sensory nerve fibres leading to the second stimulus causing long term hypersensitivity (Aley *et al.*, 2000). As stated in Chapter 1 this model has been proposed to be useful in studying the development of chronic pain. Understanding the mechanisms that lead to chronic pain will allow the development of new strategies and therapeutics with the goal of prevention and treatment of chronic pain. To establish the model two initiators of priming will be tested: carrageenan (Aley *et al.*, 2000) and TNFα (Bogen *et al.*, 2012). It will then be decided which of these produce the most robust version of the model, which will be taken forward for further study.

3.1.2 Role of Immune Cells in Inflammation

Hyperalgesic priming is induced via the intraplantar injection of carrageenan/TNFα followed by the intraplantar injection of PGE_2 (Aley *et al.*, 2000; Bogen *et al.*, 2012). Both of these stimuli are known to cause activation of a local immune response. Carrageenan causes the infiltration of macrophages, neutrophils and mast cells which then themselves release inflammatory mediators including histamine, bradykinin and further prostaglandins (Vinegar *et al.*, 1987). PGE_2 is responsible for many of the classic signs of inflammation including swelling, redness (due to increased blood-flow) and pain (Funk, 2001). PGE_2 also stimulates the migration of macrophages and neutrophils into the damaged tissue, promoting both the inflammation and sensitisation of the tissue, and the removal of damaged cellular or foreign materials (Kalinski, 2012). These immune cells release inflammatory mediators that lead to changes in receptors and channels expressed on the peripheral terminals of the sensory nerves, including prostaglandin receptors such as EP1-4 which activate protein kinase A (Hata *et al.*, 2004b) which has been shown to phosphorylate TRPV1 enhancing its sensitivity to stimuli (see **1.2.3.1**). Through these mechanisms immune cell-mediated sensitisation of sensory nerves contributes to the generation of chronic pain. Two key cells in these events are macrophages and neutrophils, as detailed in **1.2.2.1** and **1.2.2.2**, they contribute to peripheral sensitisation and a change in their function can lead to prolonged inflammation by further release of inflammatory mediators. If there is a prolonged presence of immune cells in an injured tissue it can lead to chronic inflammation causing lasting damage due to maintained release of inflammatory mediators potentially leading to self-inflicted tissue damage (Bryant, 2007).

Inhibition of immune cell recruitment has been shown to reduce damage in during periods of chronic pain the inhibition of the chemokine CX3CL1 prevents macrophage infiltration to injury sites (Huang *et al.*, 2014). To date the potential contribution of immune cells during the chronic phase of hyperalgesic priming has yet to be addressed. It is likely that cellular events in close proximity to the sensory nerve terminals play a role in the eventual establishment of hyperalgesic priming, but this still needs to be investigated.

It is well established that microglia within the central nervous system have important roles in the sensitization of spinal neurones and contribute to both the rapid and long-lasting changes in spinal cord excitability associated chronic pain states. For example in response to peripheral injury and activation of sensory nerves, microglia in the spinal cord rapidly proliferate and transition from an resting; ramified, environment sensing phenotype to an “active” amoeboid phenotype (Nakajima *et al.*, 2001b). Microglia have been demonstrated to produce several pro-inflammatory mediators including IL-1, IL-6 and TNFα. Release of these cytokines in the CNS has been linked to neuronal apoptosis due to activation of caspases and excitotoxicity through the increase of neurotransmitter release and ion channel expression (Nakajima *et al.*, 2001a; Smith *et al.*, 2012). The change in morphology of microglia to their amoeboid form does not always lead to the production of pro-inflammatory cytokines there is also evidence of the production of anti-inflammatory IL-10 and IL-4, reducing the production of inflammatory cytokines and the expression of cytokine receptors on microglia (Cherry *et al.*, 2014; Sawada *et al.*, 1999).

It has been widely documented that in many different models of pain the numbers of “activated” microglia in the dorsal horn of the spinal cord are increased. For example in osteoarthritic chronic pain there is an increase in the levels of “activated” microglia within the dorsal horn (Sagar *et al.*, 2011). In inflammatory conditions, such as rheumatoid arthritis, spinal cord microglia contribute to the increased pain behaviour exhibited in mice. Preventing microglia function via the inhibition of the P_2X_7 receptor present on microglia (see **1.1.3.3**) has successfully reduced allodynia (Nieto *et al.*, 2016). The role of microglia in the model of hyperalgesic priming has not been studied. Their involvement in the production of both pro and anti-inflammatory cytokines could be key to the changes in pain processing.

3.1.3 Lipid Mediators of Inflammation

During inflammation G-protein coupled receptors (GPCRs) coupled to G_q such as 5-HT2, adenosine receptor 1 and mGluRs are activated leading to the breakdown of phospholipids via enzymes phospholipase A_2, and phospholipase C this leads to the production of arachidonic acid (AA) (Walter, 2003). AA is converted to a large number of different eicosanoids which can have varied effects on multiple cells types. Arachidonic acid can enter one of two main pathways that go on to produce various eicosanoids (Figure 3.1). Firstly the enzymatic action of COX can convert AA into the prostaglandins (PGs). AA metabolised by COX-1 and COX-2 leads to the production of prostaglandin H_2 (PGH_2) and PGD synthase and PGE synthase convert PGH_2 to PGD_2 and PGE_2 respectively. As detailed in **1.3**, PGs stimulate both immune cells and sensitise peripheral nerve terminals.

Conversion of AA via LOX leads to the production of HETEs and the leukotrienes (Samuelsson *et al.*, 1987b). The HETEs play an important role in inflammatory responses, 5-HETE causes neutrophil infiltration (Bittleman *et al.*, 1995), and neutrophils generate other HETEs including 8-HETE, 9-HETE, 11-HETE and 12-HETE (Goetzl *et al.*, 1979). A number of the HETEs (5-HETE, 12-HETE and 15-HETE) increase the expression of COX-2 via the enhancement of IL-1 (Di Mari *et al.*, 2007), allowing for the increased metabolism of AA. As well as the sensitisation of TRPV1 (Hwang *et al.*, 2000) a crucial nociceptive receptor discussed in Chapter 1.

The leukotrienes are produced from AA which is converted to 5-HPETE by 5-lipoxygenase. The 5-HPETE is then reduced to 5-hydroxyeicosanoid which is dehydrated to produce leukotriene A_4 (LTA_4) (Haeggström *et al.*, 2011) which can be further metabolised to produce the other leukotrienes B_4, C_4, D_4 and E_4 (Salmon *et al.*, 1987). Leukotriene B_4 (LTB_4) is highly studied due to its role in mediating immune cell activity. It triggers the recruitment of neutrophils to an injured site via increasing adherence to the endothelium (Sun *et al.*, 2016). As well as the influx of macrophages to injury sites as demonstrated in models of lung inflammation. (Ee *et al.*, 2016).

Over the last few years the processes which curtail inflammatory responses have been actively studied (Libby, 2007). It has been demonstrated that the resolution of inflammation is not a passive process, but involves the generation of lipid pro-resolving mediators (Serhan, 2004). The resolvins are a group of lipid mediators that act to return a tissue to its resting state after an inflammatory reaction (Serhan *et al.*, 2004). They are crucial in the termination of an immune response acting to reduce neutrophil and macrophage infiltration and reduce their release of inflammatory mediators (Qu *et al.*, 2015). There are two main families of resolvins, the D series and E series both of which act via similar mechanisms and are produced from omega-3 fatty acids (Serhan *et al.*, 2002b) (further detail on resolvins in **5.1**).

It is becoming widely accepted that inflammatory conditions such as colitis, cardiovascular disease as well as obesity induced chronic low-grade inflammation may arise, at least in part, due to compromised resolvin responses (Neuhofer *et al.*, 2013; Qu *et al.*, 2015; Zhang *et al.*, 2012). Clearly this has similarities to the hyperalgesic priming response, which may also be influenced by the resolvin mediators.

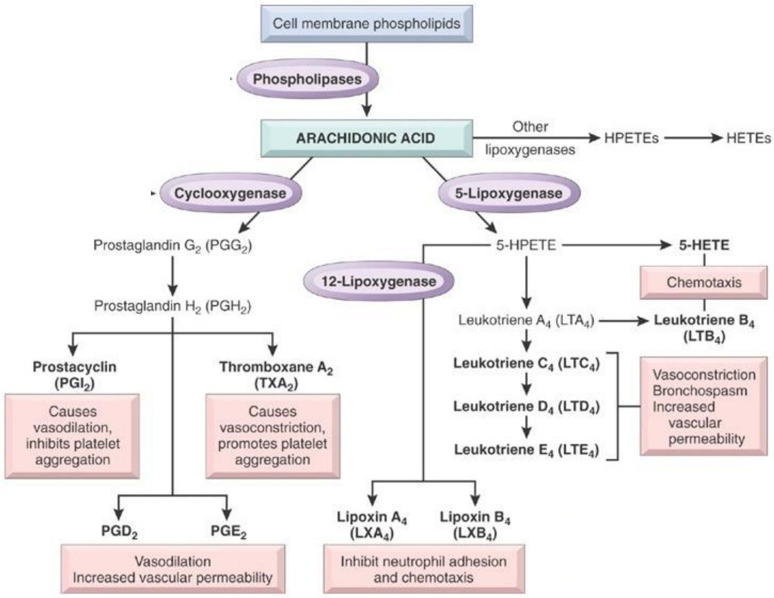


Figure 3.1 Arachidonic acid (AA) metabolism pathway. AA is produced from phospholipids broken down by phospholipases. AA can then be metabolised by two pathways. 5-LOX converts AA into 5-HPETE that is used to produce the leukotrienes and 5-HETE. The second pathway works via COX to generate the prostaglandins and thromboxanes. Adapted from Robbins & Contran's *Pathological Basis of Disease* 8th ed. (2010)

3.1.4 Aims

1. Establish a model of hyperalgesic priming using carrageenan or TNFα to identify the most robust model.

2. Investigate changes in peripheral and spinal immune cell responses at different stages of the hyperalgesic priming model

3. Investigate potential changes in plasma lipids associated with hyperalgesic priming.

3.2 Methods

3.2.1 Induction of Hyperalgesic Priming

3.2.1.1 Carrageenan and TNFα Induction of Hyperalgesic Priming

Male Sprague Dawley rats (200-250g) received an intraplantar injection of carrageenan (1% solution, 5µl)/saline or TNFα (100ng/5µl)/saline into the left hindpaw (doses from (Aley *et al.*, 1999; Aley *et al.*, 2000; Bogen *et al.*, 2012)). Behaviour testing of pain behaviour (weight-bearing and paw withdrawal thresholds (PWTs)) (detailed in **Chapter 2**) were carried out at 1 hour and 4 hours post-injection then once daily up to 7 days. Once rat pain behaviour had returned to baseline at least 72 hours after the initial injection rats were injected with PGE_2 (1µg/5µl) into the same site as previous injection. Then behavioural testing was carried out at 1 hour and 4 hours and up to 7 days post injection (timeline for carrageenan figure 3.2A, TNFα figure 3.2B).

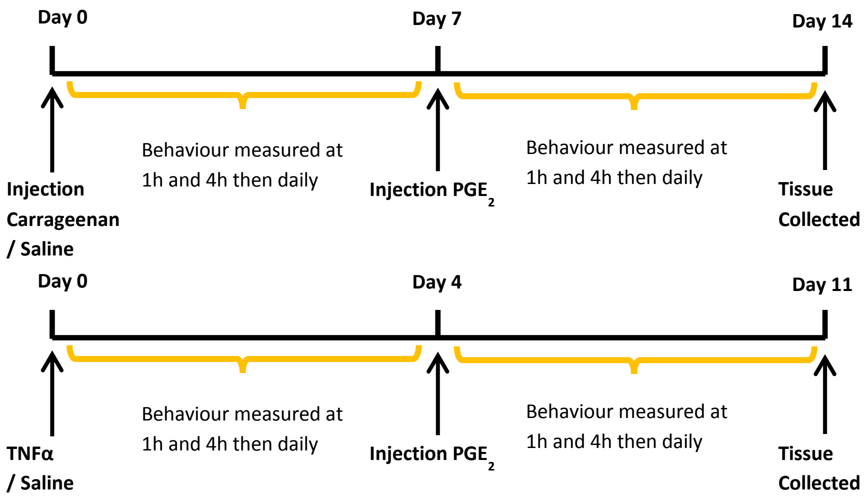


Figure 3.2 A: Timeline of carrageenan induced hyperalgesic priming protocol. On day 0 rats received an intraplantar injection of carrageenan or saline. Pain behaviour was measured at 1 and 4h then daily. On day 7 all rats were injected into the same as the previous injection with PGE_2. Pain behaviour was assessed at 1 and 4 hours then daily until day 14. Rats were humanely euthanized with sodium pentobarbital and transcardially perfused with 4% PFA. **B:** Timeline of TNFα induced hyperalgesic priming protocol. On day 0 rats received an intraplantar injection of carrageenan or saline. Pain behaviour was measured at 1 and 4h then daily. On day 4 all rats were injected into the same as the previous injection with PGE_2. Pain behaviour was assessed at 1 and 4 hours then daily until day 11. Rats were humanely euthanized with sodium pentobarbital and transcardially perfused with 4% PFA.

3.2.1.2 Carrageenan Induction at 14 days

To investigate the duration of the primed state which had been stated to last up to 21 days post initial injection (Aley *et al.*, 2000). Male Sprague Dawley rats (200-250g) received an intraplantar injection of carrageenan (1% solution, 5µl) into the left hindpaw. Pain behaviour testing of weight-bearing and PWTs was carried out at 1 hour and 4 hours post-injection then once daily up to 14 days. At 14 days post-carrageenan animals were injected with PGE_2 (1µg/5µl) into the same site as previous injection and behavioural testing was carried out at 1 hour and 4 hours then up to 7 days post injection.

3.2.2 Immunohistochemistry

3.2.2.1 Tissue Preparation

Skin and spinal cord were collected from 7 day carrageenan primed rats perfused with 4% PFA and sliced on a microtome at 40μm (spinal cord) and 30μm (plantar skin). The thickness of tissue was chosen to allow for maximum antibody penetration without losing histology (Ramos-Vara, 2011). The tissue was stored in sucrose azide and then washed in PBS prior to immunohistochemistry. Dorsal root ganglia were also collected to be used in miRNA analysis (see Chapter 6)

3.2.2.2 Macrophages: ED1 and Mannose

Skin tissue was mounted on gelatinised glass slides, and then frozen at -20°C for 20mins and then thawed. This was repeated 3 times to ensure the tissue had adhered to the slide. A border was drawn around the tissue using a hydrophobic pen to reduce loss of liquid during staining.

The tissue was then blocked using 6% goat block (60μl goat serum, 10μl triton-X in 1ml PBS) for 2 hours at room temperature. The blocking solution was removed and 1ml of primary antibody solution was added, ED1 (Serotec) and mannose (Abcam) at 1:100 and 1:300 dilutions respectively made up in blocking solution. The tissue was then incubated at room temperature for 12 hours.

The primary antibody solution was then removed and the tissue washed with 1xPBS repeated 3 times. A secondary antibody solution containing goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 568 at a 1:500 dilution in TTBS. The tissue was incubated for 2 hours at room temperature in the dark. The antibody solution was removed and the tissue was washed in ddH_2O 5 times. The fourth wash was replaced with 1:500 DAPI solution made up in 1xPBS. The tissue was left to dry in the dark and then cover-slipped with Fluoromount (for results on macrophages see appendix).

3.2.2.3 Neutrophils: Neutrophil Elastase

Skin tissue was prepared as detailed previously in **3.2.2.2**. Mounted tissue was washed in 1xPBS 3 times to remove as much sucrose azide as possible. The tissue was then blocked using 6% donkey block (60µl goat serum, 10µl triton-X in 1ml PBS) for 2 hours at room temperature. The blocking solution was removed and 1ml of primary antibody solution was added, neutrophil elastase (Santa Cruz) at a 1:100 dilution made up in blocking solution. The tissue was then incubated at room temperature for 12 hours.

The primary antibody solution was then removed and the tissue washed with 1xPBS repeated 3 times. A secondary antibody solution containing donkey anti-goat Alexa Fluoro 488 at a 1:500 dilution in TTBS. The tissue was incubated for 2 hours at room temperature in the dark. The antibody solution was removed and the tissue was washed in ddH_2O 5 times. The forth wash was replaced with 1:500 DAPI solution made up in 1xPBS. The tissue was left to dry in the dark and then protected with a coverslip and Fluoromount (for results on neutrophils see appendix).

3.2.2.4 Microglia: IBA1

Spinal cord immunohistochemistry was conducted using a free-floating method. Sections were washed in 1xPBS 3 times to remove as much sucrose azide as possible. The tissue was then blocked using 6% goat block (60µl goat serum, 10µl triton-X in 1ml PBS) for 2 hours at room temperature. The blocking solution was removed and 1ml of primary antibody solution was added, anti-IBA1 (Wako) at a 1:2000 dilution made up in blocking solution. The tissue was then incubated on a rocker at 4°C for 72 hours.

The primary antibody solution was removed and the tissue washed with 1xPBS for ten minutes repeated 3 times. A secondary antibody solution containing goat anti-rabbit Alexa Fluor 488 at a 1:500 dilution in TTBS was then added to the sections and incubated for 2 hours at room temperature in the dark. The antibody solution was removed and the tissue was washed in 0.1M PBS 5 times. Sections were then mounted onto gelatinised slides and left to dry in the dark and then cover-slipped with Fluoromount.

3.2.2.5 Immunohistochemistry Visualisation and Analysis

Immunohistochemistry was imaged using methods described in **Chapter 2**. Skin tissue was imaged at 40x magnification. Four regions of interest were taken from each slice with 5 slices imaged per animal. Macrophages and neutrophils were then counted and the mean cell number taken for each animal.

Dorsal horn images of L5 spinal cord were collected at 20x magnification. Total microglia and total activated microglia were counted. Microglial activation was determined by morphology; microglia that had entered their amoeboid form and had the majority of their processes smaller than the total size of the cell body were counted as activated.

3.2.3 Statistical Analysis

Changes in pain behaviour (weight-bearing asymmetry and PWTs) were compared using two-way ANOVA with Bonferroni post hoc test. Analyses compared changes in pain behaviour over time with changes in control group. The analysis of immunohistochemistry quantification was conducted using a one-way ANOVA with Sidak post hoc test with multiple comparisons. Lipid mediator detection rate was analysed using chi square analysis. Normalisation was confirmed using Shapiro-Wilks normality test. Group sizes were based on previously published work for hyperalgesic priming (Aley *et al.*, 2000). Data are mean ± standard error of the mean, p value of < 0.05 was accepted as significant.

3.3 Results

3.3.1 Comparison of Carrageenan and TNFα Induced Hyperalgesic Priming

3.3.1.1 Weigh-bearing Asymmetry

Injection of 5µl of 1% carrageenan subcutaneously into the plantar surface of left hind paw caused a significant decrease in ipsilateral weight-bearing 4h post-injection ($p<0.001$ compared to saline controls), which was still significantly different 1 day later ($p<0.05$). Weight-bearing differences in the carrageenan group returned to baseline levels at 3 days post-injection and remained at pre-injection levels for 7 days. Injection of 5µl of saline (0.9% NaCl; as a control for carrageenan) into the plantar surface of the left hind paw produced no significant change in ipsilateral weight-bearing from pre-injection baselines (Figure 3.3A).

In saline pre-treated rats, intraplantar injection of PGE_2, 7 days later into the same site as the previous injection, did not cause a change in ipsilateral weight-bearing. In rats pre-treated with carrageenan, intraplantar injection of PGE_2 resulted in a significant decrease in ipsilateral weight-bearing at 4 hours post PGE_2 injection ($p<0.05$, compared to control). Weight-bearing had returned to control levels 1 day after injection (Figure 3.3B).

3.1.1.2 Von Frey PWTs

Injection of 5µl of 1% carrageenan subcutaneously into the plantar surface of the hind paw caused a significant decrease in paw withdrawal thresholds (PWTs) 4h post-injection. PWTs remained significantly ($p<0.01$) lower than PWTs of saline treated rats for 3-4 days post-injection (Figure 3.3C). By 7 days PWTs had returned to baseline values in carrageenan treated rats (Figure 3.3C). Injection of 5µl of saline (0.9% NaCl) into the plantar surface of the left hind paw did not result in a significant change in PWT.

At 7 days post saline or carrageenan injection, rats received an intraplantar injection of PGE_2 that resulted in an initial decrease in PWT at 1h post injection in both groups (saline and carrageenan). In saline-treated rats, the PGE_2 evoked decrease in PWT, was short lived (<4h) and PWTs had returned to baseline at 4 hours. In rats pre-treated with carrageenan, injection of PGE_2 was associated with a significant lowering of PWTs at 4 hours post-injection ($p<0.05$) and this remained significant 7 days post PGE_2 injection (Figure 3.3D).

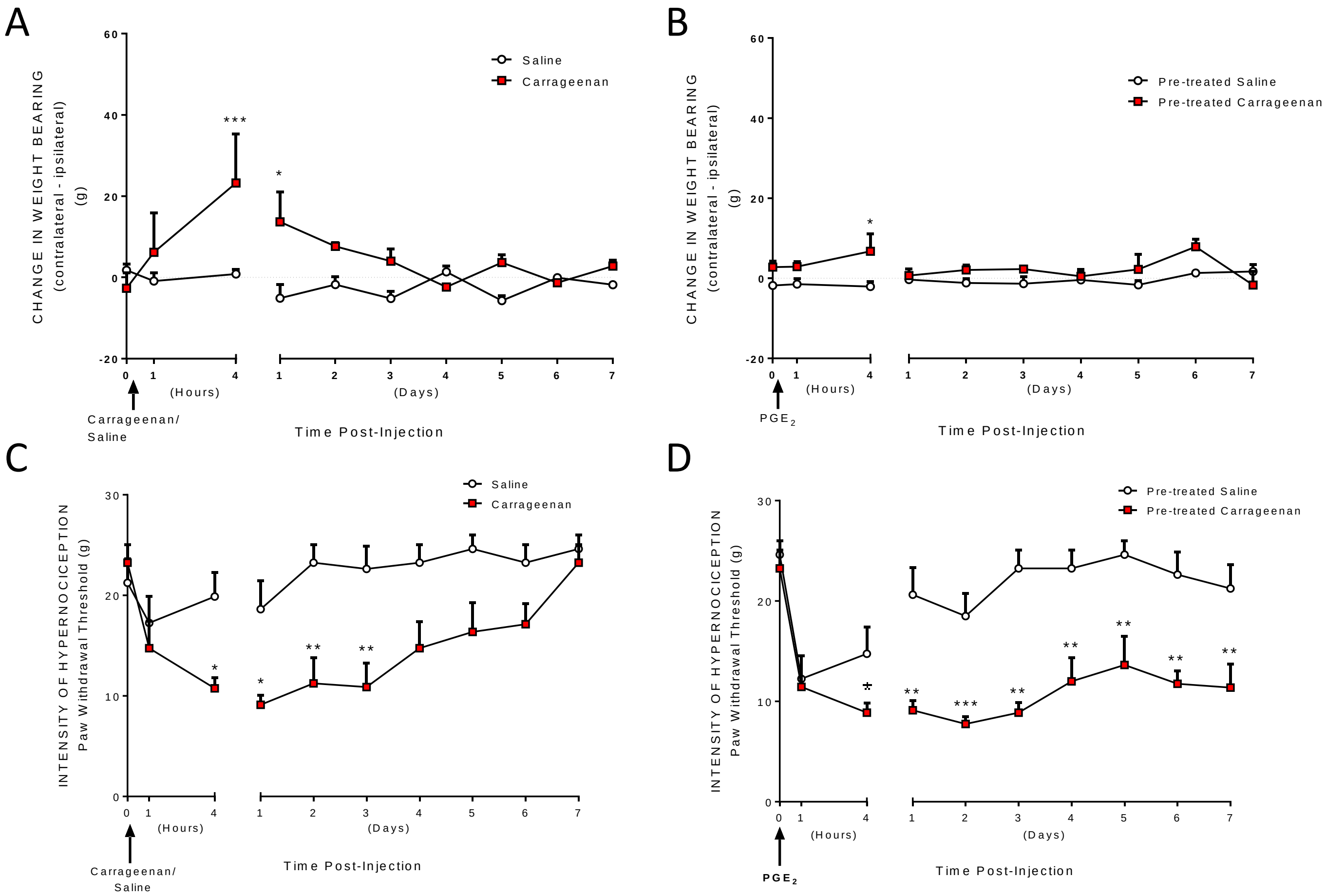


Figure 3.3 A: Carrageenan injection into the plantar surface of the left hind paw decreased ipsilateral weight bearing after 4h, which resolved by 2 days. Control rats injected with saline (0.9% NaCl, 5µl n=4) did not change in weight bearing. **B:** Seven days post initial injection rats received a single administration of PGE_2 this caused a significant decrease in ipsilateral weight bearing after 4h in rats pre-treated with carrageenan. There was no change in weight-bearing after PGE_2 injection in saline pre-treated animals. **C**: Intraplantar injection of carrageenan (5µl, 1% solution) produces a significant decrease in hindpaw withdrawal thresholds at 4 hours, which resolves by 4 days post injection in the rat. **D:** Injection of PGE_2 into both the saline and carrageenan pre-treated groups at day 7 produced an initial decrease in PWTs, which returned to baseline values at 1 day in the saline-treated group. In the carrageenan pre-treated group, PWTs are significantly lower for up to 7 days post PGE_2 injection. Data are mean ± SEM. Statistical analysis was carried out using a 2-way ANOVA test with a Bonferroni post hoc test *$p<0.05$, **$p<0.01$, ***$p<0.001$ (n=8 in both groups).

3.1.1.3 TNFα Induced Hyperalgesic Priming

In a separate group of rats injection of TNFα subcutaneously into the plantar surface of left hind paw did not alter ipsilateral weight-bearing (Figure 3.4A). Injection of TNFα resulted in a significant decrease in PWTs at 1h and 4h post injection, which then returned to baseline values at 2 days. Injection of saline did not alter PWTs (Figure 3.4C). Injection of PGE_2 into the same site as the previous injection did not alter ipsilateral weight bearing in either group of rats (Figure 3.4B). Injection of PGE_2 into the rats pre-treated with saline caused a decrease in PWT at 1h, which had recovered by 4h. Rats pre-treated with TNFα exhibited a trend towards a decreased PWT at 1-4h, which was maintained up to 7 days, but significance was not reached compared to the control group with the exception of the 6 day timepoint (Figure 3.4D).

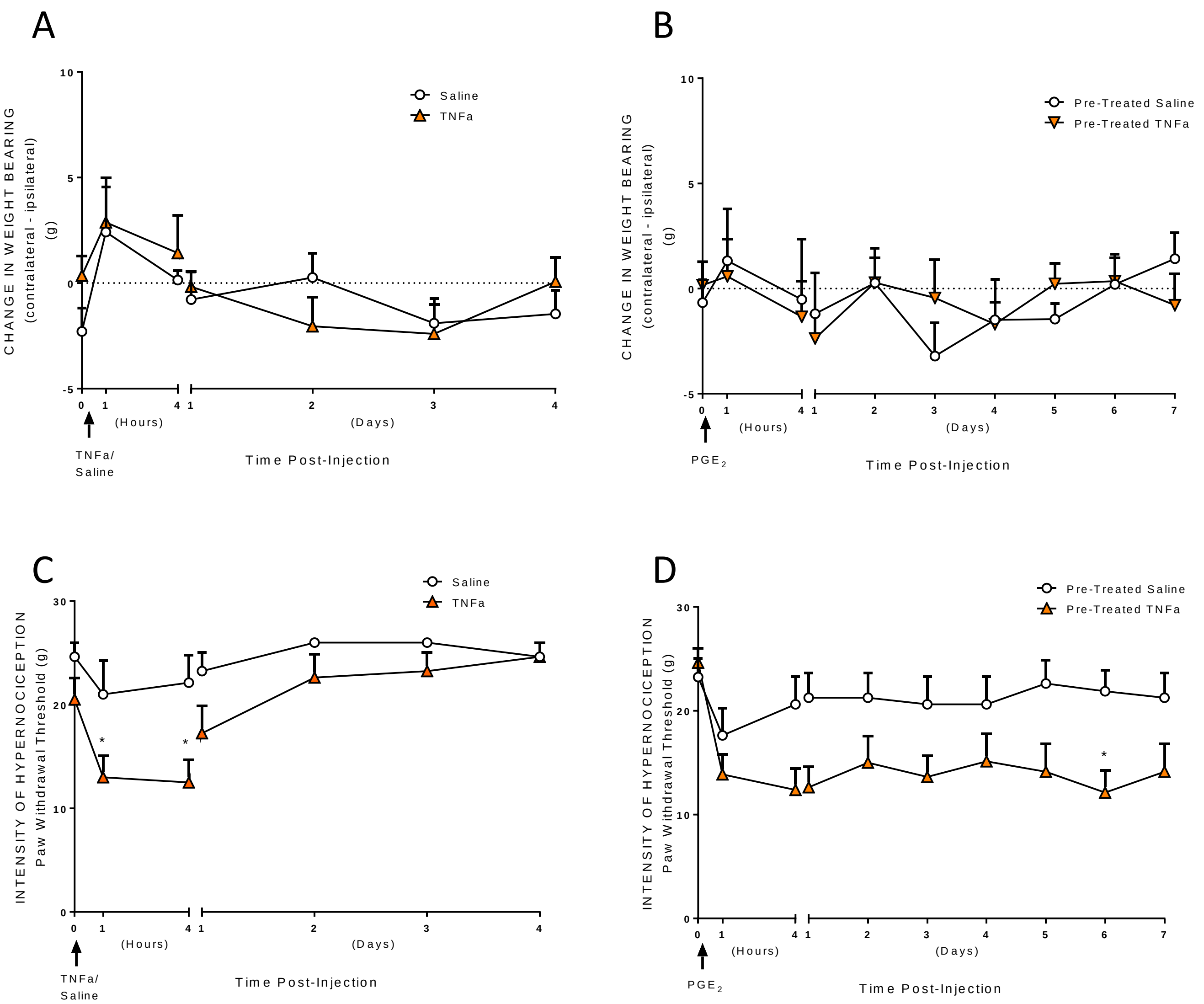


Figure 3.4 A: TNFα injection into the plantar surface of the left hind paw decreased ipsilateral weight bearing after 1h, which resolved by 4h. Control animals injected with saline (0.9% NaCl, 5μl) also exhibited a decrease in weight bearing at 1h and recovery at 4h. **B:** Four days post initial injection animals received a single administration of PGE_2 this caused a no significant change in either groups. **C**: Intraplantar injection of TNFα (5μl, 100ng) produces a significant decrease in hindpaw withdrawal thresholds at 1 and 4 hours, which resolves by 2 days post injection in the rat. D**:** Injection of PGE_2 into both the saline and TNFα pre-treated hindpaw at day 4 produces an initial decrease in hindpaw withdrawal thresholds, which has resolved by 4h in the saline-treated group. In the TNFα pre-treated group, withdrawal thresholds are lower for up to 7 days post PGE_2 injection however not significantly different. Data are mean ± SEM. Statistical analysis was carried out using a 2-way ANOVA test with a Bonferroni post hoc test *p<0.05 (n=8 in both groups).

3.3.2 Does carrageenan induced priming extend beyond 7 days?

To investigate the longevity of the primed state, the effects of injection of PGE_2 at 14 days after injection of carrageenan was also studied. As shown in the previous study injection of carrageenan caused a decrease in both ipsilateral weight-bearing and PWT at 1-4h which returned to baseline by 3 days. Responses remained at baseline until day 14 post carrageenan injection (Figures 3.5A and 3.5C). On day 14 both groups received a single injection of PGE_2 (1µg/5µl) into the same site as the previous injection. At this time-point, injection of PGE_2 was associated with an increase in ipsilateral weight bearing in the carrageenan pre-treated rats at 1 day ,which returned to baseline by day 3 (Figure 3.5B). Injection of PGE_2 in the carrageenan pre-treated rats was associated with a significant lowering of PWTs at 1, 3 and 4 days post-PGE_2 injection, compared to saline-PGE_2 group. (Figure 3.

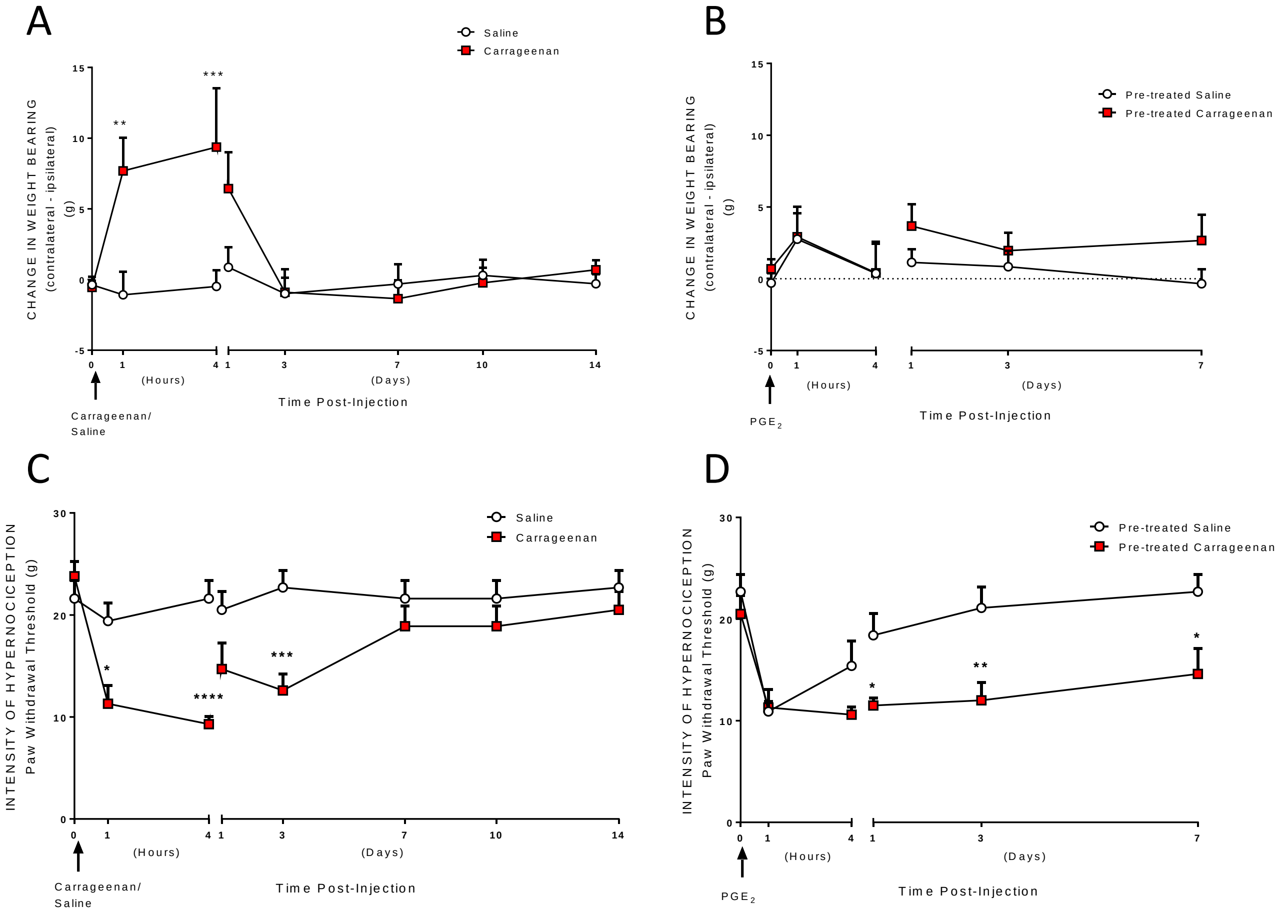


Figure 3.5 A: Carrageenan injection into the plantar surface of the left hind paw decreased ipsilateral weight bearing after 1h, which resolved by 1 day. Control rats injected with saline (0.9% NaCl, 5µl n=6) exhibited no significant change in weight bearing. **B:** Fourteen days post initial injection rats received a single administration of PGE_2 this caused a significant decrease in ipsilateral weight bear after 24h in rats pre-treated with carrageenan. There was no change weight-bearing post-PGE_2 in saline pre-treated rats. **C**: Intraplantar injection of carrageenan (5µl, 1% solution) produces a significant decrease in PWTs at 4 hours, which returned to baseline values by 1-3 days post injection in the rat. **D:** Injection of PGE2 into both the saline and carrageenan pre-treated rats at day 14 produces an initial decrease in PWTs, which has resolved by 1 day in the saline-treated group. In the carrageenan pre-treated group, PWTs were significantly lower for up to 7 days post PGE2 injection. Data are mean ± SEM. Statistical analysis was carried out using a 2-way ANOVA test with a Bonferroni post hoc test *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ (n=10 for both groups).

3.3.3 Hyperalgesic priming causes decreases in the number of microglia within the dorsal horn

Similar to the study of skin shown in the appendix, numbers of microglia in the dorsal horn of the spinal cord were studied using an IBA1 antibody at 7 days following induction of hyperalgesic priming and compared to no-primed spinal cord (carra + PGE2 versus saline + PGE2). In the control groups (carra + saline and saline + saline) numbers of, amoeboid "activated" microglia in the dorsal horn of the spinal cord were counted (Figure 3.6A-D). In the control saline + PGE2 group, numbers of activated microglia in the dorsal horn of the spinal cord were significantly increased compared to carra + saline and saline + saline groups (Figure 3.6E). This was also the case for the hyperalgesic primed group (carra + PGE2) compared to carra + saline and saline + saline groups. However, numbers of activated microglia in the hyperalgesic primed group were significantly lower than numbers in the sal + PGE2 group (Figure 3.6E).

However, the total number of microglia within the dorsal horn was also found to be significantly higher in the unprimed PGE_2 group, compared to all other groups (Figure 3.6F). As a result when numbers of activated microglia were expressed as percentage of total microglia there was no difference between the proportion of activated microglia between the primed and unprimed groups (Figure 3.6).

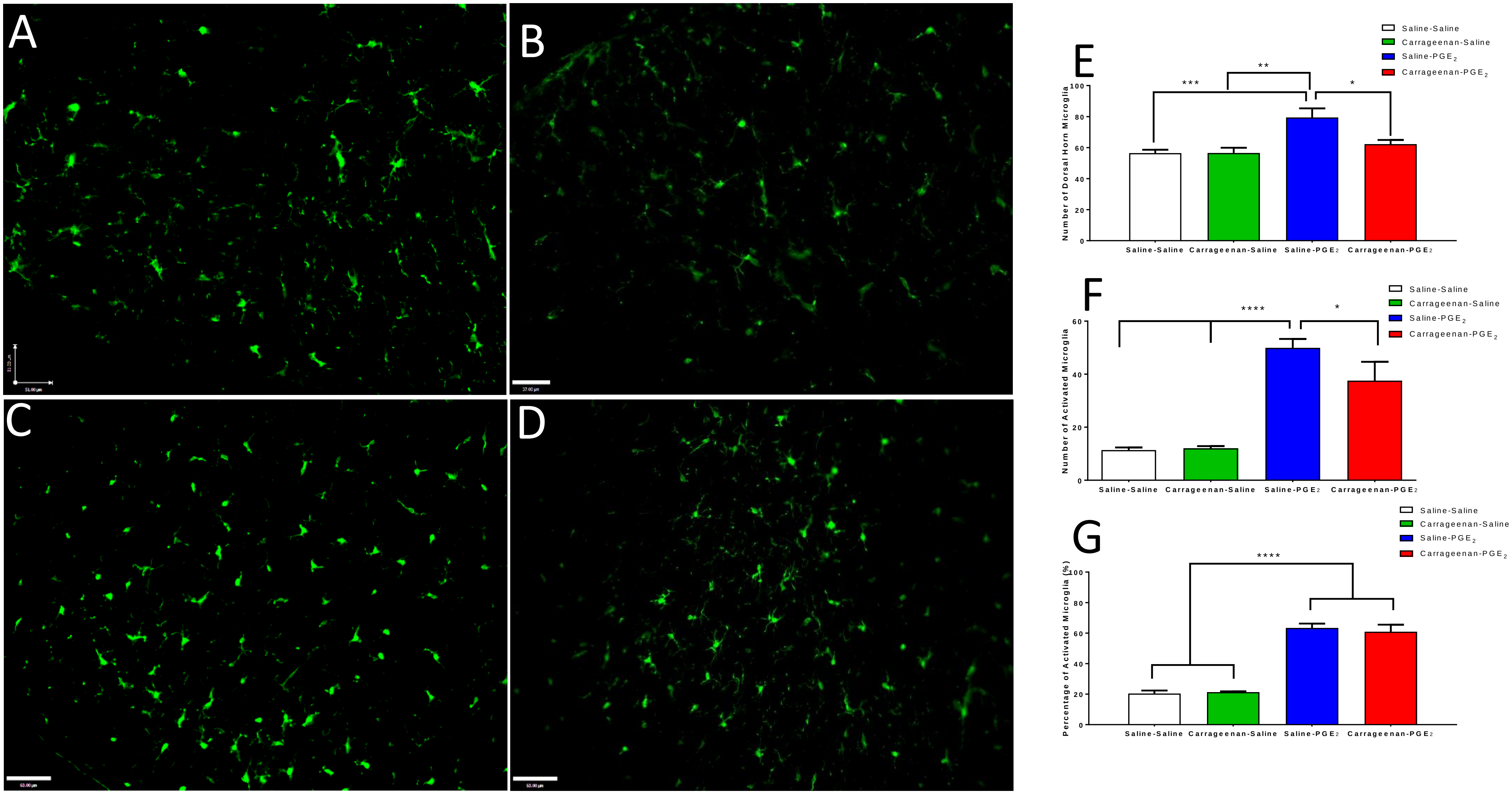

A
B
C
D
E
Saline-Saline
Carrageenan-Saline
Saline-PGE2
Carrageenan-PGE2
Number of Dorsal Horn Microglia
***
**
*
F
Number of Activated Microglia
****
*
G
Percentage of Activated Microglia (%)
****

Figure 3.6 IBA1 staining for microglia in L5 spinal cord at 20x magnification **A:** Ipsilateral dorsal horn of saline pre-treated animals 7 days post-vehicle. **B:** Ipsilateral dorsal horn of carrageenan pre-treated animals 7 days post-vehicle **C:** Ipsilateral dorsal horn of saline pre-treated animals 7 days post PGE_2 **D:** Ipsilateral dorsal horn of carrageenan pre-treated animals 7 days post PGE_2. **E:** Total number of microglia within the dorsal horn of L5 spinal cord. Saline-PGE_2 treated rats show increased microglial numbers within the dorsal horn compared to other groups. **F:** Total number of activated microglia within the dorsal horn of L5 spinal cord. Saline-PGE_2 animals show greater numbers of activated microglia compared to other groups. There are also increased activated microglia in the carrageenan-PGE_2 group compared to the control; saline-vehicle, carrageenan-vehicle groups. **G:** Percentage of activated microglia of total microglia within the dorsal horn of the spinal cord. The administration of PGE_2 caused an increase in the number of activated microglia compared to vehicle controls. There were no significant differences between saline-vehicle and carrageenan-vehicle groups or saline-PGE_2 and carrageenan-PGE_2 groups. Statistical analysis was carried out using a 1-way ANOVA test with a Sidak post hoc test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Scale bar 60µm.

3.3.4 Plasma lipid levels

In a sub-group of rats, plasma was collected at 4 hours following injection of PGE_2 and was prepared for mass spectroscopy analysis of lipid mediators and metabolites. Overall there were no significant differences in the concentration of the lipid mediators measured between primed and unprimed rats (Figure 3.7). As illustrated in Table 3.1, the concentrations of many of these lipids were very low and not all lipids were found in each sample to be above the detection level. It was evident that there were differences in the detection rate of lipids between primed and unprimed animals. In several cases lipid levels were found to be below the detection level in a greater number of samples from primed animals. Mediators such as 9-HETE and PGE_2 EA were detected in 9 out of 12 and 8 out of 12 times in unprimed animals respectively compared to 5 out of 12 and 2 out of 12 times in primed animals respectively (Table 3.1). It was found that the detection levels of PGE_2 EA were significantly lower in unprimed animals compared to primed after chi squared analysis. This could suggest a loss of these mediators during the chronic PGE_2 induced pain of hyperalgesic priming. The absence of a lipid being detected does not mean they are not present in the sample only that they are below the level of detection however further experiments can be performed looking into these lipid mediators and their potential involvement in hyperalgesic priming (see Chapter 6).

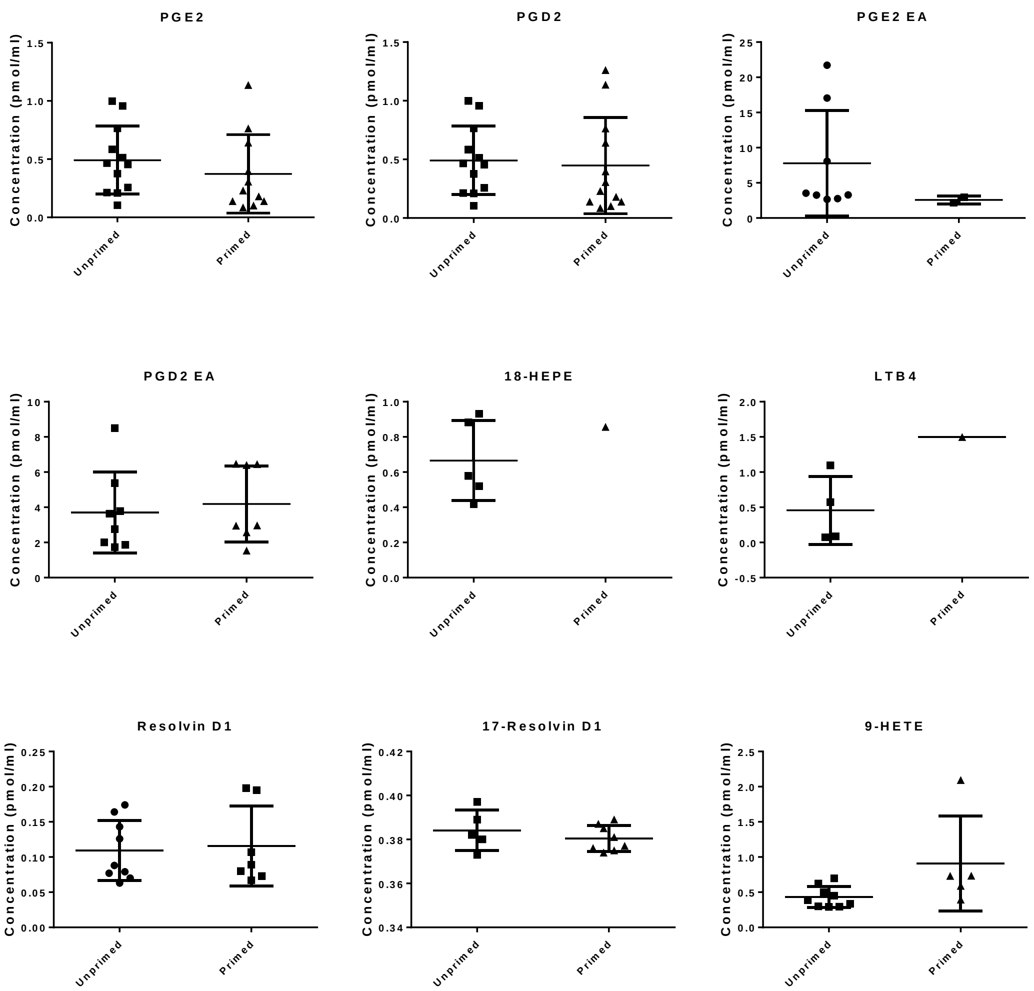


Figure 3.7 Plasma collected at 4 hours post PGE_2 administration was analysed using mass spectroscopy to identify changes in lipid mediators between primed and unprimed animals. There were no significant differences in the concentrations of various lipid mediators between primed and unprimed animals. However there were observed differences in the detection rate of several mediators including 9-HETE, 18-EPE, LTB_4 PGE_2 EA and 17-resolvin D1 summarised in table 3.1.

MEDIATOR	UNPRIMED	PRIMED
9-HETE	$^{9}/_{12}$	$^{5}/_{12}$
18-HEPE	$^{5}/_{12}$	$^{1}/_{12}$
LTB_4	$^{4}/_{12}$	$^{1}/_{12}$
PGE_2 EA	$^{8}/_{12}$ *	$^{2}/_{12}$
17-RESOLVIN D1	$^{5}/_{12}$	$^{8}/_{12}$

Table 3.1 Several lipid mediators were found to not be detected in all animals in each group. 9-HETE, 18-HEPE, LTB_4 and PGE_2 EA were found in a greater number of unprimed rats compared to primed rats. PGE_2EA was found to be detected significantly less in the primed group compared to the unprimed animals. The precursor to resolvin D1; 17-resolvin D1 was identified in a greater number of primed animals compared to unprimed animals. Data analysis performed using chi squared test * $p<0.05$

3.4 Discussion

3.4.1 Induction of Hyperalgesic Priming

The model of hyperalgesic priming has been successfully established, my data reflects previously published work by the Levine group (Aley *et al.*, 2000). Injection of 1% carrageenan induced a transient acute pain that lasted for 7 days as previously shown (Aley *et al.*, 2000; Dina *et al.*, 2008). Injection of PGE_2 into the previously injected site induced a reduction in PWTs at 1h in both the primed and unprimed groups of rats. Carrageenan-primed rats had persistent reductions in PWTs from 4h until 7 days later, consistent with previous studies (see refs in (Reichling *et al.*, 2009)). There were no significant changes in weight-bearing in primed rats, suggesting this model does not mimic ongoing pain, which would lead to a reduction in the weight placed on the injured limb. Given that this model is associated with lowered PWTs and no change to weight-bearing, the hyperalgesic priming model has the characteristics of allodynia (Sandkuhler, 2009). As demonstrated in this chapter, carrageenan induced hyperalgesic priming is a robust reproducible version of the model.

The TNFα induced version is set up in a quicker time due to the faster recovery time after the TNFα injection. Following injection of TNFα, PWTs returned to baseline by between 24-48h post-injection. However the induction of the chronic phase by injection of PGE_2 was less robust, with smaller reductions in PWTs, compared to the carrageenan induced model. These differences probably reflect the differences in the type of response to these two stimuli. Carrageenan injection causes a robust inflammatory reaction consisting of both a non-phagocytic inflammatory phase and a phagocytic inflammatory phase within the dermis and epidermis of skin. This causes infiltration of neutrophils, monocytes and mast cells producing an oedema and causing the metabolism of AA to produce the release of inflammatory mediators including PGs (Vinegar *et al.*, 1987). TNFα however activates a discrete part of the inflammatory system binding to one of two main receptors TNFR1 and TNFR2, activating the NF-κB and MAPK-p38 pathways to increase the transcription of inflammatory genes (Chen *et al.*, 2002; Wajant *et al.*, 2003). The half-life of TNFα also is relatively short having a peak effect of around 2 hours with a half-life of approximated 20mins (Oliver *et al.*, 1993). The overall duration of the inflammatory response to carrageenan is greater than that to TNFα, as well as the number of pathways activated and cells recruited being different, which could account for the differences in the chronicity of the two models of hyperalgesic priming.

Part of the work in this chapter also confirmed the optimal timepoint for mechanistic studies of hyperalgesic priming. Previous work reported the presence of hyperalgesic priming at 21 days post PGE2 injection. In this chapter I confirmed that hyperalgesic priming is evident at 14 days post-carrageenan injection, however comparison between 7 and 14 days revealed that the model was more robust at the 7 day time-point.

3.4.2 Hyperalgesic Priming: no evidence for a role of neutrophils or macrophages

Neutrophils were not present in the skin at 7 days after injection of PGE2 in either rats pre-treated with saline or carrageenan. This observation is consistent with the rapid and short lasting roles of neutrophils 5 to 90 hours post-injury (Gabrilovich, 2005). However there is the possibility that the immunohistochemistry for neutrophil elastase did not work, the protocol for the staining of neutrophils has been used to successfully stain neutrophils within skin treated with carrageenan (Burston *et al.*, 2011). Therefore it is required to carry out further controls to test the specificity of the antibody. This would involve the use of cell cultures of macrophages and neutrophils or their extraction and purification. Another method would be to use the antibody on knock-out animals who do not express ED1 or neutrophil elastase. Currently this work is preliminary and will require further work to confirm the specificity of the antibodies used. Injection of PGE_2 was associated with an increase in the number of macrophages within the rat skin tissue at 7 days post-injection, but there were no differences between the primed (carrageenan) and unprimed (saline) rats. PGE_2 injection induces macrophage infiltration as previously described (Kalinski, 2012), which is still evident 7 days later.

3.4.3 Hyperalgesic Priming Causes a Reduction in the Number of Microglia in the Dorsal Horn

There are increased numbers of activated microglia in the PGE_2 treated animals compared to those injected with vehicle. This shows that the administration of PGE_2 into the hindpaw activates microglia within the dorsal horn which is still present at 7 days post-injury. There were a larger number of “activated” (amoeboid morphology) microglia within the dorsal horn of unprimed rats compared to primed rats which would suggest a variation in activation state due to hyperalgesic priming. Unfortunately there is no suitable biomarker of microglial activation so counts have to be subjective based on the morphological shape of the observed microglia as IBA1 constitutively marks all microglia. As seen in figure 3.6B the microglia have a small cell body with many processes projecting out however in figure 3.6C the microglia no long show long process and have a more amoeboid shape. However there were differences in the total number of microglia within the dorsal horn. There were greater numbers of microglia (activated and inactivated combined) in unprimed rats compared to all other groups. Therefore the percentage of activated microglia was similar in unprimed and primed animals. This would suggest that PGE_2 causes an increase in the proliferation of microglia. Microglia have been shown to rapidly proliferate in states of chronic injury and neurodegeneration (Gómez-Nicola *et al.*, 2013; Guan *et al.*, 2016). It can therefore be concluded that hyperalgesic priming inhibits microglia proliferation. Microglia have been shown to rapidly proliferate once stimulated by injury (Gehrmann, 1996).

The higher number of microglia populations in unprimed rats could suggest a potential protective role by microglia through the production anti-inflammatory mediators such as IL-4 and IL-10 (Cherry *et al.*, 2014). PGE_2 administered peripherally has been demonstrated to promote the production of anti-inflammatory IL-10 within macrophages (MacKenzie *et al.*, 2013) so PGE_2 could potentially play a role in promoting anti-inflammatory mediator release from microglia. There is therefore a potential role for PGE_2 performing an anti-inflammatory or protective role in unprimed rats which does not occur in primed rats. Another view could be the loss of microglia in primed animals after the PGE_2 administration. PGE_2 has been shown to cause apoptosis of microglia in culture (Nagano *et al.*, 2014) this however has not been demonstrated in vivo. This duality of PGE_2 could explain the differences in microglia populations in primed compared to unprimed animals. However work needs to be done in looking at the changes in microglial populations at multiple timepoints in hyperalgesic priming.

The presence of M1 and M2 subtypes of microglia was also investigated however it was found that the CD206 did not co-localise with IBA1. This would suggest that CD206 is not expressed on rat M2 microglia within the dorsal horn however it could also be due to a lack of M2 microglia within the dorsal horn. Much of the current published work that has focused on differentiation of M1 and M2 microglia has been conducted in mice. With many of the existing antibodies being specific to the mouse antigen (Cherry *et al.*, 2014). The identification of a marker for rat M2 microglia would aid in the understanding of the activation state of the microglia in hyperalgesic priming as well as many other chronic pain models conducted in rats. However as stated in **1.1.3.3** there is debate over the terminology and existence of M1 and M2 microglia. The categorisation of microglia into two families is too simplistic as it is basely on a system used for macrophages which have been studied and found to exist in many additional classes (Ransohoff, 2016). Macrophages and microglia do share similar functions however they are different classes of cells. Therefore it is not correct to apply terminology for macrophages to microglia so lightly. Microglia require further study to truly understand their nature in different situations.

3.4.4 Potential Plasma Markers of Hyperalgesic Priming

Identification of biomarkers of hyperalgesic priming could potentially aid in the understanding of the transition from acute to chronic pain. Plasma was collected from unprimed and primed rats 4 hours post-PGE_2 injection. There were no differences in the concentrations of selected lipid mediators however there were observed differences in the detection rates within groups. It was found that 9-HETE, 18-HEPE, LTB_4 and PGE_2 EA were detected in a greater number of unprimed rats samples compared to primed rats samples.

18-HEPE is a precursor to the formation of the resolvin E series (RvE) (Oh *et al.*, 2011). The RvE1 and E2 play a crucial role in the resolution of inflamed tissues, specifically RvE1 inhibits the infiltration of macrophages and neutrophils into the damaged tissue (Arita *et al.*, 2005; Arita *et al.*, 2007; Jin *et al.*, 2009) and also aids the phenotypic switch of macrophages from an M1 (inflammatory) to M2 (anti-inflammatory) phenotype (Navarro-Xavier *et al.*, 2010). PGE_2 EA was found to be detected in a significantly greater number of unprimed animals compared to primed. PGE_2-EA is derived from PGE_2 and reduces inflammatory responses (Yu *et al.*, 1997). It is able to supress the release of TNFα from monocytes and levels of TNFα in human blood samples (Brown *et al.*, 2013). PGE_2-EA also acts to reduce cytokine-evoked epithelial damage in models of colitis (Li *et al.*, 2016; Nicotra *et al.*, 2013). The higher detection rate of many of these anti-inflammatory lipid mediators in plasma samples from unprimed rats may suggest an alteration in the primed rats' responses to inflammatory stimuli impeding their ability to recover after damage has occurred.

There was a slightly larger detection rate of the inflammatory LTB_4 in samples from unprimed rats compared to primed rats. LTB_4 targets leukocyte function promoting infiltration and granulation. LTB_4 stimulates the adherence of leukocytes to the endothelium of venules later promoting their entry into the damaged tissue. LTB_4 has a greater affinity for polymorphonuclear leukocytes such as macrophages (Samuelsson *et al.*, 1987a). However it has already been stated that the action of RvE1 promotes M1 to M2 macrophage transition (Navarro-Xavier *et al.*, 2010) therefore LTB_4 promoting macrophage infiltration may be beneficial to the recovery of the tissue.

3.5 Conclusion

Understanding the transition from acute to chronic pain may help the development of new therapeutics to both prevent and reverse chronic pain. The model of hyperalgesic priming has been shown to be replicable from previous published work on the model. There were two main models of hyperalgesic priming that had been published carrageenan induction and TNFα induction. It is shown that TNFα can induce hyperalgesic priming, however it is not as robust as the carrageenan induced version. The potential cellular changes that occur during the chronic phase of hyperalgesic priming had yet to be investigated. I have shown that hyperalgesic priming is not associated with changes in numbers of neutrophils or macrophages. I did observe changes in the total number of microglia within the lumbar region of the dorsal horn of the spinal cord. Unprimed rats had a larger number of microglia compared to primed rats, potentially suggesting an inhibition of microglial proliferation in primed rats or a loss of microglia in primed rats after injection of PGE_2. There are also several plasma lipid mediators that could be further investigated to gain a better understanding of the changes that occur during the transition from acute to chronic pain. However it is likely that the maintenance of the chronic phase has a neuronal mechanism (further discussed in Chapter 4).

Chapter 4

Investigating Central and Peripheral Nervous System Mechanisms of Hyperalgesic Priming

4.1 Introduction

4.1.1 Role of Sensory Neurones in Hyperalgesic Priming

The chronic phase of hyperalgesic priming has been shown to be dependent upon IB_4^+ C-fibres (Alvarez *et al.*, 2012b), (Joseph *et al.*, 2010) despite the EP receptors for PGE_2 being expressed throughout the periphery on sensory nerves and immune cells (Kalinski, 2012; Southall *et al.*, 2001). There could be an involvement of other fibre types in the establishment of the chronic phase of hyperalgesic priming. Despite systemic or local administration of morphine blocking carrageenan induced reduction in PWTs, PGE_2 administration is still able to cause hyperalgesia. This would suggest that the behavioural pain response of the carrageenan is not required for priming to occur (Villarreal *et al.*, 2013). Therefore the events leading to priming are not dependent on the lowering of PWT and that other factors which are not sensitive to opioid inhibition drive the local protein translation (as discussed in Chapter 1.4.3) which leads to PGE_2 induced hyperalgesia priming. However there is no investigation into whether blocking the initial PGE_2 hyperalgesia will prevent the activation of the underlying mechanisms leading to prolonged hyperalgesia.

One method of blocking sensory nerve fibres is by using local anaesthetics such as lidocaine or lignocaine. Local anaesthetics are able to enter equilibrium between a charged and uncharged form. The uncharged form is able to pass through plasma membranes and enter the axons and nerve endings of sensory nerve fibres. When the anaesthetic molecule is within the nerve fibre the charged form can enter open sodium channels blocking them and reducing the capability of the cell to depolarise therefore it is unable to generate or propagate action potentials (Figure 4.1A). The local anaesthetic binding site is within the pore of Na^+ channels. When they open the local anaesthetic can enter and block the channel (Scholz, 2002).

To investigate the involvement of different sensory nerve fibres the local anaesthetic QX-314 has been used to perform selective blockade of nerve fibres. QX-314 is an isoform of lidocaine that is permanently positively charged meaning that it is unable to pass through plasma membrane therefore cannot enter nerve fibres to internally block sodium channels (Frazier *et al.*, 1970) (Figure 4.1B). This makes QX-314 useful in the selective blockade of specific sensory nerve fibres; it has been shown that by using QX-314 in combination with capsaicin the QX-314 molecule is able to enter nerve fibres and block sodium channels via the opened TRPV1 channel. This allows for the selective block of sensory nerves that express TRPV1 which includes both peptidergic and non-peptidergic fibres. Therefore the use of QX-314 + capsaicin will block the IB_4^+ C-fibres that are involved in hyperalgesic priming. It has been shown that QX-314 + capsaicin treatment increases the threshold of rats to the application of mechanical and thermal stimuli, when compared with QX-314 alone which showed no change in threshold and capsaicin alone which caused a decrease in threshold (Binshtok *et al.*, 2007).

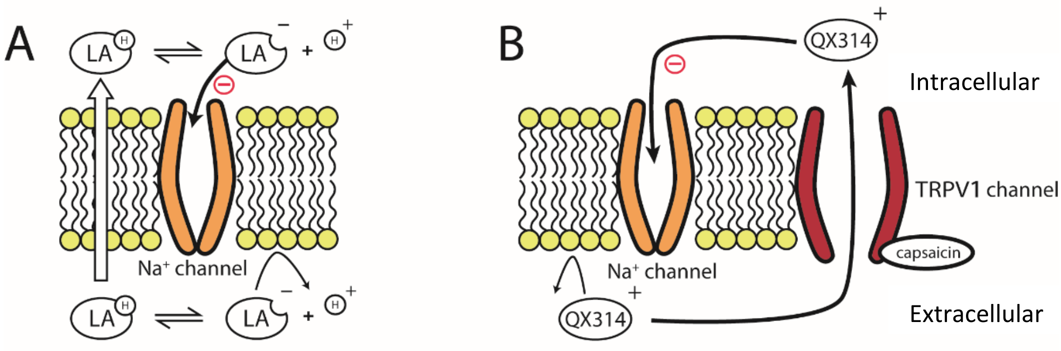


Figure 4.1: A. Mechanism of local anaesthetics. The uncharged form of local anaesthetics is able to pass through plasma membranes to block Na^+ channels from inside the axon. B. QX314 is permanently charged therefore unable to pass through the membrane. By opening an alternate channel (e.g. TRPV1) the QX314 can enter the axon and block the Na^+ channel.

QX-314 can also be co-administered with flagellin to block Aβ fibres. This works by the activation of TLR5 leading to the opening of channels that allow the entry of QX-314 (the channels that QX-314 enters through are currently unknown) (Xu *et al.*, 2015). The Aβ fibres themselves have been implicated in the maintenance of mechanical allodynia in cases of nerve injury (Campbell *et al.*, 1988; Koltzenburg *et al.*, 1992; Woolf *et al.*, 2000). As well as recently in chemotherapeutic induced hyperalgesia (Xu *et al.*, 2015).

By using QX-314 in combination with capsaicin and flagellin the sensory components of hyperalgesic priming can be better understood, elucidating whether there is involvement of other subtypes of sensory nerve fibres at different stages of hyperalgesic priming. Also investigate whether the blockade of the PGE_2 induced hyperalgesia will prevent the development of the maintained hyperalgesia post 24 hours.

4.1.2 Central Component to Hyperalgesic Priming

Hyperalgesic priming displays prolonged reduction in PWTs to a PGE_2 stimulus. Under normal control conditions the pain response to PGE_2 would normally only last for 1-2hours (Ouseph *et al.*, 1995), however it has been reported that the reduced PWTs can last up to 21 days in hyperalgesic primed rats (Aley *et al.*, 2000) (Reichling *et al.*, 2009).

There have been several studies showing evidence for peripheral plasticity in the maintenance of hyperalgesic priming. The administration of protein translation inhibitors to the site of injury such as rapamycin and cordycepin have been shown to prevent priming from being established (Ferrari *et al.*, 2013a), (Ferrari *et al.*, 2013b). This demonstrates the requirement for local protein translation in hyperalgesic priming, however this has been shown to only be required for its initiation not the maintenance of the chronic pain (Asiedu *et al.*, 2011). Local translation is important in several chronic pain states (Obara *et al.*, 2012), and therefore it is of interest to understand the factors controlling this translation (Melemedjian *et al.*, 2010) as well as the central changes that may also occur as a result.

Investigations into neuronal changes in hyperalgesic priming have been minimal (Hendrich *et al.*, 2013). Peripheral nerve recordings of hyperalgesic primed rats showed neither changes in the shape, size or duration of evoked action potentials nor any changes in threshold for their activation (Hendrich *et al.*, 2013). There was an observed difference in the resting membrane potential although it was found to be held at -70mV in primed rats compared to -50mV in controls. This evidence would suggest that the required current flow input for activation is actually higher in primed rats going against the characteristic reduction in PWT seen in hyperalgesic priming (Hendrich *et al.*, 2013). Due to a lack of reported changes in nerve activity in the periphery it is likely that the behavioural allodynia generated by hyperalgesic priming has a centrally mediated component.

Hyperalgesic priming is characterised by a change from a PKA mediated response to a PKCε, the intrathecal administration of PKC AS ODN have been shown to prevent hyperalgesic priming if given before PGE_2 (Parada *et al.*, 2003), but a study has shown that the maintenance of the chronic pain state is centrally mediated by PKMζ (Asiedu *et al.*, 2011), (Melemedjian *et al.*, 2013). This suggests that there are changes in the spinal cord that could be responsible for the behavioural allodynia in hyperalgesic priming. To distinguish whether observed changes are peripherally or centrally mediated would require the use of pharmacological agents to selectively block the central or peripheral events. This could be done with the use of selective nerve blockade with locally administer anaesthetics in combination with both peripheral and central nerve records. Combining this information with in vitro techniques such as immunohistochemistry and western blotting would allow for specific changes in each region of the pain pathway to be isolated. By collected tissue along the pain pathway including spinal cord, DRG and peripheral sciatic nerves would allow for markers to isolated and studied at each part of the pathway. Along with the potential use of gene cards to isolate markers that differ between primed and unprimed animals.

In other models of long term pain there is evidence of spinal changes that may be major contributors to enhanced sensitivity and duration of chronic pain states (Woolf, 1983; Woolf *et al.*, 2000). One of the main techniques used to determine changes in spinal excitability is the WDR single unit recording. The WDR received inputs from a range of sensory afferents allowing them to detect changes from innocuous to noxious. There are observed changes in the WDR excitability in models of long term pain such as osteoarthritis (Sagar *et al.*, 2010) leading to the investigation of WDR neurones in hyperalgesic priming. By using this method of measuring spinal excitability it should be possible to see the general changes in responsiveness of hyperalgesic primed rats to the peripheral administration of PGE_2. It is hypothesised that the administration of PGE_2 will cause an increase in the firing of WDR neurones in response to mechanical stimulation. The observed increase in firing is expected to be maintained beyond 1 hour in hyperalgesic primed rats compared to unprimed.

4.1.3 Aims

1. To establish a physiological correlate of hyperalgesic priming, so that its central mechanisms can be investigated in real time in vivo.

2. To investigate how WDR neurones in primed vs unprimed rats respond to PGE_2 administration.

3. Investigate the involvement of other sensory nerve fibres and whether their blockade has an effect on hyperalgesic priming.

4.2 Methods

4.2.1 Initiating the Primed State

Male Sprague Dawley rats (200-250g) were anaesthetised as detailed in **2.3.2** and received a subcutaneous injection into the left hindpaw of 5µl of 1% carrageenan or saline. The behavioural response to the injection was measured at intervals for up to 7 days (Figure 4.2). On day 7 rats underwent spinal electrophysiological recordings.

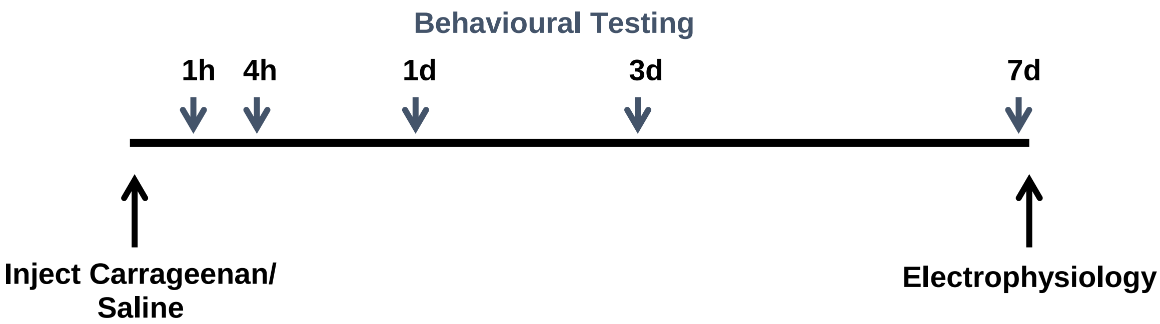


Figure 4.2: Timeline of behavioural von Frey testing conducted after injection of carrageenan/saline into the left hindpaw. On day 7 paw withdrawal threshold was measure 30mins prior to the rat undergoing surgery for electrophysiological recording.

4.2.2 Selective Sensory Nerve Blockade

4.2.2.1 Pilot Study Effect of QX-314 + Capsaicin on PGE_2 Induced Allodynia

To confirm the effect of QX-314 + capsaicin on PGE_2 induced PWTs a pilot study was conducted. QX-314 bromide (Abcam) was dissolved in saline (0.9% NaCl) at 40mg/ml to make a 4% stock solution. A stock solution containing of 2mg/ml of (E)-capsaicin (Tocris) was dissolved in 5% tween-20, 5% ethanol and 90% saline (0.9% NaCl). A 50:50 working solution of QX-314 + capsaicin was made prior to injection (containing 2% QX-314 and 50µg capsaicin). Vehicle contained 5% tween-20, 5% ethanol and 90% saline (0.9% NaCl).

Male Sprague Dawley rats were anaesthetised with isoflurane in a 2:3 oxygen nitrous oxide gas ratio (**2.3.2**). Rats then received an intraplantar injection of 50µl of QX-314 + capsaicin/vehicle (2.5% tween-20, 2.5% ethanol and 95% saline) into the left hindpaw. Rats were allowed to recover and then injected with PGE_2 (1µg/5µl) 90mins later. PWTs were then measured at 30min, 1h, 2h, 3h & 4h post PGE_2 injection.

4.2.2.2 Effect of QX-314 Pre-treatment on Hyperalgesic Priming

To determine the involvement of sensory nerve fibres on hyperalgesic priming, a combination of QX-314 plus capsaicin or QX-314 plus flagellin were used to selectively block TRPV1 versus TLR 5 expressing sensory fibres (corresponding to nociceptive and non-nociceptive fibres). Flagellin (90µg/ml) was dissolved in a vehicle of 5% tween-20, 5% ethanol and 90% saline (0.9% NaCl). Final injected concentration was 2.25µg flagellin in 50µl vehicle (2.5% tween-20, 2.5% ethanol and 95% saline (0.9% NaCl)).

Male Sprague Dawley rats (200-250g) were anaesthetised (**2.3.2**) with isoflurane and received an intraplantar injection of carrageenan (1%, 5µl) into the left hindpaw. Von Frey PWTs were measured at 1h, 4h, 1d, 3d, and 7d.

At 7 days post-carrageenan injection rats received an intraplantar injection of QX-314 + capsaicin (50µl), QX-314 + Flagellin (50µl) or vehicle (50µl) under anaesthesia. 90mins later rats received intraplantar injection of PGE_2 and PWTs were measured at 1, 2 and 4 hours and then daily for 7 days.

4.2.2.2 Effect of QX-314 on established Hyperalgesic Priming induced by PGE_2

Male Sprague Dawley rats (200-250g) were anaesthetised (**2.3.2**) with isoflurane and received an intraplantar injection of carrageenan (1%, 5µl) into the left hindpaw. Von Frey PWTs were measured at 1h, 4h, 1d, 3d, and 7d. One week later, rats received an intraplantar injection of PGE_2 and PWTs were measured at 1 and 4 hours and then daily for one week. On day 3 post PGE_2 injection, rats were anaesthetised and received an intraplantar injection of vehicle/QX-314 + capsaicin/QX-314 + flagellin. Following recovery from anaesthesia, von Frey PWTs were measured at 1h, 2h and 4h post injection and then daily until day 7.

4.2.3 Spinal Electrophysiology

Rats were set up for spinal recordings as detailed in **2.4.2.3**. Once a cell was located, it was characterised and then if it matched the required criteria for a WDR then it was carried forward for study. The cell was electrically stimulated to elucidate the fibre types that input was received from as detailed in **2.4.2.4**. The baseline neuronal responses to mechanical stimulation (see methods **2.4.2.4**) were recorded every 20mins until there were three consistent results (Figure 4.3). The receptive field of the cell was determined by application of 8g and 26g von Frey filaments to multiple regions of the hindpaw. If the stimulation elicited a response from the recorded cell it was deemed to be within the receptive field. Once stable baselines were recorded the rat was then injected with saline/PGE_2 (1µg/5µl) into the hindpaw within the receptive field of the cell. WDR neurone responses to mechanical stimulation were then recorded 10mins post drug application then repeated every 20mins, with receptive field recorded before each stimulation for 2 hours post-drug application (Figure 4.3). The response of the WDR neurone to the administration of the drug was also measured together with any post discharge that occurred due to its injection.

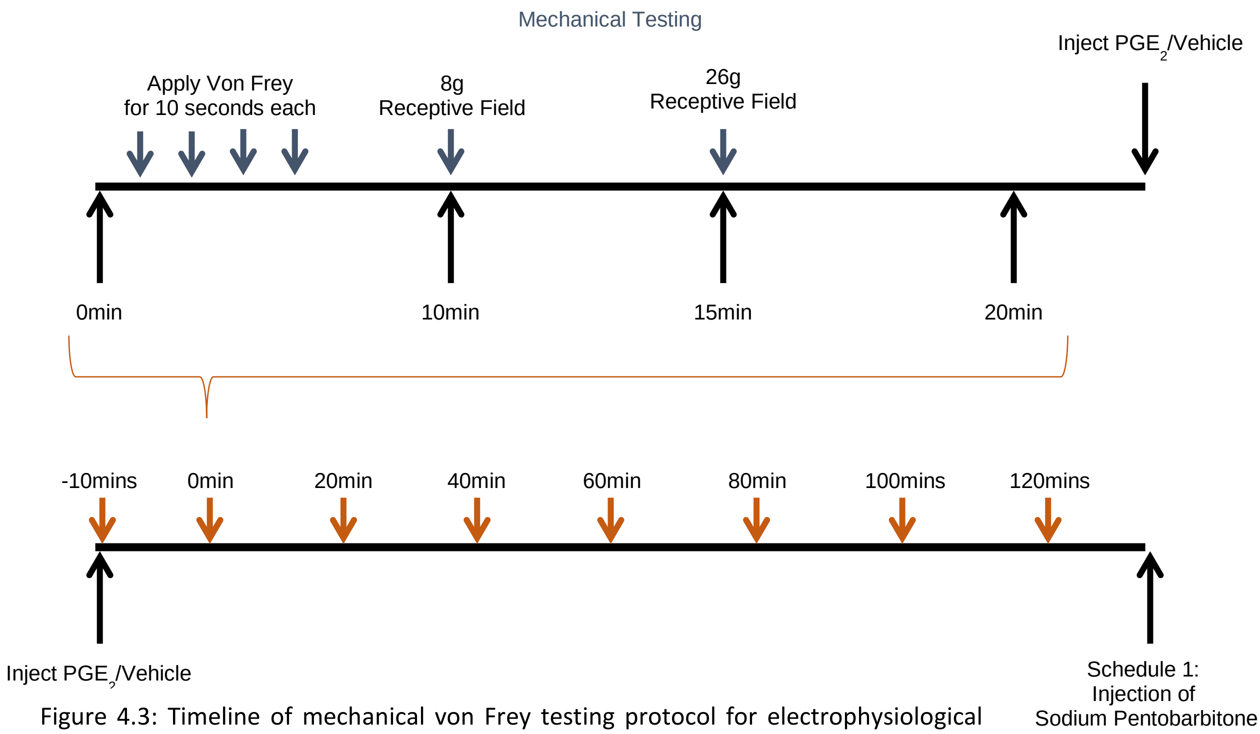


Figure 4.3: Timeline of mechanical von Frey testing protocol for electrophysiological recording of wide dynamic range neurones in the spinal cord.

4.2.4 Statistics

Changes in pain behaviour (weight-bearing asymmetry and PWTs) were compared using two-way ANOVA with Bonferroni post hoc test. Analyses compared changes in pain behaviour over time with changes in control group. The analysis of electrophysiology was conducted using a two-way ANOVA with Bonferroni post hoc test with multiple comparisons. Normalisation was confirmed using Shapiro-Wilks normality test. Group sizes were based on previously published work for hyperalgesic priming (Aley *et al.*, 2000). Data are mean ± standard error of the mean, p value of < 0.05 was accepted as significant.

4.3 Results

4.3.1 Pilot Study QX-314 + Capsaicin Inhibits PGE_2 Induced Allodynia

Intraplantar injection of PGE_2 into the left hindpaw caused a reduction in the PWTs in vehicle treated Sprague Dawley rats. The reduction lasted 1 hour and had resolved by 2 hours. The administration of QX-314 + capsaicin into the hindpaw 90mins prior to the PGE_2 administration blocked the PGE_2 response baseline measurements were made prior to PGE_2 injection (Figure 4.4).

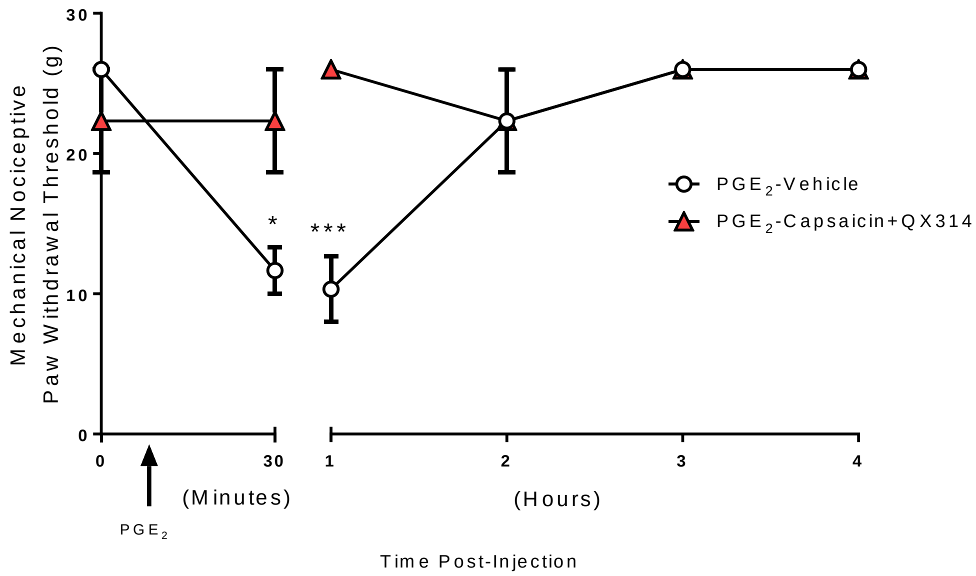


Figure 4.4 Effect of pre-treatment with QX-314 + capsaicin or vehicle 90mins prior intraplantar injection of PGE_2. All data represent mean ± SEM, n = 3 per group. Data analysed with two-way ANOVA with Bonferroni post hoc test * $p<0.05$, *** $p<0.001$.

4.3.2 QX-314 + Capsaicin Blockade of PGE_2 Induced Hyperalgesia Fails to Prevent Long-Term Hyperalgesia

Injection of 5µl of 1% carrageenan subcutaneously into the plantar surface of the hind paw caused a significant decrease in PWTs 4h post-injection. PWTs remained significantly ($p<0.01$) lower than PWTs of saline treated rats up to 3 days post-injection. By 7 days PWTs had returned to baseline values in carrageenan treated rats. Injection of 5µl of saline (0.9% NaCl) into the plantar surface of the left hind paw did not result in a significant change in PWT (Figure 4.5A).

On day 7 post-carrageenan rats received intraplantar administration of QX-314 + capsaicin/vehicle 90mins prior to intraplantar PGE_2. The QX-314 + capsaicin significantly inhibited ($p<0.0001$) the effect of PGE_2 administration when compared to vehicle treated rat with PWTs remaining between 15-26g. At 2 hours post-PGE_2 the saline pre-treated group started to show recovery with PWTs being similar to baseline at 4 hours post-PGE_2 (Figure 4.5B).

The carrageenan pre-treated group that received vehicle showed a large decrease in PWTs which remained significantly ($p<0.01$) lower than saline pre-treated rats at 4 hours post-PGE_2. The QX-314 + capsaicin delayed the onset of the long term hyperalgesia only becoming significantly lower than saline pre-treated rats by 2 days post-PGE_2 that was maintained up to 7 days later. The carrageenan pre-treated group that received vehicle showed a maintained reduction in PWTs after PGE_2 administration lasting up to 7 days later (Figure 4.5B).

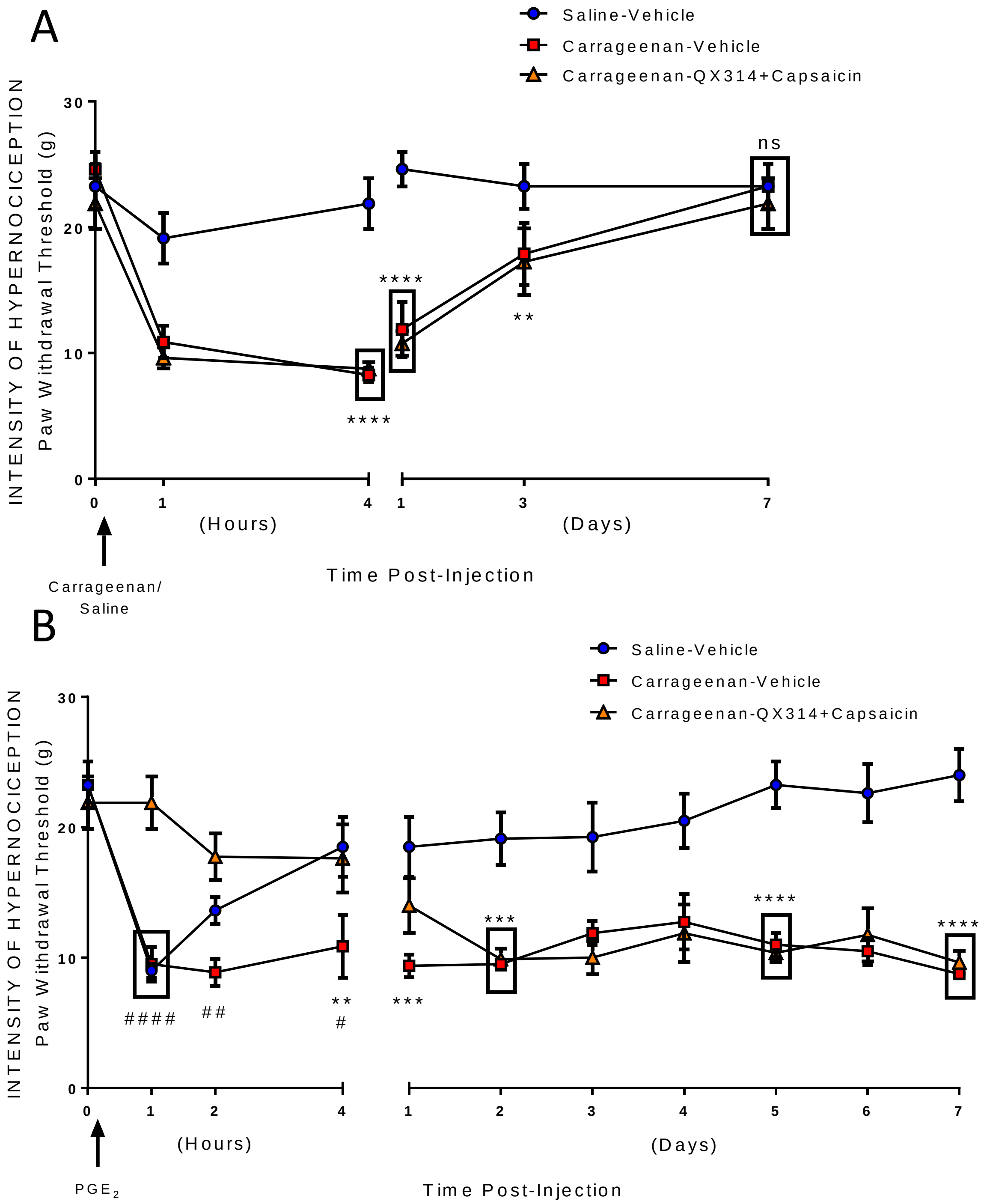


Figure 4.5 **A:** Effect of the administration of carrageenan/saline on paw withdrawal threshold. Carrageenan administration caused a significant decrease in paw withdrawal threshold that lasted up to 3 days with recovery by day 7. Saline administration caused no significant change in paw withdrawal threshold. **B:** Effect of PGE_2 administration 7 days post-carrageenan on PWTs. Intraplantar PGE_2 7 days post-carrageenan caused a reduction in paw withdrawal threshold in vehicle-treated groups. QX-314 + capsaicin treatment prevented PGE_2 induced hyperalgesia up to 4 hours. At 2 days post-PGE_2 the QX-314 + capsaicin groups showed significant differences to unprimed vehicle treated rats. Unprimed rats showed a return to baseline PWTs by 4 hours which was maintained up to 7 days. Primed rats that had received vehicle showed a reduction in paw withdrawal threshold that lasted up to 7 days. All data represent mean ± SEM, n = 8 per group. Data analysed with two-way ANOVA with Bonferroni post hoc test ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$, #$p<0.05$, ##$p<0.01$, ####$p<0.0001$. * denotes statistical comparison to saline control # denotes comparison to carrageenan-PGE_2-vehicle group. Black boxes delineate the same significant in highlighted points.

4.3.3 QX-314 + Flagellin Blockade Inhibits Second Phase of PGE_2 Hyperalgesia, but Failed to Prevent Long-Term Hyperalgesia

As shown in **4.3.**2 injection of 5µl of 1% carrageenan subcutaneously into the plantar surface of the hind paw caused a significant decrease in PWTs at 4h post-injection. PWTs remained significantly ($p<0.01$) lower than PWTs of saline treated rats up to 3 days post-injection. By 7 days PWTs had returned to baseline values in carrageenan treated rats. Injection of 5µl of saline (0.9% NaCl) into the plantar surface of the left hind paw did not result in a significant change in PWT (Figure 4.5A).

On day 7 post-carrageenan rats received intraplantar administration of QX-314 + flagellin/vehicle 90mins prior to intraplantar PGE_2. The QX-314 + flagellin significantly inhibited ($p<0.05$) the second phase of the PGE_2 induced hyperalgesia at 2 and 4 hours when compared to the primed (carrageenan pre-treated) vehicle group. At 2 hours post-PGE_2 the saline pre-treated group started to show recovery with PWTs being similar to baseline at 4 hours post-PGE_2 (Figure 4.6).

The QX-314 + flagellin group showed significantly reduced PWTs at 24h post-PGE_2 which was maintained for 7 days when compared to unprimed (saline pre-treated) vehicle treated rats. The vehicle treated primed group showed maintained PGE_2 induced hyperalgesia that last up to 7 days later (Figure 4.6B).

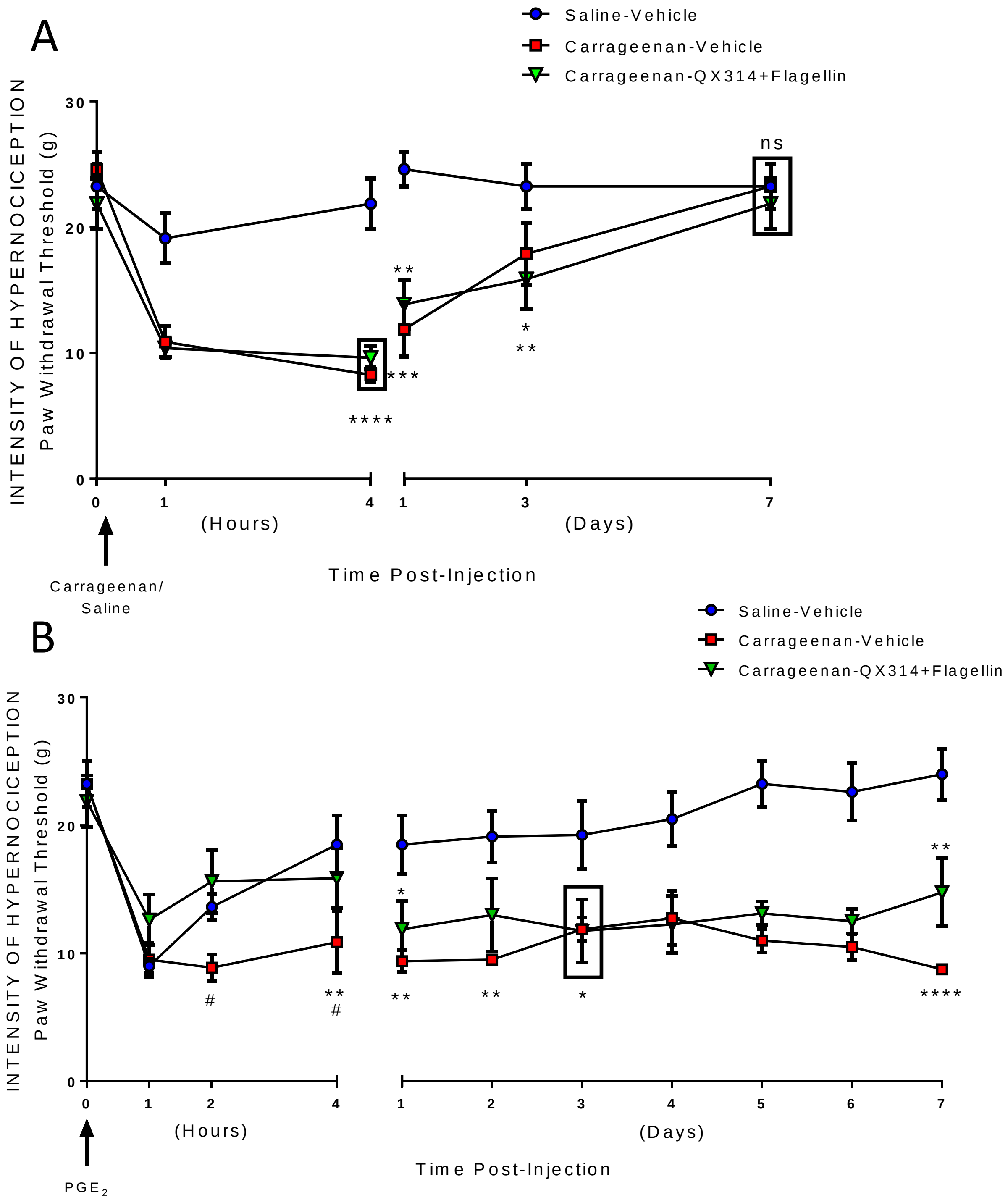
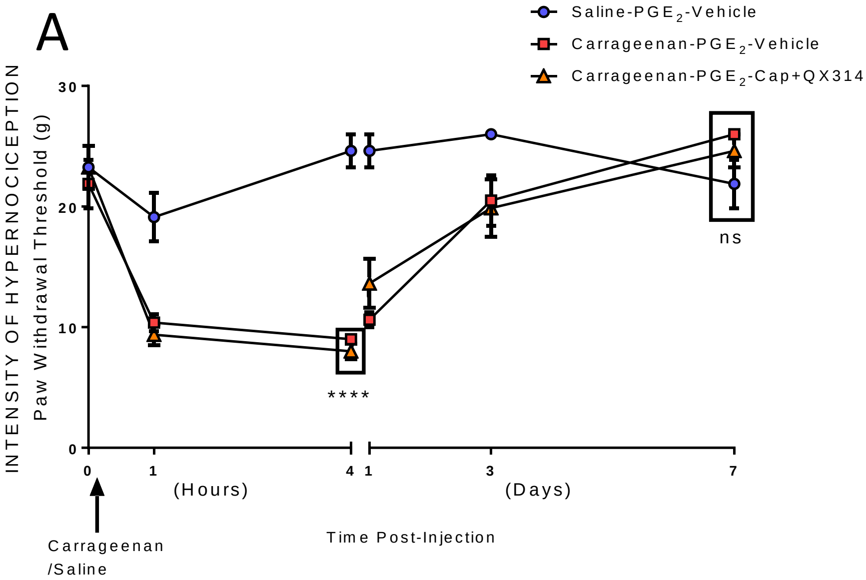


Figure 4.6 **A:** Effect of the administration of carrageenan/saline on paw withdrawal threshold. Carrageenan administration caused a significant decrease in paw withdrawal threshold that lasted up to 3 days with recovery by day 7. Saline administration caused no significant change in paw withdrawal threshold. **B:** The effect of administration of PGE_2 on paw withdrawal threshold. Intraplantar injection of PGE_2 caused a reduction in paw withdrawal threshold in all groups. The unprimed vehicle treated group and the QX-314 + flagellin group should recovery at 2 and 4 hours. The primed vehicle group showed a maintained reduction in paw withdrawal threshold lasting up to 7 days. The QX-314 flagellin group showed a reduction in paw withdrawal threshold at 1 day up to 7 days later. All data represent mean ± SEM, n = 8 per group. Data analysed with two-way ANOVA with Bonferroni post hoc test *p<0.05, **p<0.01, ****p<0.0001 #p<0.05. * denotes statistical comparison to saline control # denotes comparison to carrageenan-PGE_2-QX-314 + flagellin group.

4.3.4 QX-314 + Capsaicin Inhibits Hyperalgesic Priming induced Chronic Pain

Rats that received an intraplantar injection of carrageenan exhibited a decrease in mechanical PWTs, which returned to baseline at 7 days post-injection. Intraplantar injection of saline did not alter mechanical PWTs (Figure 4.7A). Intraplantar injection of PGE_2 caused long lasting decrease in mechanical PWTs in rats that had received previously received carrageenan. On day 3 post-PGE_2 one group of primed rats (n=8) received an intraplantar injection of QX-314 + capsaicin (50µl) and the other groups received intraplantar injection of vehicle (50µl). The administration of QX-314 + capsaicin caused a complete reversal in the PGE_2 induced hyperalgesia with values returned to pre-PGE_2 levels by 4 hours. By 24 hours following-QX-314 + capsaicin injection, PGE_2 induced hyperalgesia had returned to control values (Figure 4.7B).



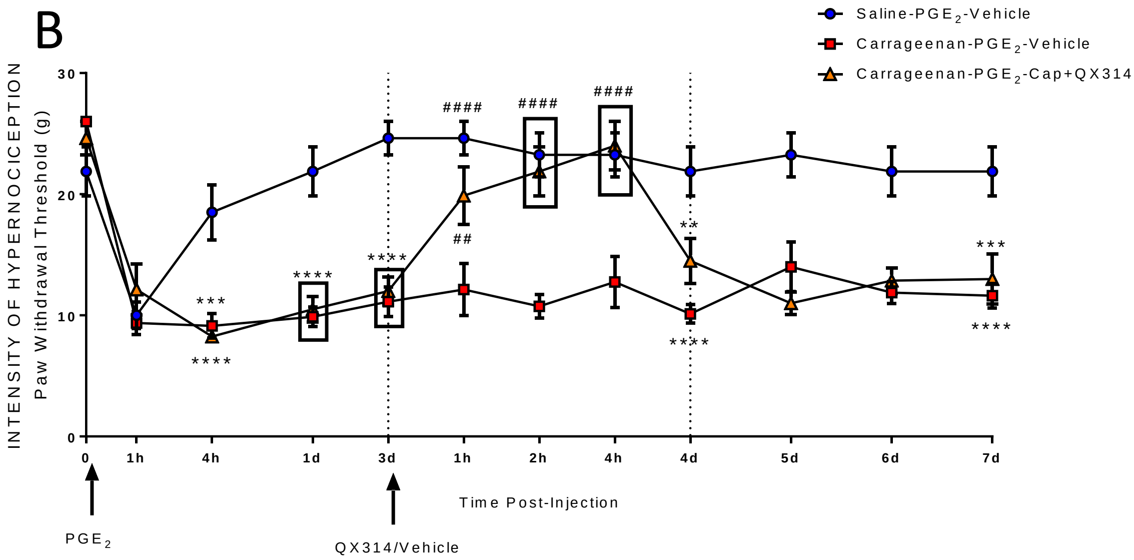


Figure 4.7 **A:** Effect of the administration of carrageenan/saline on paw withdrawal threshold. Carrageenan administration caused a significant decrease in paw withdrawal threshold that lasted up to 3 days with recovery by day 7. Saline administration caused no significant change in paw withdrawal threshold. **B:** Effect of QX-314 + capsaicin on PGE_2 induced hyperalgesic priming. The administration of QX-314 + capsaicin completely inhibited the reduction in paw withdrawal threshold by 4 hours post injection. The effect lasted until 1 day post injection before chronic pain returned. The carrageenan treated rats that received vehicle showed no change in paw withdrawal threshold. All data represent mean ± SEM, n = 8 per group. Data analysed with two-way ANOVA with Bonferroni post hoc test ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$, ##$p<0.01$, ####$p<0.0001$. * denotes statistical comparison to saline control # denotes comparison to carrageenan-PGE_2-vehicle group.

4.3.5 QX-314 + Flagellin Partially Inhibits Hyperalgesic Priming induced PGE_2 Hyperalgesia

As stated previously in **4.3.8** rats that received an intraplantar injection of carrageenan showed a decrease in PWTs in response to von Frey stimulation. The reduced threshold resolved by 7 days. Intraplantar saline showed no significant change in PWTs (Figure 4.8A). Intraplantar injection PGE_2 caused long lasting hyperalgesia in rats that had received carrageenan 7 days previous. On day 3 post-PGE_2 one group of primed rats (n=8) received an intraplantar injection of QX-314 + flagellin. The administration of QX-314 + flagellin caused a small significant reduction in the PGE_2 induced hyperalgesia at 2 hours post-injection. The hyperalgesia returned 4 hours post-QX-314 + flagellin with the vehicle having no effect on the PGE_2 hyperalgesia (Figure 4.8B).

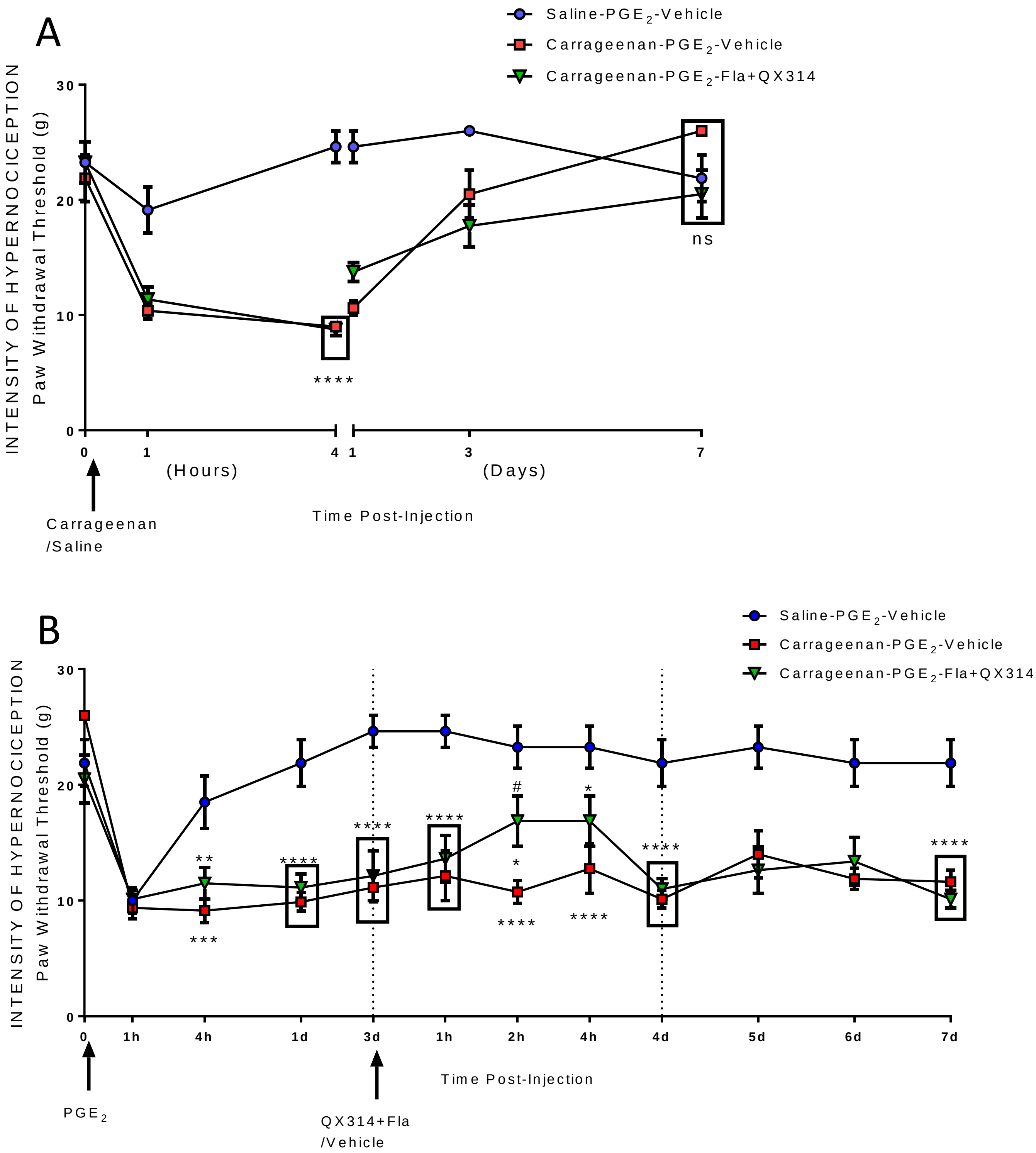


Figure 4.8 **A:** Effect of the administration of carrageenan/saline on paw withdrawal threshold. Carrageenan administration caused a significant decrease in paw withdrawal threshold that lasted up to 3 days with recovery by day 7. Saline administration caused no significant change in paw withdrawal threshold. **B:** Effect of QX-314 + flagellin on PGE_2 induced hyperalgesic priming. The QX-314 + flagellin administration caused a small reduction in PGE_2 hyperalgesia which was significantly different to vehicle treated group at 2h post injection. All data represent mean ± SEM, n = 8 per group. Data analysed with two-way ANOVA with Bonferroni post hoc test * $p<0.05$, *** $p<0.001$ #$p<0.05$. * denotes statistical comparison to saline control # denotes comparison to carrageenan-PGE_2-vehicle group.

4.3.6 Carrageenan causes Transient Hyperalgesia

Intraplantar injection of 0.9% saline (5µl) caused no change from baseline in PWTs at 1 and 4 hours post-injection that was maintained for 7 days. The intraplantar injection of carrageenan into the hindpaw lead to a reduction in PWTs. The carrageenan treated rats showed a 70% reduction in PWTs at 4h post administration. This reduction in threshold resolved over time with rats showing recovery by day 7 (Figure 4.9).

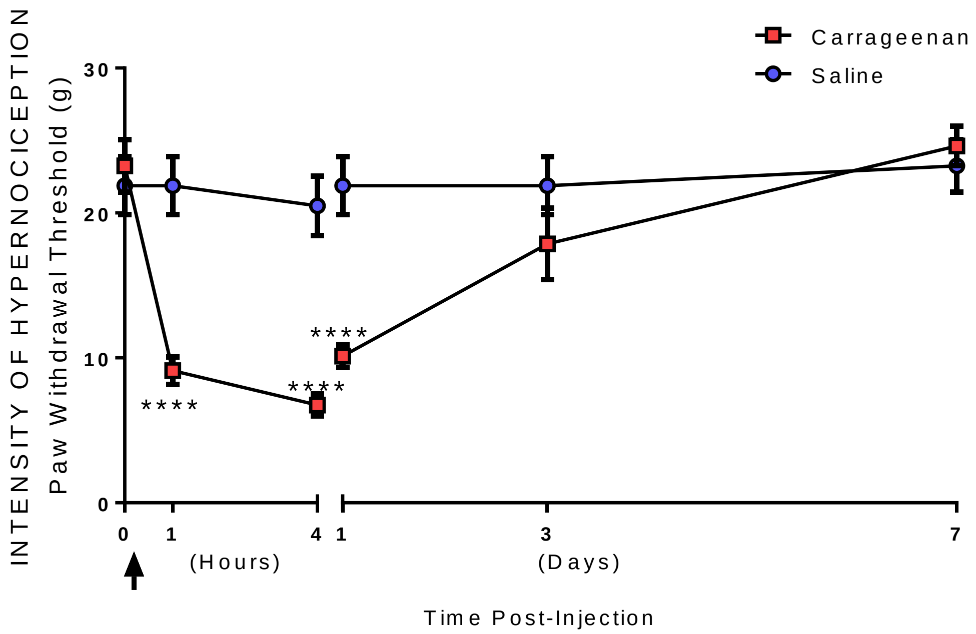


Figure 4.9: Injection of carrageenan/saline into the left hindpaw. Carrageenan causes a significant decrease in paw withdrawal threshold (****, p<0.0001). All data represent mean ± SEM Carrageenan group n = 17, saline group n = 17. Data analysed with two-way ANOVA with Bonferroni post hoc test.

4.3.7 Intraplantar PGE_2 Induces Wide Dynamic Range Neurone Firing

The administration of PGE_2 caused direct stimulation of the peripheral fibres connected to the recorded wide dynamic range neurone, this lead to neuronal firing in the absence of mechanical stimulation. The administration of vehicle caused a small amount of neuronal firing however was found to be significantly lower when compared to PGE_2 induced firing (Figure 4.10A). There was no significant difference between primed and unprimed rats. The PGE_2 also caused short-term spontaneous firing that was measured over a period of 10 seconds. There was no observed difference between primed and unprimed rats (Figure 4.10B).

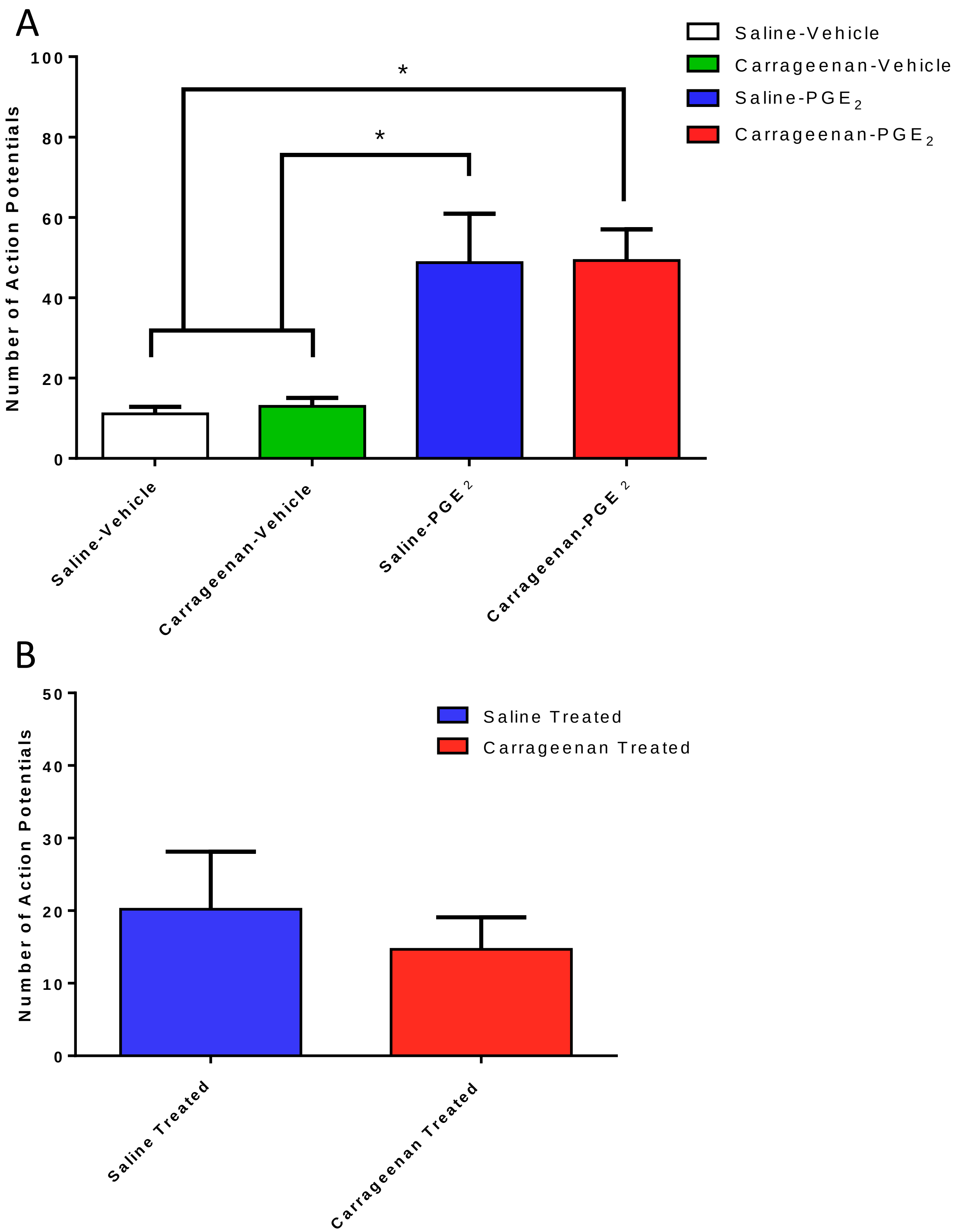


Figure 4.10: PGE_2 administration caused wide dynamic range neurone firing. **A:** Intraplantar injection of PGE_2 into the hindpaw caused direct firing of wide dynamic range neurones. The intraplantar injection of vehicle caused small amount of firing however was significantly lower that the PGE_2 elicited response. **B:** The intraplantar injection of PGE_2 lead to short duration spontaneous firing this was measured over 10 seconds and there was no difference found between primed and unprimed rat responses. All data represent mean ± SEM. Data analysed with one-way ANOVA with Sidak post hoc test. Saline-vehicle n= 7, carrageenan-vehicle n= 7, saline-PGE_2 n= 9-10, carrageenan-PGE_2 n= 8-10.

4.3.8 Intraplantar injection of PGE_2 facilitates Wide Dynamic Range Neurone Response to Low Intensity Mechanical Stimulation

Injection of PGE_2 into the left hindpaw caused a significant increase in the firing rate of recorded wide dynamic range neurones to low intensity 8 and 10g mechanical stimulation of the hindpaw in both primed ($p<0.01$) and unprimed ($p<0.05$) rats. The increased firing rate was significantly higher than vehicle controls for 30mins post PGE_2 injection, and then returned to baseline at approximately 90mins post-injection (Figure 4.11A, 4.11B). There was a small significant increase in 15g evoked neuronal firing ($p<0.05$) in PGE_2 treated rats when compared to vehicle injection (Figure 4.11C). The 26g stimulation was not significantly increased post-PGE_2 administration compared to vehicle control administration (Figure 4.11D).

The comparison between primed (carrageenan pre-treated rats) and the unprimed (saline pre-treated rats) showed no significant difference between their responses after PGE_2 to either 8g or 10g stimulation (Figure 4.11).

Prior to mechanical stimulation the cell was characterised for its C-fibre threshold, C-fibre latency and the cell depth shown in Table 4.1. This was conducted using electrical stimulation of the hindpaw, using methods described in **2.4.2.4**.

GROUP	THRESHOLD (mA)	LATENCY (ms)	DEPTH (µM)
SALINE-VEHICLE	1.70 ± 0.058	0.16 ± 0.005	723 ± 10.03
CARRAGEENAN-VEHICLE	1.95 ± 0.067	0.18 ± 0.035	726 ± 15.69
SALINE-PGE2	1.85 ± 0.052	0.17 ± 0.005	691 ± 7.43
CARRAGEENAN-PGE2	1.69 ± 0.056	0.17 ± 0.001	706 ± 11.77

Table 4.1: Characteristics of recorded cells, there were no statistical differences between groups for threshold for C-fibre stimulation or latency of firing. All cells were found within similar depth in the spinal cord.

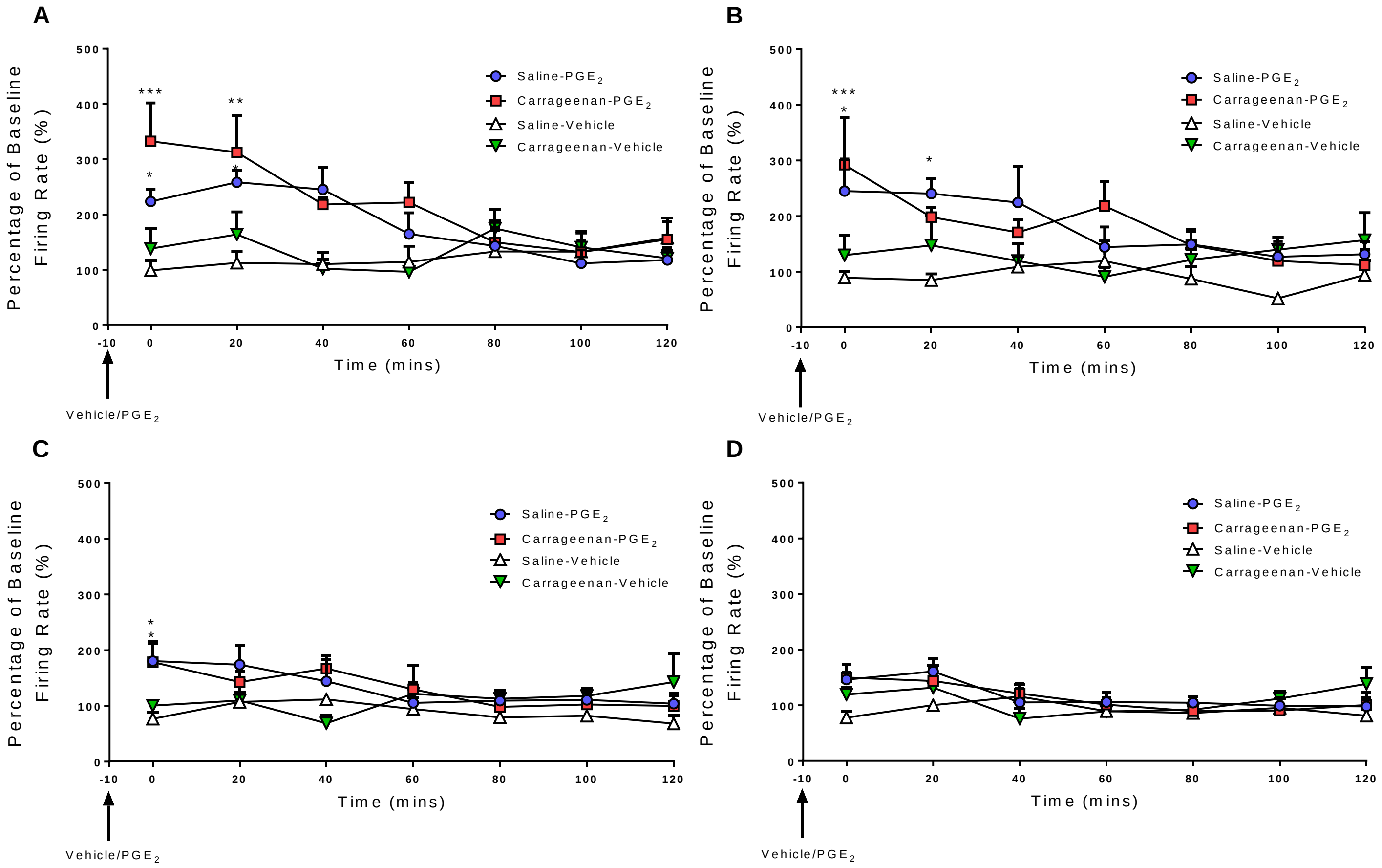


Figure 4.11: Intraplantar injection of PGE_2 caused an increase in the firing rate of wide dynamic range neurones to 8g (A), 10g (B), 15g (C) and 26g (D) mechanical stimulation of the hindpaw. **A:** Wide dynamic range neurone responses to 8g mechanical stimulation after administration of PGE_2/vehicle. **B:** Wide dynamic range neurone responses to 10g mechanical stimulation after administration of PGE_2/vehicle. **C:** Wide dynamic range neurone responses to 15g mechanical stimulation after administration of PGE_2/vehicle. **D:** Wide dynamic range neurone responses to 26g mechanical stimulation after administration of PGE_2/vehicle. All data represent mean ± SEM. Data analysed with two-way ANOVA with Bonferroni post hoc test. Saline-vehicle n= 7, carrageenan-vehicle n= 7, saline-PGE_2 n= 9-10, carrageenan-PGE_2 n= 8-10. Analysis performed with two-way ANOVA with Bonferroni post hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.3.9 Intraplantar PGE_2 Increases the Size of Wide Dynamic Range Receptive Field

The intraplantar administration of PGE_2 led to a small significant increase in the size of 8g receptive fields. The increased size of receptive field lasted for approximately 1 hour post-injection. There was no observed change in 26g receptive field after PGE_2 administration. The 26g receptive field was approximately 25% larger than the 8g receptive field before PGE_2 administration. When the 8g field increased in area the size became similar to that of the 26g field. There was no significant difference between the primed (carrageenan pre-treated) vs unprimed (saline pre-treated). The administration of saline caused no significant change in receptive field size for 8g or 26g (Figure 4.12).

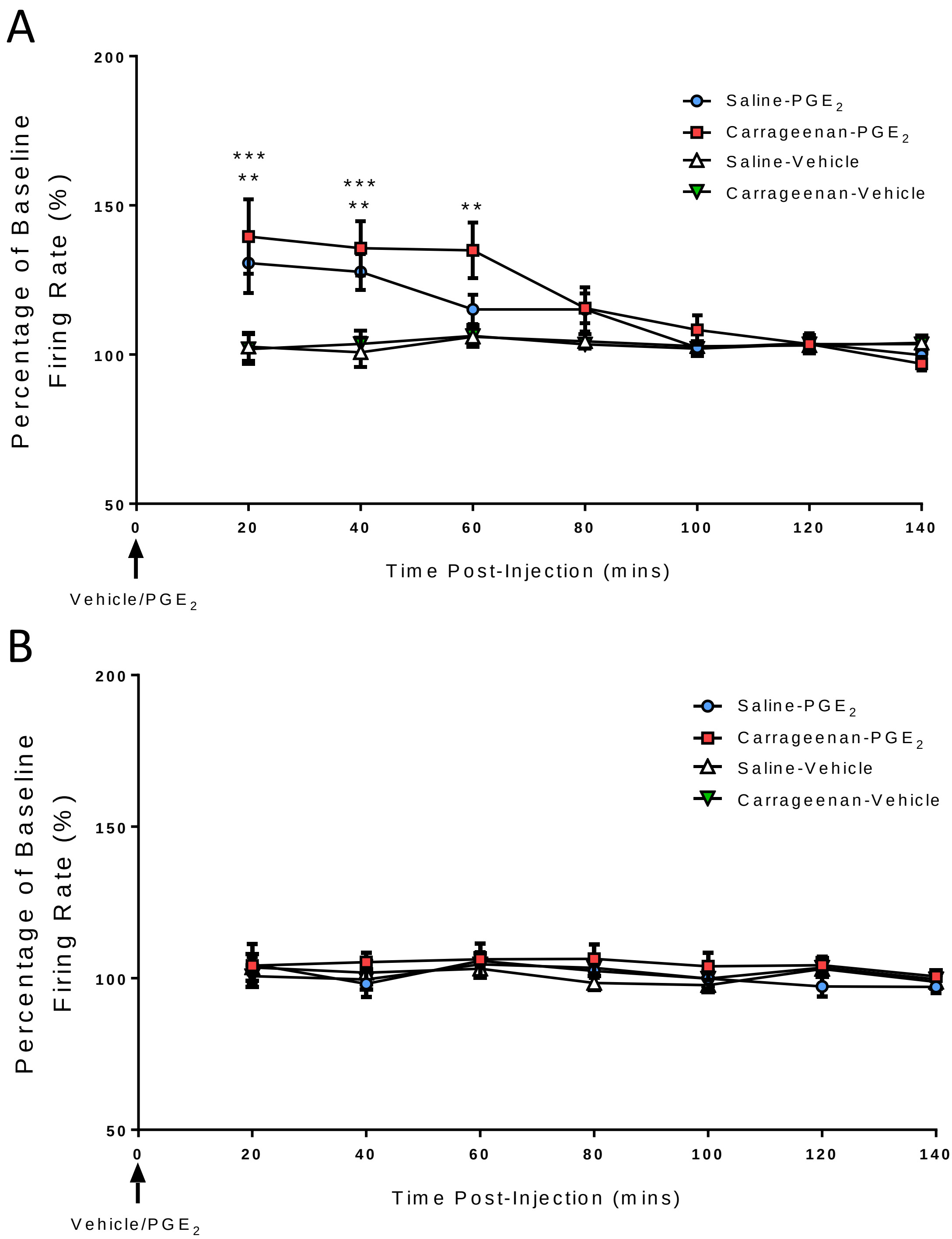


Figure 4.12: **A:** Administration of PGE_2 into the hindpaw caused an increase in the size of the receptive field of the recorded cell to 8g stimulation compared to vehicle controls. The increase last for 1 hour before returning to baseline. **B:** The administration of PGE_2 caused no significant change in the 26g receptive field. The administration of vehicle also caused no change in receptive field size. All data represent mean ± SEM. Data analysed with two-way ANOVA with Bonferroni post hoc test. Saline-vehicle n= 7, carrageenan-vehicle n= 7, saline-PGE_2 n= 9-10, carrageenan-PGE_2 n= 8-10.

4.3.10 Correlation of Wide Dynamic Range Neurone Firing in Response to Mechanical Stimulation Compared to Previous Behavioural Responses

The peak behavioural response of the rat at 4 hours following intraplantar injection of 1% carrageenan (5µl) was compared to the peak neuronal response to mechanical stimulation after PGE_2 at 10mins. The behavioural response of each rat to the intraplantar injection of 1% carrageenan was plotted against the induced firing to each von Frey filament application (8g, 10g, 15g & 26g) 10mins post-PGE_2.

The 8g stimulation produced increased neuronal firing that correlated with the observed decrease in PWT due to carrageenan administration (Figure 4.13A). There were no further observed correlations between behavioural response and mechanically induced neuronal firing to 10g (Figure 4.13B), 15g (Figure 4.13C) or 26g (Figure 4.13D) stimulations.

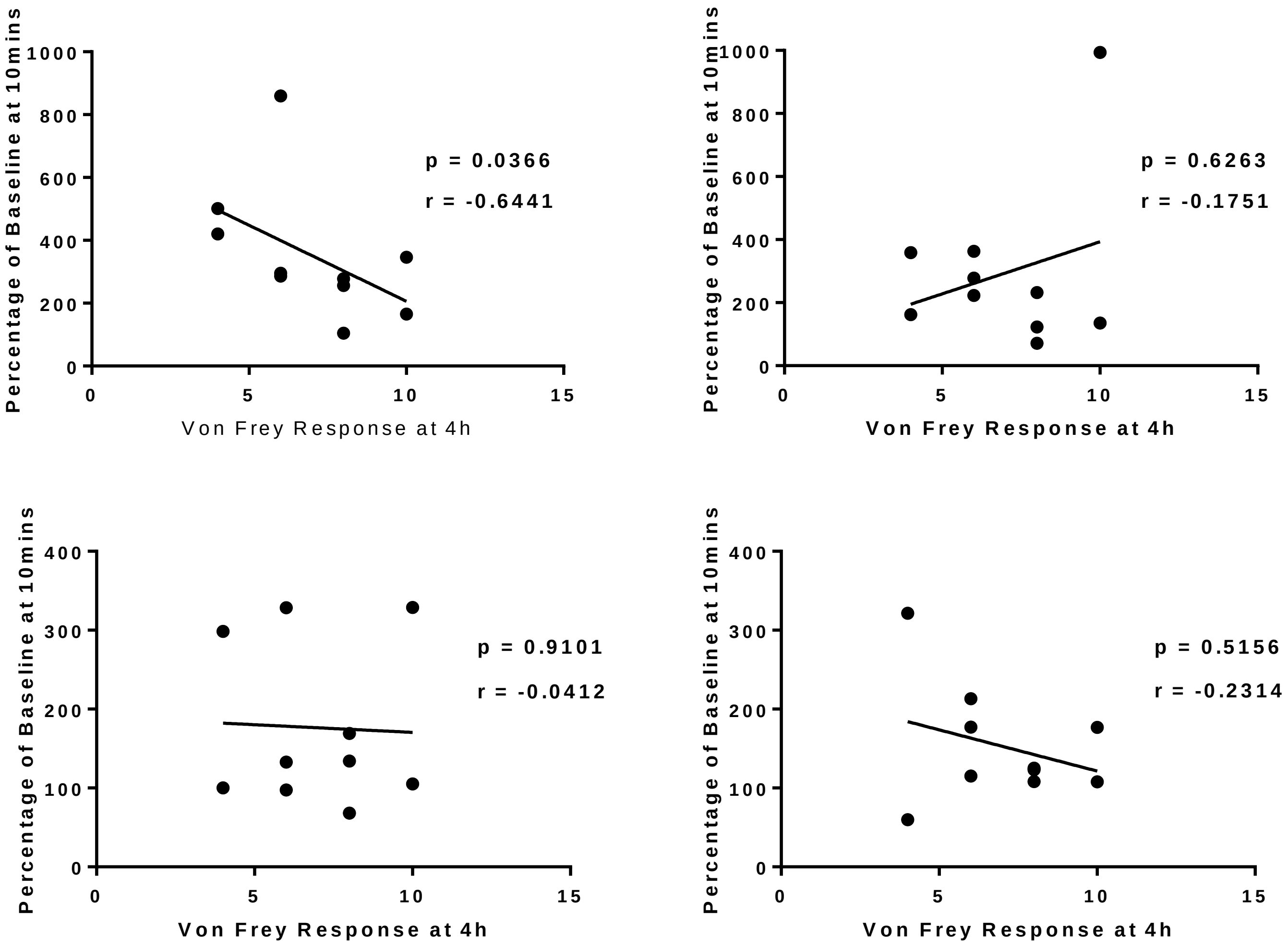


Figure 4.13: Correlation of rat pain behavioural response after carrageenan administration compared to neuronal responses to PGE_2 administration. **A:** 8g mechanical stimulation induced neuronal firing 10mins post-PGE_2 compared with carrageenan induced paw withdrawal threshold reduction at 4 hours show a negative correlation ($p<0.05$). **B:** 10g mechanical stimulation induced neuronal firing 10mins post-PGE_2 compared with carrageenan induced paw withdrawal threshold reduction at 4 hours shows no correlation. **C:** 15g mechanical stimulation induced neuronal firing 10mins post-PGE_2 compared with carrageenan induced paw withdrawal threshold reduction at 4 hours shows no correlation. **D:** 26g mechanical stimulation induced neuronal firing 10mins post-PGE_2 compared with carrageenan induced paw withdrawal threshold reduction at 4 hours shows no correlation.

4.4 Discussion

4.4.1 Blockade of Sensory Nerve Activity Inhibits PGE_2 Induced Reduction in PWTs But Does Not Prevent Chronic Pain

The early and late phases (at 1 and 4 hours respectively) of the PGE_2 response in primed rats were inhibited by the administration of QX-314 + capsaicin. Rats remained at baseline levels at 1 hour post-PGE_2 and at 4 hours. This confirms the involvement of C-fibres in the generation of hyperalgesic priming however it has been shown that hyperalgesic priming is mediated by $IB4^+$ neurones (Alvarez *et al.*, 2012a; Alvarez *et al.*, 2012b). QX-314 + capsaicin inhibits all TRPV1 expressing C-fibres showing that hyperalgesic priming may involve both non-peptidergic and peptidergic C-fibres (Woodbury *et al.*, 2004). The QX-314 + capsaicin slowed the onset of the PGE_2 allodynia, which was present at 2 days post-PGE_2, but did not prevent the chronic pain from establishing showing that the pain signal is not required to initiate the hyperalgesic response to PGE_2.

The administration of QX-314 + flagellin was unable to inhibit the early PGE_2 phase, but did significantly inhibit the reduction in PWTs at 4 hours. This does bring into question the selectivity of the QX-314 + flagellin combination and further work still needs to be done to understand the mechanisms behind this treat. Currently it is unknown what receptors the flagellin is targeting (Xu *et al.*, 2015). This may show some Aβ fibre involvement in the secondary phase of the PGE_2 response. The chronic pain was then present from 24 hours post-PGE_2 up to 7 days later. The inference of the PGE_2 using sensory blockade is unable to prevent the onset of hyperalgesic priming. There may also be some involvement with the transition from PKA to PKCε that occurs between 1 and 4 hours post-PGE_2 in primed rats (Aley *et al.*, 2000). There may be differential activation of different protein kinases in different sensory nerve. As the effect of flagellin + QX-314 only occurs at 4 hours post-PGE_2.

4.4.2 Hyperalgesic Priming Enhanced PGE_2 Response Involves both Aβ and C fibre Activation

The initiation of hyperalgesic priming requires activation of IB_4^+ neurones (Alvarez *et al.*, 2012a) however there has been no investigation into the contributions of sensory fibres types during the PGE_2 chronic pain. I have shown that the administration of QX-314 + capsaicin is able to transiently inhibit the reduction in PWTs of primed rats after PGE_2 administration. This would suggest the involvement of C-fibres however does not necessarily mean that the hyperalgesic state is mediated by IB_4^+ neurones alone. The QX-314 is acting on nerves that express TRPV1 which has been shown to be present on both peptidergic and non-peptidergic nerve fibres (Woodbury *et al.*, 2004). There is evidence that although IB_4^+ neurones are required for the initiation of priming, their activation is not required for priming to occur as NGF is itself has been shown to be able to initiate a primed state in replacement of carrageenan (Joseph *et al.*, 2010). This evidence of QX-314 + capsaicin inhibiting the PGE_2 chronic pain could suggest that both peptidergic and non-peptidergic fibres are involved in the chronic phase.

The administration of QX-314 + flagellin did not have as large an effect at the QX-314 + capsaicin however it was able to significantly inhibit the hyperalgesic response at 2 hours post-PGE_2. This would therefore point towards the potential sensitisation of Aβ in the chronic phase of PGE_2 hyperalgesic priming. This would mean that the chronic phase of the PGE_2 response leads to the sensitisation of Aβ fibres and their contribution to the enhance pain state.

4.4.3 Intraplantar Injection of PGE_2 Increases WDR Neuronal Firing

It has been shown in previous studies that PGE_2 is able to sensitise peripheral nociceptors (Pitchford *et al.*, 1991). The intraplantar administration of PGE_2 into the hindpaw lead to an increase in wide dynamic range neurone firing in response to low intensity (8g and 10g) mechanical stimulation. This shows that stimuli that under normal conditions are innocuous are now experienced as noxious. This demonstrates that the peripheral sensitisation caused by the administration of PGE_2 (Aley *et al.*, 1999) shows as increased firing of wide dynamic range neurones to previously low threshold mechanical stimuli.

The small significant increase in WDR firing to 15g mechanical stimulation post-PGE_2 signifies potential hyperalgesia as it is shown in this thesis baseline pain behaviour recordings in awake naïve rats have PWTs of between 15-26g. There was no significant change in WDR neuronal firing after PGE_2 administration to 26g mechanical stimulation.

It is therefore possible to conclude that the intraplantar injection of PGE_2 into the hindpaw triggers an allodynia effect displayed by increased firing from WDR neurones as previously innocuous 8g and 10g stimulations now elicit firing of a similar rate to 26g stimulation. With there being no change to the firing of 26g stimulation.

4.4.4 Hyperalgesic Primed Rats Do Not Show a Maintained Increase in Firing Post-PGE_2

It was hypothesized that the injection of PGE_2 into the hindpaw of rats that had been primed would induce an increase in neuronal firing in response to mechanical stimulation that would last greater than 1 hour. However it was observed that the increased WDR firing to mechanical stimulation had a duration of approximately 1 hour, before returning to baseline. This does not match the pain behavioural responses that are seen in awake rats (Chapter 3).

This could suggest that there are changes in other regions of the spinal dorsal horn such as the more superficial lamina I and II. It has been demonstrated that after persistent peripheral inflammation induced by CFA caused an augmentation in excitatory transmission due to alterations in AMPA receptor trafficking within lamina II of the dorsal horn. There were also observed decreases in tonic firing within lamina II suggesting alterations in interneurone activity (Kopach *et al.*, 2015). Therefore showing that there are changes in both excitatory and inhibitory processing in lamina II. There could therefore be changes within the superficial laminae in hyperalgesic priming. As it has been demonstrated that there are changes in $GABA_A$ activity within the dorsal horn of hyperalgesic primed mice (Kim *et al.*, 2016).

There were also several limitations of the technique to take into account, firstly due to the size of the initial injection of carrageenan being only 5µl there is potential uncertainty that the recorded cell was receiving inputs from a primed neurone. Also due to the WDR neurone receiving multiple inputs from several sensory nerves (Mendell, 1966) the changes involved in hyperalgesic priming could have lost clarity.

Desensitisation may have also been a confounding factor (Freedman *et al.*, 1996; Rang *et al.*, 2007). The stimulus protocol involved the repeated stimulation of the injured area with low and high intensity mechanical stimulation. This may have caused desensitisation of the peripheral nerve fibres particularly after the administration of PGE_2 as in mechanical behavioural tests the intensity of the stimulus never goes above the withdrawal threshold of the rat. However in the electrophysiology protocol 26g was regularly applied to the sensitised area, which could lead to desensitisation of the recorded area causing a decrease in the neuronal firing rather than the sustained increase (Rang *et al.*, 2007). Pain desensitisation has been demonstrated through the use of capsaicin based creams to reduce inflammatory pain (Derry *et al.*, 2013; Guedes *et al.*, 2016).

Finally the involvement of anaesthetic inhibition of the spinal cord may play a role. Anaesthetics are able to depress the activity of the spinal cord. However it has been shown that the inhibition due to isoflurane is far less than other previously used anaesthetics such as halothane (Cuellar *et al.*, 2005). Also it has been shown that wide dynamic range neurones are less sensitive to anaesthetic depression in comparison to dorsal horn nociceptive neurones. This does mean that there is potential loss of wide dynamic range input range than direct inhibition of the cell (Barter *et al.*, 2009). Nitrous oxide has been shown to be analgesic and anxiolytic by inhibiting glutamatergic transmission in the dorsal horn and modulatory effects on GABA receptors respectively (Georgiev *et al.*, 2008) (Emmanouil *et al.*, 2007). Hyperalgesic priming has been shown to involve changes in GABAergic plasticity (Kim *et al.*, 2016) therefore the effect of nitrous oxide may interfere with the onset of hyperalgesic priming enhanced PGE_2 responses. Future work with development of an electrophysiological correlate of hyperalgesic priming is discussed in Chapter 6.

4.5 Conclusion

Intraplantar injection of PGE_2 causes an increase in WDR neurone firing in response to low threshold mechanical stimulation demonstrating that PGE_2 causes allodynia in peripheral skin tissue. However the increase in neuronal firing was not maintained in primed rats, this suggests that there are no maintained changes in WDR processing in hyperalgesic priming.

The use of sensory blockade demonstrated that during the initiation of hyperalgesic priming there is an involvement of C-fibre activation throughout. There is however a differential effect of pre-treatment with QX-314 + flagellin the early PGE_2 phase at 1 hour was not inhibited however there was a significant reduction in PWTs at 4 hours post-PGE_2. Showing an involvement of Aβ fibres in the chronic phase of hyperalgesic priming.

Intraplantar injection of QX-314 + capsaicin into rats with established PGE_2 chronic pain inhibited the reduction in PWTs returning them to baseline levels by 2 hours and maintained up to 4 hours. This shows the involvement of C-fibres in the maintenance of the PGE_2 chronic pain. The intraplantar injection of QX-314 + flagellin transiently inhibited the PGE_2 chronic pain at 2 hours but did not return PWTs to baseline levels showing the potential sensitisation of Aβ fibres in hyperalgesic priming.

Chapter 5

Can the Resolution Pathways Modify Hyperalgesic Priming?

5.1 Introduction

As discussed in the introductory chapter (section **1.4**) amongst the many mediators that drive peripheral inflammation, PGE_2 has been shown to play a pivotal role in the hyperalgesic priming. PGE_2 acts via G-protein coupled receptors (EP receptors) to initiate a series of biological events which promote and sustain sensitisation of sensory nerves.

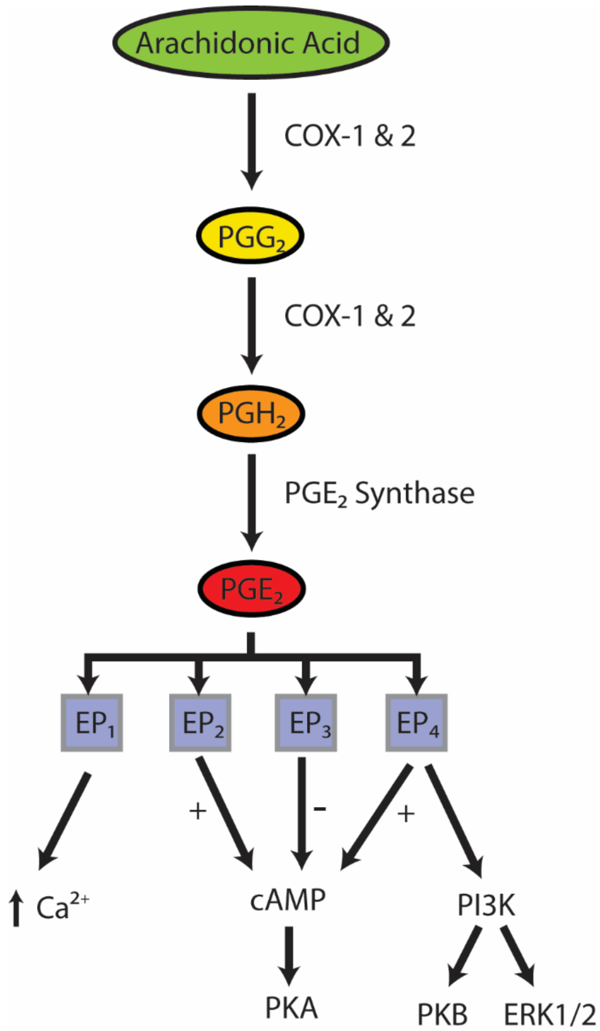


Figure 5.1: PGE_2 synthesis: prostaglandin E_2 is produced via the conversion of arachidonic acid through the enzymatic action of COX-1 & 2 to produce PGH_2. This is then converted to active PGE_2 by PGE_2 synthase, it can then go on to activate several receptor subtypes present on immune cells (adapted from (Hata *et al.*, 2004a)).

The activation of the EP_{1-4} receptors leads to several intracellular changes dependent on the G protein they are coupled to. EP_1 is G_q coupled leading to increased calcium release via the activation of phospholipase C, and causing the breakdown of phospholipids producing inositol triphosphate which activates Ca^{2+} release from the endoplasmic reticulum (Rang *et al.*, 2007). The EP_2 and EP_4 receptors are coupled to G_s leading to increased cAMP production causing activation of PKA. The EP_3 receptor has multiple splice variants having been shown to be coupled to G_i, G_s and G_q leading to a variety of intracellular changes. The coupling to G_i causes inhibition of adenylate cyclase leading to a reduction in cAMP and therefore reduced activation of PKA whereas a coupling to G_s increases cAMP. (Hata *et al.*, 2004a).

The action of PGE_2 during inflammation is to promote immune cell activity as well as mediating changes in pain perception. PGE_2 is able to stimulate the attraction and subsequent degranulation of mast cells (Kalinski, 2012). These are known to produce sensitising mediators such as histamine and bradykinin (Lee *et al.*, 1990; Morishita *et al.*, 1996) both of which have receptors on nociceptive terminals causing the generation of action potentials as well as causing sensitising receptors such as TRPV1 (Gonzalez *et al.*).

In relation to hyperalgesic priming, PGE_2 shows an extended duration of action. Under normal conditions the response of a tissue to PGE_2 is short in duration and the changes in sensitivity to mechanical stimulation are mediated through PKA activation (Aley *et al.*, 1999; Hata *et al.*, 2004b; Villarreal *et al.*, 2013). The normal half-life of PGE_2 is approximately 8h (Ishihara *et al.*, 1991) with associated pain behaviour lasting less than 2h (Aley *et al.*, 1999). In hyperalgesic priming it has been shown that there are two phases; the acute phase lasting up to 1 hour mediated via PKA, and the chronic phase lasting from 4 hours onwards mediated through PKCε (Aley *et al.*, 2000). The cause of this switch in signalling is currently unknown however there is speculation that it involves the coupling of EP receptors to adenosine A1 receptors (Ferrari *et al.*, 2015). Antagonising the negative effects of PGE_2 may aid in preventing hyperalgesic priming.

5.1.1 Resolvins

The resolvins are a group of lipid mediators that have a homeostatic role in returning a tissue to a resting state after an inflammatory reaction (Serhan *et al.*, 2002a; Serhan *et al.*, 2005). The resolvins are produced from omega-3 fatty acids, which are used to produce eicosanpentaenoic acid (EPA) and docosahexaenoic acid (DHA). DHA is converted into the resolvin D series by 15-LOX. There are several members of the resolvin D family: RvD1-RvD6. The second family of resolvins are the resolvin E series consisting of RvE1-E3, which are produced from EPA (Serhan, 2004). The resolvins can also be produced via the aspirin-triggered COX-2 pathway. Aspirin acetylates COX-2 inhibiting its ability to produce prostaglandins, acetylated COX-2 can convert DHA into 17-R-HDHA which is then converted to active AT-RvD1-3 by oxygenation via the enzyme 5-LOX (Figure 5.2) (Serhan *et al.*, 2004).

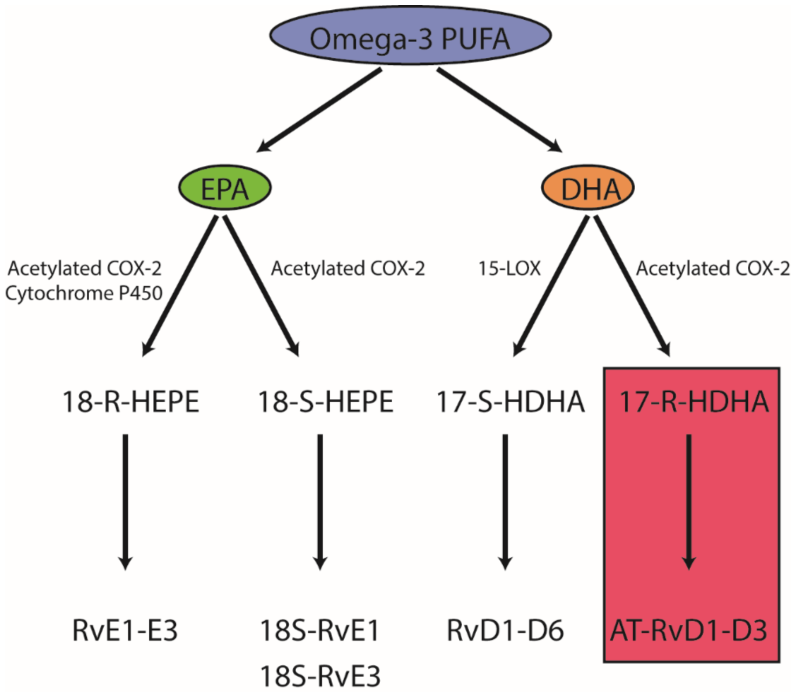


Figure 5.2: Resolvin synthesis pathway. The resolvins are produced from ω-3 fatty acids, which are converted to EPA and DHA to generate the E series and D series respectively. The aspirin triggered resolvins are highlighted in red (based on information from (Qu *et al.*, 2015)).

5.1.2 Role of Resolvins in Inflammation

The resolvins have a range of effects on various cell types including neutrophils, macrophages, microglia, and epithelial and endothelial cells, as well as directly on neurones to reduce inflammation and return the tissue to a resting state. The E series resolvins have a multitude of effects including the reduction of eosinophil (Aoki *et al.*, 2010), macrophage (Jin *et al.*, 2009) and monocyte infiltration from the circulation into the damaged tissue by a reduction in the expression of CD18 which is a crucial leukocyte adhesion molecule aiding in leukocyte translocation (Dona *et al.*, 2008; Peters *et al.*, 2006) and as well as reducing the attraction of immune cells by chemotaxis. Resolvins also act to reduce the production of pro-inflammatory cytokines from microglia such as TNFα and IL-6 (Xu *et al.*, 2013).

The resolvin D series and the aspirin-triggered resolvins have very similar effects due to the similarity of their structure and thereby activation of the same receptor. They are also able to inhibit the transmigration and infiltration of neutrophils (Bento *et al.*, 2011), eosinophils and macrophages, which reduces the production of cytokines preventing further continuation of the inflammatory response. They also act to improve epithelial and endothelial barrier integrity preventing further infiltration of immune cells (Eickmeier *et al.*, 2013). AT-RvD1 systemic administration is able to reduce the inflammation associated with urine pneumonia or colitis by the suppression of the NF-κB pathway reducing the activation of inflammatory genes (Qu *et al.*, 2015). It has also been shown that the systemic administration of AT-RvD1 reduces central sensitisation via the reversal of NMDA receptor phosphorylation (Xu *et al.*, 2010b).

The action of D series resolvins is mediated by to two main receptors ALX/FPR2 and GPR32, these are GPCRs that are expressed on a wide variety of immune cells including neutrophils and monocytes with the function of reducing the activity of the cell and inhibiting their production of inflammatory mediators. Their action has also been shown to regulate genetic transcription of inflammatory genes and meanwhile promoting pro-resolving gene transcription (Krishnamoorthy *et al.*, 2012; Spurr *et al.*, 2011).

Resolvins have been demonstrated to be successful in alleviating the pain associated with both acute and chronic inflammation (Qu *et al.*, 2015; Serhan *et al.*, 2002b). The administration of both RvD1 or RvE1 locally and/or intrathecally reduced the inflammatory pain behaviours associated with carrageenan, formalin and CFA in a dose dependent manner (Xu *et al.*, 2010a). There is evidence of the effectiveness of resolvins in the treatment of in other inflammatory disease models including adjuvant induced arthritis via the systemic administration of 17-R-HDoHE (a precursor to AT-RvD1), which was shown to reduce associated pain behaviour (Lima-Garcia *et al.*, 2011; Xu *et al.*, 2011). Previous work in our laboratory has shown a successful reduction in osteoarthritic-like pain after the systemic administration of a 17-R-HDoHE into rats previously treated with intra-articular monosodium iodoacetate (MIA), which causes inflammation through cartilage damage. The direct application of AT-RvD1 to the spinal cord during electrophysiological recordings of spinal WDRs, has been shown to reduce the WDR neuronal excitability to electrical stimulation of the hindpaw through the reduction of C-fibre and Aδ-fibre contributions (Meesawatsom *et al.*, 2016).

Hyperalgesic priming is induced by two separate inflammatory stimuli as demonstrated previously (Chapter 3), it is therefore hypothesised that the i.p administration of the precursor (17-R-HDoHE) will be able to block the generation of the PGE_2 chronic phase and inhibit the reduction in paw withdrawal threshold due to hyperalgesic priming.

5.1.3 Aim

To compare the effects of D-series resolvin precursor administration on the initiation phase versus established phase of hyperalgesic priming in vivo, and to investigate the associated underlying mechanisms of action.

5.2 Methods

5.2.1 Prevention of Hyperalgesic Priming

Male Sprague Dawley rats (200-250g) were anaesthetised as detailed in **2.3.2** and then received a subcutaneous injection into the left hindpaw of 5µl of carrageenan or saline. Pain behaviour was then assessed at several intervals for up to 7 days (Figure 5.3). On day 5, 6 and 7 rats received an i.p injection of the D-series resolvin precursor 17-R-HDoHE (300ng/300µl). Pain behaviour was performed 1 hour post precursor administration on days 5 and 6. On day 7 17-R-HDoHE was administered 1 hour prior to PGE_2 administration. On day 7 animals received a subcutaneous injection of PGE_2 (1µg/5µl) into the same site as the previous carrageenan/saline injection. Von Frey PWTs were then measured at 1 and 4 hours post-injection, and then daily up to 7 days later (timeline detailed in Figure 5.3).

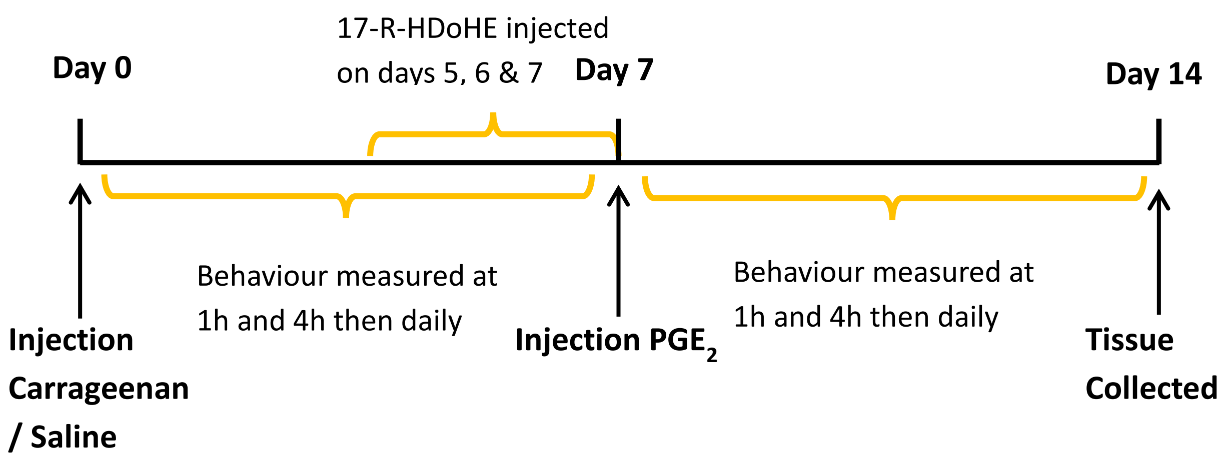


Figure 5.3: Timeline of behavioural study. Animals received an intraplantar injection of saline or carrageenan into the left hind paw. The PWTs were then measured at 1 hour and 4 hours then daily until day 7. On days 5, 6 & 7 animals received an i.p systemic dose of 17-R-HDoHE or vehicle. All groups then received an intraplantar injection of PGE_2 into the left hind paw in the same site as previous. Behavioural testing was then performed at 1 hour and 4 hours, and then daily until day 14.

On day 14 rats were humanely euthanized using an i.p overdose of sodium pentobarbitone. Animals were then perfused with 4% PFA to fix the neural tissue. Spinal cords and DRG (L4, L5, and L6) were then collected for use in immunohistochemistry and miRNA analysis.

5.2.2 Reversal of Hyperalgesic Priming Enhanced PGE_2 Chronic Pain

Male Sprague Dawley rats (200-250g) were anaesthetised as detailed in **2.3.2** rats then received a subcutaneous injection into the left hindpaw of 5µl of carrageenan or saline. Pain behaviour was assessed and then measured at several intervals for up to 7 days (Figure 5.4). On day 7 animals received a subcutaneous injection of PGE_2 (1µg/5µl) into the same site as the previous carrageenan/saline injection. PWTs were then measured at 1 and 4 hours post-injection then daily up to 7 days later. On day 12, 13 and 14 animals received an i.p administration of 17-R-HDoHE (300ng/300µl) and PWTs measured at 1 hour post precursor administration. On day 14 rats were humanely euthanized using an i.p overdose of sodium pentobarbitone (timeline detailed in Figure 5.4).

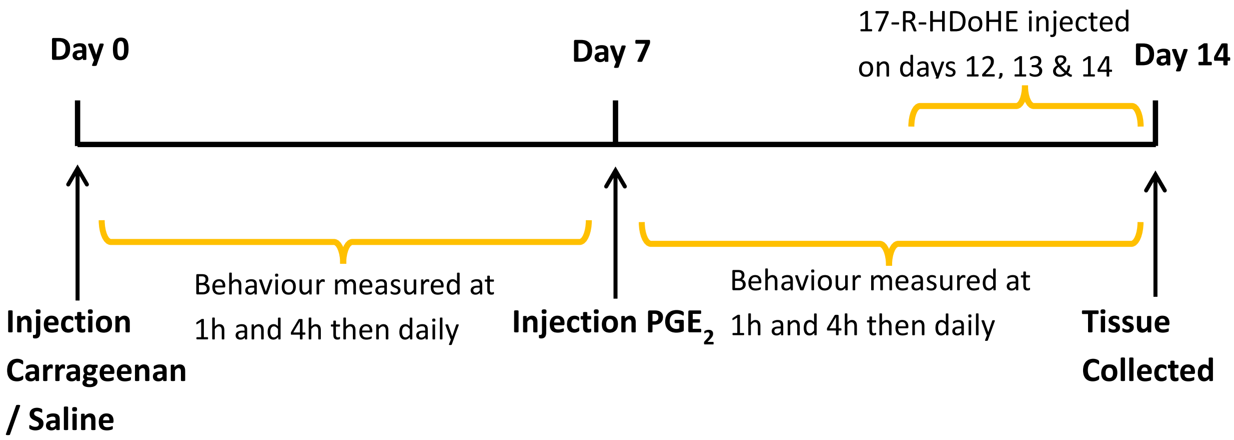


Figure 5.4: Timeline of behavioural study. Animals received an intraplantar injection of saline or carrageenan into the left hind paw. PWTs were then measured at 1 hour and 4 hours then daily until day 7. On day 7 animals received an intraplantar injection of PGE_2 into the left hind paw in the same site as previous. Behavioural testing then reoccurred at 1 hour and 4 hour then daily until day 14. On days 12, 13 & 14 animals received an i.p systemic dose of 17-R-HDoHE or vehicle.

5.2.3 Immunohistochemical Staining of Microglia

Collected spinal cord was sliced on a freezing microtome at 40µm thick slices. The tissue was washed in 1 x PBS and then blocked in a goat serum block (containing goat serum, triton X-100 and 1 x PBS in 1ml). The tissue was incubated in blocking solution for 2 hours on an automatic rocker at room temperature. Block solution was then removed and 1ml of primary antibody solution (goat blocking solution containing a 1:2000 dilution of rabbit anti-rat IBA1 (WAKO) the same antibody as used in Chapter 3) was dispensed onto the tissue, and then incubated for 72 hours at 4°C. After the incubation period the tissue was washed out with 1 x PBS 3 times.

Then a secondary antibody solution (TTBS, 488 goat anti-rabbit fluorophore) was dispensed onto the spinal cord tissue and incubated at room temperature for 2 hours. Tissue was then wash four times in 0.1 x PBS and final in distilled water to reduce the present of salts on the tissue that could potentially cause irregularities in the imaging. The spinal cord slices were mounted on gelatine coat slides and cover-slipped using Fluoromount. The tissue was then imaged on a Leica fluorescence microscope as detailed in **2.3**. Analysis was conducted as detailed in **3.2.2.6**.

5.3 Results

5.3.1 Effects of Systemic Administration of 17-R-HDoHE on Initiation of Hyperalgesic Priming

As reported previously (Figure 3.3C), intraplantar injection of carrageenan (1%, 5µl) into the left hind paw caused a decrease in PWTs compared to saline controls for up to 3 days post-injection (Figure 5.5A). The PWT in carrageenan-treated rats then returned to baseline by day 5 post-carrageenan injection. Intraplantar injection of saline did not alter PWTs (Figure 5.5A). On days 5, 6 and 7 rats received either an i.p injection of 17-R-HDoHE (300ng, 300µl) or vehicle (0.9% saline containing 0.01% EtOH, 300µl).

Injection of PGE_2 (1µg/5µl) on day 7 was associated with a reduction in PWTs in all groups of rats at 1 hour post-PGE_2 injection. At 4 hours, the group that have previously received saline (unprimed) had PWTs similar to baseline. The group that had previously received carrageenan (primed) exhibited a significant ($p<0.001$) lowering of PWTs lasting up to 7 days after injection of PGE_2.

Treatment with 17-R-HDoHE significantly altered the response to PGE2 in carrageenan primed rats. Specifically, PWTs following PGE_2 injection returned to baseline values at 4 hours following PGE_2 injection (Figure 5.5B). At 24 hours post-PGE_2 the 17-R-HDoHE treated group had significantly higher PWTs compared to the vehicle treated primed group. Overall, the 17-R-HDoHE treated primed group had PWTs closer to baseline than the vehicle treated primed group. At later timepoints the PWT in the 17-R-HDoHE treated group started to decrease and was significantly different to the un-primed group from day 6 post-PGE_2 ($p<0.01$) (Figure 5.5B).

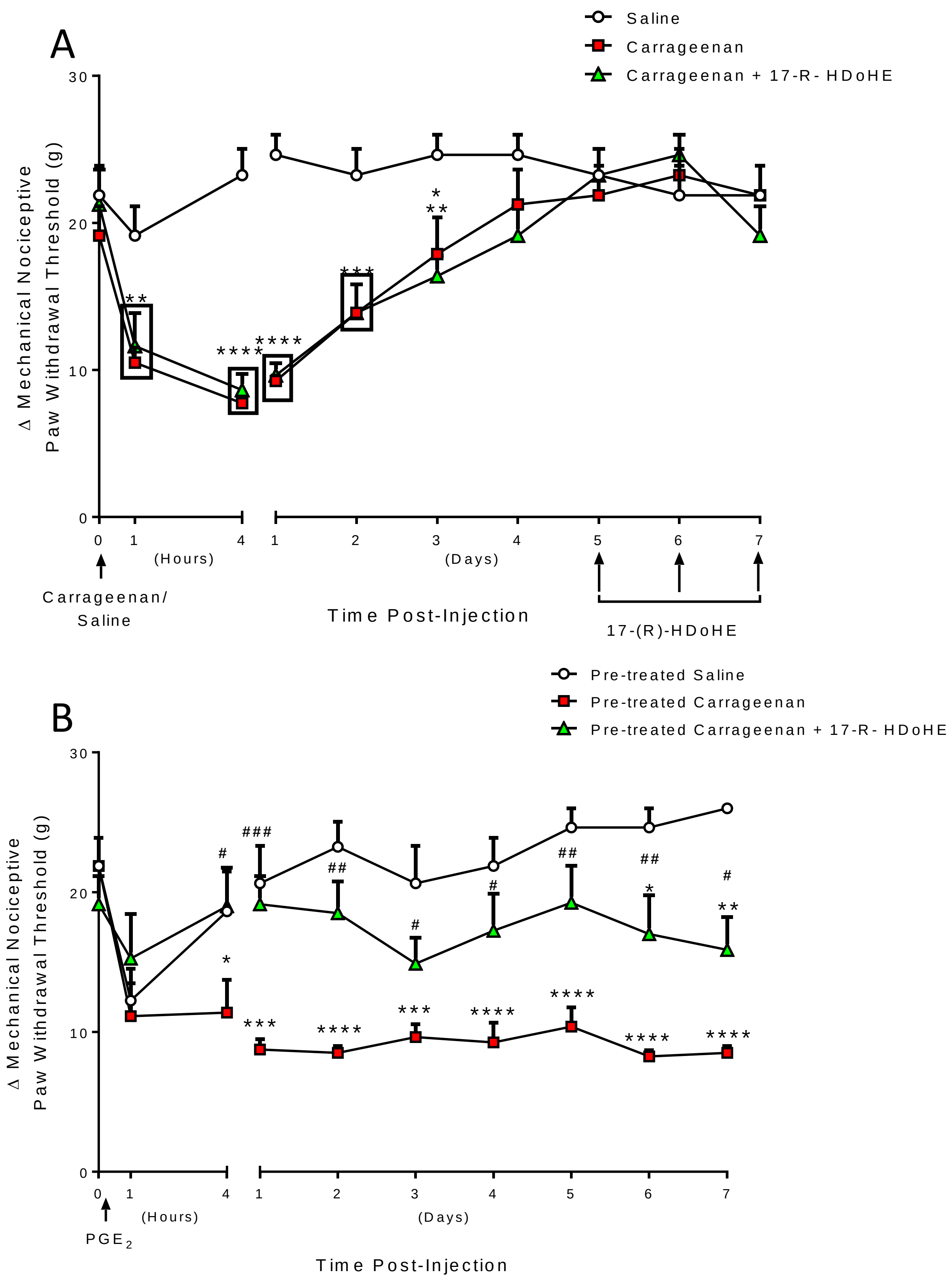


Figure 5.5: Effect of D-series resolvin precursor pre-treatment on the development of hyperalgesic priming to PGE_2. **A:** Injection of carrageenan caused a large decrease in paw withdrawal thresholds (PWTs) at 1 and 4 hours post injection, which remained for up to 3 days. **B:** Administration of PGE_2 into the left hind paw caused a decrease in paw withdrawal threshold in all groups at the 1 hour timepoint. At 4 hours the unprimed animals showed recovery. Primed rats that received vehicle showed a maintained reduction in PWTs that lasted up to 7 days post-PGE2 injection. The primed group that had systemic 17-R-HDoHE treatment had reduced PWTs, but they remained significantly higher than primed group treated with vehicle. All data represent mean ± SEM n = 8 per group. Data analysed with two-way ANOVA with Bonferroni post hoc test. * represent comparison to unprimed group *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. # represents comparison to primed-vehicle group #p<0.05, ##p<0.01, ###p<0.001. Black boxes delineate identical significance in both groups.

5.3.2 Effects of Systemic Administration of 17-R-HDoHE on the maintenance of Hyperalgesic Priming

To investigate the effect of 17-HDoHE on established hyperalgesic priming chronic pain, 17-HDoHE or vehicle were administered to groups of primed animals on days 12, 13 & 14 post-PGE_2. As previously reported intraplantar injection of carrageenan caused a decrease in PWTs in both groups that was maintained up to 3 days later. There was a recovery back to baseline levels by day 7 (Figure 5.6A). Injection of PGE_2 was associated with a lowered PWT which was maintained for up to 7 days post-PGE2 injection in this primed group. Neither systemic injection of 17-R-HDoHE or vehicle on days 12, 13 and 14 altered PWTs in carrageenan-PGE_2 primed rats there were no significant differences between either groups assessed using two-way ANOVA analysis (Figure 5.6B).

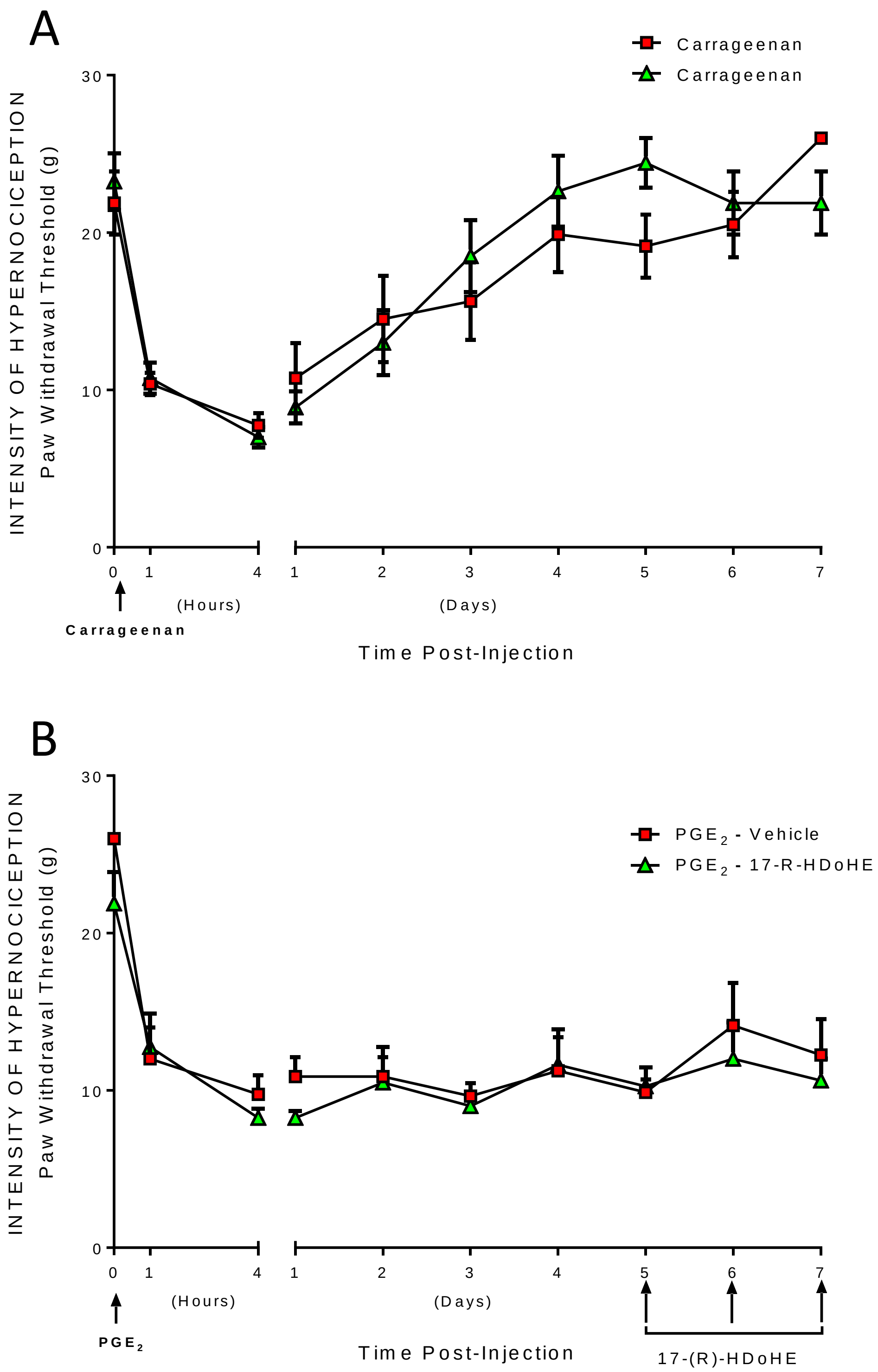


Figure 5.6: Effects of 17-R-HDoHE treatment on established hyperalgesic priming. **A:** Injection of carrageenan (1%, 5µl) caused a large decrease in PWTs at 1 and 4 hours post injection, which was maintained for up to three days. **B:** Injection of PGE_2 (1µg, 5µl) into the left hind paw caused a decrease in PWTs in all groups at 1 hour post-injection, which was maintained up to 7 days later. Systemic administration of 17-R-HDoHE (300ng, 300µl) on days 5, 6 and 7 did not alter the PGE_2 induced reduction in PWTs. All data represent mean ± SEM n = 8 per group. Data analysed with two-way ANOVA with Bonferroni post hoc test.

5.3.3 Effects of systemic administration of 17-R-HDoHE on microglia activation

Injection of PGE_2 in carrageenan primed rats was associated with an increase in the number of morphologically "activated" microglia in the dorsal horn of the spinal cord. However, the numbers of activated microglia in the L5 dorsal horn in primed rats was less than the number in unprimed rats as previously seen in Chapter 3.

Spinal cord collected from study **5.3.1** (administration of 17-R-HDoHE prior to PGE_2) was stained for microglia using IBA1 (a protein that is constitutively expressed in all microglia) (Figure 5.7A-C). Unprimed rats (saline-PGE_2) treated with vehicle had significantly higher numbers of morphologically "activated" microglia within the dorsal horn, compared to primed rats (carrageenan-PGE_2) treated with vehicle. Following treatment of primed rats with 17-R-HDoHE before PGE_2, the number of activated microglia in the dorsal horn of the spinal cord was higher than the number in primed rats treated with vehicle, however significance was not reached. Primed rats treated with 17-R-HDoHE also showed less "activated" microglia compared to the unprimed rats treated with vehicle however this was also found not to be significant (Figure 5.7D).

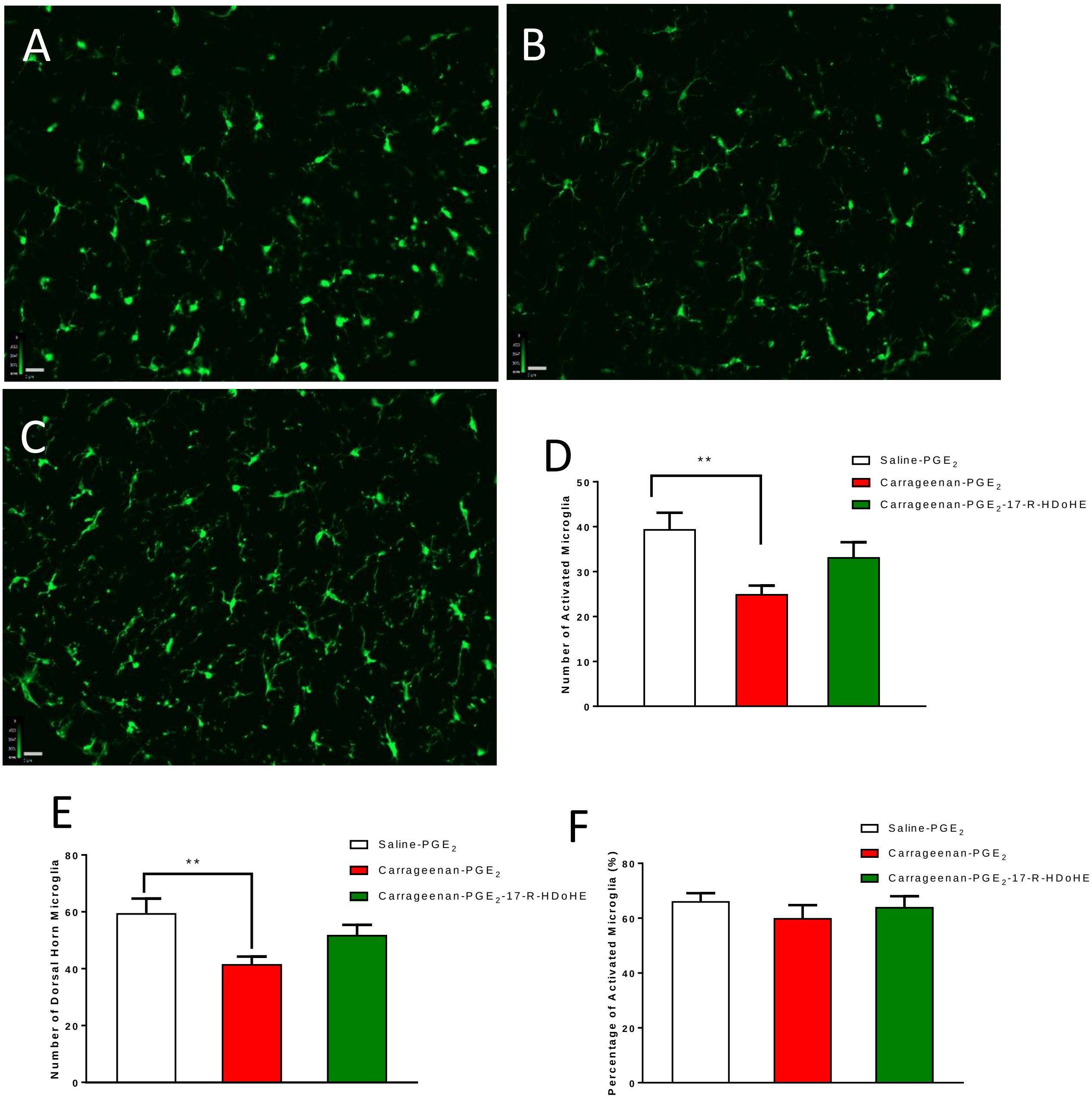


Figure 5.7: **A:** IBA1 staining of the dorsal horn of the spinal cord in unprimed animals treated with vehicle at 20x magnification. **B:** IBA1 staining of the dorsal horn of the spinal cord in primed animals treated with vehicle at 20x magnification. **C:** IBA1 staining of the dorsal horn of the spinal cord in primed animals treated with 1-R-HDoHE at 20x magnification. **D:** Total number of morphologically active microglia within L5 dorsal horn. There were significantly greater numbers of activated microglia within the dorsal horn in unprimed animals compared to primed animals with vehicle. The primed animals treated with resolvin showed no significant differences to either of the other groups. **E:** Total number of microglia within the dorsal horn of the spinal cord. There were significantly greater numbers of microglia within the dorsal horn of unprimed animals treated with vehicle compared to primed vehicle treated animals. The primed resolvin treated group so no significant differences to the other groups. **F:** Percentage of activated microglia. There were no significant differences between any groups. It was shown that the percentage of activated microglia was similar in all groups. All data represent mean ± SEM n = 8 per group. Data analysed with one-way ANOVA with Turkey post hoc test *p<0.05, **p<0.01.

5.3.4 Resolvin pre-treatment prevents changes in miRNA levels

In separate groups of rats L4, L5 and L6 DRGs were collected at several timepoints during hyperalgesic priming at 2 days post-carrageenan, 7 days post-carrageenan and 7 days post-PGE_2. Associated pain behaviour shown in Figure 5.8A and B. DRGs were investigated for changes in specific miRNAs miR-100 and miR-125 (for methods see appendix) these were selected based on their potential involvement in regulation of inflammatory genes. Tissue was collected at 2 and 7 days post carrageenan and then at 7 days post-PGE_2. Changes in this miRNAs were compared back to miRNA levels in time matched saline-treated (unprimed) rats. It was shown that there was an approximate 2 fold increase in both miR-100 and miR-125 at 2 days post-carrageenan compared to post-saline rats. This had returned to levels comparable to saline treated rats at day 7 post-carrageenan. They were both then shown to be increased by 2 fold compared to unprimed rats at 7 days post-PGE_2 during the chronic pain phase (Figure 5.8C).

The levels of miR-100 and miR-125 were then assessed in L4-6 DRGs from primed rats 7 days after PGE_2 that had been pre-treated with 17-R-HDoHE (samples obtained from **5.3.1** (Figure 5.5B)) to see whether resolvin treatment would affect their expression. They were shown to be reduced in animals that had received 17-R-HDoHE returning to levels similar to unprimed rats. They were again shown to be increased in the untreated primed group (Figure 5.8D). There were no significant differences when assessed with one-way ANOVA however comparisons need to be made to a housekeeper control gene.

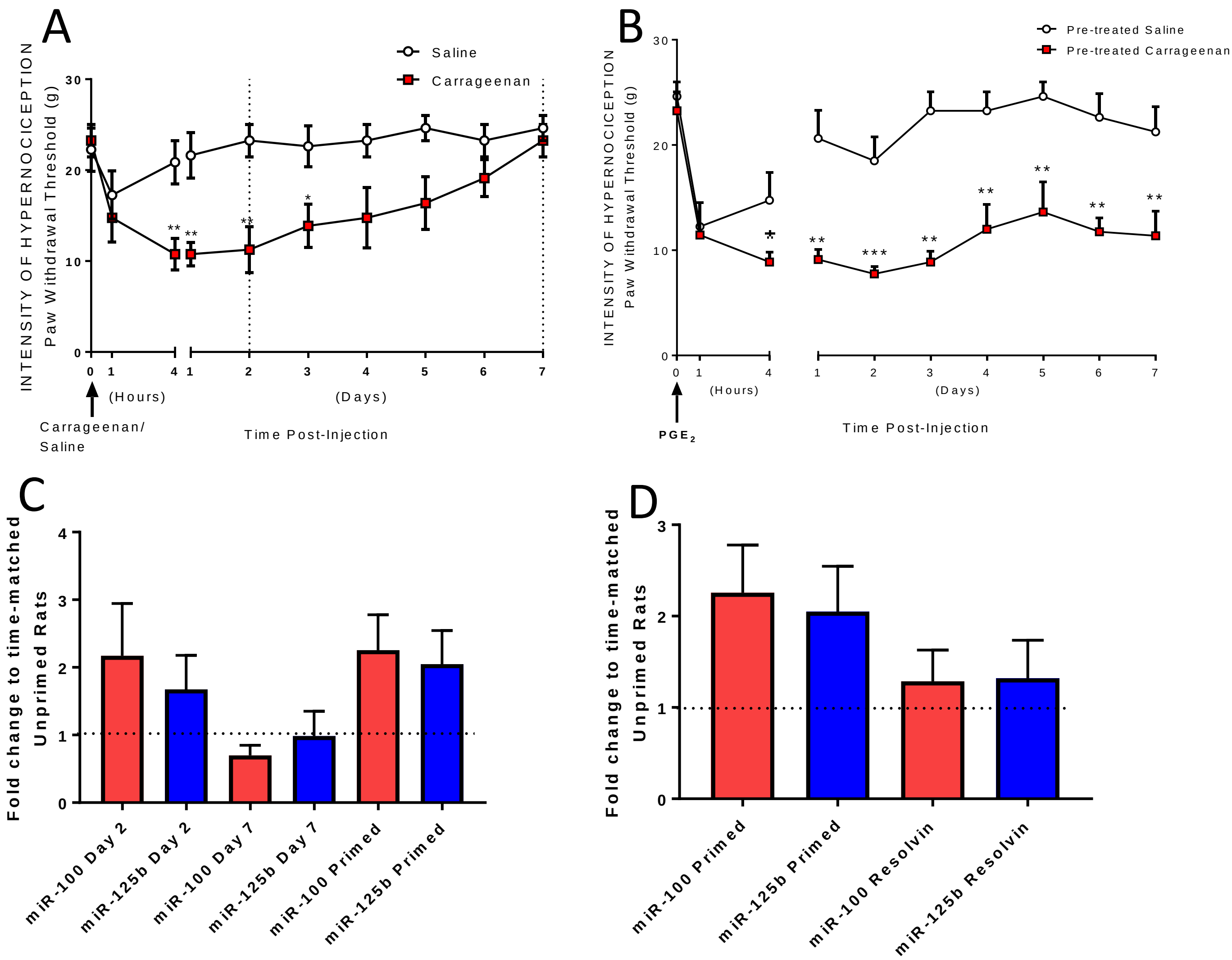


Figure 5.8 **A:** PWT change in response to intraplantar injection of carrageenan DRGs were collected at 2 and 7 days post-carrageenan delineated by dotted lines. **B:** Behavioural data from figure 3.3D. DRGs from this study were collect at day 7 post-PGE_2 for miRNA analysis. **C:** MicroRNA levels taken from L4-L6 DRGs. Changes in miR-100 and miR-125b at 2 and 7 days post-carrageenan then 7 days post-PGE_2. There were observed increases in miR-100 and miR-125b at 2 days post-carrageenan during the acute phase. At 7 days post-carrageenan these had returned to similar levels to saline controls. At 7 days post-PGE_2 the primed animals showed increases in miR-100 and mir125b compared to saline controls. **D:** Comparison between primed animals and primed animals treated with resolvin precursor. There was a reduction in the amount of miR-100 and miR-125b detected compared to primed animals. Behavioural data was analysed using two way ANOVA with Bonferroni post hoc test. *$p<0.05$, **$p<0.01$, ***$p<0.001$. MicroRNA data is expressed at fold change compared to time matched unprimed rats. All data represent mean ± SEM n = 8 per group.

5.4 Discussion

In this chapter I observed that the systemic injection of 17-R-HDoHE prior to the intraplantar injection inhibits PGE_2 induced chronic pain in primed rats. However does not return PWTs to baseline levels. The administration of 17-R-HDoHE during the established chronic pain failed to inhibit the reduction in PWTs.

The number of microglia within the dorsal horn of unprimed rats were found to be significantly higher than primed rats (as previously observed in Chapter 3) at 7 days post-PGE_2. The rats treated with 17-R-HDoHE prior to PGE_2 (with tissue collect 7 days later) showed increased numbers of microglia within the dorsal horn and were found to not be significantly different to either primed-vehicle or unprimed-vehicle rats.

There were observed changes in levels of miRNAs, it was found that levels miR-100 and miR-125b were increased during periods of both acute and chronic pain. The administration of 17-R-HDoHE prior to PGE_2 appears to have inhibited the production of miR-100 and miR-125b.

5.4.1 Resolvin Treatment Prevents Initiation of Hyperalgesic Priming Induced Chronic Pain But Fails to Reverse It

17-R-HDoHE is converted to the active resolvin D1 by 5-LOX (Serhan *et al.*, 2002b). The active aspirin triggered resolvin D1 which then promotes resolution returning the tissue to a resting state after inflammation. Injection of 17-R-HDoHE after injection of carrageenan but before injection of PGE_2 prevented the lowering of PWTs indicating that hyperalgesic priming had been blocked. However when 17-R-HDoHE was administered after PGE_2 while the chronic pain was established it failed to inhibit the reduction in PWT. This suggests that the initiation of hyperalgesic priming is mediate by inflammation however once the chronic pain is established it no longer involves activation of inflammation and may be more centrally mediated hence the resolvin has no effect. However it could also mean that the activation of the resolution pathways during this period are unable to reverse the established chronic pain or potentially an absence of the required receptors for the resolvins to act upon. Immunostaining for the resolvin D receptor ALX/FPR2 in the skin and spinal cord could reveal changes between primed and unprimed animals using an ALX/FPR2 positive antibody as shown in inflamed lung tissue (Chen *et al.*, 2013; Zhao *et al.*, 2015). However has also been successfully used in spinal cord and synovial tissue as demonstrated in (Huang *et al.*, 2016) currently under review.

Resolvins act to reduce inflammation and return a tissue to a normal state as part of a homeostasis mechanism (Serhan *et al.*, 2002b). As PGE_2 has been shown to promote the infiltration of immune cells and the sensitisation of peripheral terminals (Kalinski, 2012; Ouseph *et al.*, 1995). The inhibition of the hyperalgesic priming induced chronic pain could be due to the action of the resolvin on immune cell infiltration, by the interference with the mechanisms of PGE_2 preventing immune cell infiltration that and aiding in the reduction of cytokine release (Aoki *et al.*, 2010; Jin *et al.*, 2009).

The administration of 17-R-HDoHE before PGE_2 attenuated the induced chronic pain, but did not completely inhibit the reduction in PWTs. It has been shown that PGE_2 mediated hyperalgesic priming acts through EP_4 receptor signalling (Villarreal *et al.*, 2013). Activation of EP_4 leads to an increase in cAMP through a G_s coupled mechanism (Yokoyama *et al.*, 2013). This leads to activation of PKA however in hyperalgesic priming there is a phenotypic switch between 1 and 4 hours from PKA to PKCε. As the administration of resolvin not having an inhibitory effect at 1 hour failing to prevent the PGE_2 induced reduction in PWTs, but demonstrating inhibition of PWTs at 4 hours which has been shown to be the transition point in hyperalgesic priming from PKA to PKCε. The resolvin administration could be interfering in the switch from PKA to PKCε or direct inhibition of PKA or PKCε activation. Nobody has currently looked to the interactions with PKA or PKCε by resolvin administration. However resolvin D1 binds to and activates the ALX receptor that leads to the activation of mTOR (Keinan *et al.*, 2013; Ohira *et al.*, 2010) which has been linked to PKCε (Roh *et al.*, 2010). The role of mTOR is to regulate cell growth and protein translation. It has previously been shown that inhibition of mTOR using rapamycin prior to PGE_2 causing non-specific protein translation inhibition (Ferrari *et al.*, 2013a). The resolvins may promote the action of pro-resolvin genes through the action of mTOR. Investigation into the changes in protein expression and transcription changes during the transition from acute to chronic pain could allow for a better understanding of how the resolvins are able to inhibit hyperalgesic priming. This could be achieved through the use of techniques such as RT-PCR to assess changes in mRNA levels of various inflammatory genes that may be altered in hyperalgesic priming. The genes to be investigated could be obtained by looking at the changes in miRNA (**5.4.3**).

5.4.2 Resolvin Treatment alters Microglia Populations in Hyperalgesic Priming

As previously shown in chapter 3 that the administration of PGE_2 causes an increase in the number of morphologically “activated” microglia compared to vehicle controls. There is a larger number of activated microglia in the unprimed-vehicle group compared to the primed-vehicle group and carrageenan-resolvin group. However percentage of morphologically “activated” microglia was found to be similar across all groups this show that PGE_2 causes similar activation across all groups regardless of the initial injection or treatment.

There were differences found in the total number of microglia within the dorsal horn. There were significantly greater numbers of microglia in the unprimed-vehicle group compared to the primed-vehicle group. This could be due to PGE_2 stimulating microglia proliferation or hyperalgesic priming leads to death of microglia within the dorsal horn. However it has been shown in chapter 3 that unprimed-PGE_2 animals have greater total microglial numbers compared to both saline-vehicle and carrageenan-vehicle treated animals demonstrating a likely increase in microglia proliferation following injection of PGE_2. Injection of resolvin caused a slight increase in the total number of microglia to a similar point to the unprimed-vehicle group. This shows that the resolvin administration can potentially alter the proliferation of microglia however further research is required. Collection of spinal cords at several timepoints and identification of potential changes in proliferation during hyperalgesic priming using immunohistochemistry. However measurement of microglial numbers has been conducted using flow cytometry (Remington *et al.*, 2007) this would allow for the exact measurement of the microglia population at several timepoints and with greater accuracy compared to immunohistochemistry. Identifying changes in microglial numbers may give a better understanding of cellular changes in the spinal cord during hyperalgesic priming.

5.4.3 MicroRNA Changes Could Lead to Identification of Gene Targets in Chronic Pain

Preliminary work looking into the variation of miRNAs in hyperalgesic priming (see appendix) showed a difference in the expression of a select number of miRNAs between primed and unprimed animals. Two miRNAs; miR-100 and miR-125b as discussed have been linked to pain conditions and therefore further study was required. There are observed variations in the amounts of miR-100 and miR-125b within the DRGs throughout the stages of hyperalgesic priming. Other miRNAs were found to be altered during the chronic phase of hyperalgesic priming performed by miRNA microarray (TORAY Global, 3D-gene) (see appendix). Therefore they are potentially linked to the changes in pain behaviour. There were two fold increases in the amounts of both miR-100 and miR-125b during the acute pain phase at 2 days post carrageenan however by the recovery phase at day 7 levels have returned to saline values, this suggests their involvement in the changes in pain sensitivity, this would require further study to confirm their involvement in the mediation of pain. Their levels are then increased during the chronic pain phase after PGE_2. Therefore the genes that they modulate could be involved in the generation of hyperalgesic priming or the maintenance of the chronic pain due to the PGE_2. Previous work has shown an upregulation of miR-125b in inflammatory rheumatoid arthritis where a correlation was found between expression and level of inflammation. Where it is considered a biomarker for the measure therapeutic effectiveness (Castro-Villegas *et al.*, 2015).

These changes were found not be significantly different to each other however future work is looking into comparisons to house keeper miRNA so that more accurate measurements can be made. Further work could investigate the effect of selective knockdown via the use of antimiR oligonucleotides during periods of acute and chronic pain (Medina *et al.*, 2009; Stenvang *et al.*, 2012).

The data obtained for the miRNA analysis is preliminary work to identify potential new mechanisms that may underlie hyperalgesic priming. MicroRNAs are involved in the modulation of a larger number of genes, several have been found that are linked to potential alterations in inflammation (see appendix). As these miRNAs were isolated from DRGs the alterations to protein expression will be to the primary afferent fibres or pre-synaptic terminals in the dorsal horn. Several genes are of potential interest for future study, including $GABA_A$ subunits α1, α5, β3 and γ3. It has been shown that the recruitment of $GABA_A$ receptors is important in the generation of hyperalgesic priming (Kim *et al.*, 2016). Variations in subunit composition can alter the affinity, and opening times of $GABA_A$ receptors (Cossart *et al.*, 2005). There may therefore be alterations in the subunit composition of these $GABA_A$ receptors that lead to a change in the sensitivity of sensory neurones in hyperalgesic priming. The could be measured using patch clamp recordings of DRGs or cells of the superficial lamina to investigate changes in Cl^- currents in primed compared to unprimed rats (Zhang *et al.*, 1998). Immunohistochemistry is also a potential avenue of study, there are antibodies that are selective for the different $GABA_A$ subunits to identify changes in their composition (Dean *et al.*, 2005).

These miRNA also modulate the generation of interleukins (IL-6 and IL-10) and neurotrophins (BDNF and NGF). IL-6 has been used as an initiator of hyperalgesic priming when injection into the hindpaw (Kim *et al.*, 2015). It has also been suggested that IL-6 can aid in the transition from acute to chronic inflammation via the alteration of leukocyte infiltration (Gabay, 2006).

IL-10 is anti-inflammatory and has been discussed as a beneficial treatment for peripheral neuropathic pain (Milligan *et al.*, 2012). IL-10 deficiency has also been connected to several different chronic inflammatory conditions including inflammatory bowel disease and colitis (Glocker *et al.*, 2011; Kotlarz *et al.*, 2012). An increase in IL-6 production or a reduction in IL-10 could be a determining factor in the generation of the chronic pain associated with hyperalgesic priming.

Alterations in the levels of both NGF and BDNF are implicated in the generation of chronic pain. Both of which have been used as initiators of hyperalgesic priming (Ferrari *et al.*, 2010; Kim *et al.*, 2016). It has been shown that NGF has increased expression in damaged and inflamed tissues (McKelvey *et al.*, 2013) and acts to sensitise tissues via activation of the p75 receptor (Zhang *et al.*, 2004). It is also well documented that the injection of NGF leads to the initiation of hyperalgesia (Della Seta *et al.*, 1994) as well as augmenting the neuronal response in OA joint pain (Sagar *et al.*, 2015). BDNF is up-regulated in the DRGs of animals that have received an incision into the hindpaw and the intrathecal injection of BDNF antibodies is able to inhibit mechanical allodynia associated with the injury (Li *et al.*, 2008). Up-regulation of these neurotrophins in the model of hyperalgesic priming would lead to an alteration in the sensitivity of neurones and potentially lead to the generation of chronic pain. Reduction in miR-100 has been shown to protect against apoptosis and promote neuronal growth through a mechanism that involved the phosphorylation of TrkB (the BDNF receptor) (Kong *et al.*, 2014; Ye *et al.*, 2015).

By investigating changes in these identified genes could lead to a better understanding of the causes and mechanisms that initiate hyperalgesic priming and the enhanced PGE_2 pain response. Providing further insight into the transition from acute to chronic pain. The resolvins have also been demonstrated to modulate changes in the expression of miRNA in particular the upregulation of MiR-208a and miR-219 (Krishnamoorthy *et al.*, 2012) which could also be investigated in hyperalgesic priming.

5.5 Conclusion

The system injection of 17-R-HDoHE is able to inhibit the induction of hyperalgesic priming induced PGE_2 chronic pain potentially via the promotion of resolution pathways during the inflammatory response of PGE_2. The systemic injection of 17-R-HDoHE into primed rats that have established PGE_2 chronic pain was shown to not affect the reduction in PWTs. Therefore the activation of the resolution pathways during the established chronic pain cannot reverse the reduction in PWTs. There were larger numbers of dorsal horn microglia in unprimed rats treated with vehicle compared to primed rats treated with vehicle. The primed rats treated with resolvin shown no significant differences in the number of microglia compared to unprimed-vehicle and primed-vehicle group. It still needs to be determined whether there is an inhibition of microglia proliferation or hyperalgesic priming causes microglial apoptosis. However it can be concluded that resolvin treatment is able to inhibit this effect. There were observed increases in the expression of miR-100 and miR-125b during periods of reduced PWTs thresholds suggesting their potential involvement in the generation of hyperalgesic priming however further investigation into their role is required.

Chapter 6

General Discussion

6.1 Summary

The initial aim of this thesis was the establishment of the model of hyperalgesic priming. Chapter 3 demonstrates the successful replication of the model of hyperalgesic priming. It was found that both carrageenan and TNFα induce the primed state consistent with the published literature. However, it was found that the TNFα version of the model was not as robust as the carrageenan-induced version. It was demonstrated that hyperalgesic priming does not induce persistent pain as shown by a lack of alteration to weight-bearing asymmetry. There were observed increases in the number of macrophages at the site of injury after injection of PGE_2 within the injured area, as well as increases in the number of dorsal horn microglia, however these changes were present in unprimed and primed rats.

Single unit recording of WDRs found no differences in the responses of WDR neurones between primed and unprimed rats, but there were increases in WDR firing rate to mechanical stimulation of the hindpaw after injection of PGE_2. The i.p injection of the precursor to AT-RvD1 (17-R-HDoHE) prior to the injection of PGE_2 inhibited the reduction in PWT, but did not fully return pain responses to baseline levels. Treatment with 17-R-HDoHE during the chronic phase of priming did not alter the reduction in PWTs. Investigation into changes in miRNA levels within the DRG at several timepoints found that there were increases in two major miRNAs; miR-100 and miR-125b associated with hyperalgesic priming. These miRNAs are linked to the regulation of several inflammatory genes which could be investigated in the future to confirm their potential involvement in hyperalgesic priming.

6.2 The Model of Hyperalgesic Priming

This thesis investigated the reliability and use of the model of hyperalgesic priming as well as studying some of the cellular and molecular changes associated with it. The model had been recently developed (Aley *et al.*, 2000) so it was important to assess the reproducibility of the pain behaviour associated with hyperalgesic priming. Two versions of the hyperalgesic priming model were tested; the carrageenan and TNFα induced models. It was found that both were able to induce a primed state as evidenced by an altered behavioural nociceptive response to an intraplantar injection of PGE_2, specifically an enhanced duration of pain behaviour. Intraplantar injection of TNFα was not associated with such robust changes in nociceptive behavioural response to PGE_2, compared to the carrageenan induced model. Indeed, it was only the carrageenan model that was associated with long lasting (7 day) and significant PGE_2 evoked lowering of PWTs. On this basis the carrageenan induced version of hyperalgesic priming was used for the mechanistic studies.

The TNFα version of the model did not produce significant decreases in PWTs compared to the carrageenan induced version. This is not consistent with the published literature on the model that demonstrate similar decreases in PWTs regardless of the initiator. This potentially suggests that the model of hyperalgesic priming is not an activation of single pathway but may involve activation of multiple pathways. This is confirmed by the pre-treatment with 17-R-HDoHE which only partially inhibited the primed induced PGE_2 chronic pain. Potentially the action of the 17-R-HDoHE is to inhibit one of these pathways via the promotion of resolution. The treatment with resolvin successfully inhibited the reduction in PWTs at 4h, which is the timepoint where the switch from PKA to PKCε occurs. This requires further investigation into whether 17-R-HDoHE can inhibit the activity of PKCε which can be performed using flow cytometry (Bernsteel *et al.*, 2008). Controlled measurement of phosphorylation within in vitro cell samples using known inhibitors of PKCε followed by comparison to 17-R-HDoHE or active RvD1 administration. By identifying whether PKCε was modulated by 17-R-HDoHE would allow a better understanding of the mechanisms of resolvins as well as determining changes between the establishment of the chronic pain and its maintenance. Whereas once the chronic pain was established the treatment with 17-R-HDoHE had no effect on the PWTs.

It has been proposed that hyperalgesic priming models the mechanisms that underpin the transition from acute to chronic pain (Aley *et al.*, 2000; Ferrari *et al.*, 2013b), and that It may be useful in understanding why this occurs and identifying targets for intervention. The model does have drawbacks however, the duration of the chronic phase has been reported to be variable with some showing the absence of chronic pain after 3d (Araldi *et al.*, 2016b; Villarreal *et al.*, 2013) and others after 10 days (Kim *et al.*, 2016). The work in my thesis demonstrates that the chronic phase reliably lasts for 7 days post-PGE_2 injection in the carrageenan model of priming (Chapter 3).

However this model does have some limitations in that the protocols used do not induce priming in female rats. It has been shown that the presence of oestrogen actively inhibits the generation of hyperalgesic priming (known as type I). It has been shown that the presence of oestrogen actively inhibits the generation of type I hyperalgesic priming. Indeed if male rats are gonadectomised and treated with systemic oestrogen priming cannot be induced (Joseph *et al.*, 2003). Clinically females are statistically more likely to suffer from chronic pain (Breivik *et al.*, 2006). It has been demonstrated that type I hyperalgesic priming can be induced in females via the activation of αCaMKII which is downstream of PKCε (Ferrari *et al.*, 2013b).

The inability of hyperalgesic priming to be induced in both males and females using the same method does cast some doubt on its potential use for the development of clinical therapeutics. To overcome this potential drawback another model has recently been described (known as type II priming) in which hyperalgesic priming is induced in both males and females. The major differences between type I and type II hyperalgesic priming are illustrated in Table 6.1. Type II priming causes characteristic increases in the response to PGE_2 however does not rely on IB_4^+ expressing sensory nerves and can be induced within the 72 hour period after the initial stimulus. A major difference is that type II priming requires PKA activation for maintenance, rather than PKCε, as well as being able to be induced in females directly rather than the downstream activation of αCaMKII used to induce type I (Araldi *et al.*, 2015; Ferrari *et al.*, 2013b).

The induction of type II priming uses repeated intraplantar injection of the mu opioid agonist DAMGO to generation a primed state. Following 3 intraplantar injections of DAMGO an hour apart, a subsequent injection of PGE_2 results in a significant lowering of PWTs at 1h that was maintained at 4 hours. Interestingly, unlike type I priming type II priming was not prevented by depletion of non-peptidergic C-fibres by IB_4-saporin (Alvarez *et al.*, 2012a; Araldi *et al.*, 2015). It was also demonstrated that the chronic phase of the response was dependent on PKA with no involvement of PKCε, nor did it involve local protein translation as cordycepin did not prevent it from being established (Araldi *et al.*, 2015). This model is not only generated by opioid administration, but can also be generated by adenosine A1 agonist N-cyclopentyladenosine (CPA) (Araldi *et al.*, 2016a). Type II priming could be relevant for study in clinical chronic pain as it demonstrates increased sensitivity to subsequent stimuli after opioid treatment. There have been many studies demonstrating opioid induced hyperalgesia. The administration of repeated doses of opioids has been shown to lead to a reduction in mechanical pain thresholds (Bekhit, 2010; DuPen *et al.*, 2007). Investigation into both of these versions of hyperalgesic priming may allow for a better understanding of varied chronic pain induction as well as study into the different ways chronic pain can occur.

	TYPE I HYPERALGESIC PRIMING	TYPE II HYPERALGESIC PRIMING
REQUIRED FIBRE TYPE	IB_4	CGRP
PROTEIN KINASE MEDIATOR	PKCε	PKA
INDUCTION TIME	>72h	<72h
SEX	Male only	Male and Female

Table 6.1: Table summary of differences between Type I and Type II hyperalgesic priming (Aley *et al.*, 2000; Alvarez *et al.*, 2012b; Araldi *et al.*, 2016a; Araldi *et al.*, 2015).

6.3 Peripheral Events in Hyperalgesic Priming

Immune cells play a crucial role in the process of inflammation and it has been shown that uncontrolled immune cell activation causes chronic inflammation (Bryant, 2007). Investigations were made into differences in the presence of immune cells during the chronic phase of hyperalgesic priming specifically neutrophils and macrophages in the skin. It was found that there were elevated numbers of macrophages in the skin of both the unprimed and primed rats at 7 days post-PGE_2 injection. This suggests that PGE_2 causes similar macrophage infiltration irrespective of the pre-treatment and that they remain in the tissue for up to at least 7 days. There were no neutrophils observed within the skin samples at the same time-point, suggesting this cell type may have the tissue or been engulfed by macrophages at the end of the inflammatory response. There is however the possibility that there was no infiltration of neutrophils during the establishment of priming this means that the role of neutrophils during the initiation phase needs to determined (discussed in **6.6**). As stated in Chapter 3 and 7 there is the potential that the antibodies failed to work therefore solid conclusion cannot be made on the immune cell staining work at this time and so must be counted as preliminary. The antibodies need to be tested in positive control experiments. This would involve the use of purified or cultures macrophages and neutrophils to confirm the selectivity of the antibody therefore confirming that the absence of staining seen in these experiments is in fact true.

During inflammation there is the crucial breakdown of arachidonic acid to produce many of the inflammatory mediators that initiate the infiltration and activation of many of these immune cells (Basbaum *et al.*, 2009; Bryant, 2007). The measured plasma concentrations of each mediator was found to be similar in primed compared to unprimed animals. However there were observed differences in the detection rate of plasma lipid mediators denoting an imbalance in levels of anti-inflammatory and pro-inflammatory mediators between primed and unprimed rats. There was a greater detection rate of anti-inflammatory mediators in the plasma from unprimed rats, for example levels of 18-HEPE the precursor to the resolvin E series. RvE aids in the resolution of the tissue after an inflammatory response reducing immune cell infiltration and promoting the transition of macrophages from the inflammatory M1 subtype to the anti-inflammatory M2 subtype (Navarro-Xavier *et al.*, 2010). PGE_2-EA was also detected in a significantly greater number of samples from unprimed rats compared to primed rats, PGE_2-EA is a derivative of PGE_2 and acts to suppress immune responses via the inhibition of TNFα (Brown *et al.*, 2013). This could suggest an alteration in the endocannabinoid system as PGE_2-EA is produced from anandamide via oxygenation by COX (Brown *et al.*, 2013) The receptors at which PGE_2-EA acts are currently unknown, there is speculation that their action could be via cannabinoid receptors CB1 and CB2 however binding studies found no evidence of competitive binding for CB1 by PGE_2-EA (Pinto *et al.*, 1994). The other alternative is that PGE_2-EA binding to PGE_2 EP receptors inhibits TNFα release and is thought to be through their action on EP2 receptor thereby modulating cAMP production (Brown *et al.*, 2013). The alterations in the detection rates of different lipid mediators between primed and unprimed rats suggests that hyperalgesic priming induces a systemic release of inflammatory mediators that may contribute to changes both at the peripheral and spinal level. As lipids are able to cross the blood brain barrier (Banks, 2009; Jones *et al.*, 2007). The higher concentration of these anti-inflammatory mediators suggests alteration in their production due to the action of priming by carrageenan. These mediators could be investigated further by inhibiting their production using enzymatic inhibitors or via the promotion of their breakdown. As with the immune cell work these data have to be discussed in a preliminary form at this current stage. The lack of a particular mediator being detected can be interpreted as an absence or an extremely low concentration of the mediator in question. Therefore this data can be used to further investigate these mediators in further detail however any conclusions made at this point are solely speculative.

The roles of different sensory nerve fibres were assessed during the initiation of the PGE_2 enhanced pain and during the established chronic phase. Administration of the impermeable local anaesthetic QX-314 with capsaicin selectively blocks TRPV1 expressing nociceptors (Binshtok *et al.*, 2007), allowing the investigation of the role of the of a subset of sensory nerves. Intraplantar injection of QX-314 + capsaicin in rats with established PGE_2 induced lowering of PWTs, and resulted in a significant reversal in PWTs, which returned to baseline levels for up to 4h post injection. These data suggest that the reduction in PWT in the hyperalgesic priming model is partly mediated via TRPV1 positive nociceptors. Intraplantar injection of QX-314 + flagellin, which selectively inhibits Aβ fibres (Xu *et al.*, 2015), also caused a significant reversal of PWTs in the hyperalgesic priming model at 2 hours post-injection. However QX-314 + flagellin treatment did not completely return PWTs to baseline levels, supporting a partial involvement of Aβ fibres in hyperalgesic priming.

The roles of sensory nerve fibres during the transition from acute to chronic pain were also assessed using the same pharmacological tools. QX-314 + capsaicin was injected into the hindpaw 90mins prior to the injection of PGE_2 in primed and unprimed rats. This treatment prevented the reduction in PWT following injection of PGE_2 up to 4 hrs post-injection, however PWTs were significantly lowered at 24h post-PGE_2 injection. These data suggest that nociceptive signal driving a behavioural response is not necessary for the appearance of pain behaviour at a later timepoint. Previously, It has been shown that inhibition of the carrageenan induced pain with morphine both locally or systemically does not affect the generation of hyperalgesic priming later (Villarreal *et al.*, 2013). These data support the view that the pain signal during the initiation of hyperalgesic priming is not required for the translation changes to occur that lead to chronic pain.

The pre-treatment with QX-314 + flagellin before intraplantar injection of PGE_2 failed to inhibit the reduction in PWTs at 1 hour. However the QX-314 + flagellin treated primed group did show significantly higher PWTs compared to the untreated primed group at 2 and 4 hours post-PGE_2. This demonstrates the potential involvement of Aβ fibre sensitisation during the transition period of hyperalgesic priming however the pain was still established at 24 hour and remained up to 7 days after PGE_2 again confirming that inhibition of the pain during the PGE_2 response does not prevent PGE_2 induced hyperalgesia. The initiation of chronic pain in the absence of a PGE_2 pain response suggests the involvement of downstream signalling pathways. The switch from PKA to PKCε during the enhanced PGE_2 response is an area that requires further study, the reason for the switch in protein kinase mediation is unknown, it was suggested that there was coupling between EP receptors and A_1 adenosine receptors through lipid rafts however this has not been confirmed (Ferrari *et al.*, 2015).

Investigation into the miRNA levels within the DRG demonstrated alterations in several miRNAs (see appendix). The miRNAs, miR-100 and miR-125b have been found to modulate nerve growth and apoptosis and are upregulated in pain states and neuroinflammation (Castro-Villegas *et al.*, 2015; Kong *et al.*, 2014). There were 2 fold increases in miR-100 and miR-125b compared to saline and unprimed rats in the L4, L5 and L6 DRGs at 2 days post-carrageenan and at the later chronic phase 7 days post-PGE_2. This shows that the genetic changes that occur during the acute phase may influence or mediate the chronic phase. It has been reported that the generation of hyperalgesic priming requires protein translation to occur after the initial injection (Ferrari *et al.*, 2013a). These include changes to growth factors including NGF and BDNF (Kong *et al.*, 2014), both of which induce hyperalgesic priming (Ferrari *et al.*, 2010; Kim *et al.*, 2016). NGF levels are increased in damaged tissue (McKelvey *et al.*, 2013) and has been shown to play a role in several other pain conditions including arthritis (Halliday *et al.*, 1998) and cancer pain (Ye *et al.*, 2011). MiRNA also potentially mediate changes in the expression of receptor and channel subunits in $GABA_A$ receptors (based on predictions from mirgate database) leading to alterations in spinal excitability. Increases in $GABA_A$ receptor trafficking within the dorsal horn has been linked to the generation of hyperalgesic priming through the action of the recruiting protein neurolignin 2 (Kim *et al.*, 2016). There may therefore be alterations in the subunit composition of $GABA_A$ receptors in hyperalgesic priming within the dorsal horn this could be investigated using antibodies to determine presence of different $GABA_A$ receptor subunits within the dorsal horn (Dean *et al.*, 2005).

The effect of resolvin precursor pre-treatment on the levels of miR-100 and miR-125b in the DRGs were assessed during the chronic pain phase at 7 days post-PGE_2. They were both found to be at similar levels compared to unprimed controls. This could show that the resolvins can potentially modulate the production of these miRNAs or that the activation of the resolution pathways reduces the production of miR-100 and miR-125b. It has been shown that the D-series resolvins increase the production of pro-resolving miR-208a and miR-219 (Krishnamoorthy *et al.*, 2012). The increased expression of miR-208a up-regulates IL-10 in human macrophages (Krishnamoorthy *et al.*, 2012). Promoting the expression of miR208a in hyperalgesic primed rats may aid protecting against the generation of chronic pain.

6.4 Central Events in Hyperalgesic Priming

When studying hyperalgesic priming much of the published work has looked at the peripheral changes in primary afferent fibres during hyperalgesic priming (Aley *et al.*, 2000; Ferrari *et al.*, 2013a; Ferrari *et al.*, 2013b). However investigations into neurophysiological changes using sciatic nerve recordings have shown that there is no alteration in peripheral nerve excitability in response to hyperalgesic priming (Hendrich *et al.*, 2013), but there are changes to spinal dopamine and GABAergic innervation (Kim *et al.*, 2016; Kim *et al.*, 2015). There were alterations in the presence of $GABA_A$ receptors via increased trafficking of receptors by neurolignin 2 (Kim *et al.*, 2016). Hyperalgesic priming has been shown to involve descending dopaminergic connections from the A9 nucleus of the hypothalamus to the dorsal horn of the spinal cord. The ablation of dopaminergic input to the spinal cord prevents the generation of hyperalgesic priming (Kim *et al.*, 2015).

These studies suggested a potential alteration in the spinal processing of noxious inputs during hyperalgesic priming. To measure this electrophysiological recordings of lamina V WDR neurones were performed as this has been shown to be a useful assay of alterations in pain processing (Chapman *et al.*, 1998; Sagar *et al.*, 2010). There were increases in the firing rate of WDR neurones in response to low intensity mechanical stimulation (8g, 10g and 15g) after intraplantar injection of PGE_2 into primed and unprimed rats. This shows that PGE_2 induces changes that can be detected at the spinal level, however there were no significant changes to WDR firing in response to 26g stimulation, suggesting that PGE_2 does not cause hyperalgesia as seen in behavioural assays where the application of 26g was often shown to be noxious. There were however no significant differences found between primed and unprimed rats in their spinal responses to the intraplantar injection of PGE_2. The increased firing to mechanical stimulation after PGE_2 was maintained for between 1-2 hours post injection, this is not consistent with the awake pain behaviour where the response lasts up to 7 days post-PGE_2 (Chapter 3).

The intraplantar injection of PGE_2 did induce an increase in the receptive field size of the cell and this was likely due to the sensitisation of the surrounding tissue but also may suggest heterosynaptic activation centrally (Latremoliere *et al.*, 2009) as there is a spread of pain to other regions of the hindpaw. The increase in receptive field size only lasted as long as the increase to WDR firing to mechanical stimulation suggesting that its maintenance was due to the activity of the PGE_2. Differences between the spinal recordings and behavioural response suggests a disconnect in the processing between the periphery and the central spinal cord, however as discussed in Chapter 4 depth of anaesthesia could be a confounding factor. However changes are observed in different pain models using this technique. This does not confirm that there is no change to spinal processing during hyperalgesic priming. There is the potential for changes in spinal processing within the more superficial laminae. Lamina I and II receives much of the input from C-fibres (Millan, 1999; Todd, 2010a), as type I hyperalgesic priming is mediated by IB_4 non-peptidergic C fibres (Alvarez *et al.*, 2012a) this could be a location where changes are occurring in priming. Changes within the superficial laminae could be measured using the patch clamp technique. This would allow for the measurement of multiple neurones to obtain more information about the state of the dorsal horn in primed versus unprimed rats. There is also the potential for the use of single unit recording within lamina I and II however the use of multiunit recording would allow for a better understanding of the changes that may have occurred during hyperalgesic priming.

It has been shown that there are changes in excitatory and inhibitory nerves within the dorsal horn after administration of CFA through changes to the expression of AMPARs (Kopach *et al.*, 2015). The reason why I failed to see changes at the level of the lamina V WDR could be due to the information being directly projected to the brain from lamina I and II as explaining in Chapter 1 there are ascending connections that go direction from lamina I and II to the brain (Todd, 2010b). Also in inflammatory states using CFA there are observed differences in excitatory and inhibitory processing within lamina II (Kopach *et al.*, 2015). It is therefore important to look at a variety of neuronal connections to get a better understanding of hyperalgesic priming

The work in my thesis also shows changes to microglial populations within the dorsal horn of primed rats. However, the changes are counter-intuitive in that there were less morphologically defined activated microglia in the dorsal horn of the spinal cord in primed rats, compared to unprimed rats. In addition, the total number of microglia within the dorsal horn was lowered in primed rats, compared to unprimed rats. These data may be explained by either an inhibition of microglial proliferation or microglial apoptosis during the chronic phase of hyperalgesic priming (Gómez-Nicola *et al.*, 2013; Liu *et al.*, 2001). Microglia have been strongly implicated in changes in spinal excitability through their release of inflammatory mediators including TNFα and BDNF (Gomes *et al.*, 2013). Intrathecal BDNF has been used as an initiator of hyperalgesic priming and leads to the potentiation of NMDA receptors within primary afferent terminals contributing to the development of central sensitisation (Chen *et al.*, 2014). However it was observed that there was a decrease in the number of microglia the primed rats during the chronic pain period. This goes against the dogma that microglia potentiate pain in the chronic states (Hains *et al.*, 2006). This could mean that the microglia within the unprimed animals are performing a protective role as they have been demonstrated to release anti-inflammatory mediators (Cherry *et al.*, 2014). The controversial presence of M1 and M2 subtypes of microglia were also investigated it has been stated that the M2 subtype are anti-inflammatory and express CD206 (mannose receptor) (Stein *et al.*, 1992; Zimmer *et al.*, 2003). It was found that microglia within the rat dorsal horn do not express CD206, as most of the work on anti-inflammatory microglia has been performed in mice this could be a species difference or that rat microglia do not express CD206. However is could suggest that there are no anti-inflammatory microglia in rats treated with PGE_2. Further work could look into the optimisation of other markers of alternatively activating microglia. However as the categorisation of microglia into the M1 and M2 subtype is currently under criticism for oversimplification of microglia classification (Ransohoff, 2016). Rather than focusing on M1 or M2 classification aim to understand alterations in the release of inflammatory or anti-inflammatory mediators by microglia. Investigation the presence of the anti-inflammatory cytokines produced by microglia such as IL-4 and IL-10 or pro-inflammatory IL-6 and

IL-1β using ELISA analysis may provide a better understanding of the role of microglia in hyperalgesic priming.

6.5 Intervention in the Chronic Phase of Hyperalgesic Priming

One of the main advantages of hyperalgesic priming is the ability to investigate the changes that occur between acute and chronic pain. Other well established chronic pain models such as injection of CFA produce immediate long lasting pain, which does not mimic what is seen in cases of clinical pain (MacIntyre *et al.*, 1995; Melhorn, 1998).

The role of immune cells during the initiation phase of hyperalgesic priming could be crucial to its development. Interfering with the immune response by the pre-treatment with the RvD1 precursor 17-R-HDoHE inhibited the reduction in PWT induced by the intraplantar injection of PGE_2 into primed rats, but when injected into rats with established chronic pain failed to inhibit the reduction in PWTs. The resolvins as stated (Chapters 1 and 5) reduce inflammation via the inhibition of cytokine release and immune cell infiltration this would therefore suggest their involvement in triggering the enhanced response to PGE_2. It also suggests a potential disruption in the resolution pathways following the initial injection of carrageenan priming the system.

The site of action for the resolvin activity in hyperalgesic priming is not known. Many of the actions of resolvins are peripheral and as discussed are against the immune response (Serhan *et al.*, 2007). However there is evidence of centrally mediated resolvin activity in the spinal cord. The D-series resolvins are able to modulate spinal excitability (Meesawatsom *et al.*, 2016). The 17-R-HDoHE could be administered to intrathecally or locally to the hindpaw to determine to the location of its action. It also needs to be confirmed that the effects observed by the action of 17-R-HDoHE on chronic pain due to its conversion to AT-RvD1. By repeating the experiments using AT-RvD1 (the active version of 17-R-HDoHE) would confirm that the inhibition is due to the conversion of 17-R-HDoHE to AT-RvD1. To confirm the effects seen due to the administration of 17-R-HDoHE experiments would need to be repeated using resolvin inhibitors. As well as seeing whether hyperalgesic priming can be induced via the inhibition of tissue resolution using the same inhibitors.

Looking into other resolution mediators may give further insights into the prevention of chronic pain. The E-series of the resolvins have been extensively studied and shown a lot of potential in the reversal of chronic pain induced by CFA and formalin (Xu *et al.*, 2010b). They have also been shown to have a larger number of effects compared to the D series including inhibition of neutrophil, eosinophil, monocyte, natural killer cell, dendritic cell and mast cell infiltration (Qu *et al.*, 2015). RvE1 has also been shown to inhibit the release of pro-inflammatory cytokines from microglia (Xu *et al.*, 2013).

6.6 Limitations and Future Work

One of the major limitations with the model of hyperalgesic priming is the size and spread of the injury. The pain induced by hyperalgesic priming is close to the size of the injected 5µl bolus. The pain was not found to spread beyond the injury site this made behavioural testing difficult and also confounded on the difficulty of single unit recordings of WDRs. This also made identification and analysis of the injury site in skin tissue difficult to confirm.

To get around the problem of identifying the exact injury site and allow for further investigation into the affected sensory afferents would be to administer a dye in conjunction with the injection of PGE_2 to identify the spread of the bolus within the skin. Identification of afferent fibres that are associated with hyperalgesic priming within the skin could be performed using retrograde tracers such as DiI, which back traces to the DRG so cell bodies that receive input from the skin can be identified (Shiraishi *et al.*, 1992).

Future work could look into the action of the immune cells during the transition from acute to chronic pain at the 1 and 4h time-points after PGE_2 administration. This could be performed using similar immunohistochemistry techniques described in this thesis (Chapter 3) or fluorescence-activated cell sorting (FACS) could be used (Bonner *et al.*, 1972; Butcher *et al.*, 2011). The benefit of this approach is that multiple types of immune cell can be counted using several different membrane markers to simultaneously in samples from primed and unprimed rats, and at different timepoints. This would allow for not only distinction between M1 and M2 macrophages, but also quantification of additional types of immune cells types using markers against mast cells (Walls *et al.*, 1990), dendritic cells (Vasco *et al.*, 2010) and neutrophils.

An alternative approach to the immunohistochemistry methods employed could be to use CLARITY (Poguzhelskaya *et al.*, 2014). This technique allows for immunohistochemistry to be performed on fully intact tissue without the requirement of slicing. The process causes the tissue to become transparent through the replacement of lipids while retaining proteins and the overall structure of the tissue. This allows for immunohistochemistry to be performed in a 3D structure enabling a full 360° view of the tissue. By performing CLARITY on skin it would be possible to clearly view and analyse the movement of immune cells into the injured region through multiple tissue layers within the skin at time-points during the hyperalgesic priming transition (Epp *et al.*, 2015).

Future work should still be to aim to develop a physiological correlate of hyperalgesic priming so that pharmacological studies can be performed to better understand the mechanisms. One method could be to use the spinal electrophysiology method by administering the PGE_2 to primed and unprimed animals 24h prior to electrophysiology recordings then to characterise multiple cells to different pain modalities such as mechanical, thermal and electrical. This would allow for the investigation of whether the overall sensitivity of the animal has changed post-PGE_2, however this would only allow for the study of the chronic phase and not its onset.

A second method would be to use electromyography (EMG) recordings (Hathway *et al.*, 2009), which will allow for the measurement of the already existent behavioural output in a controlled more accurate way. With this technique it is possible to perform studies both before and after PGE_2 administration allowing for quantification of the mechanical pain modality that is used for the behavioural pain testing.

Finally the use of a multi-electrode array in lieu of single unit recording would permit the measurement of several laminae regions at once meaning changes in multiple locations could be recorded (Kajikawa *et al.*, 2011). By investigating the whole dorsal horn it could allow for more focused work on different laminar regions to then be studied in more detail. Due to evidence that the chronic phase is mediated by the activity of C-fibres (discussed in **6.3**) could suggest changes in the upper lamina when the C-fibres terminate (Light *et al.*, 1979).

In conclusion this thesis has demonstrated that hyperalgesic priming is involves altered microglial populations within the dorsal horn during the maintenance of chronic pain and there are no observed changes in the processing of pain within the

WDR neurones of lamina V. The initiation and maintenance of hyperalgesic priming induced chronic pain involves the activation of both C and Aβ fibres within the skin. Intervention with the resolvin precursor 17-R-HDoHE was able to inhibit the initiation of chronic pain, but failed to reverse established chronic pain. This model shows potential in understanding the transition from acute to chronic pain using techniques to further investigate the cellular and molecular changes that occur during hyperalgesic priming may lead to the discovery of future targets for the development of therapeutics.

Chapter 7

Appendix

7.1 Additional Methods

7.1.1 Mass Spectroscopy

All mass spectroscopy work was carried out by Petros Pousinis as part of a collaboration.

<u>7.1.1.1 Sample Preparation</u>

Rat plasma was allowed to thaw on ice. 400 µL of plasma were transferred in 1.5 ml plastic microcentrifuge tubes. Oxylipins were extracted from plasma based on a modified method previously developed in our group. Internal standards (10 µL of PGF2a-EA-d4 (2.49 µM), 10 µL of AA-d8 (1 µM), 10 µL of PGD2-d4 (1 µM), 10 µL of 15-HETE-d8 (7.6 µM) were added to each sample or blank sample (0.4 ml methanol), along with a mixture solvent comprising of; 2 µL of formic acid (98% v/v), 1 µL of a freshly prepared antioxidant butylhydroxytoluene (BHT) solution in ethanol (5 mg/ml), and 700 µL of ethanol. Samples were homogenized in microcentrifuge tubes with the addition of 703 µL of the precipitation solvent, followed by a slow vortex stage (10 min) and centrifugation (13,000 g, 10 min, 4 °C). The supernatants were transferred to glass tubes and diluted by the addition of 3 ml ddH_2O. The diluted supernatants were loaded to the Strata-X polymeric SPE column that had been preconditioned with 100% ethanol (2 ml) followed by 25% ethanol (4 ml). The SPE cartridge was then washed with ddH_2O (10 ml) and 25% ethanol (5 ml) and was allowed to run it dry. Finally, oxylipins eluted from the column with ethyl acetate containing 0.0002% BHT (*v/v*) (5 ml) and were dried in centrifugal evaporator. The samples were reconstituted in 50 % ethanol (100 µL), transferred to dark brown auto sampler vials, and stored in -80 °C until LC-MS/MS analysis. The injection volume was 20 µL.

7.1.1.2 LC-MS/MS analysis

The HPLC system used was a Shimadzu series 10AD VP LC system (Shimadzu, USA). The HPLC column used was ACE C18 (150 x 2.1 mm, 3 µm) with guard column, Security Guard cartridges ACE 3 C18 (150 x 2.1 mm column). Mobile phase A was 0.02% formic acid in Milli-Q water, and mobile phase B was 0.02% formic acid in methanol/acetonitrile (1:4, v/v),. Gradient started with 45% B, gradually increased to 100% by 12.5 min, kept at 100% for 5.5 min and re-equilibrated for 2 min. Total run time is 20 min. The flow rate was 300 µL /min and the samples were kept at 4°C. The mass spectrometer system used was an Applied Biosystem MDS SCIEX 4000 Q-TRAP hybrid triple-quadrupole–linear ion trap mass spectrometer (Applied Biosystems, USA) equipped with an ESI interface. Multiple reaction monitoring (MRM) of all analytes (43 oxylipins) detected in negative mode using specific precursor and product *m/z* ratios. Quantification was performed using Analyst 1.4.1. The identification of individual oxylipins in biofluids was confirmed by comparing their LC retention times (t_r) to that of each standard and their respective MRM transitions (Zhang *et al.*, 2007).

7.1.2 MicroRNA Extraction and Analysis

MicroRNA analysis was performed by Dr. Alex Rathbone and in collaboration with Clare Martin BSc.

7.1.2.1 tRNA extraction from PFA fixed tissue

To remove the PFA from the fixed DRGs they were washed twice with 100 % ethanol then air dried. The tissue was homogenized in digesting buffer (50 mM Tris–HCl pH 7.5, 75 mM NaCl, 5 mM $CaCl_2$ 0.5mg/ml proteinase K and 0.1% SDS) using a sterile micro-homogeniser, and digested at 55°C for 3hours. 1 mL TRIzol® Reagent was added per 50–100 mg of tissue sample and the samples homogenized again using a micro-homogeniser. The tRNA extraction was as per manufacturer's instructions for TRIzol® Reagent. The tRNA contained the miRNAs.

7.1.2.2 Q-PCR method and analysis

cDNA was synthesised from mature miRNAs using the miRCURY LNA™ Universal cDNA synthesis kit (Exiqon A/S, Denmark), which uses a poly-T primer. Samples were run in triplicate using primers for hsa-miR-100-5p (MIMAT0000098) and hsa-miR-125b-5p (MIMAT0000423) (Exiqon A/S, Denmark). Q-PCR was undertaken using the ExiLENT SYBR® Green mastermix kit (Exiqon A/S, Denmark), and the StepOne Plus thermocycler (Applied Biosystems) was used in standard mode using cycling parameters recommended by Exiqon. Data was acquired using Applied Biosystems StepOne software version 2.3. ROX™ (Life Technologies, Paisley, UK) was used as a passive reference for normalising for non-PCR related fluorescence variations, and was incorporated by the software to calculate Ct values. The Ct values were analysed using the comparative Ct method; using age matched saline control animals as the calibrator. Spike in samples containing UniSp6 were used as inter plate calibrators and variation of readings between replicate plates was <5%. GraphPad Prism 7.01 was then used to plot the fold change data with error bars showing SEM.

7.2 Additional Results

7.2.1 Effects of hyperalgesic priming on peripheral immune cells

To investigate the potential contribution of neutrophil and macrophage cells in chronic phase of hyperalgesic priming, skin tissue was collected at day 7 from carrageenan PGE_2 hyperalgesic primed rats. Sections were stained for neutrophils using an antibody against neutrophil elastase. At this timepoint, there was no cell specific neutrophil elastase staining as confirmed with DAPI staining of sections (Figure 7.1).

In contrast to the neutrophils, there was evidence for an increase in the number of macrophages in the hyperalgesic primed skin compared to control skin. Macrophages present within the hyperalgesic primed skin were positive for the mannose receptor, indicative of an M2 phenotype. However, the number of macrophages was similar in primed and unprimed rats and appeared to be related to the injection of PGE2 rather than the presence of hyperalgesic priming (Figure 7.2). This data is preliminary and will require further work to confirm the specificity of the antibodies used by testing on purified or cultured macrophages and neutrophils. The data currently cannot be used to confidently conclude and involvement of immune cells within hyperalgesic priming.

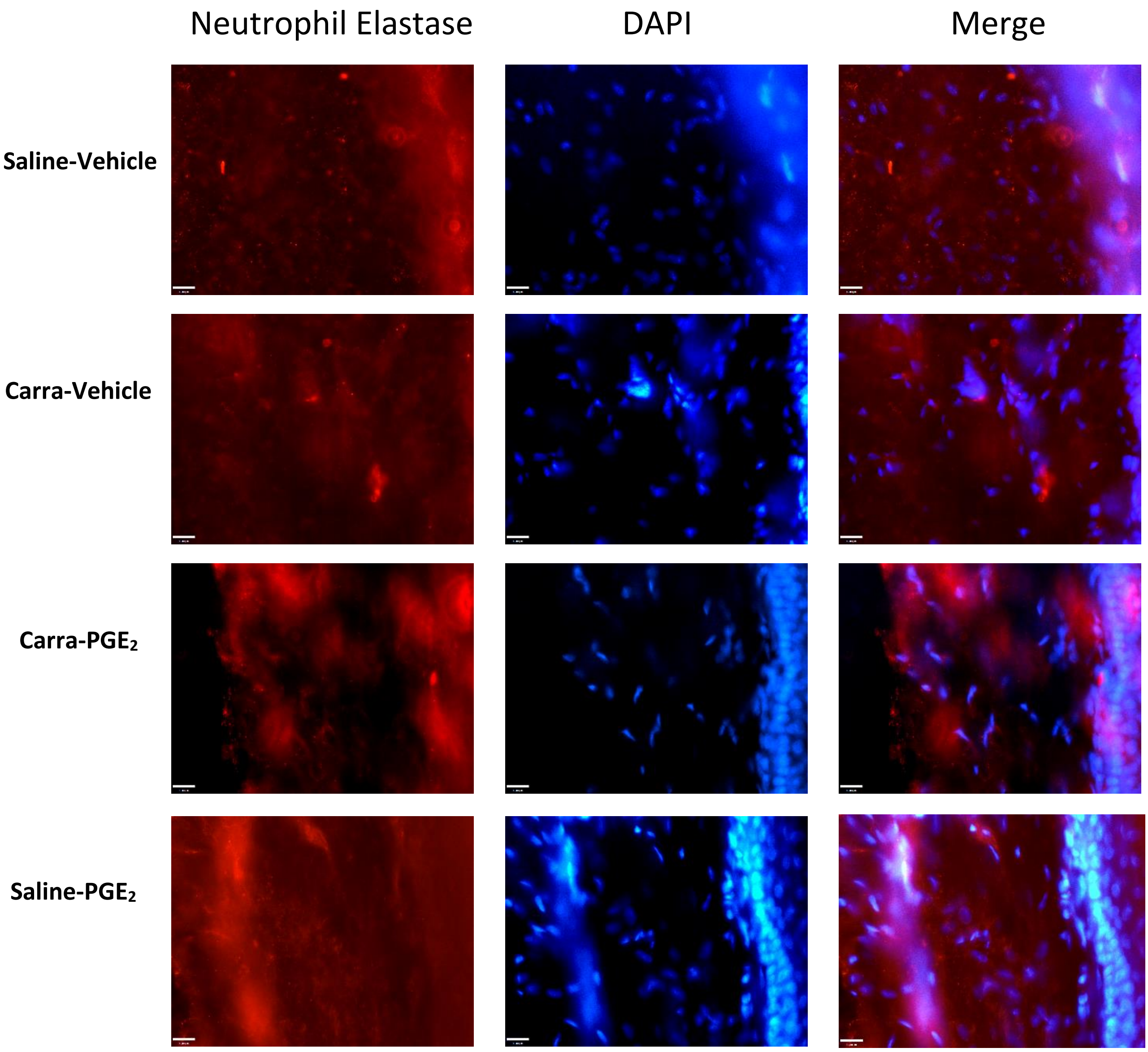
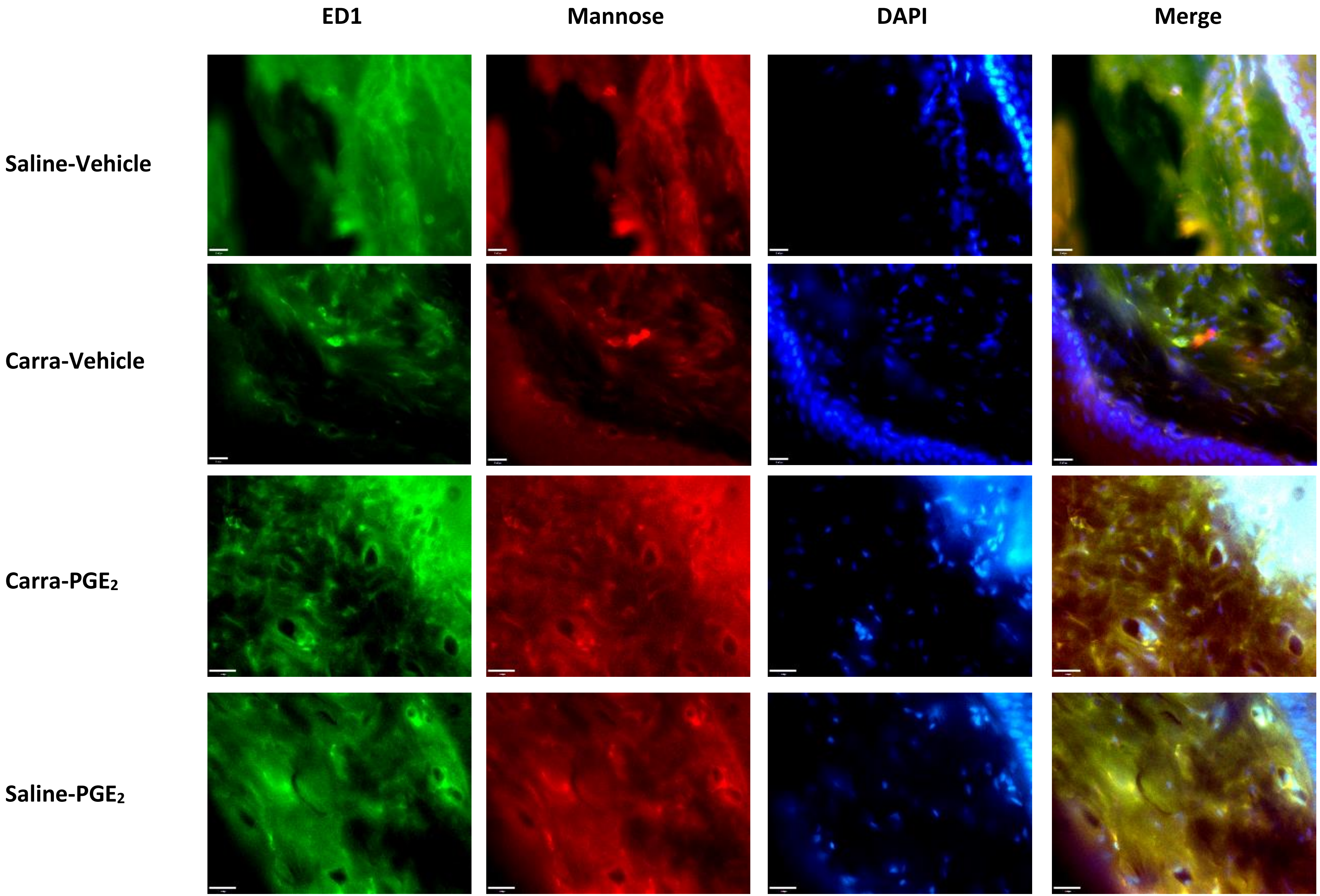


Figure 7.1 Plantar skin tissue collected at 7 days post-PGE_2/vehicle was stained for neutrophil elastase and DAPI to detect neutrophils. No neutrophils were observed in skin samples in any groups, quantification was not possible.



ED1
Mannose
DAPI
Merge
Saline-Vehicle
Carra-Vehicle
Carra-PGE2
Saline-PGE2

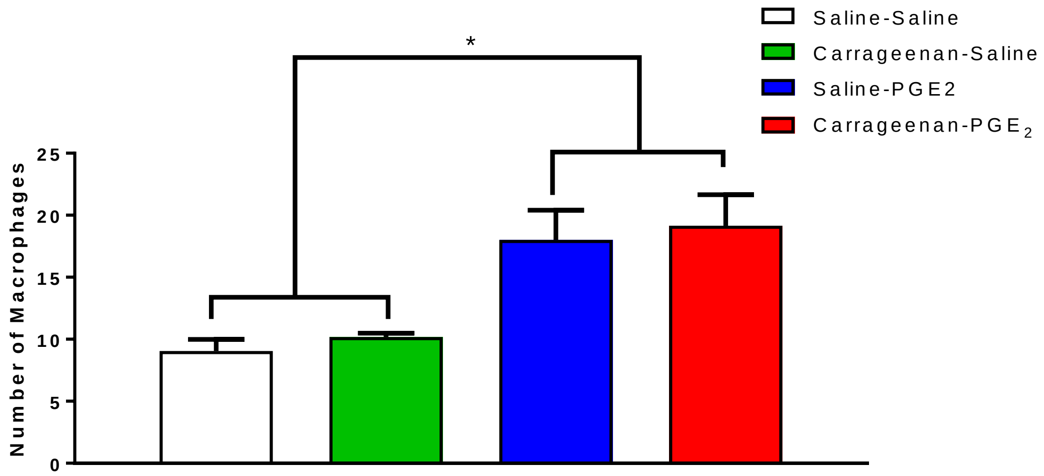


Figure 7.2 Plantar skin tissue collected at 7 days post-PGE_2/vehicle were stained for ED1, Mannose and DAPI to detect macrophages. There were significantly larger numbers of macrophages within skin samples that had previously received PGE_2 compared to vehicle treated rats. There were no differences in the number of macrophages between primed and unprimed animal or between saline-vehicle and carrageenan-vehicle rats. Data are mean ± SEM. Statistical analysis was carried out using a one-way ANOVA test with a Bonferroni post hoc test *$p<0.05$ (n=8 in both groups).

7.2.2 CD206 Mannose Staining of Dorsal Horn Microglia

Spinal cord slices were stained for mannose (CD206) and IBA1 to identify different populations of M2 activated microglia. Mannose positive cells were detected within the dorsal horn however there was no co-localisation with IBA1 demonstrating that microglia do not express CD206 (Figure 7.3).

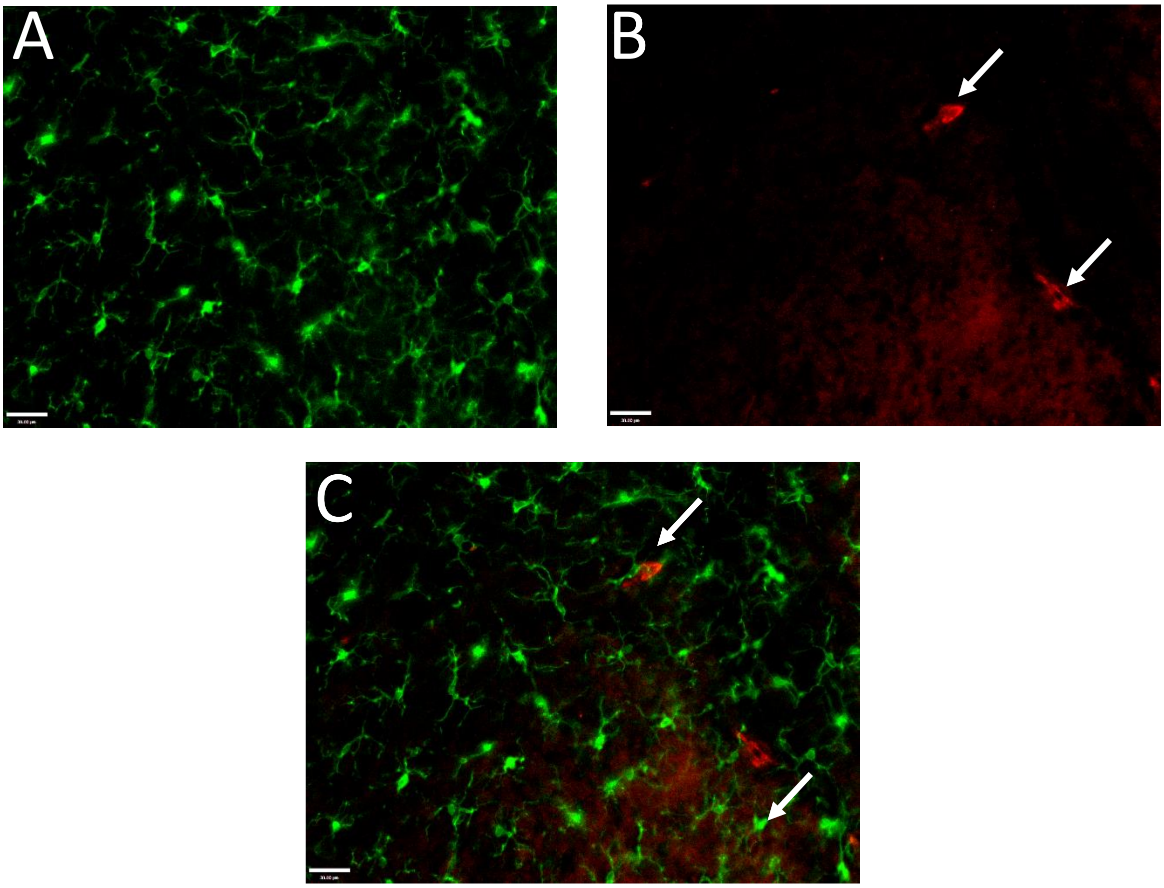


Figure 7.3 A: IBA1 staining of the dorsal horn of the spinal cord. **B:** Mannose staining of dorsal horn spinal cord. **C:** Merge of IBA1 and mannose stains. IBA1 does not co-localise with mannose positive cells.

7.2.3 Altered miRNA in Hyperalgesic Primed DRGs

L4, L5 and L6 DRGs with nerve roots removed were collected from primed and unprimed rats at 7 days post-PGE_2 analysis of miRNA levels were performed by microRNA-microarray by TORAY 3D gene. Altered miRNAs summarised in Table 7.1. There were several miRNAs that were found to be upregulated in primed animals 7 days post-PGE_2. miR-100 and miR-125b were taken forward for additional analysis.

miRNA	UPREGULATED	DOWNREGULATED
RNO-LET-7A-5P	x	
RNO-LET-7B-5P	x	
RNO-LET-7C-5P	x	
RNO-MIR-100-5P	x	
RNO-MIR-124-3P	x	
RNO-MIR-125B-5P	x	
RNO-MIR-134-5P	x	
RNO-MIR-138-5P	x	
RNO-MIR-139-3P		x
RNO-MIR-143-5P	x	
RNO-MIR-212-3P	x	
RNO-MIR-22-3P	x	
RNO-MIR-23A-3P	x	
RNO-MIR-23B-3P	x	
RNO-MIR-26A-5P	x	
RNO-MIR-291A-5P		x

Table 7.1 Summary of miRNA changes within the DRGs of rats with hyperalgesic priming induced chronic pain many miRNAs were found to be upregulated.

7.2.4 Predicted Changes in Genes Based off miRNA Analysis

To obtain information of potentially altered genes in hyperalgesic priming. Information was obtained from predicted genes that may be modulated by altered miRNAs using online database *miRGATE* and *Geneontology Pather Classification System* summarised in Table 7.2. These genes are not confirmed to be altered by the identified miRNA however they could be potential targets for further study.

MOLECULE NAME	ENTREZ GENE ID FOR RAT	PROTEIN NAME
ACHE	83817	Acetylcholinesterase
ADRA2A	25083	α2 Adrenoceptor
ADRB2	24176	β2 Adrenoceptor
ADRB3	25645	β3 Adrenoceptor
AR	24208	Androgen Receptor
ATP2C1	170699	ATPase, Ca++ transporting, type 2C, member 1
AXL	308444	AXL Receptor Tyrosine Kinase
BDNF	24225	Brain Derived Neurotrophic Factor
CA2	54231	Carbonic Anhydrase 2
CA3	54232	Carbonic Anhydrase 3
CA13	499566	Carbonic Anhydrase 13
CA5B	302669	Carbonic Anhydrase 5B, Mitochrondrial
CACNA1C	24239	Calcium Channel Subunit L-type Alpha 1C subunit
CACNA1H	114862	Calcium Channel Subunit T-type Alpha 1H subunit
CACNA2D1	25399	Calcium Channel Voltage Dependent α2/δ subunit 1
CACNA2D2	300992	Calcium Channel Voltage Dependent α2/δ subunit 2
CACNA2D3	306243	Calcium Channel Voltage Dependent α2/δ subunit 3
CACNB1		Calcium Channel Voltage Dependent β1 subunit
CACNB4	58942	Calcium Channel Voltage Dependent β4 subunit
CALCR	116506	Calcitonin Receptor
CHRNA5	25102	Nicotinic Cholinergic Receptor α 5
CNR1	25248	Cannabinoid Receptor 1
CNR2	57302	Cannabinoid Receptor 2
DAG1	114489	Dystroglycan 1
DICER1	299284	Dicer 1, Ribonuclease type III
EHMT2	361798	Euchromatic histone lysine N-methyltransferase 2
ESR1	24890	Estrogen Receptor 1
ESR2	25149	Estrogen Receptor 2
FLT4	114110	fms-related tyrosine kinase 4
GABRA1	29705	$GABA_A$ Receptor alpha 1
GABRA5	29707	$GABA_A$ Receptor alpha 5
GABRB3	24922	$GABA_A$ Receptor beta 3
GABRG3	79211	$GABA_A$ Receptor gamma 3
GAD2	24380	Glutamate Decarbonxylase 2
HDAC9	687001	Histone Deacetylase 9
HTR2C	25187	5-HT receptor 2C
HYAL1	367166	Hyaluronoglucosaminidase 1
HYAL4	404783	Hyaluronoglucosaminidase 4
IGF1	24482	Insulin Growth Factor 1
IL6	24498	Interleukin 6
IL10	25325	Interleukin 10
IL1RL1	25556	Interleukin 1 receptor-like 1
IL6R	24499	Interleukin 6 Receptor
IL6ST	25205	Interleukin 6 Signal Transducer

KCNA4	25469	Potassium channel, voltage gated shaker related subfamily A, member 4
KCNA7	365241	Potassium channel, voltage gated shaker related subfamily A, member 7
KCND3	65195	Potassium channel, voltage-gated Shal-related subfamily D, member 3
KCNIP3	65199	Kv channel interacting protein 3, calsenilin
KCNJ2	29712	Potassium channel, inwardly rectifying subfamily J, member 2
KCNJ10	29718	Potassium channel, inwardly rectifying subfamily J, member 10
KCNJ12	117052	Potassium channel, inwardly rectifying subfamily J, member 12
KDR	25589	Kinase Insert Domain Receptor
MAPK8	116554	Mitogen-activated protein kinase 8
MECP2	29386	Methyl CpG binding Protein 2
MET	24553	MET proto-oncogene receptor tyrosine kinase
MTHFR	362657	methylenetetrahydrofolate reductase (NAD(P)H)
NGF	310738	Neuronal Growth Factor
NR3C1	24413	Nuclear receptor subfamily 3, group C, member 1
NTRK2	25054	Neurotrophic tyrosine kinase receptor type 2
PDE11A	140928	Phosphodiesterase 11A
PDE4A	25638	Phosphodiesterase 4A
PDE4B	24626	Phosphodiesterase 4B
PDE7A	81744	Phosphodiesterase 7A
PDE7B	140929	Phosphodiesterase 7B
PDE8A	308776	Phosphodiesterase 8A
PTGS2	29527	Prostoglandin-endoperoxide synthase 2
RET	24716	Ret Proto-oncogene
SCN1B	29686	sodium channel, voltage-gated, type I, beta subunit
SCN2B	25349	sodium channel, voltage-gated, type II, beta subunit
SCN4A	25722	sodium channel, voltage-gated, type IV, alpha subunit
SLC12A2	83629	solute carrier family 12 (sodium/potassium/chloride transporter), member 2
SLC4A4	84484	solute carrier family 4, sodium bicarbonate cotransporter, member 4
SLC6A2	83511	solute carrier family 6 (neurotransmitter transporter), member 2
SNRPD3	687711	small nuclear ribonucleoprotein D3
TEK	89804	TEK tyrosine kinase, endothelial
TNFSF11	117516	tumor necrosis factor (ligand) superfamily, member 11
TRPV1	83810	transient receptor potential cation channel, subfamily V, member 1
TUBA8	500377	tubulin, α8
TUBG1	252921	tubulin, γ1
VAMP2	24803	vesicle-associated membrane protein 2
VDR	24873	vitamin D (1,25- dihydroxyvitamin D3) receptor

Table 7.2 Summary of predicted genes that could be potentially altered by identified miRNA.

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