THE UNIVERSITY *of* EDINBURGH

Spinal cord plasticity in peripheral inflammatory pain

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Declaration

This thesis has been composed by the candidate, Allen Charles Dickie, and the experiments were performed by the author. It is clearly stated in the text where experiments involved collaboration with others. This work has not been submitted for any other degree or professional qualification except as specified.

Signed:
Date: 23/5/2013

Abstract

Inflammatory pain is a debilitating condition that can occur following tissue injury or inflammation and results in touch evoked pain (allodynia), exaggerated pain (hyperalgesia) and spontaneous pain, yet the neural plasticity underlying these symptoms is not fully understood. However, it is known that lamina I neurokinin 1 receptor expressing (NK1R+) spinal cord output neurons are crucial for the manifestation of inflammatory pain. There is also evidence that the afferent input to and the postsynaptic response of these neurons may be altered in inflammatory pain, which could be relevant for inflammatory pain hypersensitivity. Therefore, the aim of this thesis was to study inflammatory pain spinal plasticity mechanisms by investigating the synaptic input to lamina I NK1R+ neurons. In *ex vivo* spinal cord and dorsal root preparations from the rat, electrophysiological techniques were used to assess inflammation-induced changes in and pharmacological manipulation of the primary afferent drive to lamina I NK1R+ neurons.

The excitatory input to lamina I NK1R+ neurons was examined and it was found that inflammation did not alter the relative distribution of the type of primary afferent input received and did not potentiate monosynaptic Aδ- or monosynaptic C-fibre input, the predominant input to these neurons. Spontaneous excitatory input was significantly elevated in the subset of neurons that received monosynaptic Aδ-fibre input only, regardless of inflammation.

It has recently been shown that the chemerin receptor 23 (ChemR23) represents a novel inflammatory pain target, whereby ChemR23 agonists can decrease inflammatory pain hypersensitivity, by a mechanism that involves the attenuation of potentiated spinal cord responses. This study has found that the ChemR23 agonist, chemerin, attenuated capsaicin potentiation of excitatory input to lamina I NK1R+ neurons and significantly reduced monosynaptic C-fibre input to a subset of these neurons in inflammatory pain. However, chemerin was without effect in non-potentiated conditions.

In exploring potential inflammatory pain spinal plasticity mechanisms, I have investigated a phenomenon called activity-dependent slowing (ADS), whereby repetitive stimulation of C-fibres at frequencies of 1Hz or above results in a progressive slowing of action potential conduction velocity, which manifests as a progressive increase in response latency. This is proposed to limit nociceptive input to the spinal cord, thus regulating plasticity. Results demonstrate that inflammation

significantly attenuated C-fibre ADS in isolated dorsal roots. Furthermore, ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons was significantly reduced in inflammatory pain, which could facilitate nociceptive drive to these key spinal cord output neurons and promote inflammatory pain spinal cord plasticity.

In conclusion, the major novel findings of this thesis are firstly, that chemerin can attenuate primary afferent input to lamina I NK1R+ neurons in potentiated conditions, which supports recent studies that suggest ChemR23 is a potential target for the development of new analgesics. Secondly, it was discovered that ADS in monosynaptic C-fibre inputs to lamina I NK1R+ neurons is altered in inflammatory pain, which could be relevant for inflammatory pain spinal plasticity. The findings presented in this thesis could contribute to the development of novel inflammatory pain treatments.

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Contents

List of Figures

List of Tables

Acronyms

$\boldsymbol{E_{anion}}$ anion reversal potential.

$\boldsymbol{I_h}$ hyperpolarisation-activated current.

17R-RvD1 17(R)-resolvin D1.

19-pf-RvE1 19-(p-fluorophenoxy)-RvE1.

5-HT 5-hydroxytryptamine.

5-HT3R 5-HT3 receptor.

ADS activity-dependent slowing.

AMPA α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid.

AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor.

ASIC acid-sensitive ion channel.

ATP adenosine triphosphate.

AUC area under the curve.

BDNF brain-derived neurotrophic factor.

C-LTMR unmyelinated low threshold mechanoreceptor.

CaMKII calcium/calmodulin-dependent protein kinase II.

cAMP cyclic adenosine monophosphate.

CAP compound action potential.

CCI chronic constriction injury.

CCRL2 chemokine (C-C motif) receptor-like 2.

CFA complete Freund's adjuvant.

CGRP calcitonin gene related peptide.

ChemR23 chemerin receptor 23.

ChemR23+ ChemR23 expressing.

CMKLR1 chemokine receptor-like 1.

CNS central nervous system.

COX cyclooxygenase.

D-APV D-(-)-2-Amino-5-phosphonopentanoic acid.

DRG dorsal root ganglia.

eEPSC evoked excitatory postsynaptic current.

eEPSP evoked excitatory postsynaptic potential.

EGTA ethylene glycol tetraacetic acid.

eIPSC evoked inhibitory postsynaptic current.

EPSP excitatory postsynaptic potential.

ERK extracellular signal-regulated kinase.

fMRI functional magnetic resonance imaging.

GABA γ-aminobutyric acid.

GABA$_A$R GABA$_A$ receptor.

GAD glutamic acid decarboxylase.

GFAP glial fibrillary acidic protein.

GFP green fluorescent protein.

GLT-1 glutamate transporter-1.

GlyR glycine receptor.

GPCR G-protein coupled receptor.

GPR1 G protein-coupled receptor 1.

HCN hyperpolarization-activated cyclic nucleotide-gated.

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

HFS high-frequency stimulation.

IASP International Association for the Study of Pain.

IB4 isolectin-B4.

IBA-1 ionized calcium binding adaptor molecule 1.

IL-1β interleukin-1β.

IL-6 interleukin-6.

IPSC inhibitory postsynaptic current.

ir-DIC infrared-differential interference contrast.

JSTX synthetic Joro spider toxin.

K$_v$ voltage-gated potassium channel.

KAR kainate receptor.

KCC2 potassium-chloride exporter.

LFS low-frequency stimulation.

LTD long-term depression.

LTP long-term potentiation.

MEK mitogen-activated protein kinase kinase.

mEPSC miniature excitatory postsynaptic current.

mGluR metabotropic glutamate receptor.

mIPSC miniature inhibitory postsynaptic current.

Na$_v$ voltage-gated sodium channel.

NBQX 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline.

NF200 neurofilament 200.

NGF nerve growth factor.

NK1R neurokinin 1 receptor.

NK1R+ neurokinin 1 receptor expressing.

NMDA N-methyl-D-aspartate.

NMDAR N-methyl-D-aspartate receptor.

NSAID non-steroidal anti-inflammatory drug.

PAG periaquducal gray matter.

PB parabrachial area.

PGE_2 prostaglandin E_2.

PKA protein kinase A.

PKC protein kinase C.

PKCγ protein kinase Cγ.

PPD paired-pulse depression.

PPR paired-pulse ratio.

PTX pertussis toxin.

QX-314-Cl 2(triethylamino)-N-(2,6-dimethylphenyl) acetamine chloride.

RvD1 resolvin D1.

RvD2 resolvin D2.

RvE1 resolvin E1.

RVM rostral ventromedial medulla.

SEM standard error of the mean.

sEPSC spontaneous excitatory postsynaptic current.

SIN sciatic inflammatory neuropathy.

SNI spared nerve injury.

SNL spinal nerve ligation.

SP substance P.

SP-SAP substance P – saporin.

SPME substance P methy ester.

TG trigeminal ganglia.

TMR-SP tetramethylrhodamine conjugated substance P.

TMR-SP+ TMR-SP labelled.

TNF-α tumor necrosis factor-α.

TrkB tyrosine receptor kinase B.

TRP transient receptor potential.

TRPA1 transient receptor potential subtype ankyryn 1.

TRPC1 transient receptor potential subtype canonical 1.

TRPC6 transient receptor potential subtype canonical 6.

TRPM8 transient receptor potential subtype melastatin 8.

TRPV1 transient receptor potential subtype vanilloid 1.

TRPV1+ TRPV1 expressing.

TRPV2 transient receptor potential subtype vanilloid 2.

TRPV3 transient receptor potential subtype vanilloid 3.

TRPV4 transient receptor potential subtype vanilloid 4.

TTX tetrodotoxin.

VGCC voltage-gated calcium channel.

VGLUT2 vesicular glutamate transporter 2.

VGLUT3 vesicular glutamate transporter 3.

WDR wide dynamic range.

Chapter 1

General Introduction

1.1 Pain and nociception

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (IASP Taskforce on Taxonomy 1994). Nociception is the term given to the process by which noxious stimuli, including thermal, mechanical and chemical stimuli, are detected and transduced into neural signals (Sherrington 1906). Acute pain resulting from noxious stimuli that causes, or has the potential to cause, actual tissue damage can be considered to be an adaptive sensation, in that it can elicit reflex withdrawal responses, thus removing the tissue from the noxious stimuli, while the associated unpleasant sensation is instrumental in learning to avoid potentially harmful situations (Latremoliere & Woolf 2009, Scholz & Woolf 2002).

Chronic pain is defined by The British Pain Society as "continuous, long-term pain of more than 12 weeks or after the time that healing would have been thought to have occurred in pain after trauma or surgery" (British Pain Society 2013). Chronic pain can be broadly divided into inflammatory pain, which results from tissue injury or inflammation, and neuropathic pain, which arises following injury to the peripheral and/or central nervous system. Both inflammatory and neuropathic pain features the characteristic symptoms of spontaneous pain, hyperalgesia, where responses to noxious stimuli are exaggerated and allodynia, where painful sensations result from normally innocuous stimuli such as light touch (figure 1.1). Following tissue injury, these symptoms can play an adaptive role, in that they can evoke behaviours that will protect the site of injury and aid healing by preventing further harm and/or infection. However, these advantageous properties are lost if symptoms persist after injured

tissue has healed, which can result in detrimental consequences for patients quality of life (Basbaum et al. 2009, Latremoliere & Woolf 2009, Scholz & Woolf 2002, Torsney & Fleetwood-Walker 2012).

A 2003 survey of over 46,000 adults, from 15 European countries and Israel, found that 19% of responders suffered from chronic pain, with the average duration of chronic pain being 7 years (Breivik et al. 2006). Furthermore, 64% of chronic pain suffers described the treatment of their pain as inadequate at times. In addition to these consequences on patients quality of life, chronic pain imposes a substantial financial burden upon society, with the UK costs for back pain in 1998 being estimated at £6.65 billion (Maniadakis & Gray 2000), while a more recent study has estimated the back pain costs to Germany at €48.96 billion (Wenig et al. 2009). Many analgesics that are currently used for the treatment of chronic pain lack efficacy, exhibit undesirable side effects or have the potential for abuse, therefore one of the key challenges in pain research is the development of new efficacious drugs that lack these disadvantages (Scholz & Woolf 2002, Woolf 2010).

This chapter will provide an overview of the neurobiology of pain perception and the mechanisms underlying chronic pain. This will include describing the peripheral and central organisation of the somatosensory nervous system, the changes that occur within peripheral ('peripheral sensitisation') and central ('central sensitisation') sensory circuits following injury, that can give rise to chronic pain, with a particular emphasis on central sensitisation. The main focus of this chapter will be the involvement of lamina I neurokinin 1 receptor expressing (NK1R+) projection neurons in central sensitisation mechanisms and their crucial role in chronic pain conditions. This will provide the background information for the aims of this thesis, which are detailed at the end of this chapter.

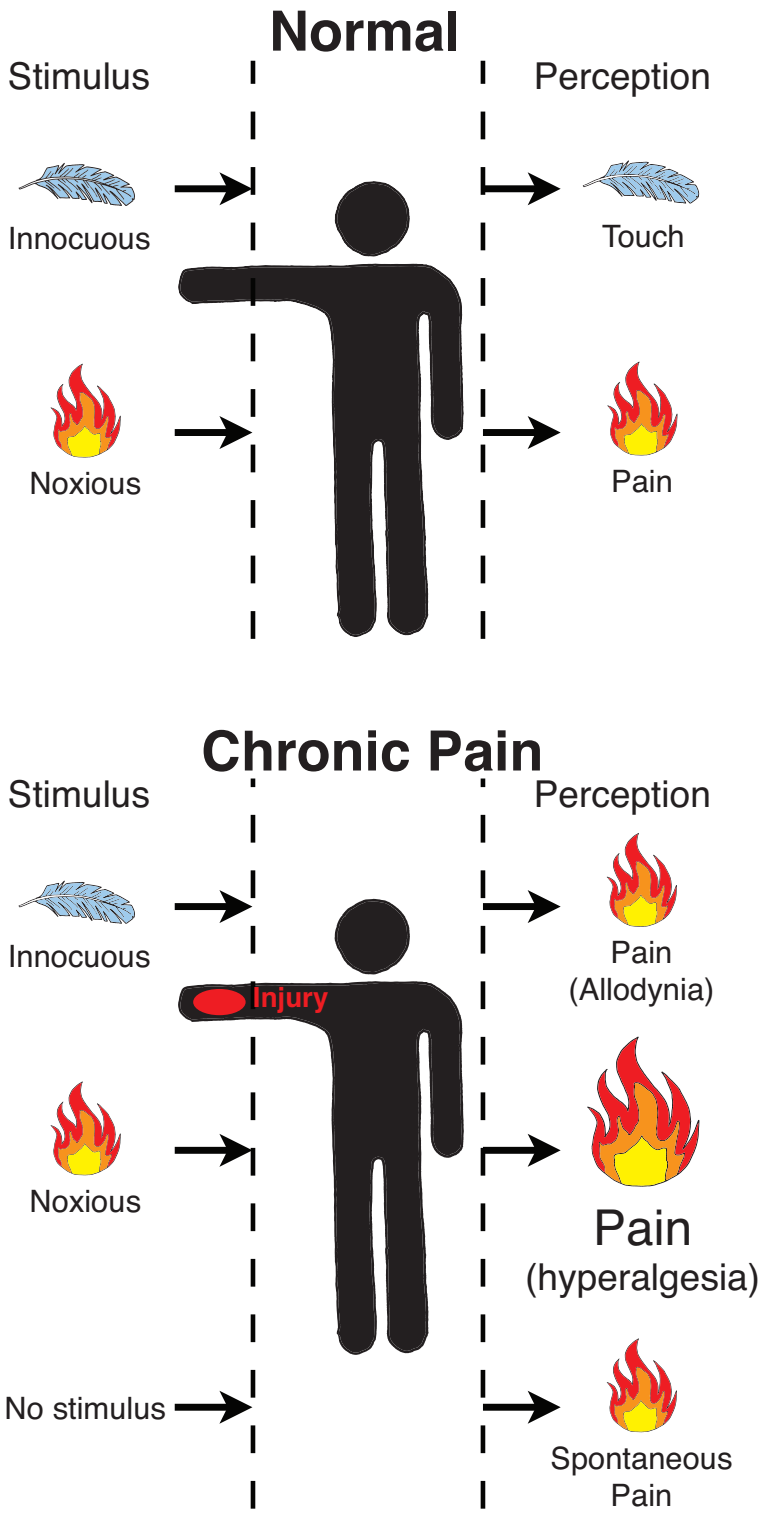

Figure 1.1: Perception of sensory stimuli. Under normal conditions innocuous stimuli are perceived as touch, while noxious stimuli give rise to the unpleasant sensation of pain. However, chronic pain conditions, which result from injury, are characterised by the symptoms of allodynia (touch-evoked pain), hyperalgesia (exaggerated pain) and spontaneous pain.

1.2 Primary afferent neurons

Primary sensory neurons of the somatosensory nervous system detect, transduce and transmit sensory information from the peripheral and visceral tissues to the dorsal horn of the spinal cord. The cell bodies of primary afferents that innervate the body are found within the dorsal root ganglia (DRG), while those innervating the face are contained within the trigeminal ganglia (TG). Primary sensory neurons are known as pseudo-unipolar neurons, in that the axons emerge from the DRG and bifurcate to form a peripheral branch that innervates tissue and viscera and a central branch (dorsal root) which forms synapses with second order neurons within the dorsal horn (Basbaum et al. 2009, Smith & Lewin 2009). Synaptic transmission between the primary afferents and dorsal horn neurons is excitatory. The excitatory neurotransmitter glutamate, is released from the central terminals of primary sensory neurons and acts upon postsynaptic ionotropic (AMPA, NMDA, kainate) and metabotropic (mGluR) glutamate receptors present on second order neurons (Larsson 2009, Larsson & Broman 2011, Latremoliere & Woolf 2009, Liu & Salter 2010). The primary afferent neurons are divided into three groups, based upon anatomical and functional properties (table 1.1), Aβ-fibres, Aδ-fibres and C-fibres and these can be further subdivided into nociceptors or non-nociceptors (Smith & Lewin 2009). Furthermore, Aβ-, Aδ- and C-fibres are known to display distinct termination patterns in the dorsal horn of the spinal cord (Todd 2010) (figure 1.2).

Fibre	Myelination	Diameter (μm)	Conduction velocity (m/s)
Aβ	myelinated	>10	5.5 – 8.8
Aδ	myelinated	2 – 6	1.6 – 5.5
C	unmyelinated	0.4 – 1.2	0.5 – 0.9

Table 1.1: Characteristics of primary afferent fibres. Diameters taken from Millan 1999. Conduction velocity values, recorded in juvenille rats, taken from Nakatsuka et al. 2000

1.2.1 Aβ-fibres

Aβ-fibres are thick diameter, heavily myelinated fibres and as such display the fastest conduction velocity of the primary afferent fibres (5.5–8.8m/s) (Nakatsuka et al. 2000). The peripheral terminals of Aβ-fibres are associated with specialised sensory complexes such as Merkel's discs or with hair follicles (Smith & Lewin 2009). Most Aβ-fibres convey low-threshold innocuous stimuli such as brush and light touch, although there is evidence that some Aβ-fibres may be involved in the transmission of noxious information (Djouhri & Lawson 2004). The central terminals of Aβ-fibres

are predominantly found in lamina II_i-V in the dorsal horn of the spinal cord (Todd 2010) (figure 1.2).

1.2.2 Aδ-fibres

Aδ-fibres are thinly myelinated, medium diameter fibres with conduction velocities ranging between 1.6–5.5m/s (Nakatsuka et al. 2000). While these fibres are myelinated, they terminate in the skin as free nerve endings, following the loss of their myelin sheath (Smith & Lewin 2009). The majority of Aδ-fibres are classified as nociceptors and respond to intense (high-threshold) mechanical, thermal and chemical stimuli. A subset of Aδ-fibres transmit non-nociceptive information resulting from the slow movement of hair and are termed D-hair afferents (Lewin & Moshourab 2004). Aδ-fibre nociceptors primarily terminate in the lamina I region of the dorsal horn, while non-nociceptive D-hair afferents terminate in lamina II_i/III (Todd 2010) (figure 1.2).

1.2.3 C-fibres

C-fibres are small diameter, unmyelinated fibres and as such display the slowest conduction velocity of the primary sensory afferents (0.5–0.9m/s) (Nakatsuka et al. 2000). The peripheral branch of C-fibres terminate in the skin as free nerve endings (Smith & Lewin 2009). As with Aδ-fibres, the majority of C-fibres can be categorised as nociceptors, on the basis that they are activated by intense (high-threshold) thermal, mechanical or chemical stimuli. C-fibres can respond to a single stimuli, e.g. heat only, or to multiple stimuli, with the latter being termed polymodal, a classification to which the majority of C-fibres fall into (Basbaum et al. 2009). It should be recognised that not all C-fibres are nociceptors. A group of unmyelinated low threshold mechanoreceptors (C-LTMRs) have been shown to mediate pleasant touch (Löken et al. 2009, Olausson et al. 2008), while C-LTMRs which express the vesicular glutamate transporter 3 (VGLUT3) may play a role in mediating mechanical allodynia in inflammatory and neuropathic pain conditions (Seal et al. 2009). Some C-fibres are also known to response to innocuous thermal stimuli only (Hunt & Mantyh 2001).

C-fibres can also be classified on a neurochemical basis into peptidergic and non-peptidergic C-fibres. Peptidergic C-fibres contain the peptides, substance P (SP) and calcitonin gene related peptide (CGRP), while non-peptidergic neurons can be identified by their ability to bind isolectin-B4 (IB4) (Basbaum et al. 2009, Woolf &

Ma 2007). The central terminals of C-fibres are largely restricted to lamina I/II, with peptidergic C-fibres terminating in lamina I/II$_o$ and most non-peptidergic in lamina II (Todd 2010) (figure 1.2).

1.3 Transduction of sensory information by primary afferent fibres

A plethora of ion channels and receptors, which are expressed on primary afferent fibres, are involved in the peripheral transduction of sensory information and the generation of action potentials (Hu, Milenkovic & Lewin 2006, Smith & Lewin 2009, Woolf & Ma 2007). Transduction occurs when stimuli activate ion channels or receptors present on the peripheral terminals of nociceptors, which drives the influx of Na^+ and Ca^{2+} through ion channels, leading to depolarisation of the terminals, which if the depolarisation is of large enough amplitude and duration will result in action potential firing and propagation along the afferent fibre towards the central nervous system (CNS).

1.3.1 Transducer receptors / channels

Transduction of thermal stimuli

Perhaps the best known transducer receptor for thermal stimuli is the noxious heat (>43°C) / capsaicin sensitive transient receptor potential subtype vanilloid 1 (TRPV1) channel, as first identified by Caterina et al. (1997). The TRPV1 channel is essential for the detection and transduction of noxious thermal stimuli, in that knockout mice lacking this channel show deficiencies in their responses to acute intense heat and fail to develop thermal hypersensitivity in the complete Freund's adjuvant (CFA) inflammatory pain model, but display normal thermal hypersensitivity following nerve injury (Caterina et al. 2000). However, Davis et al. (2000) found that the responses to acute noxious thermal stimuli were not altered TRPV1 knockout mice, although they did fail to develop thermal hypersensitivity following carrageenan hindpaw injection.

Subsequently a number of other transient receptor potential (TRP) channels have been identified that play a role in the detection of various temperatures including; transient receptor potential subtype ankyryn 1 (TRPA1) (<17°C), transient receptor potential

subtype vanilloid 2 (TRPV2) (>52°C), transient receptor potential subtype vanilloid 3 (TRPV3) (33-39°C), transient receptor potential subtype vanilloid 4 (TRPV4) (27-34°C) and transient receptor potential subtype melastatin 8 (TRPM8) (8-28°C) (Wu et al. 2010).

Transduction of mechanical stimuli

The precise channel(s) responsible for the transduction of mechanical stimuli is currently not clear (Hu, Milenkovic & Lewin 2006, Woolf & Ma 2007), however a number of candidates have been suggested including; TRPA1 (Kwan et al. 2006, 2009), TRPV4 (Alessandri-Haber et al. 2006), acid-sensitive ion channels (ASICs) (Price et al. 2001), the stretch-activated channels, transient receptor potential subtype canonical 1 (TRPC1) and transient receptor potential subtype canonical 6 (TRPC6) (Alessandri-Haber et al. 2009) and recently Piezo channels (Coste et al. 2010).

Transduction of chemical stimuli

Chemical stimuli can be detected and transduced by a number of TRP channels present on the peripheral terminals of nociceptors, including TRPV1 (capsaicin, H^+), TRPA1 (mustard oil, cinnamaldehyde, allicin, wasabi, acrolein, cadmium) and TRPM8 (menthol, icilin, eucalyptol) (Miura et al. 2013, Wu et al. 2010). Interestingly TRPA1 can also be activated by formalin and is therefore thought to mediate nociceptive responses in the formalin pain test (McNamara et al. 2007). ASICs are also involved in the detection of chemical (H^+) stimuli (White et al. 2010).

1.3.2 Voltage-gated sodium channels

Voltage-gated sodium channel (Na_v) channels allow the rapid influx of Na^+ into neurons, which drives the depolarising upstroke of action potentials and as such they play a crucial role in the initiation and propagation of action potentials (Catterall 2000, Catterall et al. 2005). Na_v channels are therefore essential for the transmission of sensory information, by primary sensory neurons, from the periphery to the dorsal horn of the spinal cord (Gold & Gebhart 2010, Liu & Wood 2011). Na_v channels are heteromultimers, made up of an α-subunit and β-subunits. The larger α-subunit is essential and sufficient to produce a functional channel, while the smaller auxiliary β-subunits confer different functional attributes to the channels, such as the

biophysical properties and channel trafficking and anchoring (Catterall 2000, Catterall et al. 2005). There are a total of nine different Na_v subtypes, named $Na_v1.1$–$Na_v1.9$. Adult rodent DRG neurons are known to express up to five Na_v channel subtypes, $Na_v1.1$, $Na_v1.6$–$Na_v1.9$ (Dib-Hajj et al. 2009, 2010). $Na_v1.3$ is embryonically expressed in DRG neurons, but undetectable in the naïve adult (Waxman et al. 1994). The expression of $Na_v1.7$–$Na_v1.9$ is largely restricted to the peripheral nervous system. $Na_v1.8$ is widely considered to be predominantly restricted to C-fibre nociceptors (Abrahamsen et al. 2008, Akopian et al. 1996, Amaya et al. 2000, Novakovic et al. 1998), however recent evidence suggests that there is also a large population of $Na_v1.8$-expressing A-fibres, that are likely to include myelinated low threshold mechanoreceptors (Shields et al. 2012).

A number of genetic studies have highlighted the importance of Na_v channels in pain. Loss of function mutations in the *SCN9A* gene, which encodes $Na_v1.7$, are known to result in congenital insensitivity to pain in humans (Ahmad et al. 2007, Cox et al. 2006, Drenth & Waxman 2007), while gain of function mutations are known to underly the painful conditions; primary erythermalgia and paroxysmal extreme pain disorder (Drenth & Waxman 2007, Fertleman et al. 2006, Yang et al. 2004). Furthermore, nociceptor-specific $Na_v1.7$ knockout mice show a reduction in sensitivity towards acute noxious mechanical and noxious thermal stimuli and fail to develop hypersensitivity in inflammatory pain models (Nassar et al. 2004). Similarly, $Na_v1.8$ global knockout mice display altered thresholds to noxious mechanical and noxious thermal stimuli and the development of inflammatory pain hypersensitivity is delayed (Akopian et al. 1999). $Na_v1.9$ global knockout mice, while exhibiting normal responses to acute noxious mechanical or noxious thermal stimuli, show deficiencies in the development of thermal, but not mechanical, hypersensitivity in inflammatory pain models. (Amaya et al. 2006, Priest et al. 2005). However, the development of neuropathic pain hypersensitivity in these $Na_v1.9$ knockout mice is no different from controls.

1.4 Peripheral sensitisation

In inflammatory pain, tissue injury or inflammation can result in an increase in the excitability of the peripheral endings of primary afferent fibres, with this hypersensitivity being termed peripheral sensitisation (Latremoliere & Woolf 2009, Woolf & Ma 2007). Peripheral sensitisation is driven by the release of a number of inflammatory mediators from damaged tissue and/or immune cells, including;

bradykinin, prostaglandins, adenosine triphosphate (ATP), 5-hydroxytryptamine (5-HT), tumor necrosis factor-α (TNF-α), nerve growth factor (NGF), H^+, interleukin-1β (IL-1β), interleukin-6 (IL-6) and proinflammatory cytokines (Gold & Gebhart 2010, Nicol & Vasko 2007, Oh et al. 2001, Sommer & Kress 2004, Stein et al. 2009, Woolf & Ma 2007). These mediators act on their corresponding receptors, that are expressed on primary afferents, to directly activate or modulate the activity of ion-channels and receptors that play a crucial role in the transduction of nociceptive signals (Basbaum et al. 2009, Bhave & Gereau 2004, Binshtok et al. 2008, Cheng & Ji 2008, Hucho & Levine 2007, Nicol & Vasko 2007). These changes can occur relatively fast, with timescales of minutes, through the activation of intracellular cascades, including protein kinase A (PKA), protein kinase C (PKC), extracellular signal-regulated kinase (ERK) and p38, which in particular can sensitise Na_v and TRP channels via their phosphorylation (Basbaum et al. 2009, Cheng & Ji 2008, Jin & Gereau 2006, Varga et al. 2006, Woolf & Ma 2007). These rapid changes allow the somatosensory nervous system to dynamically respond to injury. Longer-term changes in excitability can be driven by changes in gene expression, which can increase the expression of key transducers, such as TRP and Na_v channels and of pronociceptive neuropeptides, such as SP (Basbaum et al. 2009, Ji et al. 2002, Woolf & Ma 2007). Ultimately these changes act to increase the excitability of the primary afferent fibres through reducing activation thresholds, increasing spontaneous activity and enhancing evoked responses. This increase in excitability and spontaneous activity in primary afferent fibres is thought to be a crucial driver of central sensitisation. Peripheral sensitisation is thought to underlie thermal hyperalgesia and hypersensitivity in the zone of injured tissue (primary hyperalgesia), with central sensitisation being largely responsible for mechanical hypersensitivity and hypersensitivity in uninjured tissue surrounding the zone of injured tissue (secondary hyperalgesia) (Lewin et al. 2004).

1.5 Dorsal horn organisation

The dorsal horn of the spinal cord has been subdivided into a number of cell layers, denoted lamina I through to VI, with laminae VII to X comprising the ventral horn (Rexed 1952) (figure 1.2). The dorsal horn is broadly divided into the superficial laminae, which is comprised of laminae I and II and the deep lamina, containing laminae III to VI.

The superficial lamina predominantly receives input from nociceptive primary

afferent Aδ- and C-fibres and as such plays a crucial role in the central processing of nociceptive information (Todd 2010). Lamina I, alternatively known as the marginal layer, is composed of populations of interneurons and projection neurons. The majority (∼80%) of these lamina I projection neurons express the receptor for SP, the neurokinin 1 receptor (NK1R) (Al-Khater et al. 2008, Marshall et al. 1996, Spike et al. 2003, Todd 2010). These lamina I NK1R+ projection neurons play an essential role in the manifestation of chronic pain (Nichols et al. 1999) and are discussed in greater depth later in this chapter. Lamina II, which is subdivided into inner (II_i) and outer (II_o) layers, principally contains excitatory (glutamatergic) and inhibitory (γ-aminobutyric acid (GABA)ergic and/or glycinergic) interneurons. The termination of primary afferent input to the superficial dorsal horn shows a distinct pattern (Todd 2010) (figure 1.2). Peptidergic C-fibres terminate largely in lamina I and II_o, while the majority of non-peptidergic C-fibres target lamina II (Lorenzo et al. 2008), although a small population of non-peptidergic C-fibres form synapses in lamina I (Saeed & Ribeiro-da Silva 2012). Aδ-fibre nociceptors predominantly terminate in lamina I (Light & Perl 1979), whereas a population of peptidergic Aδ-fibres target lamina I and II_o (Lawson et al. 1997). Aδ- and some Aβ-fibre hair follicle afferents have been shown to target lamina II_i (Brown et al. 1981, Light & Perl 1979).

The deep dorsal horn contains both interneurons and projection neurons, with interneurons accounting for the majority of lamina III neurons (Todd 2010). Of the lamina III inhibitory interneurons, GABA immunoreactive neurons account for ∼40% of all cells (Polgár et al. 2003). While glycine is found in many lamina III neurons, this is typically in GABAergic neurons and very few inhibitory interneurons contain glycine only (Polgár et al. 2003, Todd & Sullivan 1990). There is a lack of a reliable way in which to identify excitatory interneurons (Todd 2010), however many lamina III, as well as lamina I/II, excitatory interneurons are know to express vesicular glutamate transporter 2 (VGLUT2) (Maxwell et al. 2007, Todd et al. 2003, Yasaka et al. 2010). There are also a subgroup of protein kinase Cγ (PKCγ) expressing excitatory interneurons that are found in lamina III, and also lamina II_i (Polgár et al. 1999), some of which receive afferent input from myelinated low-threshold mechano-sensitive fibres (Hughes et al. 2003, Neumann et al. 2008). The number of projection neurons in the deep dorsal horn, which are found throughout lamina III-VI, are much less compared to lamina I. Of these projection neurons there are two subgroups that expresses the NK1R, one of which features large dendrites, that reach as far as lamina I (Bleazard et al. 1994, Littlewood et al. 1995, Naim et al. 1997, Sakamoto et al. 1999). These lamina III-IV NK1R+ neurons are targeted by SP-containing C-fibres (Naim et al. 1997, Sakamoto et al. 1999) and are

know to be activated by noxious stimuli (Doyle & Hunt 1999, Mantyh et al. 1995, Polgár et al. 2007). The other subgroup of lamina III-IV NK1R+ neurons, have a smaller dendritic tree, although their function is unclear as they do not respond to noxious stimuli (Doyle & Hunt 1999, Mantyh et al. 1995). Torsney & MacDermott (2006) demonstrated that lamina III NK1R+ neurons predominantly receive monosynaptic Aβ-fibre input, which likely reflects this population of neurons with restricted dendritic trees. The deep dorsal horn is largely targeted by both Aβ-fibres, which transmit information relating to innocuous stimuli and Aδ-fibre hair follicle afferents (Brown et al. 1981, Light & Perl 1979) (figure 1.2.)

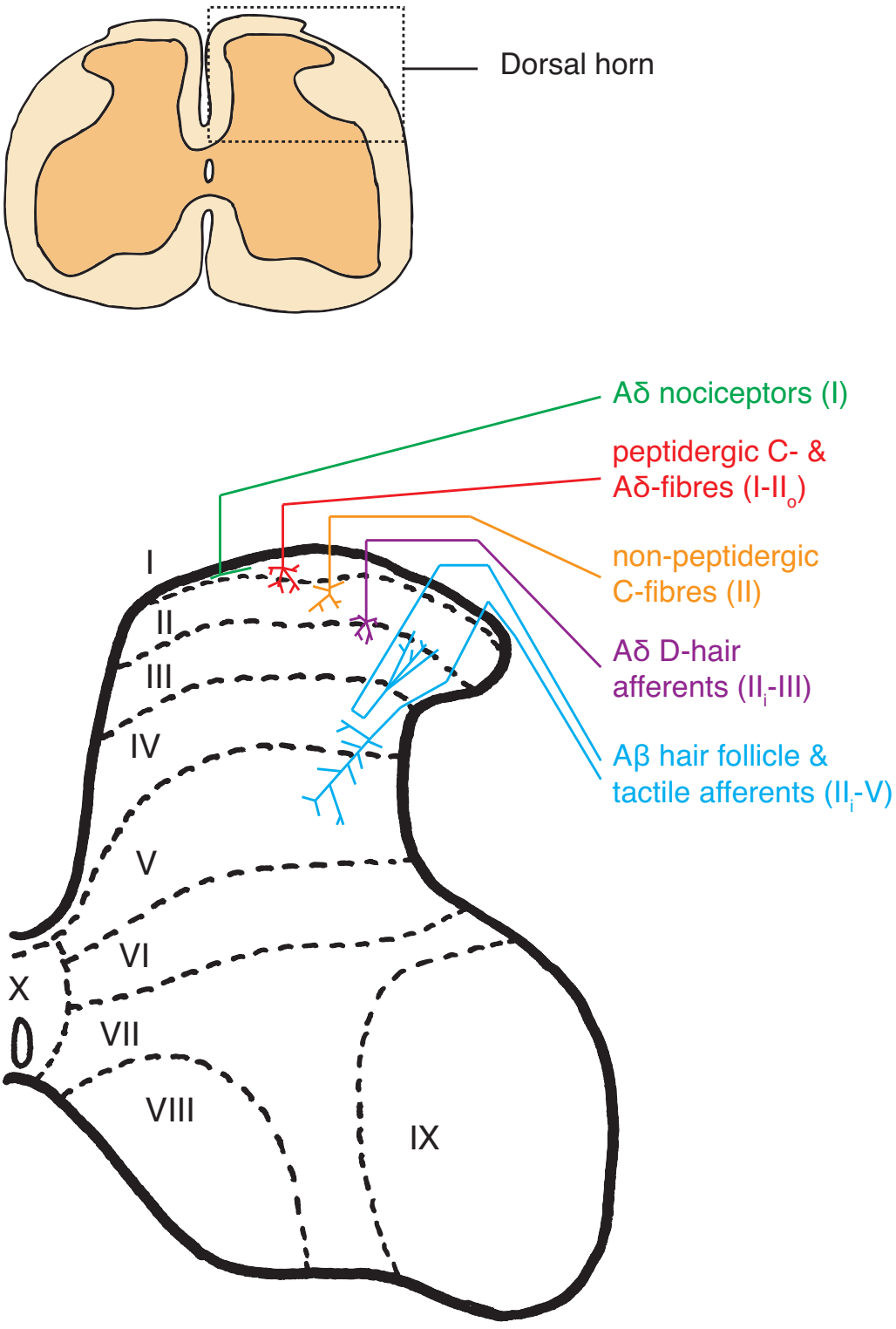

Figure 1.2: Laminar arrangement of the dorsal horn and the termination pattern of primary afferent input. Diagram based upon a traverse section from the lumbar (L4) region of the spinal cord. Adapted from Todd 2010.

1.6 Lamina I neurokinin 1 receptor expressing spinal cord projection neurons

Spinal cord projection neurons are concentrated in the lamina I region of the dorsal horn, with a smaller proportion being found in lamina III-VI (Al-Khater et al. 2008, Marshall et al. 1996, Spike et al. 2003, Todd 2010, Todd et al. 2000). In lamina II, projection neurons are scarce. The axons of many projection neurons, including those in lamina I, cross the midline and ascend to the brain in the contralateral white matter, where they target areas in the brainstem and thalamus. The pathways formed by the lamina I projection neurons in particular have been well characterised using both retrograde (Almarestani et al. 2007, Hylden et al. 1989, Lima & Coimbra 1988, Lima et al. 1991, Spike et al. 2003, Todd et al. 2000) and anterograde (Bernard et al. 1995, Gauriau & Bernard 2004, Slugg & Light 1994) tracing techniques. In the lamina I region of the dorsal horn, projection neurons have been shown to account for ∼5% of all neurons, of which ∼80% express the NK1R (Al-Khater et al. 2008, Spike et al. 2003, Todd et al. 2000). Most neurons only project contralaterally, but ∼25% have bilateral projections (Spike et al. 2003, Todd 2010). The majority of lamina I projection neurons target the parabrachial area (PB) (∼95%, figure 1.3), with smaller proportions projecting to the periaquducal gray matter (PAG) (∼30%) and thalamus (<5%), with some neurons targeting multiple sites (Spike et al. 2003, Todd 2010).

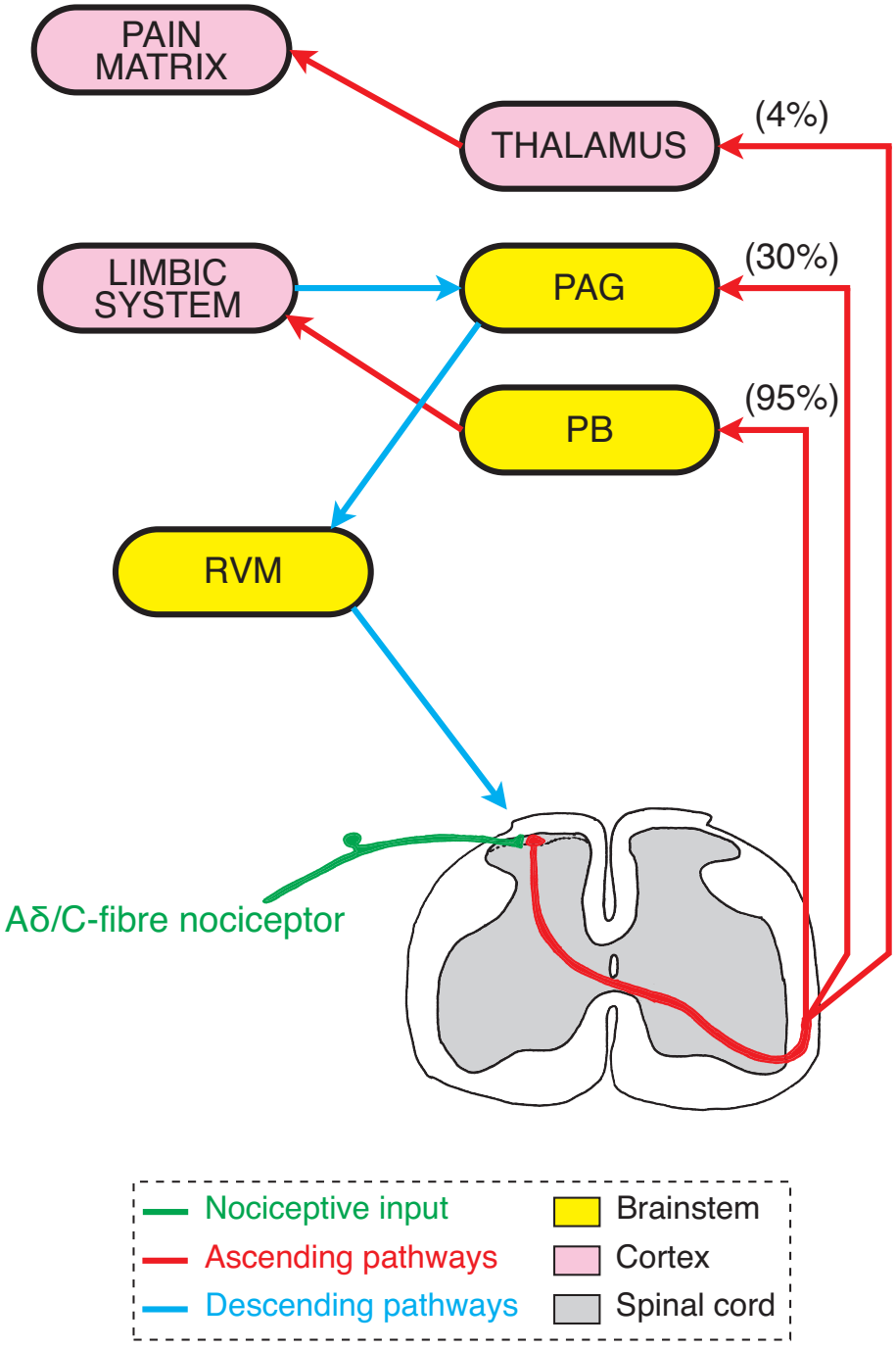

Figure 1.3: Lamina I NK1R+ neuron projections. Lamina I NK1R+ neurons receive monosynaptic input from both C-fibre and Aδ-fibre nociceptors (Torsney 2011, Torsney & MacDermott 2006). The axons of many of these neurons cross the midline and project to the brain in the lateral white matter tracts, where the main targets include the parabrachial area (PB), periaquducal gray matter (PAG) and thalamus (percentages shown in figure). The PB projections target areas in the limbic system, such as the hypothalamus and amygdala and plays a role in mediating the emotional and affective aspects of pain. The thalamic projections target areas within the 'pain matrix', such as the somatosensory cortex and anterior cingulate cortex and are involved in the discriminative aspects of pain. There are descending inhibitory and excitatory inputs from the limbic system that project to the dorsal horn, via the PAG and rostral ventromedial medulla (RVM), that play an essential role in controlling dorsal horn excitability. Adapted from Basbaum et al. 2009, D'Mello & Dickenson 2008, Todd 2010, values from Todd 2010.

Functional-anatomical studies have demonstrated that lamina I NK1R+ neurons process nociceptive information, as evidenced by NK1R internalisation in response to hindpaw capsaicin injection (Mantyh et al. 1995) and c-Fos expression induced by noxious heat, noxious cold, hindpaw formalin injection, mustard oil (topical or injection) and sciatic nerve crush injury (Doyle & Hunt 1999, Todd et al. 2002, 2005). Electrophysiological investigations have shown that lamina I NK1R+ neurons predominantly receive monosynaptic input from C-fibre nociceptors and to a lesser extent Aδ-fibres (Ruscheweyh et al. 2004, Torsney 2011, Torsney & MacDermott 2006), which includes heat / capsaicin-sensitive nociceptors (Labrakakis & MacDermott 2003, Tong & MacDermott 2006). Anatomical studies have shown that the C-fibre input to these neurons is comprised of peptidergic (Hwang et al. 2003, Todd et al. 2002) and to a lesser extent non-peptidergic afferents (Saeed & Ribeiro-da Silva 2012).

Selective ablation of NK1R+ neurons, by intrathecal administration of the cytotoxin, saporin, conjugated to SP (SP-SAP), which significantly reduces the number of NK1R+ neurons in lamina I/III (Mantyh et al. 1997), attenuates central sensitisation (Khasabov et al. 2002, Mantyh et al. 1997) and reduces hyperalgesia and allodynia in a number of animal models of inflammatory and neuropathic pain (Mantyh et al. 1997, Nichols et al. 1999, Suzuki et al. 2002). However, acute pain responses remain intact, whereby there are no differences in baseline responses to thermal and mechanical stimuli and the first phase of the formalin test is unaltered between control and SP-SAP treated rats (Mantyh et al. 1997, Nichols et al. 1999). These findings provide compelling evidence that these lamina I NK1R+ neurons play a unique role in the manifestation of chronic pain.

1.6.1 The neurokinin 1 receptor

The NK1R is a G-protein coupled receptor (GPCR) which, along with the neurokinin 2 and neurokinin 3 receptors, belongs to the family of tachykinin receptors (Łazarczyk et al. 2007, Maggi 1995). Expression of the NK1R is greatest in the lamina I region of the dorsal horn (Abbadie et al. 1996, Mantyh et al. 1997). Overall in lamina I the NK1R is found on ∼45% of all neurons (Todd et al. 1998), this includes both NK1R+ projection neurons (∼5% of all neurons) (Spike et al. 2003) and a population of likely interneurons (Todd et al. 2005), that display weak NK1R immunoreactivity (Cheunsuang & Morris 2000). In lamina II, NK1R expression is limited (Abbadie et al. 1997, Bleazard et al. 1994, Brown et al. 1995), while ∼11% of lamina III and

~28% of lamina IV neurons show NK1R immunoreactivity (Todd et al. 1998).

NK1R immunoreactivity has been shown to be increased in the dorsal horn in the CFA inflammatory pain model (Abbadie et al. 1996), specifically in the lamina I/II region (Abbadie et al. 1997, Honor et al. 1999), but levels are unchanged following hindpaw formalin or carrageenan injection (Honor et al. 1999). Nerve injury is also reported to increase the dorsal horn expression of the NK1R (Abbadie et al. 1996). Moreover, NK1R mRNA is elevated in the CFA and formalin pain models (McCarson & Krause 1994, Schäfer et al. 1993). The CFA-induced enhancement of NK1R expression is not due to the novel expression of NK1Rs in those neurons that do not express the receptor in control conditions (Abbadie et al. 1997, Honor et al. 1999).

The binding of SP to the NK1R results in receptor internalisation, with NK1R internalisation having been demonstrated in the dorsal horn following noxious mechanical, thermal and chemical (capsaicin) stimulation (Abbadie et al. 1997, Honor et al. 1999, Mantyh et al. 1995, Marvizón et al. 2003). An overview of NK1R internalisation in the superficial dorsal horn, produced by numerous stimuli, in both control and chronic pain models, is provided in table 1.2. In control conditions, in the absence of stimulation, NK1R internalisation is undetectable, while electrical activation of Aβ-fibres and innocuous stimuli do not cause internalisation (Allen et al. 1999, Honor et al. 1999, Hughes et al. 2007). However, electrical stimulation of Aδ- or C-fibres and noxious mechanical or thermal stimuli are able to drive internalisation in the superficial lamina (Abbadie et al. 1997, Allen et al. 1999, Honor et al. 1999). Notably NK1R internalisation is largely restricted to lamina I, which is unsurprising given the limited number of NK1R+ neurons in lamina II (Abbadie et al. 1997, Bleazard et al. 1994, Brown et al. 1995). Internalisation of NK1Rs is significantly increased in the formalin and carrageenan models, without further stimulation, although in the carrageenan model this response is small and only detectable 10 minutes following injection, but is not evident 3 hours after injection (Honor et al. 1999). In CFA inflammation, although CFA by itself does not result in NK1R internalisation in the superficial lamina, the degree of internalisation in response to noxious mechanical and thermal stimuli is enhanced, while innocuous mechanical stimuli can novelly drive internalisation (Abbadie et al. 1997, Honor et al. 1999). Likewise, there is an enhanced response to electrical stimulation of Aδ-fibres following CFA inflammation, in that there is a greater proportion of neurons displaying NK1R internalisation (Allen et al. 1999). However, no novel or enhanced NK1R internalisation is evident in the superficial laminae following electrical stimulation of Aβ- or C-fibres, respectively, although C-fibre stimulation results in

Model	Stimulus	NK1R Internalisation	Reference
Control	no stimulus	no	3,4
	electrical (Aβ)	no	2,4
	electrical (Aδ)	yes	2
	electrical (C)	yes	2,4
	innocuous mechanical	no	1,3
	noxious mechanical	yes	1,3
	noxious thermal	yes	1
CFA	no stimulus	no	2,3
	electrical (Aβ)	no	2
	electrical (Aδ)	increased	2
	electrical (C)	no change	2
	innocuous mechanical	increased	1,3
	noxious mechanical	increased	1,3
	noxious thermal	increased	1
Carrageenan	no stimulus	yes†	3
	innocuous mechanical	increased	3
	noxious mechanical	increased	3
Formalin	no stimulus	yes	3
CFA polyarthritis	no stimulus	no	3
Nerve transection	no stimulus	no	2
	electrical (Aβ)	no	2
	electrical (Aδ)	increased	2
	electrical (C)	no change	2
CCI	electrical (Aβ)	no	4
SNI	electrical (Aβ)	no	4

Table 1.2: Comparison of NK1R internalisation in the superficial laminae of the spinal cord in different chonic pain models. 'Increased' and 'no change' refers to the response when compared to control. † small degree of internalisation detectable for a short time following injection. References: 1; Abbadie et al. 1997, 2; Allen et al. 1999, 3; Honor et al. 1999, 4; Hughes et al. 2007.

increased internalisation in the deep dorsal horn. In a similar manner, sciatic nerve transection does not alter the response to Aβ- or C-fibre stimulation, while there is an elevated number of neurons displaying internalisation in response to Aδ-fibre activation (Allen et al. 1999). Aβ-fibre stimulation in the chronic constriction injury (CCI) and spared nerve injury (SNI) neuropathic pain models, as in control conditions, does not result in NK1R internalisation (Hughes et al. 2007).

1.6.2 Lamina III/IV neurokinin 1 receptor expressing spinal cord projection neurons

It should also be noted that there is also a population of NK1R+ projection neurons found in the lamina III/VI region of the dorsal horn. These neurons can be subdivided into two groups bases on morphological and functional differences.

One subgroup has been shown to have long dorsally directed dendrites that penetrate the superficial laminae (Bleazard et al. 1994, Littlewood et al. 1995, Naim et al. 1997, Sakamoto et al. 1999). These neurons are known to target similar sites in the brainstem and thalamus as the lamina I NK1R+ neurons (Al-Khater et al. 2008, Al-Khater & Todd 2009, Todd 2010, Todd et al. 2000) and may also project to areas in the forebrain, indicating possible roles in alertness and motor aspects of pain (Gauriau & Bernard 2004). Anatomical studies have shown that this subgroup are targeted by SP-containing C-fibre afferents, mainly onto their distal dendrites in lamina I/II$_o$, but also onto their proximal dendrites in lamina II$_i$/III (Naim et al. 1997, Sakamoto et al. 1999). However, contacts with non-peptidergic C-fibres (Sakamoto et al. 1999) and A-fibres (Naim et al. 1998) are limited. There is also evidence that these lamina III/IV NK1R+ neurons process nociceptive information, in that they demonstrate NK1R internalisation and ERK phosphorylation in response to noxious thermal, mechanical and chemical (formalin / capsaicin) stimuli (Mantyh et al. 1995, Polgár et al. 2007) and increased c-Fos expression in the formalin inflammatory pain model (Doyle & Hunt 1999).

The second subgroup of lamina III/IV NK1R+ neurons have a more restricted dendritic arbour and their function is uncertain, given they do not display NK1R internalisation or c-Fos expression in response to noxious stimuli (Doyle & Hunt 1999, Mantyh et al. 1995).

Electrophysiological studies in spinal cord slices have shown that lamina III/IV NK1R+ neurons receive minimal input from monosynaptic Aδ- or monosynaptic C-fibre nociceptors, with the majority of input (70%) arising from monosynaptic Aβ-fibres (Torsney & MacDermott 2006). However, this lack of monosynaptic input from Aδ- or C-fibres could reflect that the lamina III/IV NK1R+ neurons that Torsney & MacDermott (2006) recorded from belonged to the subgroup of neurons that have restricted dendritic arbours. Furthermore, the authors suggest that the methods used to prepare spinal cord slices for these investigations may have resulted in the loss of afferent input to the neurons and/or a reduction in the dendritic tree, which could also account for the lack of monosynaptic Aδ- or C-fibre input to these lamina III/IV

NK1R+ neurons.

1.7 Central sensitisation

Central sensitisation is a process whereby plasticity in the processing of sensory information within nociceptive pathways in the CNS leads to altered processing of nociceptive information and an enhancement of responses (Latremoliere & Woolf 2009, Woolf 1983). While peripheral sensitisation is thought to underly thermal hypersensitivity and primary hyperalgesia, central sensitisation is considered responsible for mediating mechanical hypersensitivity and secondary hyperalgesia (Lewin et al. 2004). Following tissue injury, novel spontaneous activity in C-fibre nociceptors drives changes in the dorsal horn of the spinal cord which results in spinal cord hyperexcitability / central sensitisation (Woolf 1983). This spinal cord hyperexcitability then enables Aβ- and Aδ-fibres to access nociceptive circuits that allows them to mediate mechanical allodynia and mechanical hyperalgesia, respectively (Latremoliere & Woolf 2009, Treede & Magerl 2000). However, it should be noted that the exact spinal cord circuitry that is involved in this process is not fully understood.

Central sensitisation was first described in 1983 by Clifford Woolf (Woolf 1983). Using electrophysiological recordings from rat α-motorneuron efferents to measure the flexion withdrawal reflex as a proxy of spinal cord nociceptive processing, it was discovered that repetitive noxious stimulation of the hindpaw, which was sufficient to cause inflammation, resulted in a long lasting hypersensitivity. This hypersensitivity following noxious stimulation was characterised by the following; there was an increase in novel spontaneous firing in the α-motorneurons, an enlargement of receptive fields and a reduction in activation thresholds, whereby responses could now be evoked by innocuous stimuli. Three key experiments were performed to confirm that these changes were due to changes in the central, as opposed to peripheral, processing of noxious information:

1. Selective electrical activation of Aβ-fibres, which in control conditions was without effect, was able to evoke responses in the α-motorneurons following noxious stimulation of the hindpaw.

2. Peripheral nerve block, by application of local anaesthetic (2% xylocaine) to the injured hindpaw, did not reverse the expansion of the receptive fields.

3. In the absence of peripheral injury, brief low-frequency electrical stimulation of C-fibres was sufficient to elicit the changes seen following noxious hindpaw stimulation.

What these findings elegantly show is that activation of C-fibre nociceptors drives plasticity in the dorsal horn of the spinal cord that enables low-threshold afferents to activate nociceptive pathways and enables these pathways to be activated by stimuli outwith the area of injury (secondary hyperalgesia). Subsequent studies have provided evidence that the activity of dorsal horn neurons is altered in a similar manner to the changes seen in α-motorneurons (Dougherty & Willis 1992, Lin et al. 1999, Woolf & King 1990). Interestingly, central sensitisation has also been shown to occur in higher centres in the rat, such as the amygdala (Neugebauer & Li 2003, Neugebauer et al. 2003), thalamus (Dostrovsky & Guilbaud 1990) and anterior cingulate cortex (Wei & Zhuo 2001), while human and rat neuroimaging studies suggest changes indicative of central sensitisation occur in the prefrontal cortex, PAG, PB and superior colliculus (Maihöfner et al. 2010, Mohr et al. 2008, Moylan Governo et al. 2006, Peyron et al. 2000, Shih et al. 2008).

While Woolf (1983) demonstrated that central sensitisation was driven by noxious thermal stimulation and repetitive electrical activation of C-fibres, subsequent studies have shown that central sensitisation can also be induced by capsaicin activation of TRPV1 channels (LaMotte et al. 1991, Lin et al. 1999), or by the injection of mustard oil (Woolf & King 1990, Woolf & Thompson 1991) or formalin, which both activate TRPA1 channels (Jordt et al. 2004, McNamara et al. 2007). The key properties of noxious stimuli that are required for the induction of central sensitisation are that it must be intense, repeated and sustained (Latremoliere & Woolf 2009). Furthermore, while actual tissue damage is not essential, the noxious stimulation that follows tissue damage in most cases will lead to central sensitisation.

The importance of C-fibre activity in driving inflammatory pain hypersensitivity has been further confirmed by Abrahamsen et al. (2008) using a genetic strategy. The cre-lox system was used to selectively destroy $Na_v1.8$ expressing neurons, which includes both C- and A-fibres (Shields et al. 2012). In these mice, there was a loss of $>$85% of peripherin and 13% of neurofilament 200 (NF200) expressing neurons (Abrahamsen et al. 2008), signifying the loss of the majority of C-fibres (Goldstein et al. 1991) and and some A-fibres (Perry et al. 1991), respectively. These mice failed to develop thermal or mechanical hypersensitivity in the CFA inflammatory pain model and did not display thermal hypersensitivity following hindpaw carrageenan or NGF injections. Furthermore, the second phase of the formalin test was abolished in

these mice. However, the development of mechanical and thermal hypersensitivity was unaltered following nerve injury, which demonstrates that $Na_v1.8$ expressing C-fibres are crucially required for inflammatory, but not neuropathic pain. However, one cannot rule out potential developmental changes in the circuitry of the dorsal horn in these animals, given that neonatal destruction of C-fibres is known to delay or prevent many developmental processes in the dorsal horn (Fitzgerald 2005), which could contribute to the phenotype of these mice.

1.8 Mechanisms of central sensitisation

Central sensitisation is known to involve two distinct phases, a transcription-independent early phase and a transcription-dependent late phase (Latremoliere & Woolf 2009, Woolf & Salter 2000). The early phase is largely driven by rapid changes in the activity of glutamate receptors and ion channels, via phosphorylation, while in the late phase altered gene expression can lead to long lasting changes in the expression of a large number of ion channels and receptors.

Central sensitisation lacks a single underlying mechanism and is instead considered to be a multifaceted phenomenon where the processing of somatosensory information is altered (Latremoliere & Woolf 2009). There are a number of disparate processes that are known to occur in the dorsal horn of the spinal cord that can result in increased excitability, reduced inhibition or alterations in sensory input, which in turn can give rise to the symptoms of allodynia, hyperalgesia and spontaneous pain.

One of the key players in central sensitisation is the ionotropic glutamatergic N-methyl-D-aspartate receptor (NMDAR). Under naïve conditions, NMDARs play a minimal role in fast excitatory transmission, due to the Mg^{2+} block at the channel pore at resting membrane potential (Mayer et al. 1984) (figure 1.4A). However, repetitive or high-frequency firing of C-fibres, which drives the presynaptic release of glutamate, SP and CGRP (figure 1.4B), results in a sustained depolarisation of the membrane which is sufficient to remove the Mg^{2+} block and leads to slow excitatory postsynaptic potentials (EPSPs), with a duration of tens of milliseconds. The removal of the Mg^{2+} block greatly enhances synaptic transmission and permits the influx of Ca^{2+} into the neuron, that can drive a number of intracelluar signalling pathways that are involved in central sensitisation (Latremoliere & Woolf 2009). One of the first indicators that NMDARs play a crucial role in central sensitisation was provided by Woolf & Thompson (1991), who demonstrated that the application of the NMDAR

antagonists, MK-801 or D-CCP, at concentrations that were without effect on baseline responses, prevented the induction of and reversed the hyperexcitability associated with central sensitisation.

SP, which is co-released from peptidergic C-fibre nociceptors along with glutamate (figure 1.4B), is also known to play a key role in the development of central sensitisation (Liu & Sandkühler 1997, Ma & Woolf 1995, Willis 2002). The binding of SP to its corresponding receptor, the NK1R, results in a long-lasting depolarisation of postsynaptic neurons in the dorsal horn (Henry 1976, Randić & Miletić 1977), which can act to enhance the temporal summation of C-fibre-evoked responses (Dougherty & Willis 1991, Xu et al. 1992) and the activation of a number of intracellular signalling pathways that are important in central sensitisation, such as PKA, PKC and ERK (figure 1.4C). The involvement of SP–NK1R signalling in central sensitisation was first described by Ma & Woolf (1995), who demonstrated, using electrophysiological recordings from α-motorneurons, that intrathecal application of substance P methy ester (SPME), a NK1R agonist, resulted in central sensitisation, which manifested as an increase in spontaneous activity, decrease in mechanical activation thresholds and an enhancement in the response to innocuous mechanical stimuli. Pre-treatment with the NK1R antagonist, RP67580, prevented the reduction in mechanical activation threshold and the facilitation of touch-evoked responses following SPME application, C-fibre stimulation or mustard oil application, the latter two being known to drive central sensitisation (Woolf 1983, Woolf & King 1990, Woolf & Thompson 1991). Interestingly, once central sensitisation was established, RP6780 did not reverse it (Ma & Woolf 1995). Further studies have shown that RP67580 can prevent the long-lasting potentiation of C-fibre evoked field potentials in the dorsal horn, but is without effect upon established potentiated responses (Liu & Sandkühler 1997). Therefore, SP can be considered to play a crucial role in the development, but not maintenance, of central sensitisation.

Other effectors that play an important role in central sensitisation include CGRP, bradykinin and brain-derived neurotrophic factor (BDNF). CGRP enhances the effects of SP (Woolf & Wiesenfeld-Hallin 1986) and via its actions upon CGRP1 receptors, which are expressed on postsynaptic neurons in the dorsal horn, can activate PKA and PKC (Sun et al. 2003, 2004). PKA, PKC, as well as ERK can also be activated via the actions of bradykinin (Kohno et al. 2008), which is spinally produced during intense nociceptive stimulation, acting upon the bradykinin B2 receptor, which is present on spinal neurons (Chapman & Dickenson 1992, Wang et al. 2005). BDNF acts as both a synaptic modulator and a neurotrophic factor and is produced by nociceptors, where

its activity-dependent release into the dorsal horn (Balkowiec & Katz 2000, Zhou & Rush 1996) can drive central sensitisation through the activation of PKC and ERK (Kawasaki et al. 2004, Pezet et al. 2002, Slack et al. 2005, 2004, Zhao et al. 2006) and the facilitation of NMDAR-mediated C-fibre-evoked responses (Kerr et al. 1999), through its actions upon the tyrosine receptor kinase B (TrkB). Interestingly, nociceptor-derived BDNF has been shown to regulate inflammatory, but not neuropathic pain (Zhao et al. 2006). A summary of the intracellular signalling pathways that are important in central sensitisation is provided in figure 1.4C.

A key driver of the changes that arise during central sensitisation is an increase in intracellular Ca^{2+} in postsynaptic neurons. This increase in intracellular Ca^{2+} occurs through the entry of Ca^{2+} into the neuron, predominantly via NMDARs (Latremoliere & Woolf 2009), but also through Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) (Larsson & Broman 2008, Luo et al. 2008, Vikman et al. 2008) and voltage-gated calcium channels (VGCCs) (Coderre & Melzack 1992, Woolf & Salter 2000). Release of Ca^{2+} from intracellular stores, following the activation of various metabotropic receptors can also contribute to this rise in intracellular Ca^{2+} (Guo et al. 2004, Luo et al. 2008). The increase in intracellular Ca^{2+} results in the activation of a number of kinases which can act to enhance the efficiency of synaptic transmission. PKA, PKC and calcium/calmodulin-dependent protein kinase II (CaMKII) act to phosphorylate AMPARs (Carvalho et al. 2000, Larsson & Broman 2011), while PKA, PKC as well as the tyrosine kinases, Syc and Fyn phosphorylate NMDARs (Chen & Roche 2007, Guo et al. 2002, Larsson & Broman 2011). The phosphorylation of AMPARs and NMDARs acts to enhance synaptic transmission in a number of ways. Phosphorylation of the carboxyl tail of AMPAR subunits at serine-threonine residues acts to increase channel conductance and open probability, alter the expression of AMPAR subunits at the postsynaptic membrane and regulate aspects of long-term potentiation (LTP) (Larsson & Broman 2011, Lee 2006, Liu & Salter 2010). Likewise, NMDAR phosphorylation can alter receptor function by modulating NMDAR-mediated currents, increasing the open time and open probability of the channel and regulating the trafficking of subunits to the plasma membrane (Larsson & Broman 2011, Lee 2006, Liu & Salter 2010). PKA, PKC and CaMKII also activate ERK, through its phosphorylation (figure 1.4C), which can enhance the excitability of the postsynaptic neurons by reducing inhibitory K^+ currents as a result of the ERK-mediated phosphorylation of voltage-gated potassium channels (K_vs) (Hu, Carrasquillo, Karim, Jung, Nerbonne, Schwarz & Gereau 2006, Hu & Gereau 2003, Hu et al. 2003). Furthermore, ERK, PKA and CaMKII can drive the insertion of

AMPAR subunits into the postsynaptic membrane (Galan et al. 2004, Qin et al. 2005).

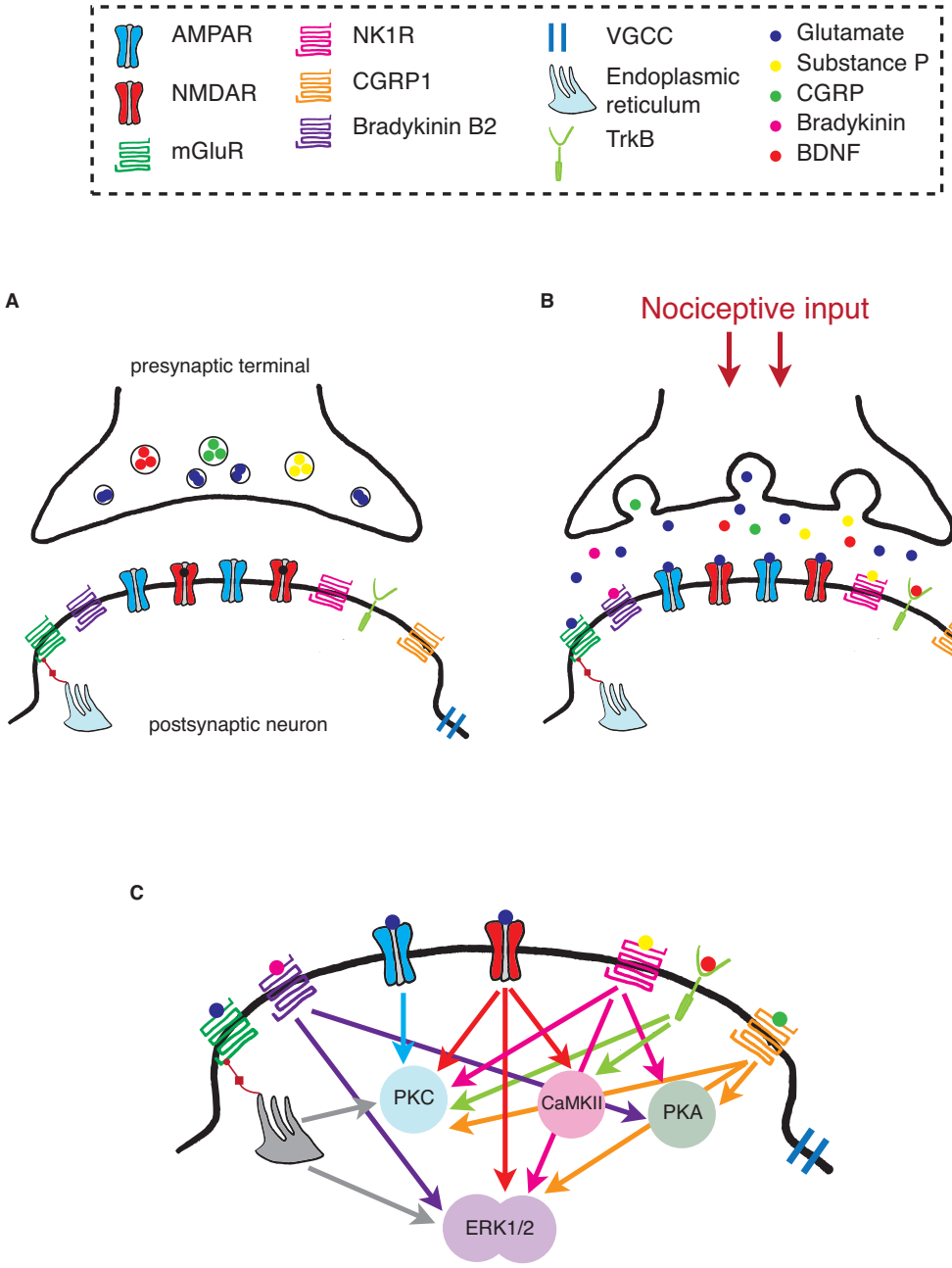

Figure 1.4: Synapse between central terminal of a nociceptor and a lamina I dorsal horn neuron. ***A*** Diagram of a synapse between the central terminal of a nociceptor and a lamina I dorsal horn neuron under naïve conditions. In this situation the pore of NMDARs are blocked by Mg^{2+} (denoted by black dot). ***B*** During nociceptive input, the central terminals of nociceptors release a number of neurotransmitters including; glutamate, that acts upon AMPARs, NMDARs (with Mg^{2+} removed due to depolarisation of the postsynaptic neuron) and mGluRs. Substance P, CGRP and BDNF are also presynaptically released and bind to NK1, CGRP1 and TrkB receptors, respectively. Spinally produced bradykinin can also activate bradykinin B2 receptors. ***C*** The binding of these neurotransmitter to their postsynaptic receptors results in the activation of a number of intracellular signalling pathways which play a key role in central sensitisation. Adapted from Latremoliere & Woolf 2009.

1.8.1 Increased excitability in central sensitisation

Synaptic transmission between primary afferent neurons and second-order neurons in the dorsal horn is excitatory, involving the release of glutamate from the central terminals of primary afferents, which acts upon ionotropic glutamate receptors (AMPAR, NMDAR and kainate receptors (KARs)) and a number of mGluRs (Larsson 2009, Larsson & Broman 2011, Latremoliere & Woolf 2009, Liu & Salter 2010). One of the key mechanisms underlying central sensitisation that occurs in the early phase is the increase in the activity and expression of postsynaptic AMPARs and NMDARs, that results from receptor phosphorylation and the insertion of receptors into the postsynaptic membrane (Ji et al. 2003, Latremoliere & Woolf 2009, Liu & Salter 2010).

NMDA receptors

NMDARs are tetrameric, non-selective cation-permeable channels that are composed of two essential GluN1 subunits along with two regulatory GluN2 (GluN2A, GluN2B, GluN2C or GluN2D) or two GluN3 (GluN3A or GluN3B) subunits (Nakanishi 1992, Paoletti & Neyton 2007). The subunit composition of NMDARs is crucial in dictating the functional characteristics of the receptor, such as ligand affinity, channel conductance and kinetics (Cull-Candy & Leszkiewicz 2004). For example, the presence of GluN2A or GluN2B subunits results in NMDARs that have greater sensitivity to Mg^{2+}, than those containing GluN2C or GluN2D subunits (Kuner & Schoepfer 1996, Monyer et al. 1994).

Anatomical studies have shown that the GluN1 subunit is present throughout the dorsal horn of the spinal cord. GluN2A is most intensely expressed in laminae III-IV, with only limited expression in laminae I/II, while the opposite is true for the expression of GluN2B, where there the expression is high in laminae I/II, but weak in lamina III-IV (Nagy et al. 2004a). Additional studies show that GluN1 and GluN2A/B mRNA shows a comparable expression pattern in the dorsal horn, although mRNA for GluN2C/D was undetectable using these methods (Shibata et al. 1999, Watanabe et al. 1994).

Using a combined electrophysiological / pharmacological approach, it has been shown that NMDARs that contain GluN2A/B and GluN2C/D subunits are functionally expressed on lamina I NK1R+ neurons (Tong et al. 2008). In addition, lamina I NK1R+ neurons express a greater percentage of NMDARs that contain

GluN2C/D subunits than lamina I neurons lacking the NK1R. Functional expression of NMDARs that contain GluN2A/B and GluN2C/D subunits has also been shown in lamina II GABAergic inhibitory interneurons, while lamina II presumed excitatory interneurons predominantly express receptors containing GluN2A/B subunits (Shiokawa et al. 2010).

In the CCI neuropathic pain model, the expression of GluN1 and GluN2B, but not GluN2A, is increased in the dorsal horn ipsilateral to the injury (Wilson et al. 2005). Western blot analysis shows that CFA inflammation causes the sustained phosphorylation of GluN2B, but not GluN2A, subunits in the lumbar dorsal horn (Guo et al. 2002). Carrageenan inflammation causes the phosphorylation of GluN1, but not GluN2A or GluN2B, subunits, without altering the overall levels of expression (Caudle et al. 2005).

AMPA receptors

AMPARs play a crucial role in both acute and chronic pain (Dickenson et al. 1997, Garry & Fleetwood-Walker 2004). AMPARs (along with KARs) are responsible for mediating fast synaptic transmission between primary afferents and second order neurons in the dorsal horn, whereby the presynaptic release of glutamate from the central terminals of primary afferents activates postsynaptic dorsal horn AMPARs, resulting in fast EPSPs with durations in the order of milliseconds (Yoshimura & Jessell 1990, Yoshimura & Nishi 1992).

AMPARs are hetromeric or homomeric tetramers that are assembled from a combination of four subunits, GluA1, GluA2, GluA3 or GluA4 and these subunits can be modified via RNA editing and alternative splicing (Hollmann & Heinemann 1994, Seeburg & Hartner 2003). The cation permeability of AMPARs is dependent upon the subunit composition (Hollmann et al. 1991, Sommer et al. 1990, 1991). In particular the Ca^{2+} permeability of AMPARs is regulated by the GluA2 subunit, whereby only those channels that lack the GluA2 subunit are permeable to Ca^{2+} (Burnashev et al. 1992, Hollmann et al. 1991).

In the dorsal horn of the spinal cord, all four AMPAR subunits have been detected (Engelman et al. 1999, Henley et al. 1993, Kerr et al. 1998, Nagy et al. 2004b, Polgár et al. 2008, Todd et al. 2009, Tölle et al. 1993). Synaptic expression of GluA1 and GluA2 subunits is widespread throughout the dorsal horn, but with greater immunoreactivity seen in lamina I/II. Furthermore, most AMPARs contain the GluA2

subunit (Nagy et al. 2004b, Polgár et al. 2008). GluA3 and GluA4 subunits are strongly expressed in lamina III-VI, but show weak expression in lamina I/II.

The Ca^{2+}-permeable AMPAR antagonist, synthetic Joro spider toxin (JSTX), has been shown to attenuate secondary mechanical hyperalgesia and secondary mechanical allodynia following gastrocnemius incision (Pogatzki et al. 2003) and first-degree burn injury (Jones & Sorkin 2004), respectively. GluA2 knockout mice, in which there is an enhancement of Ca^{2+}-permeable AMPARs, show enhanced hypersensitivity in CFA inflammation and following hindpaw formalin and capsaicin injection (Hartmann et al. 2004). While GluA1 knockout mice, where Ca^{2+}-impermeable AMPARs are enhanced, do not show an increase in ERK phosphorylation, which is considered a marker of central sensitisation (Ji et al. 1999, Karim et al. 2001), in response to C-fibre stimulation (Hartmann et al. 2004), indicating that a reduction in Ca^{2+}-permeable AMPARs correlates with a reduction in measures of spinal cord potentiation. These data indicate that Ca^{2+}-permeable AMPARs play a key role in central sensitisation.

1.8.2 Disrupted spinal cord inhibition in central sensitisation

There is evidence that altered inhibitory tone in the dorsal horn may play a role in the development of central sensitisation in inflammatory pain (Müller et al. 2003, Zeilhofer & Zeilhofer 2008). Nociceptive processing in the dorsal horn of the spinal cord is known to be influenced by spinal inhibition, in that pharmacologically mimicking disrupted inhibition in naïve animals, by spinal application of the glycine receptor (GlyR) antagonist, strychnine, or the $GABA_A$ receptor ($GABA_AR$) antagonist, bicuculline, results in the development of allodynia-like responses (Sherman & Loomis 1996, Sorkin & Puig 1996, Yaksh 1989). Studies have shown that following nerve injury there is a significant reduction in levels of immunoreactivity for GABA (Eaton et al. 1998, Ibuki et al. 1997) and its synthesising enzyme, glutamic acid decarboxylase (GAD) (Eaton et al. 1998, Moore et al. 2002), in the dorsal horn of the spinal cord, which may arise due to the death of GABAergic interneurons (Scholz et al. 2005). However, these findings are disputed by others who report that there is no loss of dorsal horn neurons following nerve injury (Polgár et al. 2004, 2005) and no change in GABA, vesicular GABA transporter or $GABA_AR$ immunoreactivity (Polgár & Todd 2008). $GABA_AR$ expression has also been shown to be increased in the CCI neuropathic pain model (Moore et al. 2002).

There is also electrophysiological evidence that spinal cord inhibition is reduced in

chronic pain conditions. GlyR-mediated miniature inhibitory postsynaptic current (mIPSC) frequency was found to be significantly reduced in the CFA inflammatory pain model (Müller et al. 2003), while $GABA_AR$-mediated mIPSC frequency is attenuated and the number of neurons displaying no evoked inhibitory postsynaptic currents (eIPSCs) is increased in neuropathic pain models (Moore et al. 2002). It is also reported that inhibitory inputs in the lamina I region of the dorsal horn may actually become excitatory following nerve injury, whereby downregulation of the potassium-chloride exporter (KCC2), which normally maintains Cl^- homeostasis, results in a disruption of the anion reversal potential (E_{anion}), transforming inhibitory hyperpolarising inputs into excitatory depolarising inputs (Coull et al. 2005, 2003). This disruption of E_{anion} has subsequently been shown to be driven by the release of BDNF from activated spinal microglia (Coull et al. 2005).

1.8.3 Altered sensory input to the spinal cord

Heterosynaptic facilitation of sensory input

Heterosynaptic facilitation, where stimulation of one group of synapses results in the augmented activity in other unstimulated synapses, normally via the sensitisation of the whole neuron, is a key component of central sensitisation (Latremoliere & Woolf 2009). Since the discovery of central sensitisation it has been clear that heterosynaptic facilitation plays a crucial role in this process. Woolf (1983) demonstrated that repetitive activity in one group of primary afferents (C-fibres) enabled a different group (Aβ-fibres) to novelly drive nociceptive spinal cord circuits. Furthermore, the expansion of receptive fields seen following nociceptive stimulation was unaffected by peripheral administration of the local anaesthetic, xylocaine, indicating that stimulation of one group of primary afferents drove central changes leading to the recruitment of a different group of afferents from outwith the area of tissue injury. It is widely considered that heterosynaptic facilitation plays an essential role in the development of inflammatory and neuropathic pain hypersensitivity, whereby repetitive activity in C-fibre nociceptors drives spinal cord hyperexcitability that enables Aβ- and Aδ-fibre afferents to access circuits by which they mediate allodynia and hyperalgesia, respectively (Latremoliere & Woolf 2009, Treede & Magerl 2000). However, while heterosynaptic potentiation is a crucial feature of central sensitisation, the underlying changes that occur in the circuitry of the dorsal horn are not well understood.

Homosynaptic facilitation of sensory input

Another facilitatory mechanism that has been proposed to be involved in central sensitisation is that of homosynaptic potentiation (Sandkühler 2010), where stimulation results in the potentiation of only those synapses that are stimulated. While heterosynaptic facilitatory mechanisms are considered to be responsible for secondary hyperalgesia and allodynia, homosynaptic potentiation by its very nature can only be involved in primary hyperalgesia (Latremoliere & Woolf 2009).

One method of altered excitatory input to the dorsal horn that has been proposed is the homosynaptic facilitation of C-fibre input, which acts to amplify nociceptive inputs and thus provides a potential mechanism for hyperalgesia. Some authors consider this potentiation to be analogous to LTP, as is typically involved in learning and memory in the cortex, since this C-fibre potentiation shares many of the same characteristics as cortical LTP (Ruscheweyh et al. 2011, Sandkühler 2010). However, the use of LTP is regarded inaccurate by others, due to unique differences in the underlying mechanisms, duration and purpose of central sensitisation compared to cortical LTP (Latremoliere & Woolf 2010).

High-frequency stimulation (HFS) (100Hz) of C-fibres significantly potentiates C-fibre-evoked field potentials in the superficial dorsal horn, which indicates summation of largely monosynaptically evoked postsynaptic currents (Liu & Sandkühler 1997). Interestingly, the induction of this potentiation was found to be NK1R-dependent, in that application of RP 67580, an NK1R antagonist, prevented the initiation of potentiation, but did not reverse established potentiation. Further evidence can be considered to support the view that homosynaptic C-fibre potentiation may be a feature of central sensitisation. In intact rats, nerve injury resulting from transection or crush (Zhang et al. 2004, Zhou et al. 2010) and hindpaw capsaicin or formalin injection (Ikeda et al. 2006) results in a potentiation of C-fibre-evoked field potentials in the superficial dorsal horn, that is comparable to that seen following electrical stimulation. While stimulation protocols similar to those used to induce long-lasting potentiation of C-fibre responses in intact rats and *ex vivo* tissue preparations, enhances pain perception in humans (Klein et al. 2004, 2008). However, these human studies cannot provide direct evidence for homosynaptic potentiation of C-fibre input, as that can only be obtained by measuring synaptic strength. Moreover, in these human studies mechanical allodynia in areas outwith the stimulated zone was reported, which strongly suggests heterosynaptic facilitatory mechanisms were involved in these responses.

1.8.4 Non-synaptic mechanisms of central sensitisation

Neuron-glia interactions

In recent years it has become clear that interactions between neurons and glial cells, namely microglia and astrocytes, in the spinal cord play an important role in the development and maintenance of central sensitisation in neuropathic and inflammatory pain.

Microglia In response to tissue or nerve injury, microglia undergo the process of microgliosis, in which a number of complex changes occur that result in microglia entering an 'activated' state (Ren & Dubner 2010, Taves et al. 2013, Tsuda et al. 2013). The activation of microglia is prominent in neuropathic pain models (Beggs & Salter 2006, 2007, Calvo & Bennett 2012), with nerve injury being associated with an increase in the number and density of spinal microglia, driven by proliferation and migration of microglia (Beggs & Salter 2007, Taves et al. 2013). However, in inflammatory pain this response is less apparent (Clark et al. 2007, Honore et al. 2000, Lin et al. 2007). Typically the activated state of microglia is determined by a morphological change in the cell from ramified to amoeboid (Streit et al. 1999). Microglia activation is driven by the release of neurotransmitters and neuromodulators, such as glutamate, CGRP, SP, ATP, BDNF and IL-6, from the presynaptic terminals of primary afferent fibres, which in addition to acting upon receptors on the postsynaptic terminal also act on receptors present on microglia (Ren & Dubner 2010). Activated microglia can release a number of mediators that can act to modulate spinal cord excitability, including BDNF, TNF-α and IL-1β as well as other proinflammatory cytokines and chemokines (Kavelaars et al. 2011, Kawasaki et al. 2008, Scholz & Woolf 2007), although the release of these mediators may occur in the absence of morphological changes (Taves et al. 2013). Notably, administration of minocycline, a specific microglia inhibitor, can significantly attenuate behavioural hypersenstivity in neuropathic (Guasti et al. 2009, Osikowicz et al. 2009, Pabreja et al. 2011, Pu et al. 2013) and inflammatory pain models (Bastos et al. 2013, Li et al. 2010).

One mechanism by which microglial activation can modulate the central processing of pain that has become apparent in recent years is by altering inhibitory tone in the dorsal horn in neuropathic pain conditions (Coull et al. 2005, Ferrini & De Koninck 2013). This microgial mediated disinhibition occurs as a result of ATP activation of

P2X4 receptors expressed on spinal cord microglia, which drives the release of BDNF from these microglia. This BDNF release, acting via TrkB, downregulates KCC2 activity, which normally maintains Cl^- homeostasis, resulting in a disruption of E_{anion}. This in turn leads to diminished $GABA_AR$ / GlyR mediated inhibition and the transformation of inhibitory hyperpolarising currents into excitatory depolarising currents (Coull et al. 2005).

Astrocytes As with microglia, astrocytes in the spinal cord can become activated following a noxious stimulus, although it should be noted that unlike microglia, astrocytes are not quiescent under normal conditions and 'activation' in this context refers to a state of enhanced function (Cao & Zhang 2008, Dong & Benveniste 2001, Watkins & Maier 2003). This activation is typically associated with a morphological change, whereby astrocytes display a hypertrophied soma with thick processes, in addition to displaying increased expression of the astrocyte marker, glial fibrillary acidic protein (GFAP) (Cao & Zhang 2008, Raghavendra et al. 2003, Watkins & Maier 2003). Astrocytes play a key role in maintaining glutamate homeostasis, in that they are responsible for the uptake of the majority of extrasynaptic glutamate through the glutamate transporter-1 (GLT-1) (McMahon & Malcangio 2009, Tanaka et al. 1997), with injury induced downregulation of GLT-1 resulting in a disruption of glutamate homeostasis (Ren & Dubner 2010). In response to an increase in intracellular Ca^{2+}, astrocytes can release glutamate and D-serine, which can modulate central sensitisation via actions on synaptic and extrasynaptic NMDARs, respectively (McMahon & Malcangio 2009, Ren & Dubner 2010). Activated astrocytes are also known to release a number of chemokines and cytokines, including IL-1β, IL-6 and TNF-α (Dong & Benveniste 2001), which are known to play a key role in central sensitisation (Kawasaki et al. 2008).

Astrocytes are highly connected via gap junctions, therefore increases in intracellular Ca^{2+} concentration can lead to signalling throughout the astrocyte network, via a 'Ca^{2+} wave', enabling long-range signalling (McMahon & Malcangio 2009, Wu et al. 2012a). Interestingly, expression of the gap junction protein connexin-43 is increased in the brainstem following facial nerve injury, indicating greater connectivity between astrocytes and a possible facilitation of Ca^{2+} signalling (Rohlmann et al. 1994). Further evidence that gap junctions play a role in chronic pain hypersensitivity is provided by the finding that intrathecal administration of carbenoxolone, which acts to reversibly decouple gap junctions, at a low dose abolished 'mirror-image' mechanical allodynia in the contralateral hindpaw in the CCI and sciatic inflammatory

neuropathy (SIN) models, while a higher dose bilaterally reversed mechanical allodynia. Carbenoxolone also abolishes thermal hyperalgesia, contralaterally at low dose and bilaterally at high dose, in the CCI model (Spataro et al. 2004). Similarly, carbenoxolone has also been shown to attenuate hypersensitivity in the formalin test (Qin et al. 2006) and in mustard oil-induced hypersensitivity (Chiang et al. 2010).

1.9 Lamina I neurokinin 1 receptor expressing projection neurons and central sensitisation

Lamina I NK1R+ neurons are essential for the manifestation of inflammatory pain (Nichols et al. 1999), therefore it is unsurprising that many of the mechanisms of central sensitisation discussed above have been investigated in the context of these neurons.

1.9.1 Lamina I neurokinin 1 receptor expressing neurons are at the origin of a spinal-bulbo-spinal loop

Studies show that lamina I NK1R+ neurons are at the origin of a spinal-bulbo-spinal loop, that controls excitability in the dorsal horn (Suzuki et al. 2002). Projections from the PB, which the majority of lamina I NK1R+ neurons target (Spike et al. 2003, Todd 2010), terminate in brain areas, including the amygdala and hypothalamus, that are implicated in the emotional and autonomic components of pain (Bernard et al. 1993, Bernard & Bandler 1998, Bester et al. 1997, Fulwiler & Saper 1984, Hunt 2000). Projections from the hypothalamus and amygdala, target the PAG, which in turn projects to the rostral ventromedial medulla (RVM), with both excitatory and inhibitory monoaminergic pathways descending from the RVM to the spinal cord, thus closing the loop (Bernard & Bandler 1998, D'Mello & Dickenson 2008, Heinricher et al. 2009, Hunt 2000, Urban & Gebhart 1999) (figure 1.3). Importantly, descending serotoninergic axons have been shown to preferentially innervate lamina I NK1R+ neurons (Polgár et al. 2002).

Disruption of the descending component of the loop provides evidence that this spinal-bulbo-spinal loop is driven by activity in lamina I NK1R+ neurons and plays a key role in controlling spinal cord excitability. The 5-HT3 receptor (5-HT3R) is expressed in the superficial lamina of the dorsal horn (Laporte et al. 1996) and the actions of 5-HT upon this receptor are known to mediate pronociceptive responses

(Ali et al. 1996, Green et al. 2000, Zeitz et al. 2002). Blocking this serotonergic signalling by intrathecal administration of the selective 5-HT3R antagonist, ondansetron, attenuates the second phase of the formalin test and mechanical and thermal hypersensitivity in the CFA inflammatory pain model (Suzuki et al. 2002). Interestingly, the reduction in hypersensitivity seen during ondansetron treatment is comparable to that following ablation of superficial NK1R+ neurons (SP-SAP treatment) and ondansetron is without effect in SP-SAP-treated rats, suggesting that these effects are mediated via NK1R+ neurons. Additional evidence shows that depletion of endogenous 5-HT in the spinal cord, by injection of the selective 5-HT neurotoxin, 5,7di-hydroxytryptamine, significantly reduces mechanical and cold allodynia in rats following nerve injury (Rahman et al. 2006), further detailing the importance of descending serotonergic input in controlling spinal cord excitability.

Electrophysiological data shows that in SP-SAP treated rats, wide dynamic range (WDR) neurons in the deep dorsal horn display significantly reduced receptive fields, less firing in the second phase of the formalin test and attenuated 'wind-up' (Suzuki et al. 2002), where repetitive C-fibre stimulation results in enhanced action-potential firing. While ondansetron was without effect on receptive fields and wind-up, it was shown to reduce the number of spikes in response to mechanical and thermal stimulation in control rats (Bee & Dickenson 2008, Suzuki et al. 2002), but not SP-SAP treated rats (Suzuki et al. 2002), suggesting the descending serotonergic pathways are not active in the absence of NK1R+ projection neurons. Ondansetron also reduced the second phase of the formalin test in control rats in a manner that mimicked the effects of SP-SAP treatment (Suzuki et al. 2002). Furthermore, spinal depletion of 5-HT reduces the number of action potentials in response to thermal and mechanical stimulation and decreases the receptive field sizes in WDR neurons (Rahman et al. 2006). Similarly, ablation of RVM neurons, which are a component of these descending pathways, reduces the number of spikes evoked by thermal, mechanical and electrical C-fibre stimulation in both control and nerve injured rats and attenuates wind-up and after-discharge in control rats (Bee & Dickenson 2008).

Data from human neuroimaging studies provides additional evidence that activity in brain areas known to be involved in descending inhibitory pathways correlates with a modulation of pain (Tracey 2008, 2010). Tracey et al. (2002) demonstrated, using functional magnetic resonance imaging (fMRI) in health human participants, that when subjects were instructed to either focus on or distract themselves from a noxious thermal stimuli, pain intensity ratings were significantly reduced during 'distraction', with this condition also exhibiting increased PAG activation. Furthermore, the

magnitude of the increase in this PAG activation was predictive of the change in the perception of pain. This finding has been supported by further evidence that when subjects are distracted, by performing the Stroop-test, they report lower pain scores following a noxious insult, which correlated with greater PAG activation in fMRI measurements (Valet et al. 2004).

1.9.2 Increased excitability in lamina I neurokinin 1 receptor expressing neurons

As discussed previously in this chapter, enhanced activity and expression of postsynaptic AMPARs and NMDARs plays a significant role in central sensitisation (Ji et al. 2003, Latremoliere & Woolf 2009, Liu & Salter 2010). This altered AMPAR and NMDAR expression / activity has been described in lamina I NK1R+ neurons.

The AMPAR subtypes that are responsible for mediating synaptic transmission between primary afferent fibres and lamina I NK1R+ neurons include a mixture of those that contain or lack the GluA2 subunit, as revealed by calculating the rectification index of α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) evoked excitatory postsynaptic currents (eEPSCs) and the ability of the Ca^{2+}-permeable AMPAR antagonist, JSTX, to reduce the amplitude of AMPA-mediated eEPSCs (Tong & MacDermott 2006). Anatomical studies using the kainate-induced cobalt uptake technique have also indicated the presence of Ca^{2+}-permeable AMPARs in lamina I NK1R+ neurons (Engelman et al. 1999). Interestingly, in CFA inflammation, AMPA eEPSCs in lamina I NK1R+ neurons showed greater inward rectification and enhanced sensitivity to 1-naphthylacetylspermine (a JSTX analogue), indicating an increase in the contribution of Ca^{2+}-permeable AMPARs (Vikman et al. 2008). As Ca^{2+}-permeable AMPARs play a key role in central sensitisation (Hartmann et al. 2004, Jones & Sorkin 2004, Pogatzki et al. 2003), this increase in Ca^{2+}-permeable AMPARs seen in lamina I NK1R+ neurons in inflammation (Vikman et al. 2008) could play a role in the development of inflammatory pain hypersensitivity.

Using a combined electrophysiological / pharmacological approach, it has been shown that NMDARs that contain GluN2A/B and GluN2C/D subunits are functionally expressed on lamina I NK1R+ neurons (Tong et al. 2008). In addition, lamina I NK1R+ neurons express a greater percentage of NMDARs that contain GluN2C/D subunits than lamina I neurons lacking the NK1R. Application of the non-competitive GluN2B antagonist, CP-101,606, reduces the peak amplitude of

NMDAR-mediated eEPSCs in lamina I NK1R+ neurons, with this antagonist effect being significantly attenuated in CFA inflammation (Vikman et al. 2008). Furthermore, CFA significantly reduces the rectification index of NMDAR-mediated eEPSCs, suggesting the N-methyl-D-aspartate (NMDA) current in inflammation displays a reduction in Mg^{2+} sensitivity. It is suggested that this reduced Mg^{2+} sensitivity could result in enhanced NMDAR-mediated synaptic transmission in the dorsal horn during inflammatory pain and play a role in central sensitisation (Vikman et al. 2008).

1.9.3 Lamina I neurokinin 1 receptor expressing neurons and disrupted inhibition

The effect of disrupted inhibition, which has been implicated in chronic pain hypersensitivity (Coull et al. 2005, 2003, Müller et al. 2003, Sherman & Loomis 1996, Sorkin & Puig 1996, Yaksh 1989, Zeilhofer & Zeilhofer 2008), upon the synaptic input to lamina I NK1R+ neurons has been investigated. Pharmacologically mimicking disrupted inhibition in an *ex vivo* spinal cord slice preparation, by application of the $GABA_AR$ and GlyR antagonists, bicuculline and strychnine, respectively, unmasks novel and enhanced polysynaptic Aβ- and polysynaptic Aδ-fibre input to these neurons (Torsney & MacDermott 2006). These novel and enhanced inputs are NMDAR-dependent, in that application of the NMDAR antagonist, D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV), blocks the appearance of novel A-fibre inputs and the enhancement of A-fibre eEPSCs during disinhibition. Although it should be noted that while these polysynaptic inputs are NMDAR-dependent, they are blocked by the AMPAR antagonist, 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline (NBQX), indicating they are not driven solely by NMDARs. The authors propose that in control conditions some or all of the excitatory interneurons that mediate this polysynaptic input receive inhibitory input, which could comprise tonic and/or afferent-driven inhibition (Narikawa et al. 2000). This inhibition is proposed to hyperpolarise these interneurons, preventing the removal of the Mg^{2+} block from NMDARs and given that this pathway is NMDAR-dependent this prevents the transmission of novel A-fibre input via these excitatory interneurons to lamina I NK1R+ neurons. It is proposed that in chronic pain states these novel polysynaptic inputs would be revealed as a result of reduced inhibition (Müller et al. 2003, Zeilhofer & Zeilhofer 2008) or inhibition that has become excitatory (Coull et al. 2005, 2003). These novel polysynaptic A-fibre inputs, which are largely polysynaptic Aβ-fibre inputs, that are

unmasked during disinhibition could provide a pathway that allows innocuous information, which is conveyed by Aβ-fibres, to drive 'nociceptive-specific' neurons in the dorsal horn and thus presents a potential mechanism by which allodynia is mediated. Furthermore, the enhanced polysynaptic Aδ-fibre input may provide additional high-threshold input and therefore could contribute to hyperalgesia. However, further research suggests that the unveiling of this polysynaptic Aβ-fibre input may not be relevant in the case of inflammatory pain (Torsney 2011), but may be more relevant for neuropathic pain (discussed below).

In vivo single unit recordings from lamina I projection neurons in the rat show that normally these neurons only respond to noxious pinch. However, following peripheral nerve injury these neurons now respond to innocuous touch and brush stimuli and show enhanced responses to pinch (Keller et al. 2007). Interestingly, these responses could be mimicked in naïve rats by the application of bicuculline, which suggests that the novel and enhanced A-fibre inputs to lamina I NK1R+ neurons that are revealed when local spinal cord inhibition is diminished (Torsney & MacDermott 2006) could play a role in the mediation of mechanical allodynia in neuropathic pain.

1.9.4 Heterosynaptic facilitation of synaptic input to lamina I neurokinin 1 receptor expressing neurons

Heterosynaptic facilitation, which is a crucial component of central sensitisation (Latremoliere & Woolf 2009), of the synaptic input to lamina I NK1R+ neurons has been demonstrated in the CFA inflammatory pain model, using patch-clamp electrophysiology in an *ex vivo* spinal cord preparation (Torsney 2011). In control tissue these neurons predominantly receive monosynaptic C-fibre input, with a smaller proportion receiving monosynaptic Aδ-fibre input. However, following CFA inflammation there is a significant change in the relative distribution of the type of primary afferent input received and specifically the incidence of monosynaptic Aδ-fibre input is increased 2-fold, without any change in the incidence of monosynaptic C-fibre input. Moreover, a proportion of control neurons, which lacked conventional monosynaptic Aδ-fibre input, received inputs from monosynaptic Aδ-fibres that formed 'silent' (pure-NMDA) synapses. In addition, the amplitude of monosynaptic Aδ-fibre eEPSCs was found to be significantly potentiated in a subset of neurons that received monosynaptic Aδ-fibre only input.

This increase in the incidence of and potentiation of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons is interesting for a number of reasons. Punctate mechanical

hyperalgesia is known to be mediated by a subset of capsaicin-insensitive Aδ-fibres (Fuchs et al. 2000, Magerl et al. 2001, Ziegler et al. 1999), while secondary, and possibly also primary, mechanical hyperalgesia is recognised to be centrally mediated (Klede et al. 2003, Klein et al. 2008, Lewin et al. 2004). As such, the novel monosynaptic Aδ-fibre inputs that are revealed during inflammation could mediate mechanical hyperalgesia, with the unmasking of these inputs, possibly through the 'activation' of Aδ-fibre silent synapses, forming part of the central process by which mechanical hyperalgesia is established.

1.9.5 Homosynaptic facilitation of synaptic input to lamina I neurokinin 1 receptor expressing neurons

Several studies by Jürgen Sankühler's group have investigated homosynaptic potentiation of the monosynaptic C-fibre input to lamina I SP-responsive, retrogradely labelled projection neurons, which are presumably NK1R+ projection neurons. Electrical HFS (100Hz) and low-frequency stimulation (LFS) (2Hz) of monosynaptic C-fibre input to lamina I projection neurons induces long-lasting potentiation of C-fibre eEPSCs in these neurons (Ikeda et al. 2003, 2006). However, this effect is dependent on which subpopulation, defined on the basis of the region to which they project, of neurons involved. Specifically, those neurons that project to the PB are potentiated by HFS but not LFS, while those projecting to the PAG are potentiated by LFS, but not HFS. Furthermore, this potentiation is a Ca^{2+} dependent process, which requires the co-activation of NK1Rs, NMDARs and T-type VGCCs. The potentiation of C-fibre eEPSCs appears to be specific to lamina I projection neurons, as these HFS and LFS protocols do not induce potentiation in unidentified dorsal horn neurons. It is known that C-fibre nociceptors mediate inflammatory pain hypersensitivity (Abrahamsen et al. 2008), therefore this homosynaptic potentiation of the C-fibre input to lamina I projection neurons, which are known to be essential for the manifestation of inflammatory pain (Nichols et al. 1999), could be involved in the development of inflammatory pain hypersensitivity.

1.10 Summary

Inflammatory pain is a debilitating condition, which imposes substantial human and financial costs upon society (Basbaum et al. 2009, Breivik et al. 2006, Latremoliere & Woolf 2009, Maniadakis & Gray 2000, Scholz & Woolf 2002, Torsney &

Fleetwood-Walker 2012, Wenig et al. 2009). There is a pressing need for more efficacious inflammatory pain treatments, that are free from the undesirable side effects which beset many current analgesics (Scholz & Woolf 2002, Woolf 2010). The characteristic symptoms of inflammatory pain; hyperalgesia, allodynia and spontaneous pain, arise largely as a result of altered central processing of nociceptive information, driven by central sensitisation (Latremoliere & Woolf 2009). However, the neuronal plasticity that underlies these changes is not fully understood. Lamina I NK1R+ neurons are known to be essential for the manifestation of inflammatory pain (Nichols et al. 1999) and there is compelling evidence that the primary afferent input to and the postsynaptic responses of these neurons are altered in inflammatory pain (Ikeda et al. 2006, Torsney 2011, Torsney & MacDermott 2006), which could be relevant for inflammatory pain hypersensitivity. Gaining a greater understanding of spinal cord inflammatory pain plasticity is therefore crucial to further our knowledge of the underlying mechanisms of this condition and to inform the development of novel inflammatory pain treatment strategies.

1.11 Thesis aims and hypotheses

The experiments presented in this thesis were designed to investigate spinal cord plasticity in inflammatory pain. Given recent evidence that synaptic input to lamina I NK1R+ neurons, which are crucial for the manifestation of inflammatory pain (Nichols et al. 1999), is altered following inflammation (Torsney 2011) or when the monosynaptic C-fibre input to these neurons is electrically stimulated to mimic the firing pattern seen during inflammation (Ikeda et al. 2006), both inflammation-induced changes in and pharmacological modulation of the primary afferent input to these neurons in the CFA inflammatory pain model were investigated to address the following aims and hypotheses.

Aims

- Chapter 2: Assess the impact of inflammatory pain on the type of primary afferent input received by and the spontaneous excitatory drive to lamina I NK1R+ neurons.
- Chapter 3: Investigate the ability of chemerin, an agonist of the novel inflammatory pain target, the chemerin receptor 23 (ChemR23), to modulate primary afferent input to lamina I NK1R+ neurons.
- Chapter 4: Evaluate inflammation-induced changes in the phenomenon of activity-dependent slowing (ADS) in primary afferent input to lamina I NK1R+ neurons.

Hypotheses

- Chapter 2: CFA inflammation will alter the relative distribution of the type of primary afferent input that lamina I NK1R+ neurons receive.
- Chapter 3: The ChemR23 agonist, chemerin, will attenuate the capsaicin potentiation of synaptic input to lamina I NK1R+ neurons and monosynaptic C-fibre input to a subset of these neurons in CFA inflammation.
- Chapter 4: ADS in isolated dorsal roots and in the monosynaptic C-fibre input to lamina I NK1R+ neurons will be altered in CFA inflammation, which could have functional consequences for the activity of these neurons.

The individual aims and hypotheses are discussed in greater detail in the corresponding chapters.

Chapter 2

Synaptic input to lamina I neurokinin 1 receptor expressing neurons in inflammatory pain

2.1 Introduction

2.1.1 Effects of inflammation on primary afferent fibres

The Aβ-, Aδ- and C-fibre primary afferent components of dorsal roots can be defined based on activation threshold and conduction velocity (Nakatsuka et al. 2000, 1999, Park et al. 1999, Torsney 2011). Previous studies have established that the complete Freund's adjuvant (CFA) inflammatory pain model does not alter activation threshold, conduction velocity or amplitude of Aβ-, Aδ- or C-fibres in isolated dorsal roots, using both intracellular recordings from individual dorsal root ganglia (DRG) neurons (Nakatsuka et al. 1999) and extracellular population recordings (Baba et al. 1999, Torsney 2011). Differences exist in the electrically evoked properties of dorsal roots isolated from adult and juvenile rats, namely juveniles exhibit significantly increased activation thresholds and reduced conduction velocity for all primary afferent fibre types, presumably due to increased myelination and/or fibre diameter in adult rats (Nakatsuka et al. 2000, Park et al. 1999). Notably, CFA inflammation has no effect on these properties of dorsal roots in both adults (Baba et al. 1999, Nakatsuka et al. 1999) and juveniles (Torsney 2011).

A major focus of this thesis is to investigate the spinal mechanisms of inflammatory pain, by examining the synaptic inputs to lamina I neurokinin 1 receptor expressing (NK1R+) neurons. It is therefore important to verify that the electrical stimulation

intensities used in previous studies (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006) to activate the different primary afferent fibre types are appropriate. Furthermore, it is important to confirm that inflammation does not alter the electrically evoked properties of isolated rat dorsal roots to ensure that, where dorsal root evoked excitatory postsynaptic currents (eEPSCs) are recorded, any changes resulting from CFA inflammation presented here can be attributed to central rather than peripheral changes.

2.1.2 Altered patterns of synaptic input to lamina I neurokinin 1 receptor expressing neurons in inflammatory pain

Under naïve conditions, lamina I NK1R+ neurons predominantly receive monosynaptic C- and/or monosynaptic Aδ-fibre input, with a smaller proportion receiving monosynaptic Aβ-fibre or purely polysynaptic input (Torsney 2011, Torsney & MacDermott 2006). Following peripheral inflammation there is a loss of inhibition in the dorsal horn of the spinal cord (Müller et al. 2003, Zeilhofer & Zeilhofer 2008). Pharmacologically mimicking this disrupted inhibition by applying the glycine receptor (GlyR) and $GABA_A$ receptor ($GABA_AR$) antagonists strychnine and bicuculline, respectively, reveals novel and enhanced polysynaptic A-fibre input to lamina I NK1R+ neurons (Torsney & MacDermott 2006). This increased A-fibre input to lamina I NK1R+ neurons is largely the result of novel polysynaptic Aβ-fibre input (figure 2.1A), thereby unveiling a pathway that enables low-threshold (touch) input to access 'nociceptive specific' neurons and thus provides a potential model for the development of allodynia. Interestingly, a similar facilitation of polysynaptic Aβ-fibre input has also been reported in unidentified lamina II neurons (Baba et al. 1999). This increased polysynaptic Aβ-fibre input to lamina I NK1R+ neurons does not appear to be implicated in inflammatory pain (Torsney 2011), but may be relevant in neuropathic pain (Keller et al. 2007), where spinal cord disinhibition is a dominant feature (Costigan et al. 2009).

Following CFA inflammation, monosynaptic Aδ-fibre input to lamina I NK1R+ neurons is significantly increased by around two fold, without any change in the incidence of monosynaptic Aβ- or monosynaptic C-fibre input. Furthermore, monosynaptic Aδ-fibres were shown to form pure-N-methyl-D-aspartate (NMDA) or 'silent' synapses with a portion of control cells that were classified as lacking conventional monosynaptic Aδ-fibre input, but which typically received polysynaptic A-fibre input (figure 2.1B). It is proposed that the 'activation' of these silent synapses

could account for the novel monosynaptic Aδ-fibre inputs observed following CFA inflammation and form part of the mechanism responsible for mechanical hyperalgesia associated with inflammatory pain (Torsney 2011).

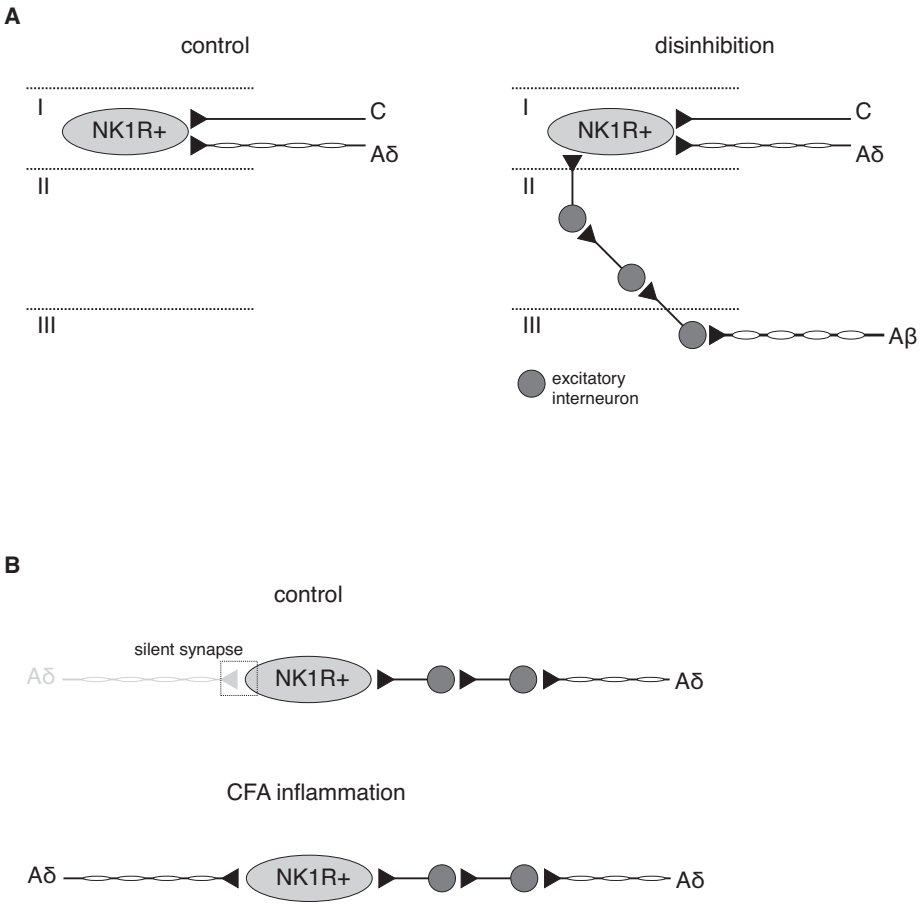

Figure 2.1: Changes in synaptic input to lamina I NK1R+ neurons. ***A*** Left: in control conditions lamina I NK1R+ neurons typically receive input from monosynaptic Aδ- and/or monosynaptic C-fibres. Right: pharmacologically mimicking disinhibition reveals a novel polysynaptic Aβ-fibre pathway to these neurons, adapted from Torsney & MacDermott 2006. ***B*** Top: in control conditions a subset of lamina I NK1R+ neurons which do not receive conventional monosynaptic Aδ-fibre input, receive input from monosynaptic Aδ-fibre silent synapses. Bottom: following CFA inflammation lamina I NK1R+ neurons receive novel monosynaptic Aδ-fibre input, which may result from the activation of these silent synapses, adapted from Torsney 2011

This increased monosynaptic Aδ-fibre drive to lamina I NK1R+ neurons is interesting for the following reasons. A subset of capsaicin-insensitive Aδ-fibres have been shown to mediate punctate mechanical hyperalgesia (Fuchs et al. 2000, Magerl et al. 2001, Ziegler et al. 1999) and secondary mechanical hyperalgesia and potentially also primary mechanical hyperalgesia has been established to be a centrally mediated process (Klede et al. 2003, Klein et al. 2008, Lewin et al. 2004). Therefore, these novel Aδ-fibre inputs to these neurons during inflammation could potentially mediate mechanical hyperalgesia, with the unmasking of these inputs being part of central

processes involved in establishing mechanical hyperalgesia.

2.1.3 Potentiation of synaptic inputs to lamina I neurokinin 1 receptor expressing neurons in inflammatory pain

C-fibre potentiation

A number of studies have investigated ways in which monosynaptic C-fibre input to lamina I NK1R+ neurons may be potentiated, which could have implications for the development of allodynia and hyperalgesia. The effect of high-frequency stimulation (HFS) and low-frequency stimulation (LFS) of monosynaptic C-fibre input to lamina I projection neurons, identified by retrograde labelling and that were substance P (SP) responsive and thus likely to be NK1R+ neurons, has been assessed (Ikeda et al. 2003, 2006). HFS (100Hz) of monosynaptic C-fibre input to a subset of these neurons that project to the parabrachial area (PB) produces homosynaptic potentiation of C-fibre eEPSCs (Ikeda et al. 2003). Furthermore, this potentiation was demonstrated to be a Ca^{2+} dependent process, which is reliant on neurokinin 1 receptor (NK1R), N-methyl-D-aspartate receptor (NMDAR) and T-type voltage-gated calcium channel (VGCC) co-activation. Incidentally, this phenomenon occurred only in these projection neurons and the stimulation protocol was without effect in unidentified spinal cord neurons. However, the physiological relevance of this finding is questionable due to the fact that stimulation of C-fibres in this manner does not reflect the continuous low-frequency firing patterns observed in inflammatory pain (Djouhri et al. 2006, Puig & Sorkin 1996, Xiao & Bennett 2007). Furthermore, increasing the C-fibre stimulation frequency correlates with a reduction in the ability of these fibres to repetitively fire action potentials (Nakatsuka et al. 2000), so the degree to which C-fibres can sustain action potential firing when stimulated at 100Hz is debatable. Subsequent investigation into this phenomenon revealed that LFS (2Hz), that more closely mimics the spontaneous C-fibre firing pattern seen during inflammation, resulted in homosynaptic potentiation of C-fibre eEPSCs in a subset of neurons that project to the periaquducal gray matter (PAG) (Ikeda et al. 2006). As with HFS, LFS potentiation was dependent on the concurrent activation of NMDARs, NK1Rs and T-type VGCCs and was Ca^{2+}-dependent. In summary, as detailed above it was shown that different lamina I NK1R+ neuron subpopulations respond differently to identical stimulation protocols. While HFS of C-fibre inputs potentiates C-fibre synapses onto PB projecting neurons, it is without effect on those projecting to the PAG (figure 2.2A) (Ikeda et al. 2003, 2006). Conversely, LFS of C-fibres has no effect in

PB projecting neurons, but potentiates C-fibre synapses onto PAG projecting neurons (figure 2.2B) (Ikeda et al. 2006). It is worth noting that C-fibre activity is essential for the development of inflammatory pain (Abrahamsen et al. 2008), therefore this C-fibre potentiation could play a crucial role in the development of inflammatory hypersensitivity. However, potentiation of C-fibre synapses onto these neurons does not appear to occur following CFA inflammation (Torsney 2011). As C-fibre eEPSCs are the focus of several investigations in later chapters of this thesis, it was important to establish whether these C-fibre synapses onto lamina I NK1R+ neurons are potentiated following CFA inflammation in these particular studies.

A-fibre potentiation

Unlike monosynaptic C-fibre input to lamina I projection neurons, *in vitro* LFS of monosynaptic A-fibre inputs on to PAG projecting neurons, at C-fibre intensity, does not potentiate eEPSCs (Ikeda et al. 2006). However, following CFA inflammation monosynaptic Aδ-fibre input to a subset of lamina I NK1R+ neurons that received only Aδ-fibre input displayed a significantly increased eEPSC amplitude (Torsney 2011). Aδ-fibre eEPSCs were used in experimental protocols in later chapters, therefore it was important to establish whether this Aδ-fibre potentiation was present in the data presented here.

Monosynaptic Aβ-fibre input to lamina I NK1R+ neurons has been shown to be minimal (Torsney 2011, Torsney & MacDermott 2006). Polysynaptic Aβ-fibre input to lamina I NK1R+ neurons is predicted to be enhanced under conditions of disinhibition (Torsney & MacDermott 2006), however this does not appear to be the case in CFA inflammation (Torsney 2011).

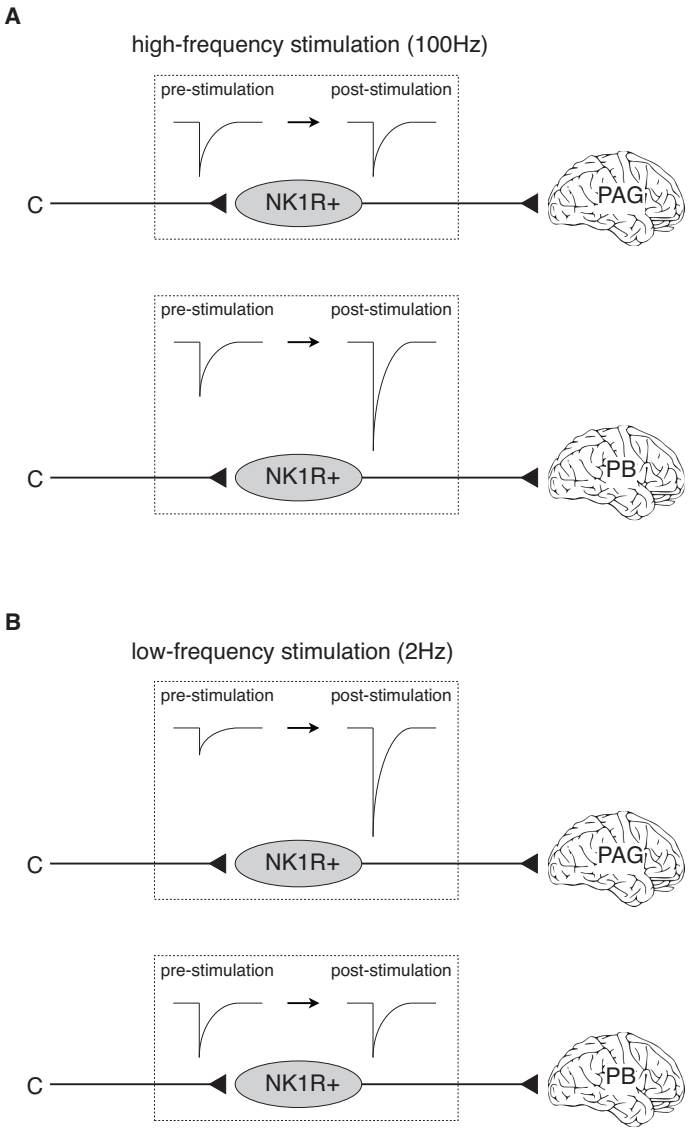

Figure 2.2: Repetitive electrical stimulation of C-fibre input to lamina I NK1R+ projection neurons potentiates eEPSCs in different subsets, depending on stimulation frequency. ***A*** Stimulation of C-fibre inputs to NK1R+ neurons at high frequency (100Hz) has no effect on PAG projecting neurons (top), but potentiates eEPSCs in those that project to the PB (bottom) (Ikeda et al. 2003, 2006). ***B*** Low frequency stimulation of C-fibre inputs to the subset of lamina I NK1R+ neurons that project to the periaquducal gray matter (PAG) causes a long lasting potentiation of eEPSCs (top), however is without effect in neurons projecting to the parabrachial area (PB, bottom) (Ikeda et al. 2006).

2.1.4 Spontaneous excitatory input to lamina I NK1R+ neurons

The evidence presented above suggests that evoked excitatory input to lamina I NK1R+ neurons is likely to be potentiated following inflammation (Ikeda et al. 2006, Torsney 2011). Others have studied the effects of inflammation on spontaneous excitatory input to lamina II neurons, with conflicting results. CFA inflammation is reported to significantly increase spontaneous excitatory postsynaptic current

(sEPSC) frequency and amplitude (Park et al. 2011), however others have demonstrated CFA is without effect (Lappin et al. 2006). It is not known whether spontaneous excitatory input to lamina I NK1R+ neurons is altered in inflammation. Given that these neurons play such a crucial role in the manifestation of inflammatory pain (Nichols et al. 1999), it is important to establish whether the spontaneous input they receive is altered in inflammation. It is known that different lamina II neuronal subtypes show different levels of altered spontaneous excitatory input in neuropathic pain (Balasubramanyan et al. 2006). Given that the pattern of primary afferent input to lamina I NK1R+ neurons is likely to be altered in inflammation (Torsney 2011), it is of interest to determine whether spontaneous excitatory input to these neurons varies with the type of input received.

2.2 Chapter aims and hypotheses

The experiments presented in this chapter were conducted to determine the changes in primary afferent input to lamina I NK1R+ neurons following CFA inflammation and to address the following aims and hypotheses.

Aims

1. Confirm that CFA inflammation does not alter the electrical activation threshold, conduction velocity or amplitude of Aβ-, Aδ- and C-fibre compound action potentials recorded in isolated dorsal roots.
2. Verify the appropriate electrical stimulation intensities required to activate the different primary afferent fibre types for use in patch-clamp studies of synaptic input to lamina I NK1R+ neurons.
3. Confirm the previously reported increased incidence and potentiation of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons during inflammation.
4. Determine whether potentiation of C-fibre synapses with lamina I NK1R+ neurons occurs during CFA inflammation.
5. Investigate the spontaneous excitatory input to lamina I NK1R+ neurons and determine whether this is altered by CFA inflammation and/or the type of primary afferent input a neuron receives.

Hypotheses

1. CFA inflammation will not alter the electrical activation thresholds, conduction velocity or amplitude of Aβ-, Aδ- and C-fibre compound action potentials recorded in isolated dorsal roots.
2. The appropriate electrical stimulation intensities for activation of different primary afferent components, for use in subsequent patch-clamp studies, will be in agreement with stimulation intensities reported in previous studies (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006).
3. The incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons will be significantly increased following CFA inflammation.

4. CFA inflammation will not result in potentiation of monosynaptic C-fibre input to lamina I NK1R+ neurons.

2.3 Methods

2.3.1 Animals

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986, and the International Association for the Study of Pain (IASP) ethical guidelines for animal research (Zimmermann 1983). Juvenile Sprague Dawley rats of both sexes, aged approximately postnatal day 21 (P21), that were bred within and obtained from the University of Edinburgh Biological Research Resources were used in all experiments. Animals were housed in cages at 21°C and 55% relative humidity, with a 12h light-dark cycle. Food and water were provided *ad libitum*. Importantly, previous studies have shown that juvenile rats of this age develop inflammation-induced behavioural hypersensitivity and spinal cord hyperexcitability that is comparable to adult rats (Torsney 2011, Torsney & Fitzgerald 2002)

2.3.2 Complete Freunds's adjuvant inflammatory pain model

The CFA inflammatory pain model is a commonly used experimental model of persistent inflammatory pain (Ren & Dubner 1999). CFA contains heat attenuated *mycobacterium tuberculosis* suspended in a mineral oil. CFA injection provokes an immune response and when injected subcutaneously into the plantar surface of a rats paw it causes localised tissue inflammation and oedema (Nakatsuka et al. 1999, Xiao & Bennett 2007). About 24 hours following injection, rats develop a persistent mechanical and thermal hypersensitivity, in both adults (Lin et al. 2007, Nakatsuka et al. 1999, Raghavendra et al. 2004, Xiao & Bennett 2007) and juveniles (Torsney 2011).

In these studies, CFA was injected into the plantar surface of the left hindpaw (0.5mg/ml, 1µl/g body weight, Sigma, figure 2.3) under brief isoflurane anaesthesia. Injections were performed 2-6 days (∼P18) prior to electrophysiological recordings at ∼P21 (Torsney 2011). Control rats were untreated.

Figure 2.3: CFA hindpaw injection, showing location of CFA injection.

2.3.3 Isolated dorsal root electrophysiology

Isolated dorsal root preparation

Isolated dorsal roots were prepared using previously established methods (Bardoni et al. 2004, Labrakakis et al. 2003, Lee et al. 2002, Torsney 2011). Control untreated or CFA treated rats, aged ~P21 were anaesthetised with isoflurane and decapitated. Spinal cords with dorsal roots attached were removed and placed in ice-cold dissection solution that was continuously bubbled with 95% O_2 / 5% CO_2. Lumbar (L4/5) dorsal roots, with DRGs removed, were cut near to the dorsal root entry zone and placed in oxygenated recovery solution for 1 hour at 37°C and were then maintained at room temperature prior to recording. In the case of CFA treated animals, dorsal roots from the inflamed (left) side only were prepared. It was not always possible to retain DRGs, therefore to ensure all preparations were comparable, any DRGs were removed. Dorsal roots of comparable length were used in both groups (control: 4.4 $\pm$ 0.3mm, CFA: 4.2 $\pm$ 0.2mm, P=0.761, unpaired t-test).

Dorsal roots were transferred to the recording chamber of an upright microscope (Zeiss Axiokop II) and continually perfused with oxygenated Krebs solution at a rate of 1-2ml/min at room temperature. The composition of the Krebs solution was as follows (in mM); 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, 1 $MgCl_2$, 2 $CaCl_2$, pH 7.4. A high Mg^{2+}, low Ca^{2+} Krebs solution was used minimise

excitotoxic damage during the dissection and recovery. To further minimise excitotoxicity during dissection, the ionotropic glutamate receptor antagonist, kynurenic acid, was added to the dissection solution. The recovery solution was composed of the following (in mM); 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, 6 $MgCl_2$, 1.5 $CaCl_2$, pH 7.4. The composition of the dissection solution was as follows (in mM); 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose, 6 $MgCl_2$, 1.5 $CaCl_2$, 1 kynurenic acid, pH 7.4. All chemicals were obtained from Sigma.

Compound action potential electrophysiology

Compound action potential (CAP) recordings were used to evaluate the influence of CFA inflammation on the electrically evoked properties of isolated dorsal roots. Recordings were made using glass suction electrodes placed at either end of the dorsal root, one for stimulating and the other for recording (Figure 2.4) (Baba et al. 1999, Bardoni et al. 2004, Labrakakis et al. 2003, Lee et al. 2002, Torsney 2011). Dorsal roots were electrically stimulated 10 times at a frequency of 0.2Hz and a stimulus duration of 0.1ms, with an ISO-Flex Stimulus Isolator (A.M.P.I. Intracel). To determine the activation threshold for each primary afferent component, stimulation intensities were increased in a stepwise manner as follows (in μA): 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 25, 30-100 (in 10μA steps) and 150-500 (in 50μA steps) (Nakatsuka et al. 2000, Torsney 2011). Data were acquired and recorded using a Cygnus ER-1 differential amplifier (Cygnus Technologies Inc.) and pClamp 10 software (Molecular Devices).

The main components of the CAPs were differentiated as fast (Aβ), medium (Aδ) and slow (C) conducting components, with each displaying a characteristic triphasic (positive-negative-positive) response (figure 2.6B). Small intermediate components were occasionally observed, as has been reported previous (Géranton et al. 2009, Torsney 2011), however these were not analysed. The data from roots in which the identity of any of the three components was ambiguous were excluded from analysis. Activation threshold was defined as the lowest stimulation intensity at which the negative component of the triphasic response was first clearly identifiable (figure 2.5). Amplitude and conduction velocity were measured from averaged traces (average of 10 traces) at stimulation intensities of 20, 100 and 500μA for Aβ-, Aδ- and C-fibre components, respectively, as these were the intensities selected to activate these fibre types in subsequent dorsal root eEPSC experiments, as described in section 2.4.1.

Amplitude was measured as the distance between the positive and negative peak of the response (figure 2.6B). In the case of Aδ- and C-fibres, typically the second positive peak was chosen, as the first positive peak sometimes overlapped with the previous component (example in figure 2.6A), despite using dorsal roots that were as long as possible. The latency between the stimulus artefact and the negative peak of the triphasic response (figure 2.6A) and the distance between the recording and stimulating electrodes (dorsal root length) were measured and conduction velocity calculated using the following formula:

$$conduction\ velocity = \frac{root\ length}{response\ latency} \qquad \text{(a)}$$

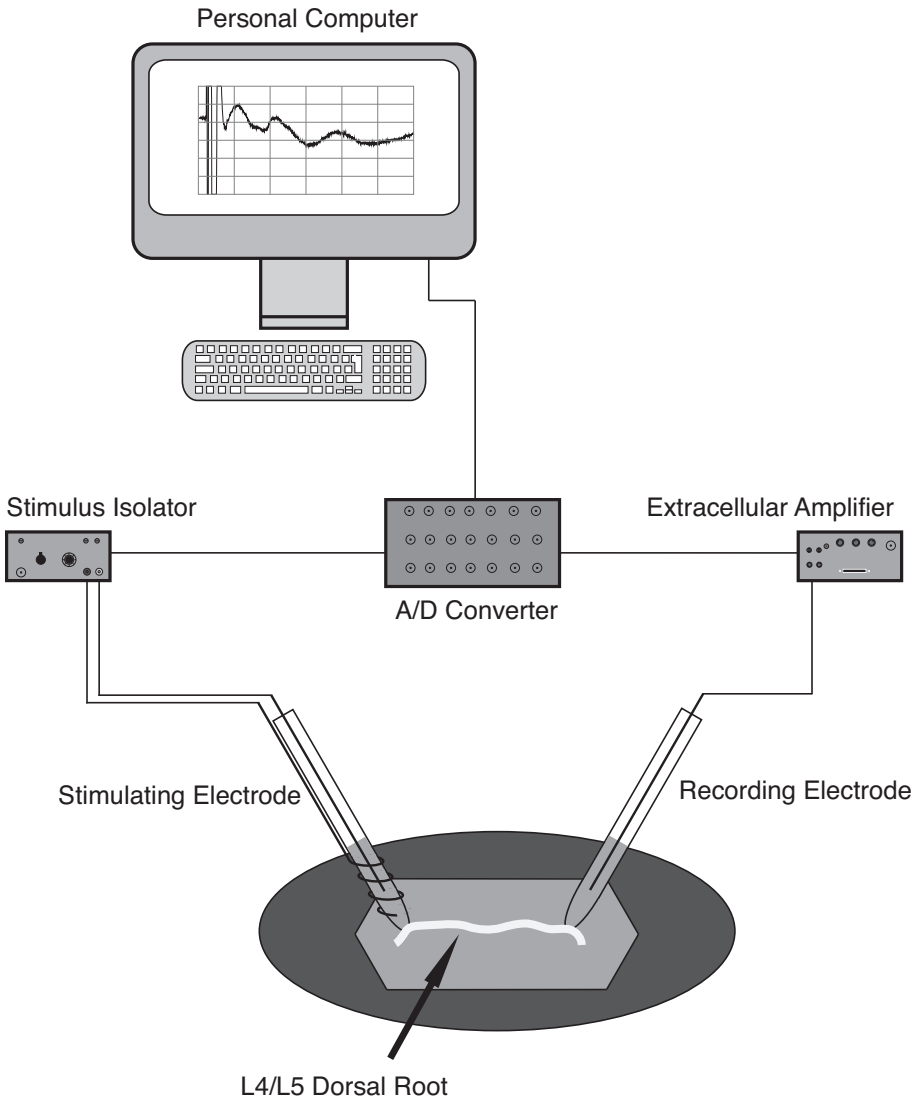

Figure 2.4: Compound action potential recording set-up.

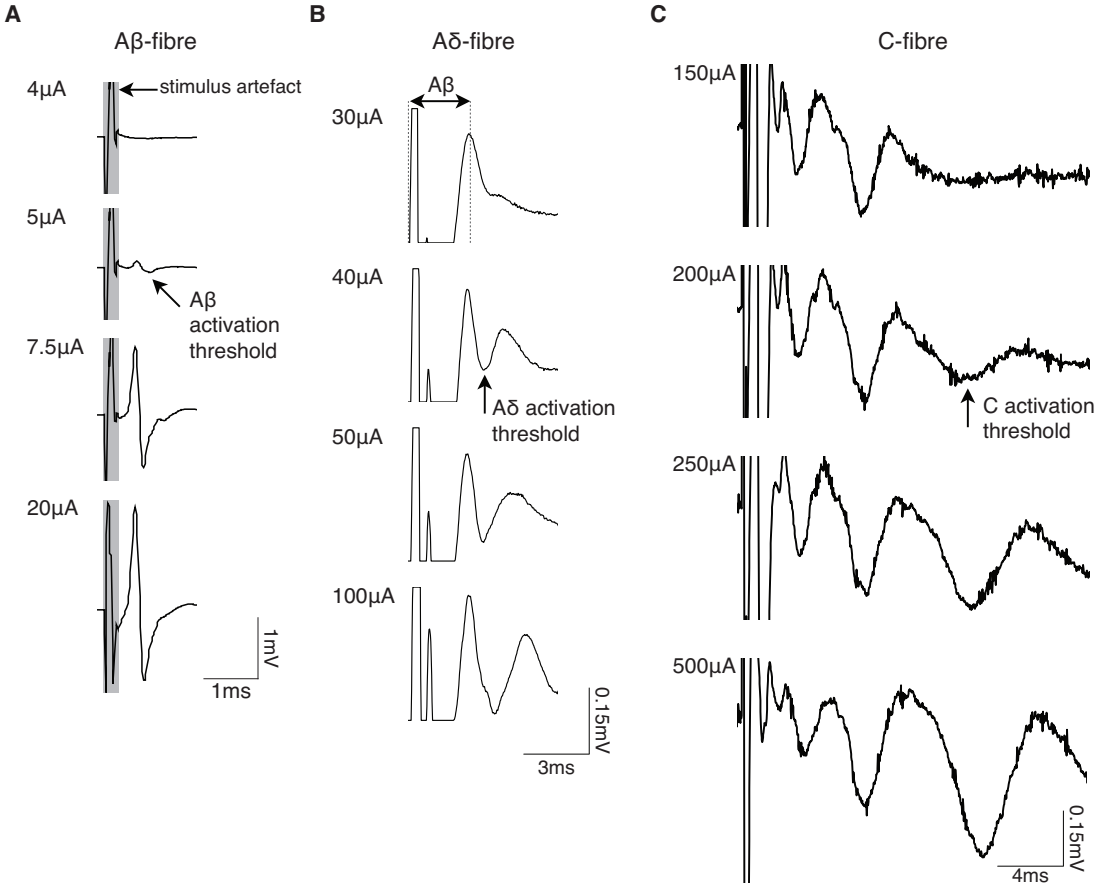

Figure 2.5: Identification of primary afferent activation thresholds. Examples of identification of activation thresholds for Aβ- (***A***), Aδ- (***B***) and C-fibres (***C***) in isolated dorsal roots. Activation thresholds were defined as the stimulation intensity at which the negative peak of the triphasic response is first clearly identifiable, indicated on the traces by arrows. The stimulus artefact is highlighted in ***A*** by the grey box. In ***B*** the first positive peak of the Aδ-fibre response is shown overlapping with the second positive peak of the Aβ-fibre response. Numbers to left of traces indicate stimulation intensity used. All traces shown are an average of 10 traces.

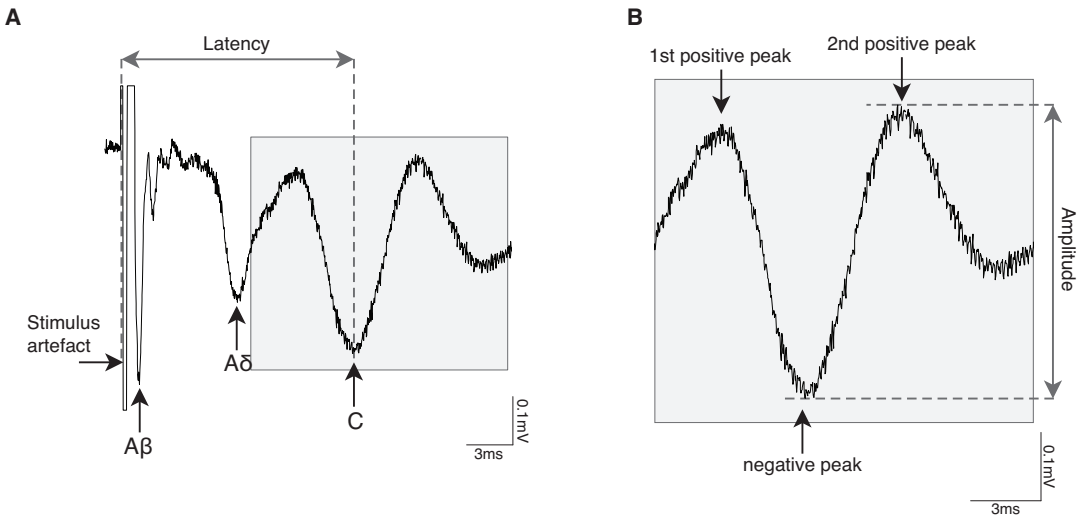

Figure 2.6: Examples of how compound action potential latency and amplitude was measured. ***A*** Response latency was measured as the time between the stimulus artefact and the negative peak of the triphasic response. This example indicates the latency for the C-fibre component. The negative peaks of the Aβ-, Aδ- and C-fibre components are indicated by arrows. ***B*** Example trace shows C-fibre component from ***A*** (denoted by box), where the negative and 2 positive peaks of the triphasic response can be clearly seen. Amplitude was measured as the difference between the negative and second positive peak. Traces shown are an average of 10 traces.

2.3.4 Spinal cord slice electrophysiology

Spinal cord slice preparation

Spinal cord slices with dorsal roots attached were prepared using previously established methods (Bardoni et al. 2000, 2004, Nakatsuka et al. 1999, Tong et al. 2008, Tong & MacDermott 2006, Torsney 2011, Torsney & MacDermott 2006). Spinal cords were removed from control or CFA treated rats and placed in ice-cold dissection solution in the manner detailed in section 2.3.3. Following removal of the ventral roots, DRGs, dura mater and arachnoid membrane, the lumbar (L4/5) spinal cord was embedded in 3% low-melting-point agar (Invitrogen) and transverse slices (350μm) with dorsal roots attached (left side only in CFA tissue) were cut using a vibrating blade microtome (Intracell) and placed in oxygenated recovery solution for 1 hour at 37°C. Slices were then incubated with tetramethylrhodamine conjugated substance P (TMR-SP) (Enzo Life Sciences), as described below and were then kept in recovery solution at room temperature prior to use (Labrakakis & MacDermott 2003, Torsney 2011, Torsney & MacDermott 2006). Slices were transferred to the recording chamber of an upright microscope (Zeiss Axiokop II) equipped with infrared-differential interference contrast (ir-DIC) (Hamamatsu) for electrophysiological recordings and fluorescence (Cairn Research) for identification of TMR-SP labelled (TMR-SP+) neurons. Slices were fully submerged and held in place with a small weight made of sliver wire (Harvard Apparatus) and continually perfused with oxygenated Krebs solution at a flow rate of 1-2ml/min at room temperature.

Neurokinin 1 receptor expressing neuron identification

To enable the targeting of lamina I NK1R+ neurons for electrophysiological investigation, neurons were pre-labelled with a fluorescent SP ligand, as has been reported elsewhere (Labrakakis & MacDermott 2003, Takazawa & MacDermott 2010, Tong et al. 2008, Tong & MacDermott 2006, Torsney 2011, Torsney & MacDermott 2006). After spinal cord slices had been cut and placed in oxygenated recovery solution for 1 hour at 37°C, as described above, slices were incubated in 35nM TMR-SP, in oxygenated recovery solution, for 30 minutes at room temperature. Following incubation in TMR-SP, slices were kept in oxygenated recovery solution at room temperature prior to use. This method for pre-identification of lamina I NK1R+ neurons is regarded to have minimal impact on the synaptic

responses of these neurons. TMR-SP is one of the least biologically active of the fluorescent SP conjugates, as evidenced by the fact it does not alter neuronal M-type K^+ currents at nM concentrations or elevate Ca^{2+} in Chinese hamster ovary cells that express the NK1R (Bennett & Simmons 2001). Importantly, TMR-SP has also been shown to have no effect on the synaptic responses of lamina I NK1R+ neurons (Tong & MacDermott 2006). While it is reported that ∼45% of lamina I neurons display NK1R immunoreactivity (Todd et al. 1998) and only ∼5% of all lamina I neurons are projection neurons, the majority of which express the NK1R (Spike et al. 2003), it is argued that TMR-SP labelling will preferentially label lamina I NK1R+ projection neurons (Torsney & MacDermott 2006). TMR-SP labelling in the spinal cord slice preparation labels a small proportion of neurons in the lamina I region, far less than 45% (Torsney & MacDermott 2006). Given that the majority of lamina I NK1R+ projection neurons display moderate to strong levels of NK1R immunoreactivity (Spike et al. 2003), it is argued that TMR-SP labelling is more likely to identify these projection neurons (Torsney & MacDermott 2006) as opposed to the population of likely interneurons that display weak NK1R immunoreactivity (Cheunsuang & Morris 2000, Todd et al. 2005).

Patch-clamp electrophysiology

Whole-cell patch-clamp recordings were made from TMR-SP+ / presumptive NK1R+ neurons in the lamina I region of the dorsal horn. A suction electrode was used to electrically stimulate the dorsal root and activate primary afferent inputs (figures 2.7 & 2.8A). The lamina I region was identified visually as the thin area between the white matter and lamina II, the latter being easily distinguished as a translucent band across the dorsal horn. Furthermore, in lamina II there are very few projection neurons (Todd 2010) and limited numbers of NK1R+ neurons (Bleazard et al. 1994, Brown et al. 1995). To confirm that the recorded neurons were located in lamina I, images, using a X5 objective, showing the dorsal horn and position of the recording electrode were obtained (figure 2.8A).

Patch electrodes, with a tip resistance of 4–6MΩ when filled with intracellular solution, were pulled from thick wall borosilicate glass (GC150F-7.5, Harvard Apparatus) using a Flaming/Brown model P-97 microelectrode puller (Sutter Instruments). The composition of the intracellular solution used in all eEPSC and sEPSC recordings is as follows (in mM): 120 Cs-methylsulfonate, 10 Na-methylsulfonate, 10 ethylene glycol tetraacetic acid (EGTA), 1 $CaCl_2$, 10

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5 2(triethylamino)-N-(2,6-dimethylphenyl) acetamine chloride (QX-314-Cl), 5 Mg-ATP, pH adjusted to 7.2 with CsOH, osmolarity ~290mOsm. In addition, 1μM Alexa Fluor 488 hydrazide was added to the recording pipette. This filled the neuron recorded from and confirms it was the targeted TMR-SP+ neuron (figure 2.8B). All chemicals were obtained from Sigma, except from EGTA (Fluka), QX-314-Cl (Alomone Labs) and Alexa Fluor 488 (Molecular Probes). All recordings were made at a holding potential of -70mV, which is the anion reversal potential (E_{anion}) in lamina I neurons (Coull et al. 2003) and as such blocks inhibitory inputs on to these neurons. This enables the study of excitatory input without the influence of inhibition. It is important to note that while the E_{anion} is developmentally regulated, lamina I neurons in rats of the age used in these studies display an E_{anion} comparable to adults (Cordero-Erausquin et al. 2005). Junction potential was corrected prior to recording. Data were recorded and acquired with an Axopatch 200B amplifier and pClamp 10 software (Molecular Devices). Data were filtered at 5kHz and digitised at 10kHz. Cells were excluded if any of the following criteria were met: access resistance $>$25MΩ; holding current $>$100pA or $<$-100pA; membrane resistance $<$300MΩ. Membrane resistance was monitored by measuring current responses to a -20mV voltage step.

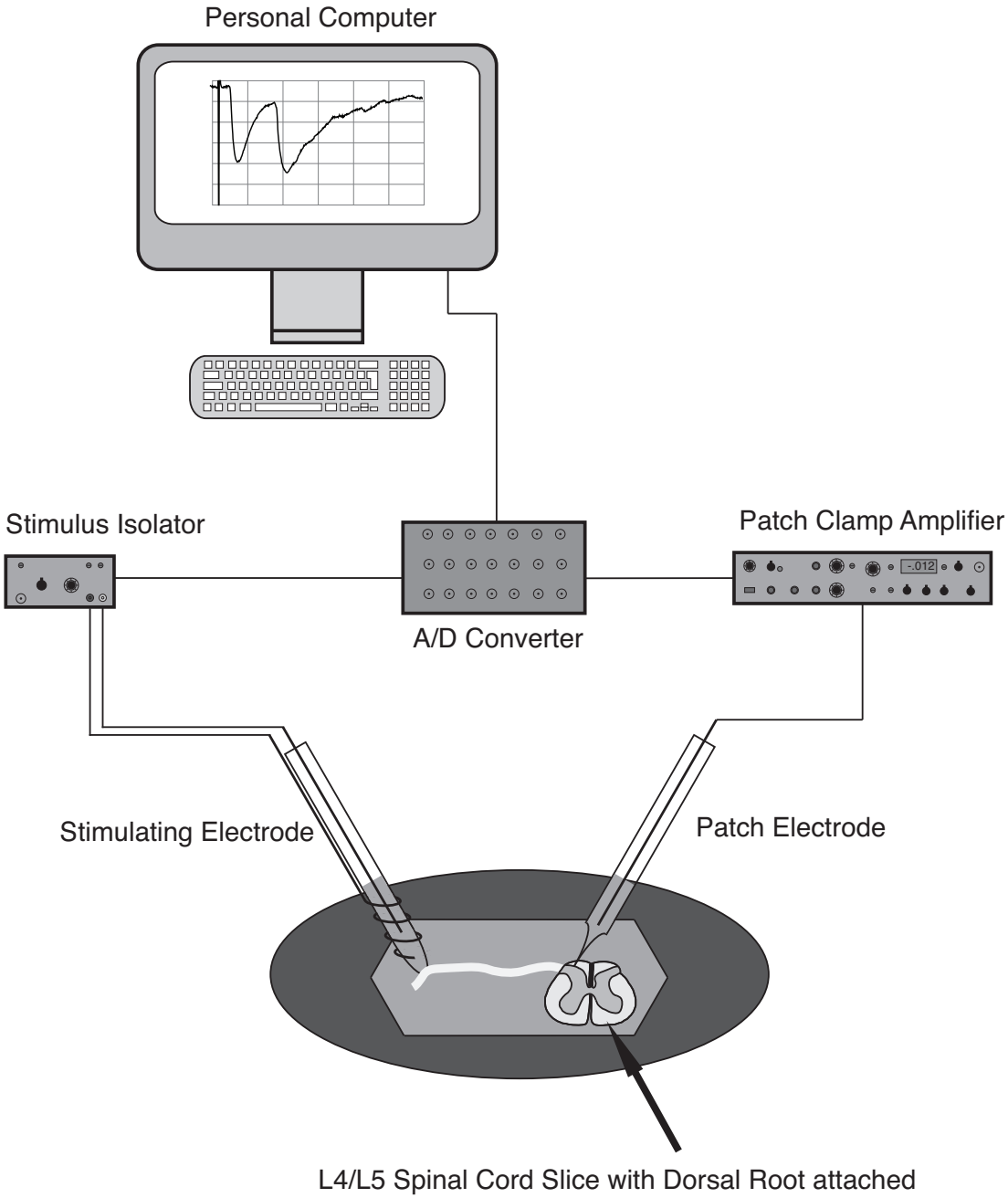

Figure 2.7: Patch-clamp electrophysiology recording set-up.

2.3.5 Primary afferent input characterisation

Synaptic input to lamina I NK1R+ neurons was characterised in tissue isolated from control and CFA treated rats to confirm the previously reported alterations seen following inflammation (Torsney 2011). To characterise input the dorsal root was stimulated three times at low frequency (0.05Hz), with a stimulus duration of 0.1ms at intensities of 20, 100 and 500μA to activate Aβ-, Aδ- and C-fibre inputs respectively. At this stimulation frequency, both monosynaptic and polysynaptic primary afferent

input displays a stable latency, therefore responses which were stable and thus time locked with the stimulus were classified as 'evoked'. Whereas responses that lacked stability and were thus unconnected with the stimulus were considered 'spontaneous' (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006). The monosynaptic / polysynaptic nature of stable afferent input was determined in the manner previously reported (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006). The dorsal root was stimulated 20 times, with a stimulus duration of 0.1ms at the intensities and frequencies detailed below in table 2.1.

Input	Stimulation intensity (μA)	Stimulation frequency (Hz)
Aβ	20	20
Aδ	100	2
C	500	1

Table 2.1: Stimulation parameters used to determine the monosynaptic / polysynaptic nature of primary afferent input to lamina I NK1R+ neurons

These frequencies were selected because action potential firing fails in Aδ- and C-fibres at stimulation frequencies >2Hz and >1Hz, respectively, while Aβ-fibres show no failures at frequencies of up to 20Hz (Nakatsuka et al. 2000). Therefore, if frequencies greater than these were used, it would have been impossible to differentiate between synaptic failures and a failure of action potential firing in the primary afferent inputs. A-fibre eEPSCs were classified as monosynaptic if there was an absence of synaptic failures and a stable latency ($\leq$2ms) and polysynaptic where synaptic failures occurred and/or latency variability was >2ms. C-fibre eEPSCs were classified as monosynaptic if no synaptic failures occurred, regardless of latency variability, with the presence of synaptic failures indicating a polysynaptic input (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006). Neurons that received both monosynaptic and polysynaptic input were classified on the basis of the monosynaptic input only, while those that received only polysynaptic input were classified as polysynaptic only, regardless of whether this was polysynaptic Aβ-, Aδ- or C-fibre input. Neurons that displayed no input in response to dorsal root stimulation were excluded as it was impossible to be certain that these neurons received no input, it being most likely that the input was cut in the process of preparing the spinal cord slices.

The amplitude of monosynaptic responses was determined by measuring the difference between baseline and the negative peak of the response (figure 2.15A). The latency between the stimulus artefact and the onset of the monosynaptic response was measured (figure 2.15C), as was the distance between the stimulating electrode and the point at where the dorsal root entered the dorsal horn and estimated conduction

velocity calculated using equation (a). This method of determining conduction velocity provides an imperfect estimate, as it ignores the portion of the conduction pathway within the spinal cord and does not take into account the synaptic delay. However, as the primary afferent inputs have such different conduction velocities (Géranton et al. 2009, Nakatsuka et al. 2000, Torsney 2011), this measure is still informative. The amplitude and estimated conduction velocity of inputs were calculated using responses recorded at intensities of 20, 100 and 500μA for Aβ-, Aδ- and C-fibres respectively.

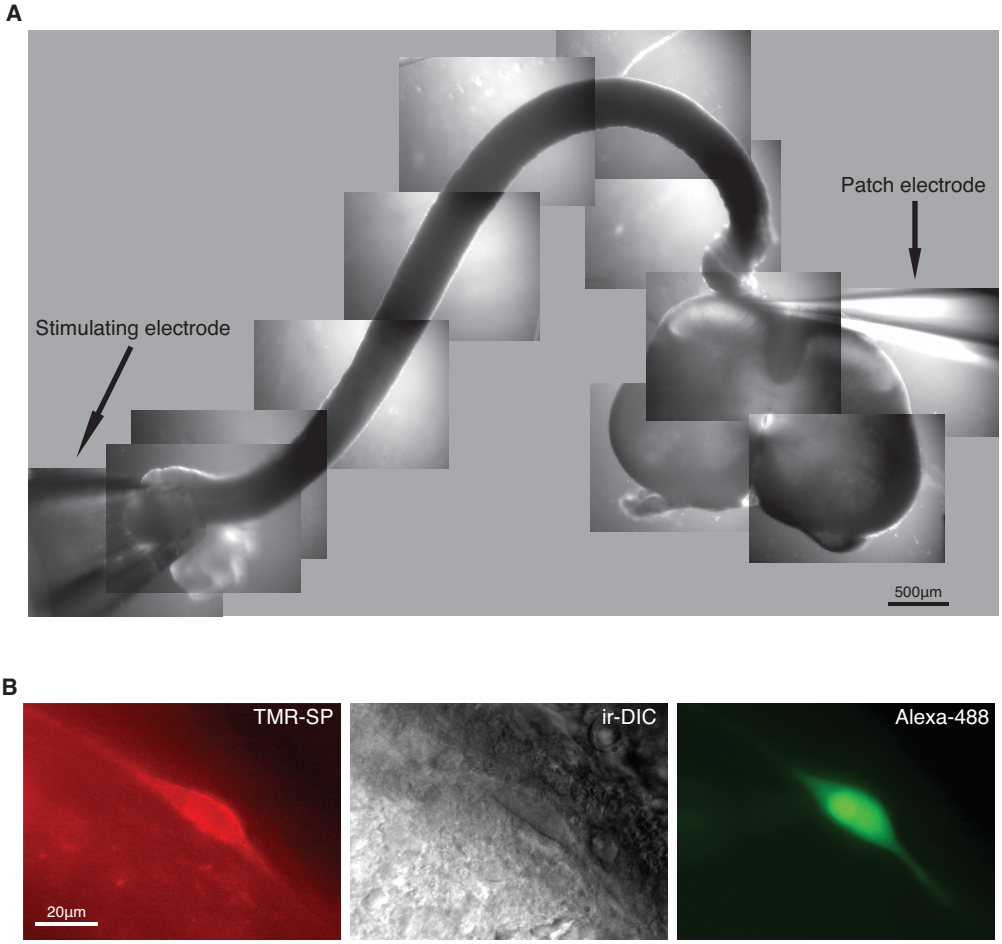

Figure 2.8: Spinal cord slice and lamina I NK1R+ neuron images. ***A*** ir-DIC image of spinal cord slice preparation employed to record dorsal root eEPSCs in lamina I NK1R+ neurons. ***B*** Identification of a lamina I NK1R+ neuron using TMR-SP labelling. Neuron visualised with TMR-SP fluorescence (left panel), ir-DIC (middle panel) and filled with Alexa Fluor 488 hydrazide (right panel)

2.3.6 Spontaneous excitatory input

Spontaneous excitatory input to lamina I NK1R+ neurons was investigated to determine if this was altered by CFA inflammation and/or whether the type of

synaptic input received was predictive of the level of excitatory drive to these neurons. Patch-clamp electrophysiology (as described in section 2.3.4) was used to record sEPSCs in neurons, from tissue isolated from control or CFA treated rats. At least 5 minutes after establishing whole-cell configuration, sEPSCs were recorded for 5 minutes prior to characterising the input received, to avoid any potential influence from the characterisation stimulation protocol used. Recordings were made at a holding potential of -70mV which, is the E_{anion} in these neurons (Coull et al. 2003), therefore no inhibitory blockers were required to block direct inhibitory inputs.

The influence of primary afferent input type on sEPSCs was assessed only in neurons that received monosynaptic input. Neurons receiving polysynaptic input were not included in this analysis as it was not possible to be certain that these neurons truly lacked monosynaptic input, as monosynaptic input could had been severed in the process of preparing spinal cord slices. To confirm that sEPSCs recorded in lamina I NK1R+ neurons are mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and NMDA receptors, in a subset of cells the AMPA and NMDA antagonists 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline (NBQX) (10μM, Abcam), and D-(-)-2-Amino-5-phosphonopentanoic acid (D-APV) (30μM, Abcam), respectively, were bath applied for 13 minutes.

Mini Analysis software (Synaptosoft Inc.) was used to analyse sEPSCs. To assess the influence of CFA inflammation / input received on sEPSCs, the final 2 minutes of the recording were analysed. Where NBQX / D-APV was applied, the final 2 minutes prior to drug application and the final 2 minutes of drug application were analysed. sEPSC events were automatically detected by the software and were then accepted or rejected following further visual examination.

2.3.7 Statistical analysis

All data were assessed for normality using D'Agostino & Pearson omnibus normality tests, to establish whether it was appropriate to use parametric or non-parametric statistical tests, or whether data should be transformed prior to performing parametric tests.

CAP data were analysed using 2-way repeated measures ANOVA. The amplitude data were log transformed prior to analysis. Recording location and the overall pattern of primary afferent input, in spinal cord slice eEPSC experiments was assessed using the Chi-squared test. Changes in the incidence of individual input types were evaluated

using Fischer's exact test. 2-way ANOVA was performed on rank transformed estimated conduction velocity and amplitude data. The influence of CFA inflammation on sEPSC frequency and amplitude within the overall neuronal population was statistically analysed using a Mann Whitney U-test. Statistical analysis of the effect of CFA and input type on sEPSC frequency and amplitude in cells that received monosynaptic primary afferent input were performed using 2-way ANOVA followed by Tukey's multiple comparison post-tests on log transformed data. All data are presented as mean $\pm$ standard error of the mean (SEM). All statistical analysis was performed using Prism 6 (Graphpad Software).

2.4 Results

2.4.1 Electrophysiological properties of isolated dorsal roots

To confirm that CFA inflammation does not alter the activation threshold, conduction velocity or amplitude of Aβ- Aδ- and C-fibres (Baba et al. 1999, Nakatsuka et al. 1999, Torsney 2011) and verify the appropriate electrical stimulation intensities to activate these components in subsequent eEPSC studies, CAPs were recorded from dorsal roots isolated from control and CFA treated rats. It is well established that the three different primary afferent components can be distinguished on the basis of threshold (Aβ $<$ Aδ $<$ C) and conduction velocity (Aβ $>$ Aδ $>$ C) (Meyer et al. 2005), as illustrated in example figure 2.9A. At Aβ-fibre stimulation intensity (20μA) only a single component with a characteristic triphasic response is visible and as this is the first component following the stimulus artefact, and thus the fastest conducting, this can be considered to be the Aβ-fibre component. When the stimulation intensity is increased to Aδ- and C-fibre strength (100 & 500μA respectively) a second (Aδ) and then third (C) slower component becomes identifiable. The activation threshold was significantly different between Aβ- Aδ- and C-fibres ($P<0.0001$, 2-way repeated measures ANOVA), however CFA inflammation was without effect ($P=0.422$, 2-way repeated measures ANOVA, figure 2.9B, table 2.2) and there was no interaction between these factors ($P=0.826$, 2-way repeated measures ANOVA). These data were used to determine the appropriate stimulation intensities to use in subsequent dorsal root eEPSC patch-clamp studies to selectively activate the different primary afferent inputs. Stimulation intensities which were above that required to activate the component of interest, but which did not activate any higher threshold components (e.g. activated all Aβ- but no Aδ-fibres) were selected. Therefore, 20, 100 and 500μA were chosen to activate Aβ-, Aδ- and C-fibre components respectively (as detailed in section 2.3.5). Conduction velocity of the primary afferent components was significantly altered by fibre type, but unaffected by CFA inflammation, with no interaction between these factors ($P<0.0001$, $P=0.979$ & $P=0.688$, respectively, 2-way repeated measures ANOVA, figure 2.9C, table 2.2). Similarly, fibre type significantly altered the response amplitude, but CFA was without effect and there was no interaction ($P<0.0001$, $P=0.465$ & $P=0.432$, respectively, 2-way repeated measures ANOVA on log transformed data, table 2.2).

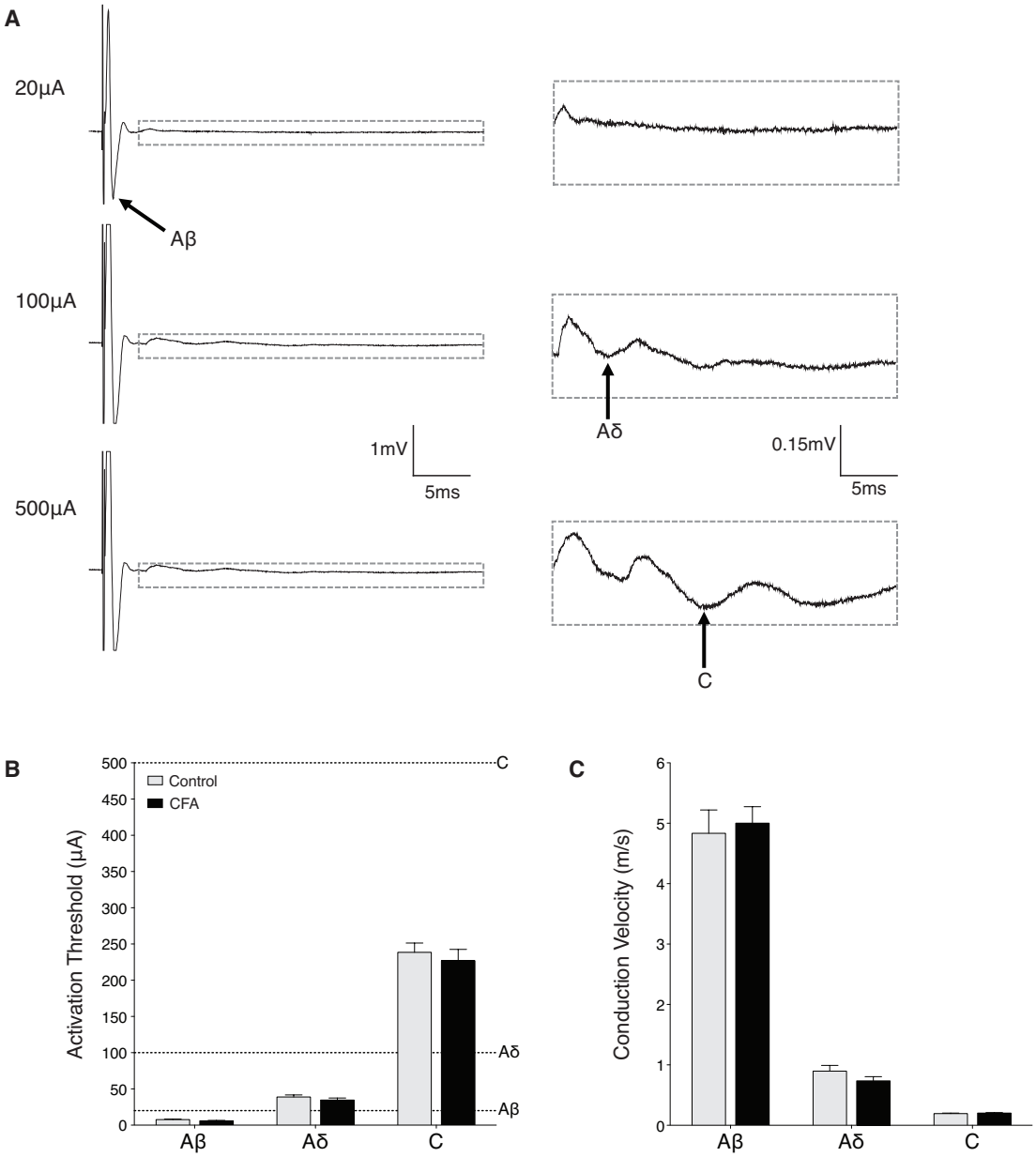

Figure 2.9: CFA inflammation does not alter activation thresholds or conduction velocity of primary afferents. ***A*** Example traces from control dorsal roots, left shows low magnification, right shows magnified portion of trace, as indicated by box on left trace, average of 10 traces shown, arrows denote negative peak of 'triphasic' component. Stimulation intensities used were: Aβ-fibre (top); 20μA, Aδ-fibre (middle); 100μA, C-fibre (bottom); 500μA. ***B*** Electrical activation thresholds for Aβ-, Aδ- and C-fibres recorded from dorsal roots isolated from control and CFA treated rats. There was a significant effect of fibre type ($P<0.0001$), but no significant effect of CFA (P=0.422) on activation threshold. There was no interaction between these factors (P=0.826) Dotted lines indicate the dorsal root stimulation intensities to be employed in subsequent patch-clamp eEPSC studies to selectively activate the different fibre types. ***C*** Conduction velocities for Aβ-, Aδ- and C-fibre components. CFA inflammation had no effect (P=0.979), while fibre type significantly affected conduction velocity ($P<0.0001$), with this effect being independent of CFA (P=0.688). All statistics: 2-way repeated measures ANOVA, n=13 in all groups.

		Threshold (pA)	Conduction velocity (m/s)	Amplitude (mV)
Control	Aβ	7.73 ± 0.64	4.83 ± 0.39	1.52 ± 0.23
	Aδ	38.85 ± 2.78	0.90 ± 0.10	0.10 ± 0.02
	C	238.46 ± 12.85	0.19 ± 0.01	0.11 ± 0.02
CFA	Aβ	6.04 ± 0.57	5.00 ± 0.27	2.34 ± 0.42
	Aδ	34.62 ± 2.80	0.74 ± 0.07	0.10 ± 0.02
	C	226.92 ± 15.62	0.20 ± 0.01	0.14 ± 0.03

Table 2.2: Electrically evoked properties of isolated dorsal roots. Activation threshold, conduction velocity and amplitude of Aβ-, Aδ- and C-fibre components recorded from dorsal roots isolated from control of CFA treated rats. All values shown as mean ± SEM, all groups n=13.

2.4.2 Effect of inflammation on primary afferent input to lamina I neurokinin 1 receptor expressing neurons

To confirm the previously reported increased incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons seen during inflammation (Torsney 2011) and determine whether monosynaptic C-fibre input to these neurons is potentiated in inflammatory pain, patch-clamp recordings were made from lamina I NK1R+ neurons pre-labelled with TMR-SP.

The location of the lamina I NK1R+ neurons targeted for patch-clamp recordings was analysed to ensure that recordings were made from comparable anatomical locations in tissue isolated from control and CFA treated rats. Primary afferent input was characterised in neurons situated predominantly in the middle region of the dorsal horn, with smaller proportions in medial and lateral regions (as defined in figure 2.10A). This result could reflect the fact that the central portion of lamina I is thicker and displays more dense NK1R immunoreactivity (Todd et al. 1998). The overall pattern of cell location showed no difference between control and CFA groups (P=0.320, Chi-squared, 2.10B).

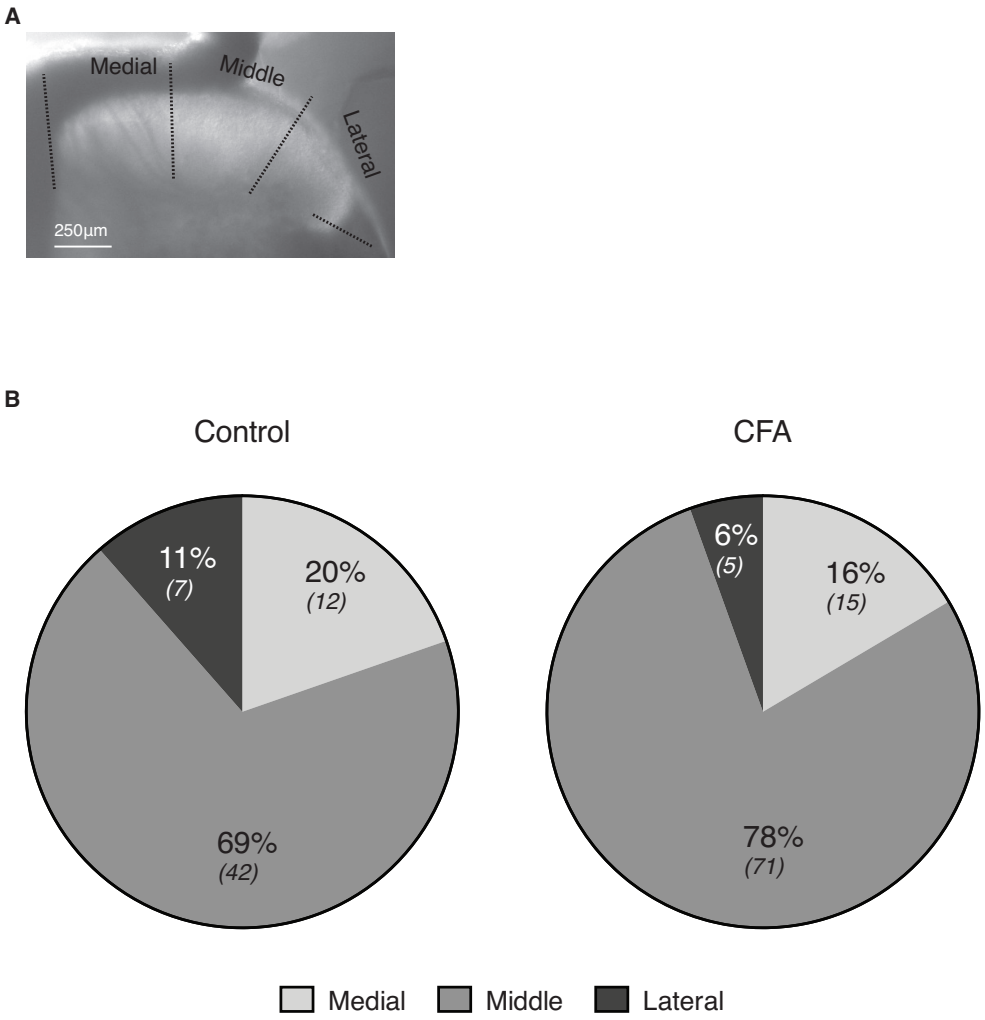

Figure 2.10: Location of patch clamp recordings. ***A*** Image of dorsal horn showing definition of medial, middle and lateral regions. ***B*** Patch clamp recordings were made predominantly in the middle region of lamina I of the dorsal horn, with a smaller proportion in the medial and lateral regions. There was no significant difference in the pattern of recording location between control and CFA tissue, P=0.320 (Chi-squared). Control n=61, CFA n=91. Actual sample sizes in parentheses.

Primary afferent input to lamina 1 NK1R+ neurons was classified on the basis of the monosynaptic input they received, regardless of whether they received additional polysynaptic input, as follows. Neurons with only polysynaptic input were classified as 'polysynaptic only':

- 'C' = monosynaptic C-fibre only
- 'Aδ' = monosynaptic Aδ-fibre only
- 'Aδ + C' = monosynaptic Aδ-fibre with monosynaptic C-fibre
- 'Aβ' = monosynaptic Aβ-fibre only

- 'Aβ + C' = monosynaptic Aβ-fibre with monosynaptic C-fibre
- 'Aβ + Aδ' = monosynaptic Aβ-fibre with monosynaptic Aδ-fibre
- 'Aβ + Aδ + C' = monosynaptic Aβ-fibre with monosynaptic Aδ-fibre and monosynaptic C-fibre
- 'Polysynaptic only' = polysynaptic only

Example traces in figures 2.11 and 2.12 show lamina I NK1R+ neurons that receive: 2.11A; monosynaptic C-fibre only input, 2.11B; monosynaptic Aδ-fibre with monosynaptic C-fibre and a small polysynaptic Aβ-fibre input, 2.12A; monosynaptic Aδ-fibre only input, although this example also has a small polysynaptic Aβ- and polysynaptic C-fibre input, 2.12B; monosynaptic Aβ-fibre only input. In these example traces it can be seen that these monosynaptic inputs do not show failures when stimulated at high frequency and exhibit stable latencies. Figure 2.13 shows an example of a neuron that receives polysynaptic only input, where there is clear evidence of failures and a large latency variability. Overall, CFA inflammation did not alter the pattern of input that lamina I NK1R+ neurons received (P=0.507, Chi-squared test, figure 2.14). In spinal slices isolated from control and CFA treated animals, the predominant input type was monosynaptic C-fibre, with almost three quarters of all neurons receiving this type of input. In addition, neurons received smaller but similar proportions of monosynaptic Aδ-fibre and polysynaptic input. The incidence of monosynaptic Aδ- or monosynaptic C-fibre, or polysynaptic input was not affected by CFA inflammation (all: $P>0.05$, Fischer's exact test). However, CFA inflammation resulted in a 5 fold increase in neurons that received monosynaptic Aβ-fibre input, although this did not reach statistical significance (P=0.051, Fischer's exact test).

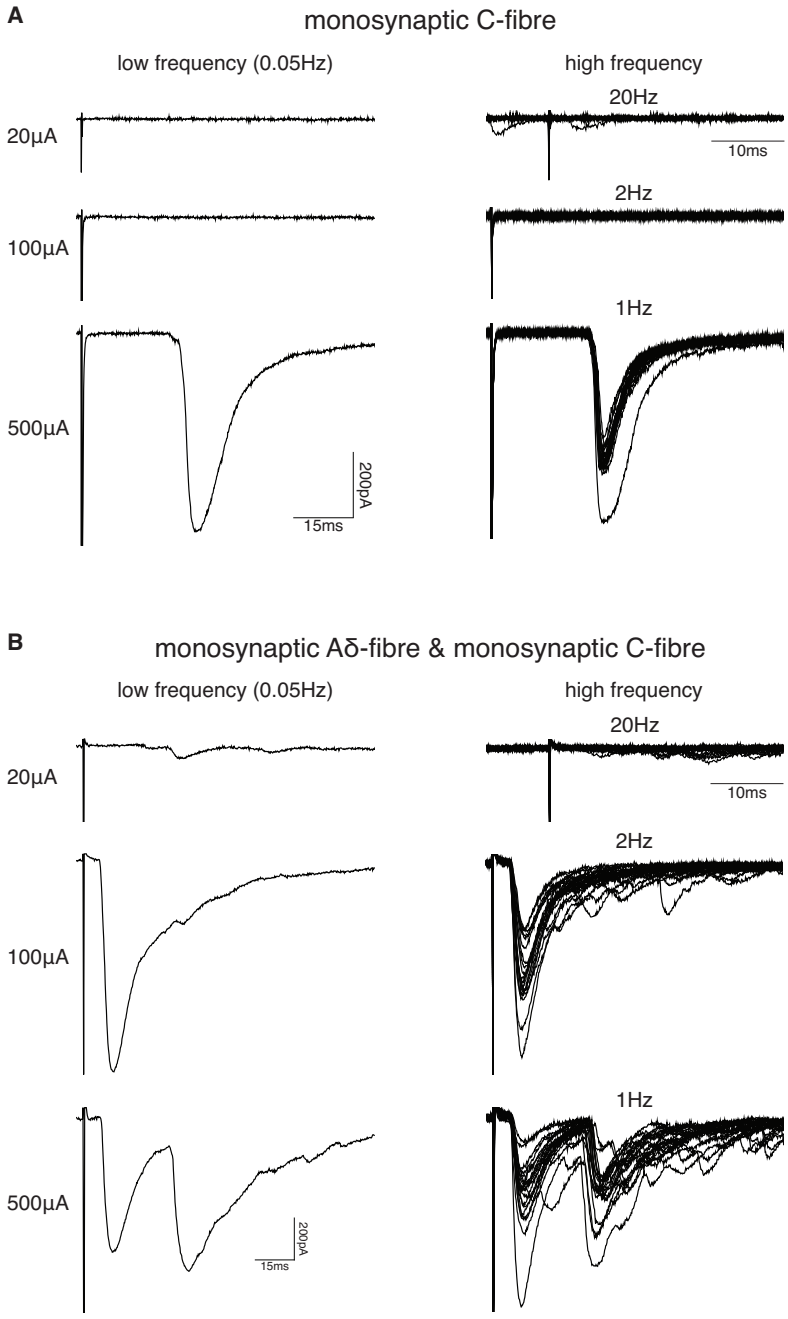

Figure 2.11: Monosynaptic primary afferent input to lamina I NK1R+ neurons. Examples of characterisation of synaptic input to lamina I NK1R+ neurons: ***A*** CFA neuron with monosynaptic C-fibre input, ***B*** control neuron with both monosynaptic Aδ- & monosynaptic C-fibre input. Left traces show example of eepsc using low frequency (0.05Hz) stimulation at Aβ- (20μA), Aδ- (100μA) and C-fibre (500μA) stimulation intensities, average of 3 traces shown. Right traces show example of eEPSCs using high frequency stimulation (20μA / 20Hz, 100μA / 2Hz, 500μA / 1Hz), 20 superimposed traces shown.

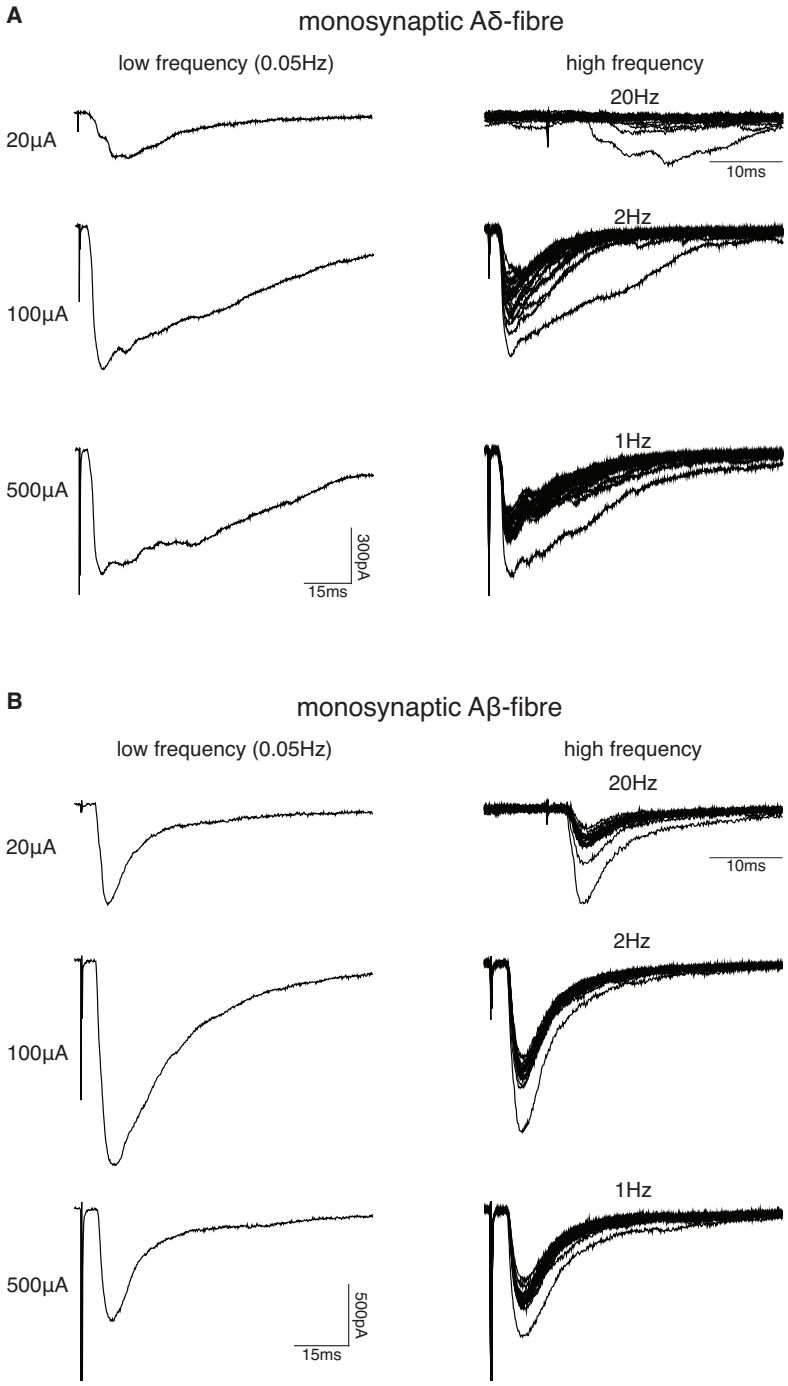

Figure 2.12: Monosynaptic primary afferent input to lamina I NK1R+ neurons. Examples of characterisation of synaptic input to lamina I NK1R+ neurons: ***A*** CFA neuron with monosynaptic Aδ-fibre input and a small polysynaptic Aβ-fibre input, ***B*** CFA neuron with Aβ- input. Left traces show example of eEPSCs recorded during low frequency (0.05Hz) stimulation at Aβ- (20μA), Aδ- (100μA) and C-fibre (500μA) stimulation intensities, average of 3 traces shown. Right traces show example of eEPSCs using high frequency stimulation (20μA / 20Hz, 100μA / 2Hz, 500μA / 1Hz), 20 superimposed traces shown.

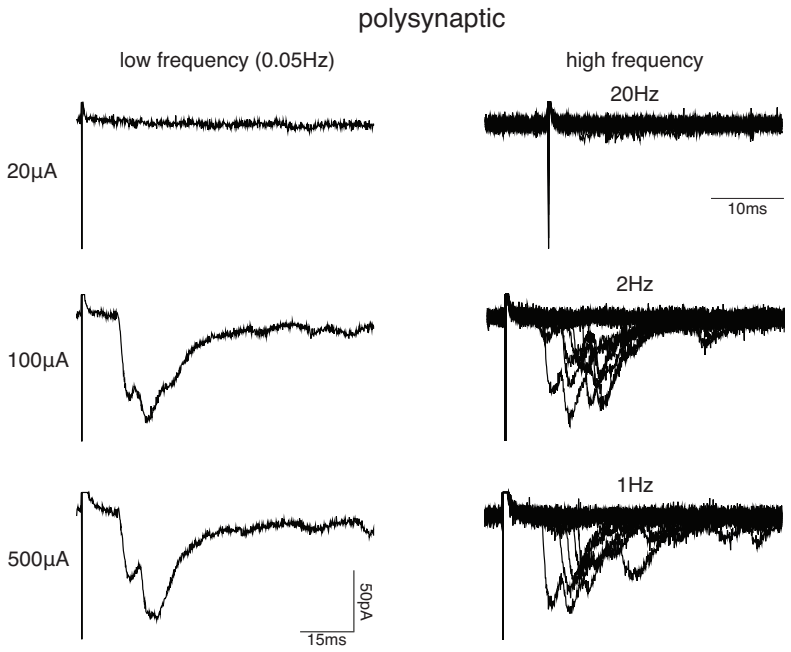

Figure 2.13: Polysynaptic primary afferent input to lamina I NK1R+ neurons. Example of characterisation of synaptic input to a control lamina I NK1R+ neuron that received polysynaptic input. Left traces show example of eEPCSs using low frequency (0.05Hz) stimulation at Aβ- (20μA), Aδ- (100μA) and C-fibre (500μA) stimulation intensities, average of 3 traces shown. Right traces show example of eEPSCs using high frequency stimulation (20μA / 20Hz, 100μA / 2Hz, 500μA / 1Hz), 20 superimposed traces shown.

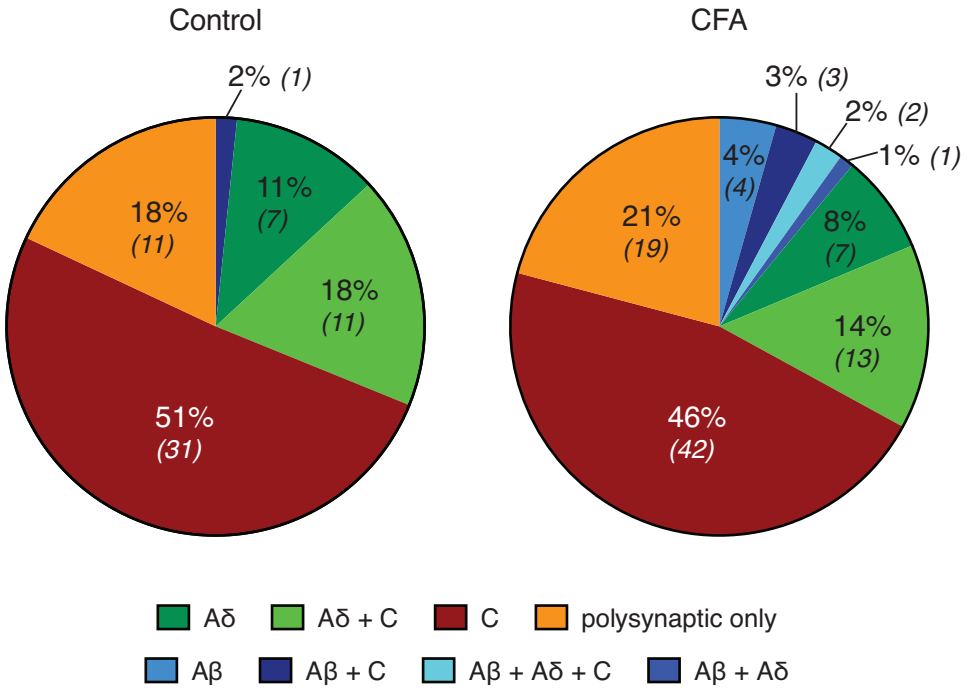

Figure 2.14: CFA inflammation increases the incidence of monosynaptic Aβ-fibre, but not monosynaptic Aδ- or monosynaptic C-fibre input to lamina I NK1R+ neurons. CFA inflammation had no impact on the overall pattern of input received by lamina I NK1R+ neurons (P=0.507, Chi-squared). The incidence of monosynaptic Aβ-fibre input was increased following CFA inflammation, however this just failed to reach significance (P=0.051, Fischer's exact test). CFA inflammation did not not alter the incidence of monosynaptic Aδ- or C-fibre, or polysynaptic input (all P>0.05, Fischer's exact test). Control n=61, CFA n=91. Actual sample sizes in parentheses

CFA inflammation did not alter the peak amplitude of monosynaptic Aδ- or monosynaptic C-fibre inputs, regardless of whether neurons received Aδ-fibre only, both Aδ- & C-fibre or C-fibre only input (P=0.388, 2-way ANOVA on rank transformed data, figure 2.15B). Amplitude was not significantly difference between input types and there was no interaction between CFA and input type P=0.999 & P=0.217, respectively, 2-way ANOVA on rank transformed data). Neurons that received Aδ-fibre inputs are displayed as two groups, depending on whether C-fibre input was also received, because it has previously been shown that monosynaptic Aδ-fibre inputs are potentiated following inflammation in those neurons which receive Aδ-fibre only input, but not Aδ- with C-fibre input (Torsney 2011). The amplitude of monosynaptic C-fibre inputs was not altered by CFA inflammation, regardless of whether Aδ-fibre input was also received and there was no interaction between these factors (P=0.386, P=0.103 & P=0.168, respectively, 2-way ANOVA, data not shown). The estimated conduction velocity of Aδ-fibre inputs was significantly greater than C-fibre inputs (P<0.0001, 2-way ANOVA on rank transformed data, figure 2.15D) as would be expected. CFA inflammation did not alter conduction velocity and there was

no interaction between input type and CFA (P=0.937 & P=0.723, respectively, 2-way ANOVA on rank transformed data). Aβ-fibre inputs displayed similar amplitudes to Aδ- and C-fibres but faster conduction velocities (table 2.3), however these were not statistically analysed due to the small sample size in the control group. Occasionally there was an overlapping of components that made it difficult to analyse peak amplitude and/or estimated conduction velocity, in such cases that parameter was excluded from the final analysis.

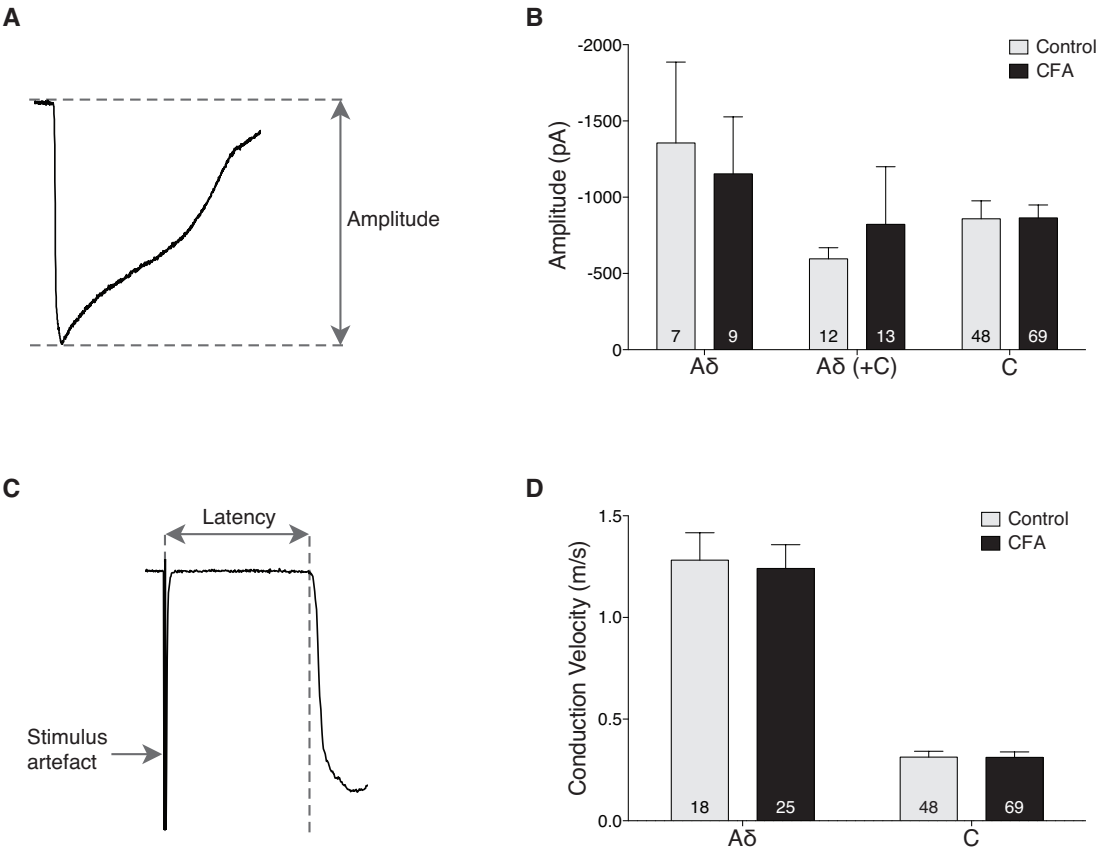

Figure 2.15: Amplitude and estimated conduction velocity of monosynaptic input to lamina I NK1R+ neurons is not altered by CFA inflammation. ***A*** Example of how peak eEPSC amplitude was measured. ***B*** Both input type and CFA inflammation had no significant effect on eEPSC peak amplitude and there was no interaction between these factors (P=0.099, P=0.388 & P=0.217, respectively). ***C*** The response latency, used to calculate estimated conduction velocity, was measured as the time between the stimulus artefact and the onset of the monosynaptic eEPSC response. ***D*** Conduction velocity was significantly altered by input type (P<0.0001), but was unaffected by CFA inflammation (P=0.937). There was no significant interaction between input type and CFA (P=0.723). All statistics: 2-way ANOVA on rank transformed data, sample sizes indicated by numbers on bars.

		Conduction Velocity (m/s)	Amplitude (pA)
Control	Aβ	2.21 (*1*)	-1272.60 (*1*)
	Aδ	1.28 ± 0.17 (*18*)	-875.92 ± 209.68 (*19*)
	C	0.31 ± 0.03 (*48*)	-858.14 ± 118.05 (*48*)
CFA	Aβ	1.68 ± 0.17 (*10*)	-917.36 ± 378.91 (*10*)
	Aδ	1.24 ± 0.12 (*26*)	-957.45 ± 226.93 (*25*)
	C	0.31 ± 0.03 (*71*)	-864.83 ± 84.56 (*69*)

Table 2.3: Amplitude and estimated conduction velocity of monosynaptic Aβ-, Aδ- and C-fibre inputs to lamina I NK1R+ neurons in tissue isolated from control and CFA treated rats. All values shown as mean ± SEM, sample sizes in parentheses.

2.4.3 Spontaneous excitatory input to lamina I neurokinin 1 receptor expressing neurons

To assess whether the spontaneous excitatory input to lamina I NK1R+ neurons was altered by CFA inflammation and/or the type of primary afferent input a neuron receives, sEPSCs were recorded from pre-labelled lamina I NK1R+ neurons is tissue isolated from control or CFA treated rats. Figure 2.16A shows example sEPSC traces recorded in neurons receiving monosynaptic: Aδ-fibre only, Aδ- & C-fibre and C-fibre only input, in tissue from both control (Aδ only) and CFA treated rats (Aδ & C, C only). CFA inflammation did not influence sEPSC frequency (P=0.460, 2-way ANOVA on log transformed data), but frequency was significantly altered by input type (P=0.002, 2-way ANOVA on log transformed data). There was no significant interaction between inflammation and input type (P=0.745, 2-way ANOVA on log transformed data). Post-hoc analysis revealed that neurons receiving monosynaptic Aδ-fibre only input displayed significantly greater sEPSC frequency that those receiving C-fibre only input ($P<0.01$, 2-way ANOVA on log transformed data, Tukey's multiple comparison post-test, figure 2.16B). Both input type and CFA inflammation were without effect on sEPSC amplitude, and there was no interaction between these two factors (P=0.512, P=0.444 & P=0.950, respectively, 2-way ANOVA on log transformed data, figure 2.16C). In the overall population of neurons, CFA inflammation did not influence sEPSC frequency or amplitude (P=0.661 and P=0.159, respectively, Mann Whitney, table 2.4). The addition of NBQX and D-APV, to tissue isolated from both control and CFA treated rats, abolished sEPSC events, as shown in the representative traces in figure 2.17A. NBQX and D-APV reduced sEPSC frequency from 1.61 ± 0.67Hz to 0 ± 0Hz (figure 2.17B), while sEPSC amplitude was reduced from 31.46 ± 5.20pA to 0 ± 0pA (figure 2.17C).

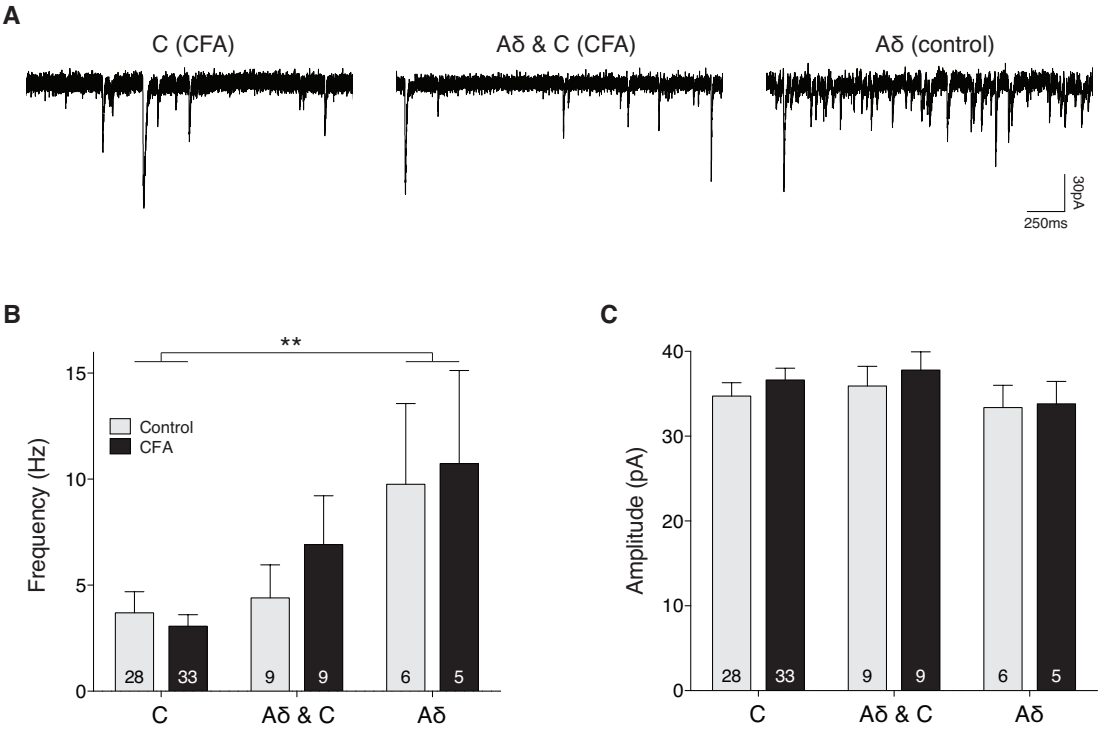

Figure 2.16: Lamina I NK1R+ neuron sEPSC frequency, but not amplitude, is altered by primary afferent input type. ***A*** Example sEPSC traces from cells receiving monosynaptic C-fibre (left), Aδ & C-fibre (middle) and Aδ-fibre (right) input. ***B*** The frequency of sEPSCs was significantly altered by input type (P=0.002). Cells with monosynaptic Aδ only input showed significantly higher frequency that those with C-fibre input ($P<0.01$). CFA inflammation was without effect on sEPSC frequency (P=0.460) and there was no interaction between input type and CFA (P=0.745). ***C*** Input type and CFA inflammation had no significant effect on sEPSC amplitude (P=0.515 & P=0.444, respectively) and there was no interaction between these factors (P=0.950). All stats = 2-way ANOVA on log transformed data, followed by Tukey's multiple comparison post-test $**P<0.01$. Sample sizes indicated by numbers on bars.

		Frequency (Hz)	Amplitude (pA)	*n*=
Control	Aδ	9.76 ± 3.80	33.37 ± 2.63	*6*
	Aδ & C	4.39 ± 1.56	35.91 ± 2.32	*9*
	C	3.69 ± 0.99	34.72 ± 1.57	*28*
	Aβ†	17.07	35.14	*1*
	Poly	4.38 ± 1.56	34.83 ± 3.37	*7*
	Overall	**4.89 ± 0.84**	**34.80 ± 1.08**	***51***
CFA	Aδ	10.74 ± 4.39	33.80 ± 2.63	*5*
	Aδ & C	6.91 ± 2.31	37.77 ± 2.15	*9*
	C	3.07 ± 0.54	36.60 ± 1.39	*33*
	Aβ‡	5.06 ± 1.21	43.86 ± 3.75	*9*
	Poly	2.65 ± 3.20	32.49 ± 1.90	*17*
	Overall	**4.21 ± 0.57**	**36.49 ± 1.01**	***73***

Table 2.4: sEPSC frequency and amplitude values recorded in lamina I NK1R+ neurons in tissue isolated from control and CFA treated rats. CFA did not alter the overall frequency (P=0.661) or amplitude (P=0.159) of sEPSCs, Mann Whitney. All values shown as mean ± SEM. † Includes monosynaptic Aβ- & C-fibre. ‡ Includes monosynaptic Aβ-fibre only; Aβ- & Aδ-; Aβ- & C-; Aβ- & Aδ- & C-fibre.

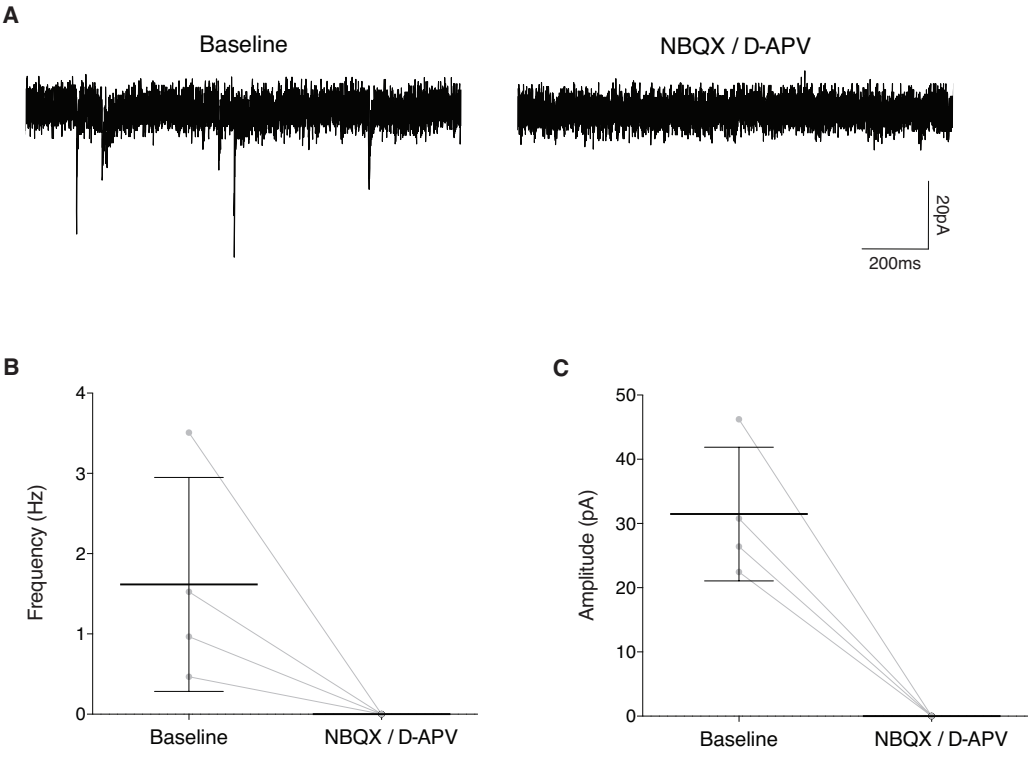

Figure 2.17: sEPSCs are blocked by NBQX + D-APV. ***A*** Representative sEPSC traces recorded in a lamina I NK1R+ neuron, in tissue isolated from a CFA treated rat, in the absence (left) and presence of the AMPA and NMDA antagonists, NBQX and D-APV, respectively (right). Application of NBQX and D-APV blocked sEPSC events, as seen in the frequency (***B***) and amplitude (***C***) data. All groups n=4 (control n=2, CFA n=2).

2.5 Discussion

2.5.1 Electrically evoked responses of primary afferent fibres in isolated dorsal roots

CAP recordings in isolated dorsal roots established that activation threshold, conduction velocity and response amplitude for Aβ-, Aδ- and C-fibres were not affected by CFA inflammation, which confirms the findings of earlier studies in juvenile (Torsney 2011) and adult rats (Baba et al. 1999, Nakatsuka et al. 1999). Therefore, identical stimulation parameters can be used to electrically activate primary afferent inputs in dorsal root eEPSC studies in tissue isolated from control and CFA treated rats. Importantly, the conduction velocity values reported here are comparable to those described in other studies where extracellular CAP recordings were performed in *ex vivo* tissue (Daniele & MacDermott 2009, Géranton et al. 2009, Labrakakis et al. 2003, Torsney 2011), which can be considered to further confirm the identity of the afferent components examined in this study.

From these data, stimulation intensities of 20, 100 and 500μA were selected to activate Aβ-, Aδ- and C-fibre inputs, respectively, in dorsal root eEPSC studies. These intensities are largely in agreement with published studies (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006), however a slightly lower intensity was chosen to activate Aβ-fibres, 20μA versus 25μA. The reason for this choice is that in 2 dorsal roots recorded here, the activation threshold for Aδ-fibres was 25μA. Therefore, to avoid potentially misidentifying Aδ-fibre input as Aβ-fibre input, this lower intensity was selected.

It could be argued that the method of determining the activation threshold of the primary afferent components was subjective as it relied upon the investigator to determine when the negative peak of the triphasic response was first clearly identifiable based upon a visual inspection of the resulting traces. As extracellular population recordings, these were reliant on the simultaneous activation of a critical mass of afferents for response detection. Response amplitude varies with stimulation intensity, as more or less afferents are activated and so activation thresholds are not a clear all or nothing response. However, the activation thresholds reported here are comparable to published values obtained by intracellular recording from individual neurons (Nakatsuka et al. 2000), where these issues do not exist. This can be considered to support the findings reported here.

2.5.2 Synaptic input to lamina I neurokinin 1 receptor expressing neurons in inflammatory pain

The overall pattern of synaptic input to lamina I NK1R+ neurons was unaltered by CFA inflammation, as was the incidence of monosynaptic Aδ- and/or monosynaptic C-fibre and polysynaptic input. The incidence of monosynaptic Aβ-fibre input was increased, although this did not reach a level of statistical significance. These findings do not confirm previous work that reports that the overall pattern of input is significantly altered and specifically that the incidence of monosynaptic Aδ-fibre input is increased following inflammation (Torsney 2011). It is unclear why these previous findings were not replicated in the results presented here.

In the experiments presented here a stimulation intensity of 20μA was employed to activate Aβ-fibre inputs, as an intensity of 25μA was found to be the Aδ-fibre activation threshold in a few cases in CAP recordings. In the work that established that the incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons is increased in CFA inflammation, a stimulation intensity of 25μA was used to activate Aβ-fibre inputs (Torsney 2011). This finding is unlikely to be influenced due to misidentification of inputs resulting from using an Aβ-fibre stimulation intensity of 25μA. If monosynaptic Aδ-fibre inputs were wrongly identified as Aβ-fibre, arguably a corresponding increase in the incidence of monosynaptic Aβ-fibre input would be expected, although this was not the case. Any Aδ-fibres identified at a stimulation intensity of 25μA would have been stimulated at 20Hz to determine their monosynaptic / polysynaptic nature. As Aδ-fibres fail to fire action potentials at frequencies of >2Hz (Nakatsuka et al. 2000), this is highly likely to have lead to failures and thus any misidentified Aδ-fibre inputs would likely have been classed as polysynaptic Aβ-fibre. Notably, polysynaptic Aβ-fibre input was not altered by CFA inflammation (Torsney 2011).

One possibility is that the previously described increased incidence of monosynaptic Aδ-fibre input (Torsney 2011) occurs in a subpopulation of lamina I NK1R+ neurons and that this present study did not include enough of that subpopulation for the effect to be detected. There is evidence that different lamina I NK1R+ subpopulations may receive different types of input and respond differently to identical patterns of stimulation. Lamina I projection neurons that target the PB have a greater incidence of monosynaptic Aδ-fibre input than those that target the PAG (9% versus 2%) (Ruscheweyh et al. 2004), however it is unknown whether this is altered following inflammation. As previously discussed, electrical stimulation of C-fibre inputs to

these neurons drives synaptic plasticity, with the outcome depending on the neuronal subpopulation involved (Ikeda et al. 2006), so clearly different subpopulations have the capacity to respond differently to the same input. The extent to which altered C-fibre responses impact upon Aδ-fibre inputs is unclear. However, it is proposed that C-fibre activity drives spinal cord hyperexcitability, which in turn allows A-fibres access to circuits, that enable them to mediate hyperalgesia and allodynia (Latremoliere & Woolf 2009, Treede & Magerl 2000), thereby providing a potential mechanism by which altered C-fibre activity in a particular neuronal subpopulation could impact upon the Aδ-fibre input in those neurons. The method used here and elsewhere (Torsney 2011) for identifying lamina I NK1R+ neurons does not enable the identification of subpopulations so it is not possible to conclude whether this could account for the differences in input pattern reported.

Different lamina I NK1R+ neuron subpopulations are known to display different levels of NK1R immunoreactivity, specifically neurons that project to the PAG show lower NK1R expression (Spike et al. 2003). Incidentally, this PAG projecting subpopulation also receive a lower incidence of monosynaptic Aδ-fibre input than those projecting to the PB (Ruscheweyh et al. 2004). The TMR-SP used to identify lamina I NK1R+ neurons in this study was obtain from a different supplier than has been used in previous reports (Torsney 2011). Therefore, the failure to replicate the previously reported increase in the incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons in inflammation (Torsney 2011) could be due to differences in the sensitivity of the TMR-SP used. For example, a reduction in TMR-SP sensitivity could have resulted in the preferential targeting of neuronal subpopulations that display greater NK1R immunoreactivity. If the inflammation induced increase in the incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons (Torsney 2011) occurs in a specific neuronal subpopulation, then a change in TMR-SP sensitivity could have altered the proportion of that subpopulation in the data presented here.

The possibility of addressing the hypothesis that changes in input pattern are a subpopulation specific process are restricted with current techniques for identifying lamina I NK1R+ neurons. While TMR-SP labelling of neurons (Labrakakis & MacDermott 2003, Torsney 2011, Torsney & MacDermott 2006), ensures the neuron recorded from expresses the NK1R, it does not identify subpopulations. Retrograde labelling of neurons, as used by others (Ikeda et al. 2003, 2006, Ruscheweyh et al. 2004), identifies subpopulations, however only ∼80% of lamina I projection neurons display NK1R immunoreactivity (Al-Khater et al. 2008, Spike et al. 2003, Todd et al.

2000) so this approach will include data from non-NK1R+ neurons. However, one approach that has been used to negate this possibility is to confirm whether retrograde labelled neurons respond to application of SP (Ikeda et al. 2003, 2006). Although, it should be recognised that retrograde labelling of spinal cord neurons is a more time consuming and invasive procedure.

One potential approach to overcome these limitations would be to use genetic labelling techniques. Such approaches have already been employed to dissect different populations of peripheral (Cavanaugh et al. 2011, Li et al. 2011, Takashima et al. 2010) and central (Cavanaugh et al. 2011, Mesnage et al. 2011, Nowak et al. 2011, Paul et al. 2012, Torsney et al. 2006, Zeilhofer et al. 2005) sensory neurons. A transgenic mouse model that expresses the human NK1R with a green fluorescent protein (GFP) marker gene has been developed (Vasiliou et al. 2007). However, the utility of such a model is questionable as the expression pattern of GFP expressing neurons is not comparable with the NK1R+ neuron pattern established by numerous groups (Al-Khater et al. 2008, Brown et al. 1995, Mantyh et al. 1997, Spike et al. 2003, Todd 2010, Todd et al. 2000). Furthermore, this transgenic model (Vasiliou et al. 2007) does not identify subpopulations of these neurons. To date no other study has applied genetic labelling approaches to study the different lamina I NK1R+ neuron subpopulations.

The increased monosynaptic Aβ-fibre input to lamina I NK1R+ neurons reported here just failed to reach a level of statistical significance and there is conflicting evidence to support this finding. Previous studies that have examined the synaptic input to lamina I NK1R+ neurons have shown there to be minimal monosynaptic Aβ-fibre input to these neurons in both control and inflamed conditions (Torsney 2011, Torsney & MacDermott 2006). Normally, light touch / brush stimulation of the hindpaw does not result in NK1R internalisation in lamina I neurons, however following CFA inflammation and after the initial CFA-induced NK1R internalisation has subsided, the same stimuli now cause NK1R internalisation (Abbadie et al. 1997, Honor et al. 1999). This effect is thought to be mediated by a phenotypic switch in Aβ-fibres, whereby there is novel SP expression in these fibres in inflammation (Neumann et al. 1996) as opposed to novel monosynaptic Aβ-fibre input. Furthermore, electrical stimulation of the sciatic nerve at Aβ-fibre strength fails to evoke NK1R internalisation in lamina I neurons regardless of inflammation (Allen et al. 1999). Incidentally, NK1R internalisation in response to electrical stimulation of Aδ-fibres (Allen et al. 1999) and noxious mechanical stimuli (Abbadie et al. 1997, Honor et al. 1999), which is known to be Aδ-fibre mediated (Treede & Magerl 2000),

is increased following CFA inflammation. This can be considered to support the previously reported finding that the incidence of Aδ-fibre input to lamina I NK1R+ neurons is increased in CFA inflammation (Torsney 2011). Following nerve injury Aβ-fibres sprout dorsally into lamina I (Woolf et al. 1992, 1995) which could form the basis of novel monosynaptic Aβ-fibre input. However, there is limited evidence that this sprouting occurs in inflammation (Ma & Tian 2002) and this finding has been strongly disputed by others (Hughes et al. 2003, Shehab et al. 2003, Tong et al. 1999), so collateral sprouting is unlikely to account for the increase in Aβ-fibre input reported here.

Under control conditions a portion of lamina I NK1R+ neurons which do not receive conventional monosynaptic Aδ-fibre input, receive monosynaptic Aδ-fibre input which forms pure-NMDA or 'silent' synapses (Torsney 2011). It is suggested that inflammation results in the activation of these synapses, presumably through α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor (AMPAR) insertion into the post-synaptic membrane and provides a possible mechanism for the unveiling of novel monosynaptic Aδ-fibre inputs, which could be relevant for mechanical hyperalgesia (Torsney 2011). However, limited evidence was found for the existence of silent monosynaptic Aβ-fibre input to lamina I NK1R+ neurons (Torsney, unpublished data). Furthermore, while other studies provide clear evidence for the presence of silent synapses in the dorsal horn (Baba et al. 2000, Bardoni et al. 1998, Jung et al. 2005, Li & Zhuo 1998), none of these studies identify the pre- or post-synaptic populations involved. However, it is possible that these reported silent synapses were formed with Aβ-fibres, as the minimum stimulation protocol used by these studies to identify silent synapses enables the identification of silent synapses with low-threshold afferents only (Baba et al. 2000, Bardoni et al. 1998, Li & Zhuo 1998). The existence of silent synapses in the dorsal horn of the spinal cord has recently been disputed (Yasaka et al. 2009). However, as part of the silent synapse identification protocol, presynaptic stimulation was paired with depolarisation of the postsynaptic neuron to confirm the pharmacological inhibition of inhibitory postsynaptic currents (IPSCs) (Yasaka et al. 2009). This pairing protocol has been reported to drive AMPAR expression at silent synapses (Durand et al. 1996, Isaac et al. 1995, Liao et al. 1995). Therefore, this finding is inconclusive as the protocol used to identify silent synapses may have unintentionally altered any silent synapses (Yasaka et al. 2009). In summary, if the novel monosynaptic Aβ-fibre input reported here is a real effect, the mechanism by which these inputs are unmasked is unclear.

Inflammation did not result in any alteration in the amplitude of monosynaptic inputs

to lamina I NK1R+ neurons. This finding does not confirm the previously reported CFA induced potentiation of monosynaptic Aδ-fibre input amplitude, in neurons which receive monosynaptic Aδ-fibre only input (Torsney 2011). However, the failure of this study to detect novel monosynaptic Aδ-fibre input following inflammation could account for this difference. This suggests that the increased incidence of monosynaptic Aδ-fibre input following inflammation is correlated with this amplitude potentiation (Torsney 2011). Therefore, it could be argued that the novel Aδ-fibre inputs that are unmasked during inflammation display higher amplitudes than those that are functional in control conditions. As such it is possible that the novel inputs are responsible for the increased Aδ-fibre eEPSC amplitude, as opposed to there being a potentiation of synapses which are functional in control conditions. Furthermore, *in vitro* electrical stimulation of Aδ-fibre inputs to these neurons in tissue isolated from control rats does not result in potentiation (Ikeda et al. 2006), which could be considered to support this postulate.

It has been demonstrated that *in vitro* electrical stimulation of monosynaptic C-fibre input on to lamina I NK1R+ neurons, to mimic the firing pattern seen during peripheral inflammation can drive potentiation at these C-fibre synapses (Ikeda et al. 2006). This previous finding is not not supported by data reported here or by others (Torsney 2011). However, this C-fibre potentiation was only present in a subset of neurons which project to the PAG, which account for approximately a third of these neurons (Spike et al. 2003). This population was not specifically targeted, therefore a failure to reveal a similar effect could reflect the fact that data were recorded from neurons that project to other targets (Al-Khater et al. 2008, Marshall et al. 1996, Spike et al. 2003, Todd et al. 2000). The population of neurons that project to the PAG have been demonstrated to display lower levels of NK1R immunoreactivity (Spike et al. 2003), therefore an alternative explanation is that TMR-SP labelling may not be sufficient for identification of this subpopulation. To address these questions, it would be necessary to record from pre-identified lamina I NK1R+ neuronal subpopulations. If the subset of PAG projecting neurons were incorporated in this study, the failure to observe C-fibre potentiation could be indicative of the different timescales employed, with recordings made minutes following artificial electrical stimulation (Ikeda et al. 2006) versus days after natural stimulation through inflammation (Torsney 2011). To establish whether C-fibre potentiation is a feature of early inflammatory processes, C-fibre eEPSCs could be recorded in tissue isolated from rats within a period that is shorter than the 2 - 6 days after CFA injection used here.

2.5.3 Spontaneous excitatory input to lamina I neurokinin 1 receptor expressing neurons

This study has novelly examined the spontaneous excitatory input to lamina I NK1R+ neurons and investigated whether this is influenced by CFA inflammation and/or the type of monosynaptic primary afferent input received. CFA inflammation had no significant effect on sEPSC frequency or amplitude in the overall population of lamina I NK1R+ neurons or when the neuronal subpopulations receiving monosynaptic Aδ-only, Aδ- & C- or C-fibre only input are considered independently. Interestingly, the type of monosynaptic input on to these neurons significantly predicted sEPSC frequency, but not amplitude, with neurons that received monosynaptic Aδ-fibre only input displaying significantly greater sEPSC frequency than those receiving monosynaptic C-fibre only input. The effect of input type on sEPSCs was investigated only in neurons that received monosynaptic Aδ- and/or monosynaptic C-fibre input as it was not possible to be certain that neurons classified as having polysynaptic input only would not have had monosynaptic input *in vivo* as it may have been the case that monosynaptic input was cut in the process of the spinal cord slice preparation. Neurons that received monosynaptic Aβ-fibre input were not analysed in this manner as the low numbers in the control group made statistical analysis unsound.

Although the data presented here did not find any change in the incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons in CFA inflammation, previous reports have suggested CFA results in an increase in monosynaptic Aδ-fibre input to these neurons (Torsney 2011). Given that those neurons receiving monosynaptic Aδ-fibre input display an elevated sEPSC frequency, if previous reports are correct and this type of input is increased in inflammation, then it could be hypothesised that there will also be an increase in the spontaneous excitatory drive to these neurons.

It should be recognised that sEPSCs arise from both action-potential dependent and action-potential independent neurotransmitter release (Engelman & MacDermott 2004). Although the increased sEPSC frequency in neurons receiving monosynaptic Aδ-fibre only input cannot be directly attributed to the Aδ-fibre input, this seems a plausible explanation given the previously reported potentiation in neurons with this type of input (Torsney 2011). However, it is possible that this increased excitatory input was driven from elsewhere in the spinal cord network. Additionally, while the neuron in which sEPSCs were recorded was clamped at the E_{anion} (-70mV), thus blocking direct inhibitory inputs, it is possible that varying levels of inhibition within

the wider spinal cord network impact differently on sEPSCs in particular subgroups of neuron. One approach to undertake a more complete investigation into the link between primary afferent input and spontaneous excitatory input to lamina I NK1R+ neurons, would be to record miniature excitatory postsynaptic currents (mEPSCs) in addition to sEPSCs. Recording in tetrodotoxin (TTX) to block network activity would enable the direct study of spontaneous neurotransmitter release from synaptic terminals directly onto these neurons. However, this would include excitatory input from excitatory interneurons, so would be unable to give a definitive answer to the influence of monosynaptic primary afferent input in setting sEPSC frequency in lamina I NK1R+ neurons.

To my knowledge this is the first study that has investigated the effect of CFA inflammation on spontaneous excitatory input to lamina I NK1R+ neurons. Others have studied the effects of CFA inflammation on unidentified lamina II neurons and report no effect on sEPSCs in rat (Lappin et al. 2006), but significantly increased frequency and amplitude in mice (Park et al. 2011). Similarly there are conflicting reports on the effect of nerve injury on sEPSCs. No effects were found in unidentified neurons in the lamina I/II (Spicarova et al. 2011) and II_o regions (Kohno et al. 2003). Nerve injury has also been reported to cause changes in sEPSC frequency and/or amplitude in lamina II neurons, although the effect was different depending upon the action potential discharge pattern that the neuron displayed, where for example 'tonic' neurons show reduced sEPSC frequency while 'phasic' firing neurons show increased frequency (Balasubramanyan et al. 2006).

2.6 Conclusion

The results presented here demonstrates that CFA inflammation does not alter the electrical activation threshold, conduction velocity or amplitude of Aβ-, Aδ- or C-fibre components in rat dorsal roots (figure 2.9, table 2.2). These findings confirm previous reports showing inflammation does not alter these properties in juveniles (Torsney 2011) and adults (Baba et al. 1999, Nakatsuka et al. 1999). These findings mean that identical parameters can be used to activate the different primary afferent fibres in eEPSC studies performed in spinal slices with dorsal roots attached, isolated from control and CFA treated rats. These data also confirm the stimulation intensities used to activate Aδ- and C-fibres in previous studies (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006). However, it was established that a slightly lower intensity should be used to activate Aβ-fibres, to prevent potential misidentification of primary afferent inputs.

In the data presented here, CFA inflammation did not alter the overall pattern of primary afferent input to lamina I NK1R+ neurons or the incidence of monosynaptic and polysynaptic inputs, apart from an increase in monosynaptic Aβ-fibre input that did not reach statistical significance (figure 2.14). This did not replicate the previously reported alterations in monosynaptic Aδ-fibre input to these neurons during inflammation (Torsney 2011). Similarly, the potentiation of a subset of monosynaptic Aδ-fibre inputs in inflammation (Torsney 2011) was not found in these data presented here (figure 2.15, table 2.3). Inflammation did not increase C-fibre eEPSC amplitude, which confirms that potentiation of C-fibre synapses with lamina I NK1R+ neurons is not a feature of CFA inflammation (Torsney 2011).

These data presented here have novelly shown that CFA inflammation does not alter sEPSC frequency or amplitude in lamina I NK1R+ neurons, either in the entire population or when the neuronal subpopulations receiving monosynaptic Aδ- only, Aδ- & C- or C-fibre only input are considered independently (figure 2.16, table 2.4). Furthermore, neurons that received monosynaptic Aδ-fibre only input displayed significantly greater sEPSC frequency that neurons receiving monosynaptic C-fibre only input. This suggests that if the previously reported increase in the incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons following inflammation (Torsney 2011) is a genuine effect, then this could be associated with an increase in spontaneous excitatory input to these neurons.

Chapter 3

Chemerin modulation of potentiated primary afferent input to lamina I neurokinin 1 receptor expressing neurons

3.1 Introduction

Many currently available treatments for inflammatory pain lack efficacy and/or exhibit undesirable side effects (Scholz & Woolf 2002). Commonly used pharmacological interventions for inflammatory pain include opioids and cyclooxygenase (COX) inhibitors, however use of these drugs can result in complications. Non-selective COX inhibitors can lead to kidney damage and gastrointestinal bleeding, while selective COX-2 inhibitors are known to elevate the risk of cardiovascular disease (Mattia & Coluzzi 2005, Mukherjee et al. 2001, Sommer & Birklein 2010). Opioids often cause sedation, nausea, respiratory depression, constipation, cognitive disturbances and in cases of long-term use can lead to addiction (Noble et al. 2010, Sommer & Birklein 2010, Xu & Ji 2011). Development of new analgesics that are both efficacious and lack such side effects is therefore a key challenge for pain research. The goal of this chapter is to investigate whether chemerin, an agonist of the chemerin receptor 23 (ChemR23), can modulate potentiated primary afferent input to lamina I neurokinin 1 receptor expressing (NK1R+) neurons. The findings from the studies presented here could provide insight into whether ChemR23 represents a promising target for the development of novel inflammatory pain treatment strategies, as recent studies have suggested (Xu et al. 2010).

3.1.1 The use of novel lipid mediators in the treatment of inflammatory pain

In recent years there has been a growing interest in the use of novel lipid mediators, including lipoxins, protectins / neuroprotectins and resolvins, in the treatment of inflammatory diseases (Recchiuti & Serhan 2012, Serhan 2010, Serhan & Chiang 2008, Serhan et al. 2008). In the case of inflammatory pain, particular attention has been focussed on the resolvins, which includes resolvin E1 (RvE1), resolvin D1 (RvD1) and resolvin D2 (RvD2) (Ji et al. 2011, Lee 2012, Park et al. 2011, Sommer & Birklein 2010, Xu & Ji 2011, Xu et al. 2010). Resolvins are endogenously synthesised from the omega-3 fatty acids docosahexaenoic acid (RvD1 and RvD2) and eicosapentaenoic acid (RvE1) (Serhan et al. 2008, 2002, Spite et al. 2009). Interestingly, dietary intake of these omega-3 fatty acids can provide relief from inflammatory joint pain (Goldberg & Katz 2007, Lee et al. 2012, Tokuyama & Nakamoto 2011) and there is some evidence that omega-3 fatty acid supplements can attenuate neuropathic pain (Ko et al. 2010) and reduce non-steroidal anti-inflammatory drug (NSAID) intake following surgery (Maroon & Bost 2006) and in rheumatoid arthritis (Galarraga et al. 2008). In addition to exhibiting anti-inflammatory properties, resolvins have been shown to play a unique role in the resolution of inflammation, which in recent years has been demonstrated to be an active homeostatic process that is distinct from inflammation (Lawrence et al. 2002, Serhan & Chiang 2008, Serhan et al. 2008). The pro-resolution activity of resolvins is in contrast to many commonly used inflammatory pain treatments, such as COX-2 inhibitors and local anaesthetics, that actually inhibit key resolution processes (Chiang et al. 2008, Gilroy et al. 1999, Schwab et al. 2007, Serhan et al. 2008). It has been speculated that this inhibition of natural resolution may actually extend the period of inflammatory pain (Ji et al. 2011).

Resolvins have been suggested to be a promising novel treatment for inflammatory pain in part because they are proposed to exhibit limited side effects due to a number of factors (Ji et al. 2011, Xu et al. 2010). As naturally occurring endogenous mediators they are likely to be safe. These compounds are also highly potent at low concentrations, exhibiting comparable analgesic activity to morphine and COX-2 inhibitors at much lower doses (Xu et al. 2010). The resolvins RvE1, RvD1 and RvD2 do not alter baseline thermal or mechanical withdrawal thresholds, while RvE1 and RvD2 are without effect on the first phase of the formalin-induced pain. Therefore, resolvins attenuate maladaptive / chronic pain but do not alter protective acute pain responses. (Huang et al. 2011, Park et al. 2011, Xu et al. 2010).

There is a growing body of evidence to support the use of resolvins in the treatment of inflammatory pain, where it has been shown that resolvins have both peripheral and central mechanisms of action (Xu et al. 2010).

Peripheral administration of RvE1 significantly reduces oedema in the carrageenan inflammatory pain model (Xu et al. 2010). Specifically, pretreatment with RvE1 significantly reduced the carrageenan-induced increases in neutrophil infiltration and proinflammatory cytokine and chemokine expression (Xu et al. 2010).

Central (intrathecal) administration of resolvins has been shown to be highly effective in attenuating thermal, mechanical and chemical hypersensitivity in a number of inflammatory and neuropathic pain models, as shown in table 3.1. The fact that resolvins can attenuate mechanical hypersensitivity, which is known to be a centrally mediated process (Klede et al. 2003, Lewin et al. 2004) and the second phase of formalin-induced hypersensitivity, which is typically considered to be driven by central sensitisation (Ji et al. 1999, Yamamoto & Yaksh 1992), indicates that resolvins have a central mechanism of action.

The central activity of RvE1 has been investigated in relation to its effects on;

1. Tumor necrosis factor-α (TNF-α) signalling: which is crucial in centrally mediated inflammatory pain processes (Kawasaki et al. 2008)
2. Transient receptor potential subtype vanilloid 1 (TRPV1) channels: that are known to be essential for thermal hypersensitivity (Caterina et al. 2000, Davis et al. 2000) and which can be sensitised by TNF-α and other inflammatory mediators, with this sensitisation contributing to inflammatory pain (Basbaum et al. 2009, Cheng & Ji 2008, Palazzo et al. 2012, Pingle et al. 2007).

RvE1 abolished both the capsaicin and TNF-α mediated potentiation of spontaneous excitatory postsynaptic current (sEPSC) frequency, but not amplitude, in unidentified lamina II spinal cord neurons (Xu et al. 2010). The authors suggest that this finding indicates a presynaptic mechanism of action, however sEPSCs were recorded, where network activity is not blocked by tetrodotoxin (TTX), so this cannot be concluded from their results. These effects were further demonstrated to be mediated via extracellular signal-regulated kinase (ERK) signalling. Inhibition of the ERK pathway, with the mitogen-activated protein kinase kinase (MEK) inhibitors PD98059 and U0126, prevented the capsaicin potentiation of sEPSCs. RvE1 also reduced capsaicin / TNF-α driven ERK phosphorylation (pERK) in dorsal root ganglia (DRG)

cultures, which support a presynaptic mechanism of action (figure 3.1A) (Xu et al. 2010).

The postsynaptic effects of RvE1 have been investigated in unidentified lamina II neurons, where it was shown that RvE1 attenuates the TNF-α-mediated potentiation of postsynaptic N-methyl-D-aspartate (NMDA) currents and phosphorylation of ERK (Xu et al. 2010), the latter being considered a marker of central sensitisation (Ji et al. 1999, Karim et al. 2001).

Notably, RvE1 alone did not alter sEPSCs or the phosphorylation of ERK in DRG or dorsal horn neurons (Xu et al. 2010). Therefore, the evidence provided by Xu et al. (2010) indicates that resolvins reduce inflammatory pain by two mechanisms;

1. By reducing the peripheral inflammatory response.
2. By 'normalising' potentiated presynaptic and postsynaptic spinal cord responses.

Stimulus	Model	RvE1	RvD1	RvD2	Reference
Thermal	CFA	✓	✓	✓	Park et al. 2011, Xu et al. 2010
	carrageenan	✓	✓	✓	Park et al. 2011, Xu et al. 2010
	SNL	✓			Xu et al. 2013, 2010
Mechanical	CFA	✓			Xu et al. 2010
	carrageenan	✓	✓	✓	Abdelmoaty et al. 2011, Park et al. 2011
	TNFα	✓			Xu et al. 2010
	post-operative pain	✓	✓		Huang et al. 2011, Xu et al. 2010
	CCI	✓			Xu et al. 2013
	SNL	✓			Xu et al. 2013
Chemical	formalin (2^{nd} phase)	✓	✓	✓	Park et al. 2011, Xu et al. 2010
	capsaicin (*i.t.*)	✓			Xu et al. 2010

Table 3.1: Attenuation of behavioural hypersensitivity by resolvins.

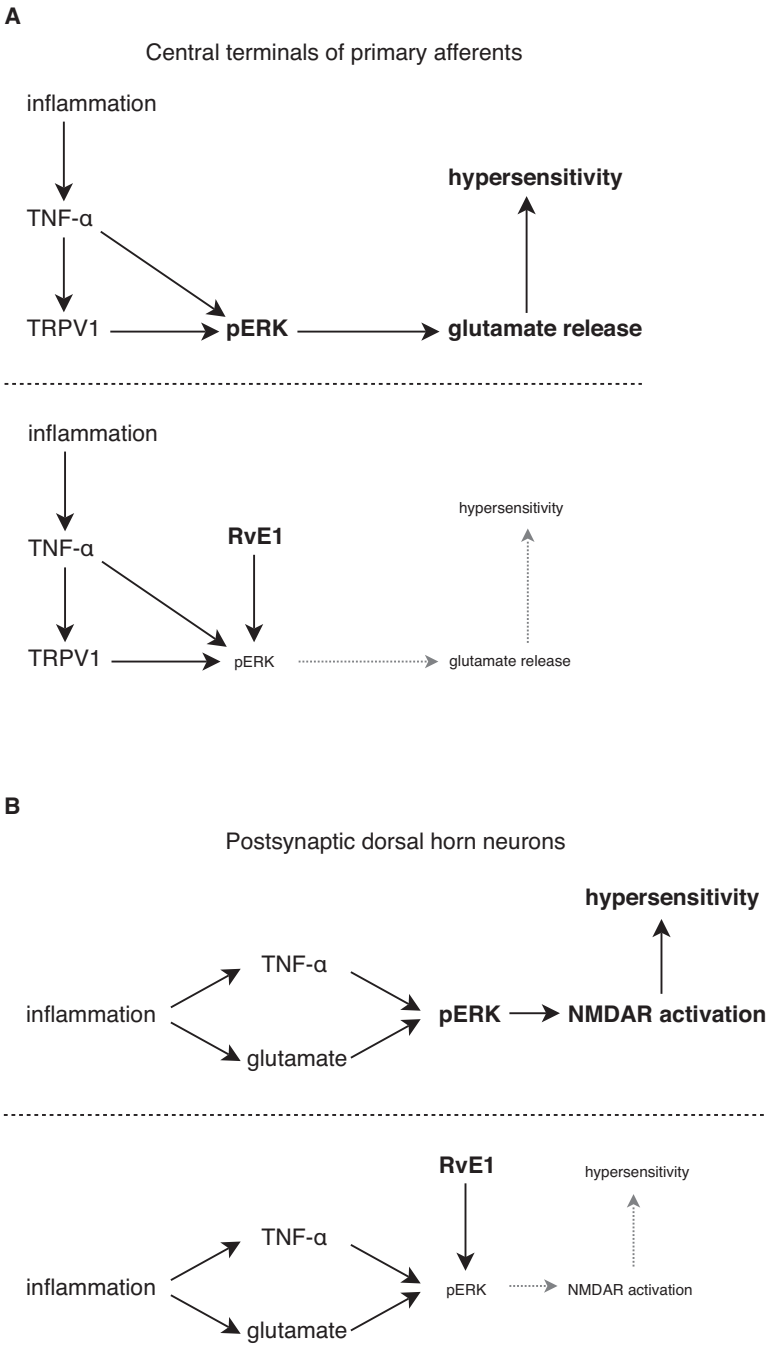

Figure 3.1: Schematic diagram of RvE1 mediated attenuation of inflammatory pain. ***A*** In the central terminals of primary afferent fibres, RvE1 attenuates inflammatory pain hypersensitivity via a reduction in ERK phosphorylation leading to a reduction in the presynaptic release of glutamate. ***B*** In poststynaptic dorsal horn neurons, RvE1 attenuates inflammatory pain through the inhibition of potentiated NMDA currents by suppressing ERK phosphorylation. Adapted from Xu et al. 2010.

Resolvins have also demonstrated an ability to regulate a number of transient receptor potential (TRP) channels, including TRPV1, transient receptor potential subtype vanilloid 3 (TRPV3), transient receptor potential subtype vanilloid 4 (TRPV4) and transient receptor potential subtype ankyryn 1 (TRPA1) (Bang et al. 2012, 2010, Park

et al. 2011). Notably, these channels are known to be involved in the inflammatory pain response (Alessandri-Haber et al. 2005, Basbaum et al. 2009, Caterina et al. 2000, Davis et al. 2000, Mandadi & Roufogalis 2008, McMahon & Wood 2006). A list of TRP channels that are inhibited by resolvins is shown in table 3.2. All resolvins were shown to be particularly potent inhibitors of these TRP channels and highly active at low nM concentrations. Interestingly, RvE1 was reported to inhibit TRPV1 channels to a much greater extent than the commonly used TRPV1 antagonist AMG9810, with an IC_{50} of 1nM versus 163nM for AMG9810 (Park et al. 2011). The exact mechanisms by which resolvins inhibit TRP channels is not well understood. However, current evidence suggests that the resolvin inhibition of TRP channels is mediated via G-protein coupled receptors (GPCRs) as opposed to direct interactions with the channels (Ji et al. 2011, Park et al. 2011, Xu et al. 2010). Specifically, the RvE1 mediated inhibition of TRPV1 is thought to result from RvE1 acting via the GPCR, ChemR23 (also known as chemokine receptor-like 1 (CMKLR1)) to inhibit the phosphorylation of ERK and presynaptic release of glutamate (Xu et al. 2010). It is also worth noting that the omega-3 fatty acid, resolvin precursors, have been reported to modulate the activity of TRP channels (Leonelli et al. 2011).

Channel	RvE1	RvD1	RvD2	17R-RvD1	Reference
TRPV1	✓		✓		Park et al. 2011, Xu et al. 2010
TRPV3		✓		✓	Bang et al. 2012, 2010
TRPV4		✓			Bang et al. 2010
TRPA1		✓	✓		Bang et al. 2010, Park et al. 2011

Table 3.2: Inhibition of TRP channels by resolvins.

The evidence discussed above strongly suggests that resolvins, particularly RvE1, are effective at attenuating inflammatory pain through a mechanism whereby potentiated spinal cord responses are normalised. However, these findings were made in unidentified lamina II neurons, so it is unclear which neuronal subtypes are involved. Lamina I NK1R+ neurons are known to be essential for the development of inflammatory pain (Nichols et al. 1999), which is driven by C-fibres (Abrahamsen et al. 2008), some of which may be potentiated in inflammation (Ikeda et al. 2006). Therefore, it is possible that some of the effects of the resolvins could be mediated through an attenuation of potentiated primary afferent input to these key spinal cord output neurons.

3.1.2 ChemR23 as a potential target for the treatment of inflammatory pain

RvE1 has been reported to act through the $G_{\alpha}i$ coupled GPCR, ChemR23 (Arita et al. 2005). This finding has been disputed as the result has not been replicated by other groups (Bondue et al. 2011). However, the ability of RvE1 to attenuate hyperalgesia in the carrageenan and formalin pain models was abolished by application of the $G_{\alpha}i$ inhibitor, pertussis toxin (PTX) and ChemR23 siRNA, with PTX also blocking the RvE1 attenuation of potentiated spinal cord responses (Xu et al. 2010). Therefore, ChemR23 clearly plays a role in mediating the RvE1 attenuation of inflammatory pain responses and represents a potential target for the development of novel inflammatory pain treatments.

ChemR23 is largely expressed in adipocytes (Bozaoglu et al. 2007, Goralski et al. 2007, Huang et al. 2010, Roh et al. 2007) and immune cells including monocytes, macrophages, dendritic cells, microglia and natural killer cells (Bondue et al. 2011, Graham et al. 2009, Luangsay et al. 2009, Parolini et al. 2007, Samson et al. 1998, Vermi et al. 2005). While the expression data for ChemR23 in pain pathways is not comprehensive, anatomical studies have demonstrated that ChemR23 is expressed both peripherally in DRG cell bodies and centrally in the central terminals of primary afferent fibres and also in spinal cord neurons (Xu et al. 2010). Further evidence from microarray studies corroborates that ChemR23 mRNA is expressed in DRG neurons and in the dorsal horn (Abdelmoaty et al. 2011, Rodriguez Parkitna et al. 2006). However, as these were microarray studies of DRG tissue it is not possible to attribute ChemR23 expression level changes to any particular subpopulation of DRG neurons. ChemR23 mRNA expression is not altered in DRG or spinal cord neurons following complete Freund's adjuvant (CFA) inflammation (Rodriguez Parkitna et al. 2006), but is increased in dorsal horn neurons by carrageenan inflammation (Abdelmoaty et al. 2011) and nerve injury (Rodriguez Parkitna et al. 2006). Almost a third of all DRG neurons express ChemR23 and there is a large degree of co-expression between ChemR23 and TRPV1, with 61% of TRPV1 expressing (TRPV1+) DRG neurons also expressing ChemR23 (Xu et al. 2010). In the superficial lamina of the dorsal horn, ChemR23 is expressed on substance P (SP) containing axon terminals (Xu et al. 2010). These findings are particularly interesting for the following reasons. The majority of C-fibres and some Aδ-fibres express TRPV1 (Amaya et al. 2003, Kobayashi et al. 2005, Michael & Priestley 1999, Yu et al. 2008), while around a half of C-fibres and a fifth of Aδ-fibres contain SP (Lawson et al. 1997, 1993, McCarthy & Lawson 1989). Lamina I NK1R+ neurons receive monosynaptic input from C- and

Aδ-fibres (Torsney 2011, Torsney & MacDermott 2006), that are known to include TRPV1+ (Hwang et al. 2003, Labrakakis & MacDermott 2003, Tong & MacDermott 2006) and SP containing (Hwang et al. 2003, Todd et al. 2002) inputs. Therefore, given the co-expression of ChemR23 with TRPV1 and SP (Xu et al. 2010) it is possible that lamina I NK1R+ neurons will receive input from primary afferents that express ChemR23. Therefore, it is hypothesised that ChemR23 is likely to be functionally expressed on primary afferent input to lamina I NK1R+ neurons and that activation of these ChemR23 receptors could attenuate potentiated primary afferent input to these key spinal cord output neurons that are crucial for the manifestation of inflammatory pain (Nichols et al. 1999).

Chemerin

The peptide chemerin has been identified as the natural ChemR23 ligand (Meder et al. 2003, Wittamer et al. 2003). It is currently unclear which cell types are responsible for the endogenous production and release of chemerin, however endothelial cells, keratinocytes, chondrocytes, platelets and osteoclasts have been proposed as possible sources (Berg et al. 2010, Bondue et al. 2011, Du et al. 2009, Luangsay et al. 2009, Nagpal et al. 1997, Vermi et al. 2005). Chemerin plays a key role in a number of physiological processes including adipocyte generation and metabolism (Goralski et al. 2007) and the chemotaxis of macrophages and dendritic cells (Wittamer et al. 2003). The role of endogenous chemerin in the modulation of inflammatory pain is currently unknown. However, like RvE1, chemerin application has been shown to significantly reduce the second phase of formalin-induced pain and inhibit the capsaicin potentiation of spinal cord responses (Xu et al. 2010). It is therefore possible that chemerin activation of ChemR23 receptors could attenuate primary afferent input to a subset of lamina I NK1R+ neurons.

3.1.3 Summary

The evidence discussed above demonstrates that activation of ChemR23 with the agonists chemerin and RvE1 can attenuate the behavioural hypersensitivity associated with inflammatory pain, by a mechanism that involves the normalisation of potentiated spinal cord responses. Furthermore, ChemR23 is expressed on TRPV1+ and SP containing primary afferents (Xu et al. 2010). Lamina I NK1R+ neurons are essential for the manifestation of inflammatory pain (Nichols et al. 1999) and some of

the monosynaptic C- and monosynaptic Aδ-fibre input they receive may be potentiated in inflammation (Ikeda et al. 2006, Torsney 2011). In addition, these neurons are known to receive input from TRPV1+ and SP containing afferents (Hwang et al. 2003, Labrakakis & MacDermott 2003, Todd et al. 2002, Tong & MacDermott 2006). Therefore, it is hypothesised that ChemR23 is likely to be functionally expressed on a subset of inputs to these neurons. ChemR23-mediated analgesia could in part be due to a normalisation of potentiated monosynaptic C- and/or monosynaptic Aδ-fibre excitatory input to these neurons. As such, the application of chemerin could attenuate primary afferent input to a subset of these neurons in inflammatory pain. Notably, RvE1 is not commercially available, therefore it was not possible to investigate the effect of this compound upon primary afferent input to lamina I NK1R+ neurons.

3.2 Chapter aims and hypotheses

The experiments presented in this chapter were performed to determine whether chemerin, an agonist of the novel inflammatory pain target ChemR23 (Xu et al. 2010), can attenuate potentiated primary afferent input to lamina I NK1R+ neurons and to address the aims and hypotheses detailed below.

Aims

1. Using miniature excitatory postsynaptic current (mEPSC) recordings, establish whether the ChemR23 agonist, chemerin, modulates excitatory input to lamina I NK1R+ neurons in non-potentiated conditions.
2. Determine whether chemerin can attenuate the capsaicin potentiation of primary afferent input to these neurons, as measured using mEPSC recordings.
3. Ascertain whether ChemR23 is functionally expressed on monosynaptic C- and/or monosynaptic Aδ-fibre input to lamina I NK1R+ neurons, using dorsal root evoked excitatory postsynaptic current (eEPSC) recordings.
4. By employing dorsal root eEPSC recordings, investigate the ability of chemerin to modulate monosynaptic C- and/or monosynaptic Aδ-fibre input to these neurons and establish whether chemerin effects are dependent upon CFA inflammation.

Hypotheses

1. In non-potentiated conditions, chemerin will not alter mEPSCs in lamina I NK1R+ neurons.
2. Chemerin will attenuate the capsaicin potentiation of mEPSCs in lamina I NK1R+ neurons.
3. ChemR23 will be functionally expressed on a subset of monosynaptic C-fibre and to a lesser extent a subset of monosynaptic Aδ-fibre input, to lamina I NK1R+ neurons.
4. In CFA inflammation, chemerin will attenuate monosynaptic C-fibre input to a subset of lamina I NK1R+ neurons and to a lesser extent will attenuate monosynaptic Aδ-fibre input to a subset of these neurons.

3.3 Methods

3.3.1 Animals

As described in section 2.3.1.

3.3.2 Complete Freunds's adjuvant inflammation

As described in section 2.3.2.

3.3.3 Capsaicin potentiation

Spinal cord slice preparation

Spinal cords were removed from control rats only and spinal cord slices were made as described in section 2.3.4, except all dorsal roots were removed prior to embedding the spinal cord in agar.

Miniature excitatory postsynaptic current (mEPSC) recordings

Whole cell patch-clamp configuration, as described in section 2.3.4, was used to record mEPSCs from NK1R+ neurons in lamina I. Bath application of 0.5μM TTX (Alomone Labs) was used to block action potential dependent events. Inhibition was blocked by recording at a holding potential of -70mV, which is the anion reversal potential (E_{anion}) in lamina I neurons (Coull et al. 2003) and bath application of the $GABA_A$ receptor ($GABA_AR$) and glycine receptor (GlyR) antagonists, bicuculline (10μM, Tocris) and strychnine (1μM, Sigma), respectively. The internal solution composition was as follows (in mM, all Sigma); 110 K-methanesulfonate, 10 NaCl, 10 EGTA, 1 $CaCl_2$, 10 HEPES, 5 Mg-ATP, 0.5 Na-GTP, pH adjusted to 7.2 with KOH, osmolarity ∼290mOsm. 1μM Alexa Fluor 488 hydrazide (Molecular Probes) was also included in the recording pipette. Junction potential was corrected prior to recording. Data were acquired and recorded using an Axopatch 200B amplifier and pClamp 10 software (Molecular Devices). Data were filtered at 5kHz and digitised at 10kHz. Neurons were excluded if any of the following criteria were met: access resistance; >25MΩ, holding current; >100pA or <-100pA, membrane resistance; <300MΩ.

Capsaicin potentiation of excitatory input to lamina I neurokinin 1 receptor expressing neurons

Baseline mEPSCs were recorded for 5 minutes, following which the TRPV1 agonist, capsaicin (1μM, Sigma) was bath applied for a further 5 minutes to pharmacologically potentiate primary afferent input (Yang et al. 1998). TRPV1 is only found on primary afferents in the spinal cord (Caterina et al. 1997, Cavanaugh et al. 2011, Tominaga et al. 1998), with the majority of C- and some A-fibres expressing the channel (Amaya et al. 2003, Kobayashi et al. 2005, Michael & Priestley 1999, Yu et al. 2008). One study has suggested that TRPV1 may be expressed on a subset of lamina II GABAergic interneurons (Kim et al. 2012), however this finding has not been validated by recent studies using transgenic TRPV1 reporter mice, that clearly demonstrate that TRPV1 is not expressed on dorsal horn neurons and is only present in the central terminals of primary afferent fibres in the spinal cord (Cavanaugh et al. 2011). Therefore, applying capsaicin to spinal cord slices should potentiate primary afferent input only, by increasing the release of glutamate from the central terminals (Kim et al. 2009). Importantly, lamina I NK1R+ neurons are known to receive input from TRPV1+ afferents (Labrakakis & MacDermott 2003). Potentiation was assessed by comparing mEPSCs recorded at baseline and during capsaicin application. To assess whether ChemR23 activation modulates this capsaicin potentiation, recombinant mouse chemerin (100ng/ml, R&D Systems) was applied for 10 minutes prior to and throughout capsaicin application, in a separate group of lamina I NK1R+ neurons. It was not possible to carry out a single neuron analysis of the chemerin modulation of capsaicin mEPSC potentiation because capsaicin mEPSC potentiation shows significant reductions with repeated capsaicin application (data not shown). Therefore, separate neuronal groups were studied to assess the effect of capsaicin alone, chemerin & capsaicin and chemerin alone. Recombinant mouse chemerin was used because rat chemerin is not commercially available, however there is a high degree of homology between rat and mouse chemerin and cross-species activity has been reported (Busmann et al. 2004). Although much of the interest in targeting ChemR23 revolves around the use of RvE1 (Ji et al. 2011, Lee 2012, Park et al. 2011, Sommer & Birklein 2010, Xu & Ji 2011, Xu et al. 2010), this compound is not commercially available so it was not possible to investigate its potential. The effect of ChemR23 activation in non-potentiated conditions was similarly assessed by applying chemerin alone. In all cases mEPSC frequency and amplitude was measured during the final 2 minutes of baseline or drug application and were analysed using Mini Analysis (Synaptosoft). mEPSC events were automatically detected by the software

and were then accepted or rejected following further visual examination.

3.3.4 Inflammatory pain

Spinal cord slice preparation

Spinal cords were isolated from control and CFA treated rats and spinal slices with dorsal roots attached were prepared as described in section 2.3.4.

Effects of chemerin application on dorsal root evoked primary afferent input to lamina I neurokinin 1 receptor expressing neurons

To assess the ability of chemerin to modulate primary afferent input to lamina I NK1R+ neurons in an inflammatory pain model, the effect of chemerin was assessed on monosynaptic C- and monosynaptic Aδ-fibre eEPSCs in tissue isolated from control and CFA treated rats.

Monosynaptic C- and monosynaptic Aδ-fibre input to lamina I NK1R+ neurons was identified in the manner described previously in section 2.3.5. Following input characterisation, dorsal roots were then stimulated at an intensity of 500μA, with a stimulus duration of 0.1ms and a frequency of 0.05Hz and eEPSCs recorded. eEPSCs were recorded for a baseline period of 10 minutes, followed by a further 15 minutes minutes where chemerin (100ng/ml) or vehicle (Krebs only) was applied. A vehicle control was recorded because previous observations in our laboratory have shown that repetitive stimulation of monosynaptic C- and monosynaptic Aδ-fibres in this manner can result in a gradual 'run-down' of the response (unpublished data), this can also be observed in figure 3.5A. Therefore, by comparing chemerin data to vehicle control this enabled the accurate determination of the amplitude changes resulting from chemerin treatment independently from any changes that may occur due to run-down. Peak eEPSC amplitude was measured for each sweep, as shown in figure 2.15A and mean peak amplitude per minute was calculated. All data were normalised to minute 2, because in many neurons there was a large degree of run-down in the peak eEPSC amplitude between minute 1 and minute 2, after which the response generally stabilised. Neurons in which the peak amplitude could not be clearly identified were excluded. To compare the effect of chemerin / vehicle between control and CFA treated tissue, the amplitude change between the mean eEPSC amplitude for baseline (10 minutes) and final 5 minutes of chemerin / vehicle application was calculated as

follows:

$$\Delta amplitude = \left(\frac{chemerin/vehicle\ mean\ amplitude}{baseline\ mean\ amplitude} \right) 100 \quad \text{(a)}$$

It was hypothesised that chemerin would modulate primary afferent input to a subset of lamina I NK1R+ neurons, given that ChemR23 is only expressed on a subset of TRPV1+ and a subset of SP containing afferents (Xu et al. 2010). To identify this subgroup, linear regression analysis was performed on vehicle data to calculate 95% prediction bands. A neuron was classified as a responder if the peak eEPSC amplitude fell below the lower limit of the 95% prediction bands for at least the final 5 minutes of chemerin application. To evaluate the validity of this classification method and to assess whether responders and non-responders were two distinct subpopulations, frequency histograms of mean peak amplitude in the final 5 minutes of chemerin / vehicle application were plotted.

To determine the pre- / post-synaptic nature of chemerin effects, paired-pulse recordings were conducted in a subset of neurons that received monosynaptic C-fibre input in tissue isolated from CFA treated rats. eEPSCs were recorded in the manner described above, however each dorsal root evoked event featured 2 stimuli given in close succession, with an interstimulus interval of 500ms (figure 3.8A). The duration of this interstimulus interval is longer than is typically been used in paired-pulse recordings (Bardoni et al. 2007, Li & Baccei 2009), however stimulation of C-fibres at frequencies of $>$1Hz, which is equivalent to an interstimulus interval of $<$1000ms, results in failures to fire action potentials (Nakatsuka et al. 2000). Therefore, 500ms was chosen to balance the need for the two stimuli to be given close enough so that the second release event is influenced by the first, with the need to avoid action potential failures. Paired-pulse ratio (PPR) was calculated for the 5 minutes prior to ('pre-chemerin') and the final 5 minutes of chemerin application using the following equation, so as to correct for misleading facilitation that can be caused by random amplitude fluctuations (Kim & Alger 2001):

$$paired\text{-}pulse\ ratio = \frac{mean\ eEPSC\ 2}{mean\ eEPSC\ 1} \quad \text{(b)}$$

To examine whether the initial eEPSC amplitude was predictive of the chemerin response, the correlation between initial amplitude at minute 2 and the amplitude change, recorded in the final 5 minutes of chemerin application was analysed. As it was hypothesised that chemerin would reduce potentiated inputs, this analysis was

performed to provide insight into whether neurons that received inputs with greater initial eEPSC amplitudes (possibly signifying potentiated inputs) displayed enhanced chemerin responses.

3.3.5 Statistical analysis

All data were assessed for normality using D'Agostino & Pearson omnibus normality tests, or Kolmogorov-Smirnov tests if sample sizes were insufficient to use the former, to establish whether it was appropriate to use parametric or non-parametric statistical tests, or whether data should be transformed prior to performing parametric tests.

Paired t-tests were used to assess changes in mEPSC frequency and amplitude following application of chemerin alone, capsaicin alone and chemerin & capsaicin, except for frequency following chemerin alone where a Wilcoxon test was used. To determine whether chemerin altered the capsaicin potentiation of mEPSC frequency or amplitude, 2-way repeated measures ANOVA, followed by Bonferroni post-tests were used. To establish whether chemerin alone, capsaicin alone or chemerin & capsaicin caused significant changes in the probability distribution of mEPSC inter-event interval or amplitude within individual neurons, a 2 sample Kolmogorov-Smirnov test was used.

The influence of time and chemerin on monosynaptic C- and monosynaptic Aδ-fibre eEPSCs in tissue isolated from both control and CFA treated rats was assessed by 2-way repeated measures ANOVA followed by Bonferroni post-tests. The difference in amplitude change between control and CFA groups was assessed using 2-way ANOVA followed by Bonferroni post-tests and differences in amplitude change between CFA vehicle, chemerin responder and chemerin non-responder groups were analysed with 1-way ANOVA followed by Bonferroni post-tests. To determine whether there were differences in the frequency distribution of peak amplitude in the final 5 minutes of chemerin application between vehicle, chemerin responder and chemerin non-responder groups, 1-way ANOVA followed by Bonferroni post-tests were used. The effect of chemerin on PPR in neurons classified as chemerin responders and chemerin non-responders was statistically analysed by 2-way repeated measures ANOVA followed by Bonferroni post-tests. To assess whether there was any correlation between initial eEPSC amplitude and the chemerin reduction in amplitude, Pearson's r test was used.

All data are presented as mean ± standard error of the mean (SEM). All statistical

analysis was performed using Prism 6 (Graphpad Software).

3.4 Results

3.4.1 Chemerin alone does not alter mEPSC frequency or amplitude in lamina I neurokinin 1 receptor expressing neurons

To assess whether chemerin alone altered synaptic input to lamina I NK1R+ neurons, mEPSCs were recorded in the absence ('baseline') and presence of chemerin. Representative traces of baseline and chemerin recordings are shown in figure 3.2A. Chemerin did not alter the probability distribution of inter-event intervals in 6/11 neurons. In 2/11 neurons there was a significant leftward shift, indicating shorter inter-event intervals / increased frequency. In 3/11 neurons there was a significant rightwards shift, indicating longer inter-event intervals / reduced frequency (all statistics, Kolmogorov-Smirnov 2-sample test, example shown in figure 3.2B). Overall chemerin had no significant effect on mEPSC frequency (P=0.824, Wilcoxon, n=11, figure 3.2C). The cumulative probability distribution of mEPSC amplitude was not altered by chemerin in 5/11 neurons, but significantly leftward shifted, indicating reduced amplitude, in 1/11 and significantly rightward shifted, indicating increased amplitude, in 5/11 (all statistics, Kolmogorov-Smirnov 2-sample test, example shown in figure 3.2D). The summary data show that chemerin alone did not significantly alter mEPSC amplitude in lamina I NK1R+ neurons (P=0.654, paired t-test, n=11, figure 3.2E)

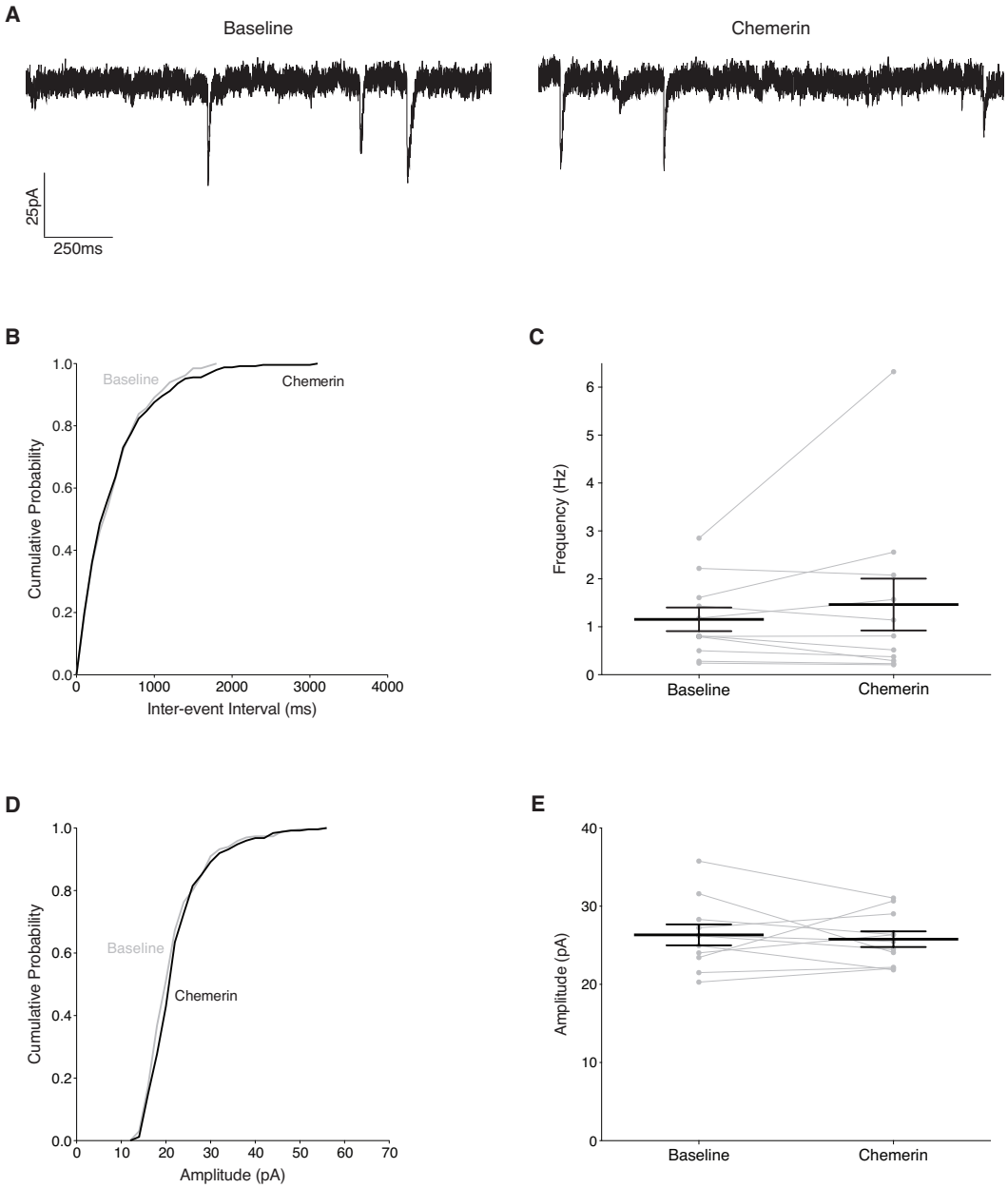

Figure 3.2: Chemerin does not alter lamina I NK1R+ neuron mEPSC frequency or amplitude. ***A*** Representative mEPSC traces recorded prior to (baseline, left) and during chemerin application (right). ***B*** Example cumulative probability curve shows chemerin does not alter the distribution of mEPSC inter-event intervals in an individual neuron (P=0.992, 2-sample Kolmogorov-Smirnov test). ***C*** Summary data shows that chemerin did not significantly alter mEPSC frequency (P=0.824, Wilcoxon, n=11). ***D*** Example cumulative probability curve shows chemerin had no effect on mEPSC amplitude distribution in an individual neuron (P=0.171, 2-sample Kolmogorov-Smirnov test). ***E*** Summary data demonstrates that chemerin did not alter mEPSC amplitude (P=0.654, paired t-test, n=11). Black lines in ***C*** & ***E*** denote mean $\pm$ SEM, grey points & lines show trajectories for individual neurons.

3.4.2 Chemerin attenuates capsaicin potentiation of primary afferent input to lamina I neurokinin 1 receptor expressing neurons.

To determine whether chemerin could attenuate capsaicin potentiation of primary afferent input to lamina I NK1R+ neurons, mEPSCs were recorded prior to and during capsaicin treatment, in two populations of neurons in either the presence (n=10) or absence (n=12) of chemerin. Figure 3.3A shows representative traces recorded in the absence of chemerin, before ('baseline') and during capsaicin treatment ('capsaicin'), 3.3D displays traces recorded in the presence of chemerin, prior to ('chemerin') and during capsaicin treatment ('chemerin & capsaicin'). Capsaicin alone resulted in a significant leftwards shift in the distribution of inter-event intervals in 10/12 neurons, while in 2/12 there was no effect (all statistics, Kolmogorov-Smirnov 2-sample test, example shown in figure 3.3B). Summary data show that capsaicin alone resulted in a large significant potentiation of mEPSC frequency (P=0.002, paired t-test, n=12, figure 3.3C). Capsaicin applied in the presence of chemerin resulted in a significant leftwards shift in inter-event interval distribution in 9/10 neurons, while there was no effect in 1/10 (all statistics, Kolmogorov-Smirnov 2-sample test, example shown in figure 3.3D). Overall, capsaicin applied in the presence of chemerin caused a significant increase in mEPSC frequency (P=0.008, paired t-test, n=10, figure 3.3F). When the effect of chemerin on the capsaicin potentiation was assessed, it was discovered that capsaicin, in the presence and absence of chemerin, significantly increased the frequency of mEPSCs (P=0.0001, 2-way repeated measures ANOVA), however this capsaicin potentiation was significantly reduced by chemerin (P=0.031, 2-way repeated measures ANOVA, Bonferroni post-test, figure 3.3G).

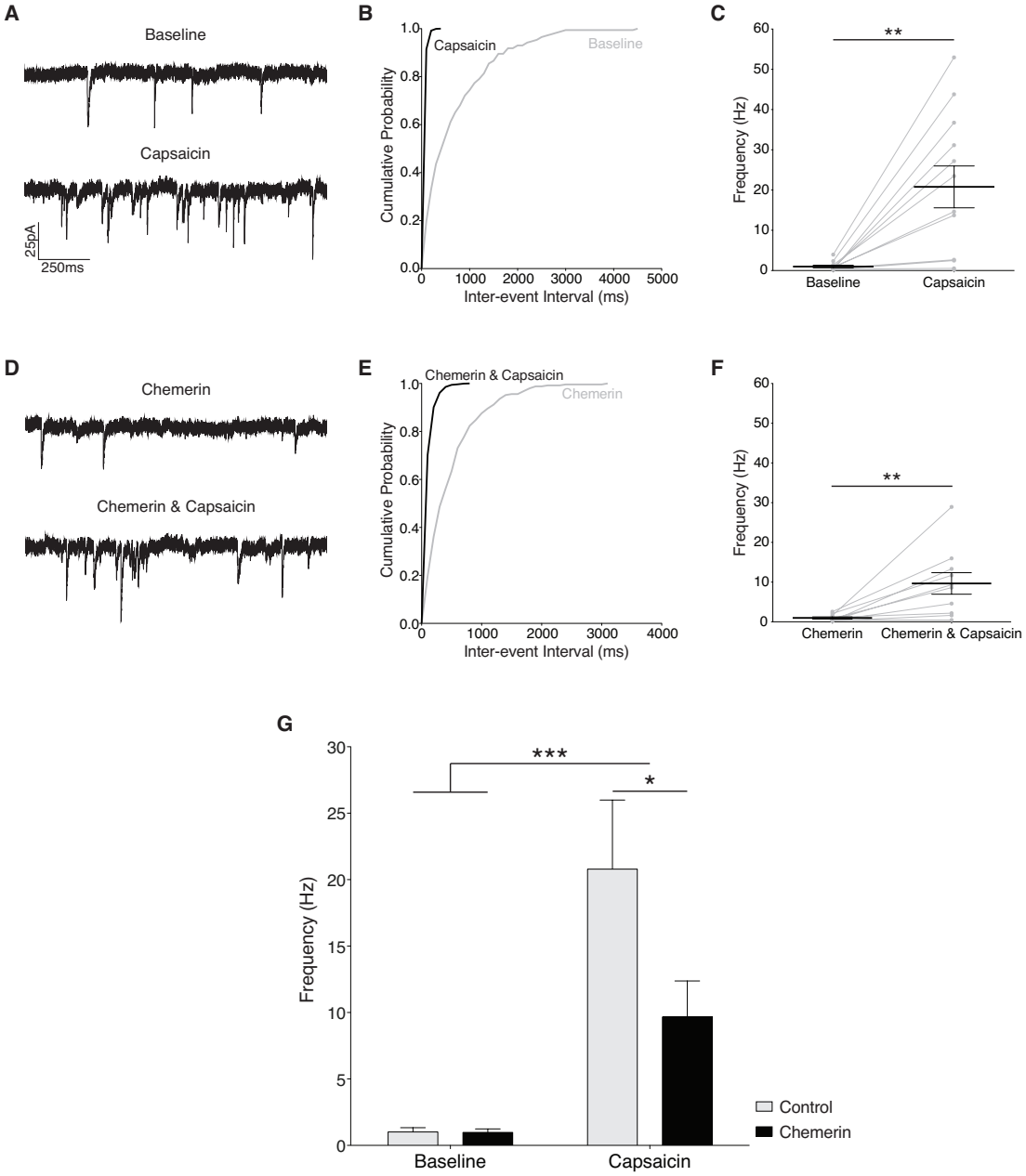

Figure 3.3: Chemerin significantly attenuates the capsaicin potentiation of mEPSC frequency in lamina I NK1R+ neurons. Representative mEPSC traces recorded before (top) and during (bottom) capsaicin application in the absence (***A***) and presence (***D***) of chemerin. Example cumulative probability plots from individual neurons show capsaicin applied in the absence (***B***) and presence (***E***) of chemerin resulted in a significant leftwards shift in the inter-event interval distribution (both $P<0.0001$, 2-sample Kolmogorov-Smirnov test). Summary data show that mEPSC frequency was significantly increased by capsaicin treatment in the absence (***C***, P=0.002, paired t-test, n=12) and presence of chemerin (***F***, P=0.008, paired t-test, n=10). ***G*** Capsaicin significantly increased mEPSC frequency in the presence and absence of chemerin (P=0.0001, 2-way repeated measures ANOVA), however this increase was significantly attenuated by chemerin (P=0.031, 2-way repeated measures ANOVA, Bonferroni post-tests). $**$ $P<0.01$, $***$ $P<0.001$. Scale bars in ***A*** apply to ***D***. Black lines in ***C*** & ***F*** denote mean $\pm$ SEM, grey points & lines show trajectories for individual neurons. Error bars in ***G*** = SEM.

Capsaicin alone caused a significant rightwards shift in mEPSC amplitude distribution in 10/12 neurons, while in 2/12 it was without effect (all statistics, Kolmogorov-Smirnov 2-sample test, example shown in figure 3.4A). Figure 3.4B demonstrates that capsaicin significantly increases mEPSC amplitude in lamina I NK1R+ neurons (P=0.0003, paired t-test, n=12). Capsaicin applied in the presence of chemerin resulted in a significant rightwards shift in mEPSC amplitude distribution in 5/10 neurons, while in 2/10 there was a significant leftwards shift and 3/10 no change (all statistics, Kolmogorov-Smirnov 2-sample test, example shown in figure 3.4C). Summary data show that capsaicin applied in the presence of chemerin did not significantly alter mEPSC amplitude (P=0.194, paired t-test, n=10, figure 3.4D). Figure 3.4E demonstrates that capsaicin significantly potentiated mEPSC amplitude in the presence and absence of chemerin (P=0.002, 2-way repeated measures ANOVA), while chemerin was without effect and there was no interaction between chemerin and capsaicin (P=0.168 & P=0.443, respectively, 2-way repeated measures ANOVA).

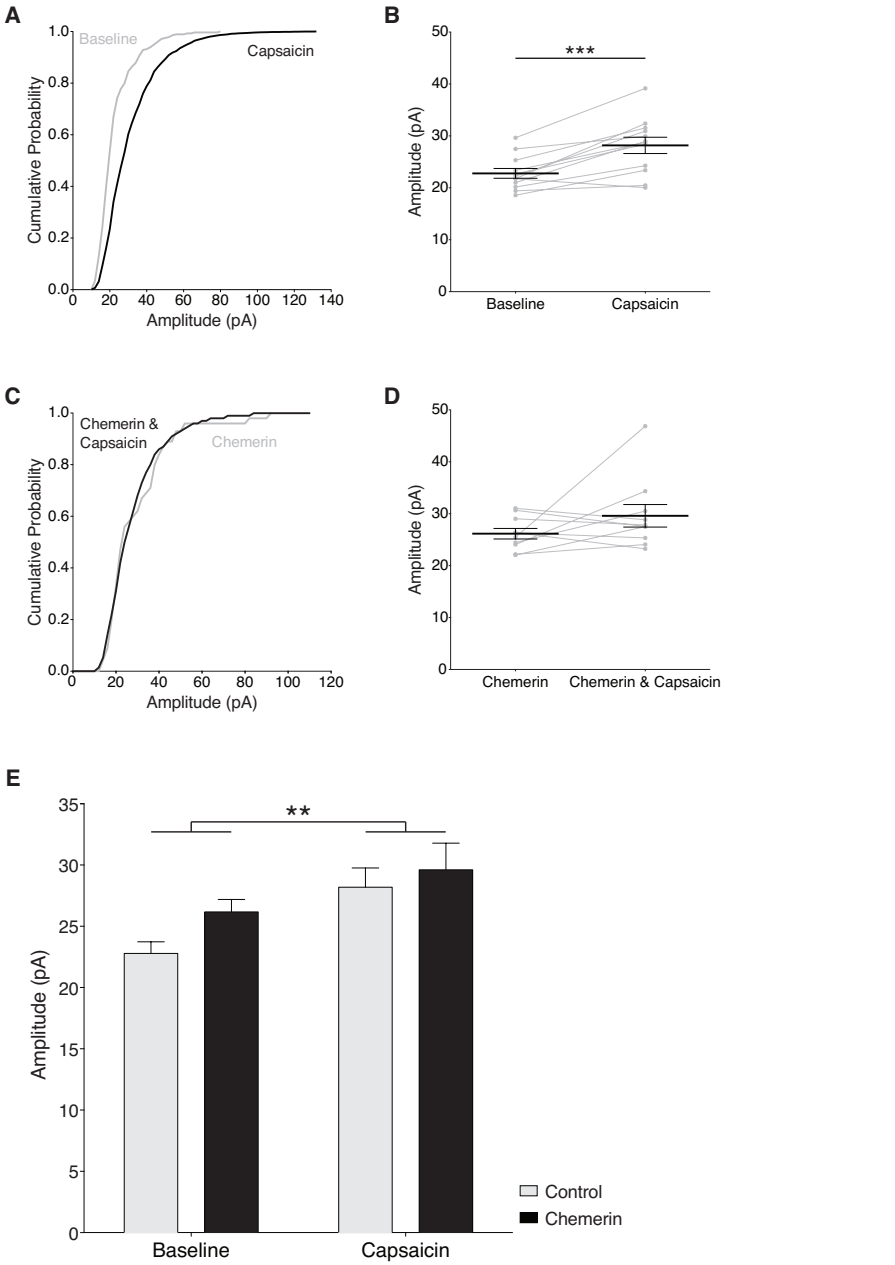

Figure 3.4: Chemerin does not alter the capsaicin potentiation of mEPSC amplitude. ***A*** Example cumulative probability plot demonstrates that in an individual neuron capsaicin caused a significant rightward shift in the amplitude distribution, indicating an increase in amplitude ($P<0.0001$, 2-sample Kolmogorov-Smirnov test). ***B*** Overall, capsaicin caused a significant increase in mEPSC amplitude ($P=0.0003$, paired t-test, $n=12$). ***C*** Cumulative probability plot example shows capsaicin applied in the presence of chemerin did not alter the mEPSC amplitude distribution in an individual neuron ($P=0.647$, 2-sample Kolmogorov-Smirnov test). ***D*** Capsaicin applied in the presence of chemerin did not significantly alter mEPSC amplitude ($P=0.194$, paired t-test, $n=10$). ***E*** Capsaicin in the presence and absence of chemerin significantly increased mEPSC amplitude ($P=0.002$, 2-way repeated measures ANOVA), chemerin did not significantly alter amplitude ($P=0.168$, 2-way repeated measures ANOVA) and there was no interaction between chemerin and capsaicin ($P=0.443$, 2-way repeated measures ANOVA). $**$ $P<0.01$ $***$ $P<0.001$. Black lines in ***B*** & ***D*** denote mean $\pm$ SEM, grey points & lines show trajectories for individual cells. Error bars in ***E*** = SEM.

3.4.3 Chemerin attenuates monosynaptic C-fibre input to a subset of lamina I neurokinin 1 receptor expressing neurons in inflammatory pain

To evaluate the ability of chemerin to modulate monosynaptic C-fibre input to lamina I NK1R+ neurons, in tissue isolated from control or CFA treated rats, C-fibre eEPSCs were recorded before and during the application of chemerin or vehicle. In neurons from control tissue, there was a significant change in eEPSC amplitude over time (P=0.013, 2-way repeated measures ANOVA, figure 3.5A), but chemerin was without effect (P=0.502, 2-way repeated measures ANOVA) and there was no interaction between time and chemerin (P=0.251, 2-way repeated measures ANOVA). Similarly, in neurons from tissue isolated from CFA treated rats, eEPSC peak amplitude significantly declined over time (P<0.0001, 2-way repeated measures ANOVA, 3.5B), but there was no difference between chemerin and vehicle groups (P=0.152, 2-way repeated measures ANOVA). There was however a significant interaction between time and chemerin (P=0.0006, 2-way repeated measures ANOVA) indicating that the significant decline in amplitude that occurred was influenced by chemerin, which could be consistent with an effect in a subset of neurons. Figure 3.5C reveals that the change in eEPSC amplitude between baseline and chemerin was not significantly altered by chemerin (P=0.156, 2-way repeated measures ANOVA), CFA inflammation did not alter the response and there was no interaction between chemerin and CFA (P=0.931 & P=0.434, respectively, 2-way repeated measures ANOVA).

It was hypothesised that chemerin would attenuate primary afferent input to a subset of lamina I NK1R+ neurons, as ChemR23 is only expressed on a subset of TRPV1+ and a subset of SP containing afferents (Xu et al. 2010), with these neurons being known to be targeted by TRPV1+ (Hwang et al. 2003, Labrakakis & MacDermott 2003, Tong & MacDermott 2006) and SP containing (Hwang et al. 2003, Todd et al. 2002) primary afferents. To identify this subset, linear regression analysis was performed on vehicle data to calculate 95% prediction bands. Neurons were classified as responders if the peak eEPSC amplitude fell below the lower 95% prediction band for at least the final 5 minutes of chemerin treatment. Neurons where this did not occur were classified as non-responders. Figure 3.6A shows an example of a non-responder recorded in CFA tissue, where the peak amplitude of the C-fibre eEPSC largely remains within the 95% prediction bands. Figure 3.6B shows an example of a chemerin responder recorded in CFA treated tissue, where the peak amplitude of the response clearly falls below the lower 95% prediction band during the final 5 minutes of chemerin application. Using this criteria it was discovered that

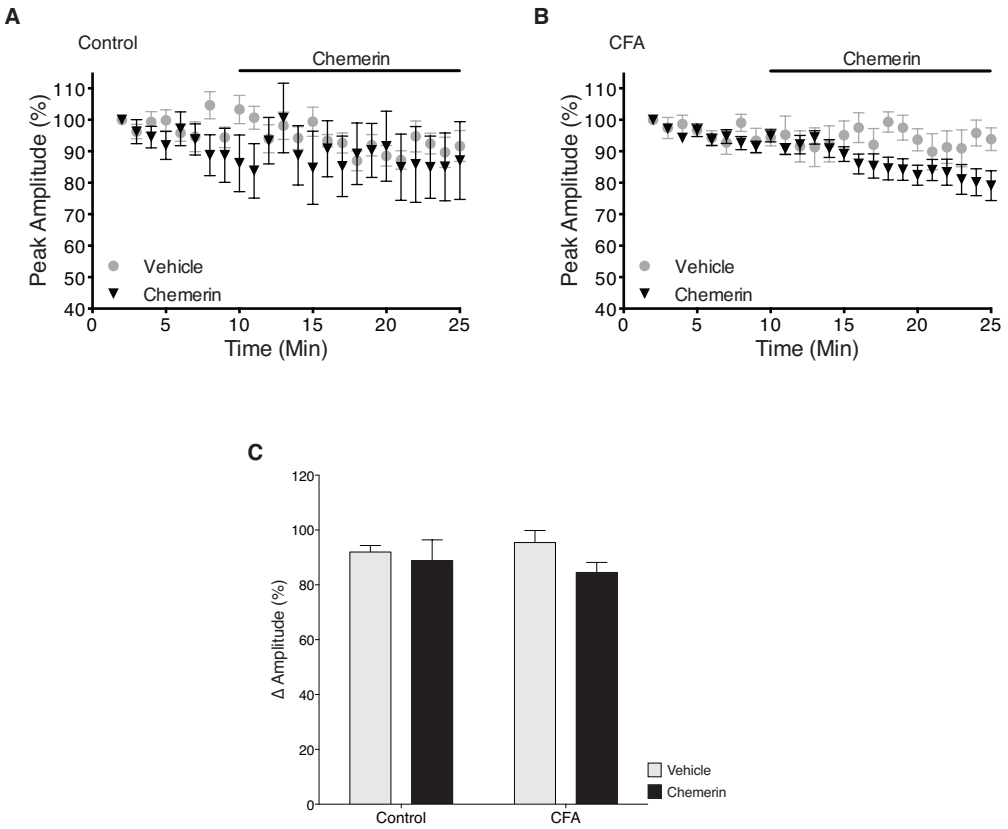

Figure 3.5: Chemerin does not alter the peak amplitude of monosynaptic C-fibre eEPSCs in the overall lamina I NK1R+ neuron population. ***A*** In control tissue, chemerin did not alter the C-fibre eEPSC peak amplitude (P=0.502, chemerin n=7, vehicle n=8). ***B*** In CFA inflammation, no differences in peak eEPSC amplitude were detected between chemerin and vehicle groups (P=0.152, chemerin n=16, vehicle n=7), while amplitude significantly declined over time (P<0.0001). There was a significant interaction between time and chemerin (P=0.0006), indicating that the reduction in amplitude was dependent on chemerin. ***C*** Both chemerin (P=0.156) and CFA inflammation (P=0.931) was without effect on the amplitude change and there was no interaction between these factors (P=0.434). ***A*** & ***B***: 2-way repeated measures ANOVA, ***C***: 2-way ANOVA. Error bars = SEM.

in C-fibre eEPSCs recorded in tissue isolated from CFA treated rats, 7/16 neurons were classified as responders, while 9/16 were non-responders. In recordings from control tissue, 1/7 neurons were classified as responder, while 6/7 were non-responders.

To assess the validity of this classification criteria and ensure that responders and non-responders were distinct populations, frequency histograms of the mean amplitude during the final 5 minutes of chemerin / vehicle application recorded in CFA tissue were produced. As shown in figure 3.6C, there was a significant difference between the amplitude distribution of vehicle, chemerin responder and chemerin non-responder groups (P<0.0001, 1-way ANOVA). The distribution of chemerin responders was significantly different from chemerin non-responders and

vehicle (both $P<0.0001$, 1-way ANOVA, Bonferroni post-tests), while there were no differences between the vehicle and non-responder groups ($P>0.999$, 1-way ANOVA, Bonferroni post-tests).

To assess whether the initial amplitude of C-fibre eEPSCs was predictive of the degree to which chemerin reduced the amplitude of C-fibre responses, correlation between the initial amplitude, recorded in minute 2 and the amplitude change recorded in the final 5 minutes was assessed in chemerin treated neurons from CFA tissue only. Figure 3.6E demonstrates that there was no significant correlation between initial amplitude and amplitude change ($P=0.886$, Pearson's r test). Therefore, chemerin effects were independent of the initial peak amplitude of C-fibre eEPSCs.

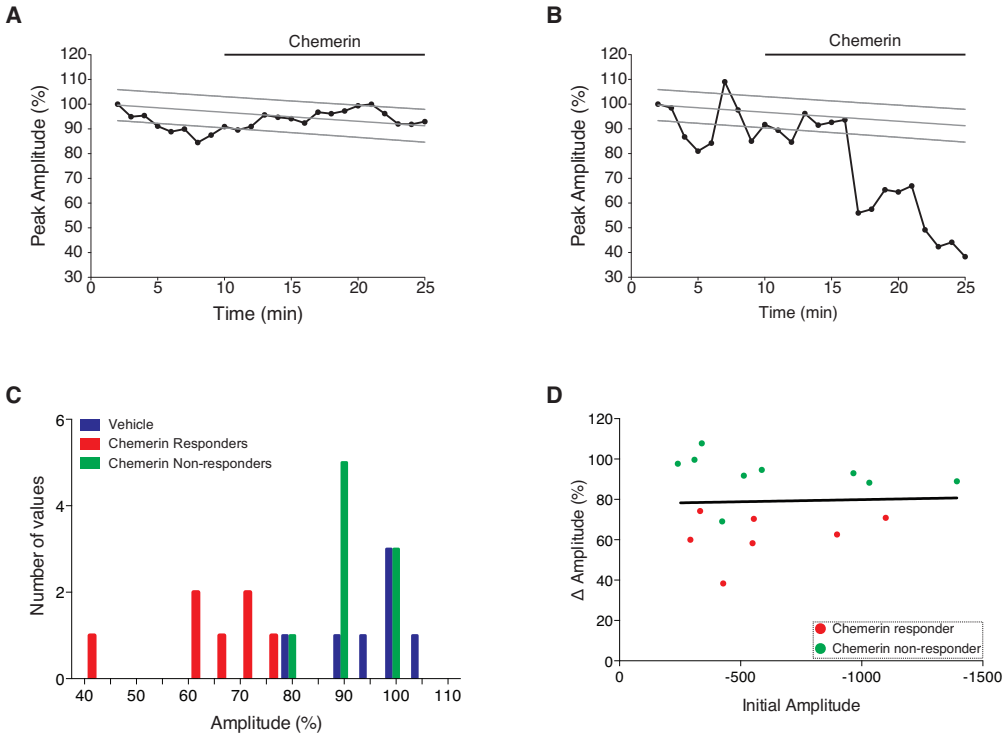

Figure 3.6: Dissecting a subpopulation of chemerin responders. ***A*** Example of a chemerin non-responder demonstrates the peak amplitude of the C-fibre eEPSCs largely fall within the 95% prediction bands (designated by the upper and lower grey lines). ***B*** An example of a chemerin responder, where the peak amplitude of the C-fibre eEPSCs clearly falls below the lower 95% prediction band for at least the final 5 minutes of chemerin treatment. ***C*** Frequency histogram of the mean peak eEPSC amplitude during the final 5 minutes, shows a significant difference in the frequency distribution of vehicle, chemerin responder and chemerin non-responder groups ($P<0.0001$, 1-way ANOVA) and post-tests revealed that chemerin responders were significantly different from chemerin non-responders and vehicle (both $P<0.0001$, 1-way ANOVA, Bonferroni post-tests), while there was no significant difference between vehicle and non-responders ($P>0.999$, 1-way ANOVA, Bonferroni post-tests). ***D*** To assess whether the initial amplitude (minute 2) was predictive of the amplitude change that resulted from chemerin (measured in the final 5 minutes) Pearson's r test for correlation was performed on chemerin data from CFA tissue only (responders and non-responders were combined) and revealed no significant correlation, $P=0.886$. Vehicle n=7, chemerin responders n=7, chemerin non-responders n=9. Examples in ***A*** & ***B*** recorded in CFA tissue.

Representative traces of C-fibre eEPSCs recorded pre-chemerin and during chemerin, in neurons classified as non-responders and responders are shown in figures 3.7B & D, respectively. In the subgroup of neurons classified as non-responders, chemerin had no significant effect on peak amplitude when compared to vehicle ($P=0.802$, 2-way repeated measures ANOVA, figure 3.7A), there was no change in amplitude over time ($P=0.172$, 2-way repeated measures ANOVA) and no interaction between these factors ($P=0.705$, 2-way repeated measures ANOVA). In chemerin responders, peak amplitude significantly declined over time ($P<0.0001$, 2-way repeated measures ANOVA, figure 3.7C) and chemerin significantly attenuated C-fibre eEPSC peak

amplitude (P=0.001, 2-way repeated measures ANOVA), with this effect being influenced by time (P<0.0001, 2-way repeated measures ANOVA). Post-hoc testing revealed chemerin significantly reduced eEPSC amplitude in this subset of neurons from 16 to 25 minutes (P<0.05 to <0.001, 2-way ANOVA, Bonferroni post-tests). Analysis of the amplitude change in vehicle, chemerin responders and chemerin non-responders revealed there to be a significant difference between these groups (P<0.0001, 2-way repeated measures ANOVA). Post-hoc tests found that chemerin responders showed a significantly greater change in amplitude than vehicle and non-responders (P=0.0003 & P=0.0002, respectively, 2-way ANOVA, Bonferroni post-tests, figure 3.7E), while there was no significant difference between vehicle and chemerin non-responders (P=0.996, 2-way ANOVA, Bonferroni post-tests).

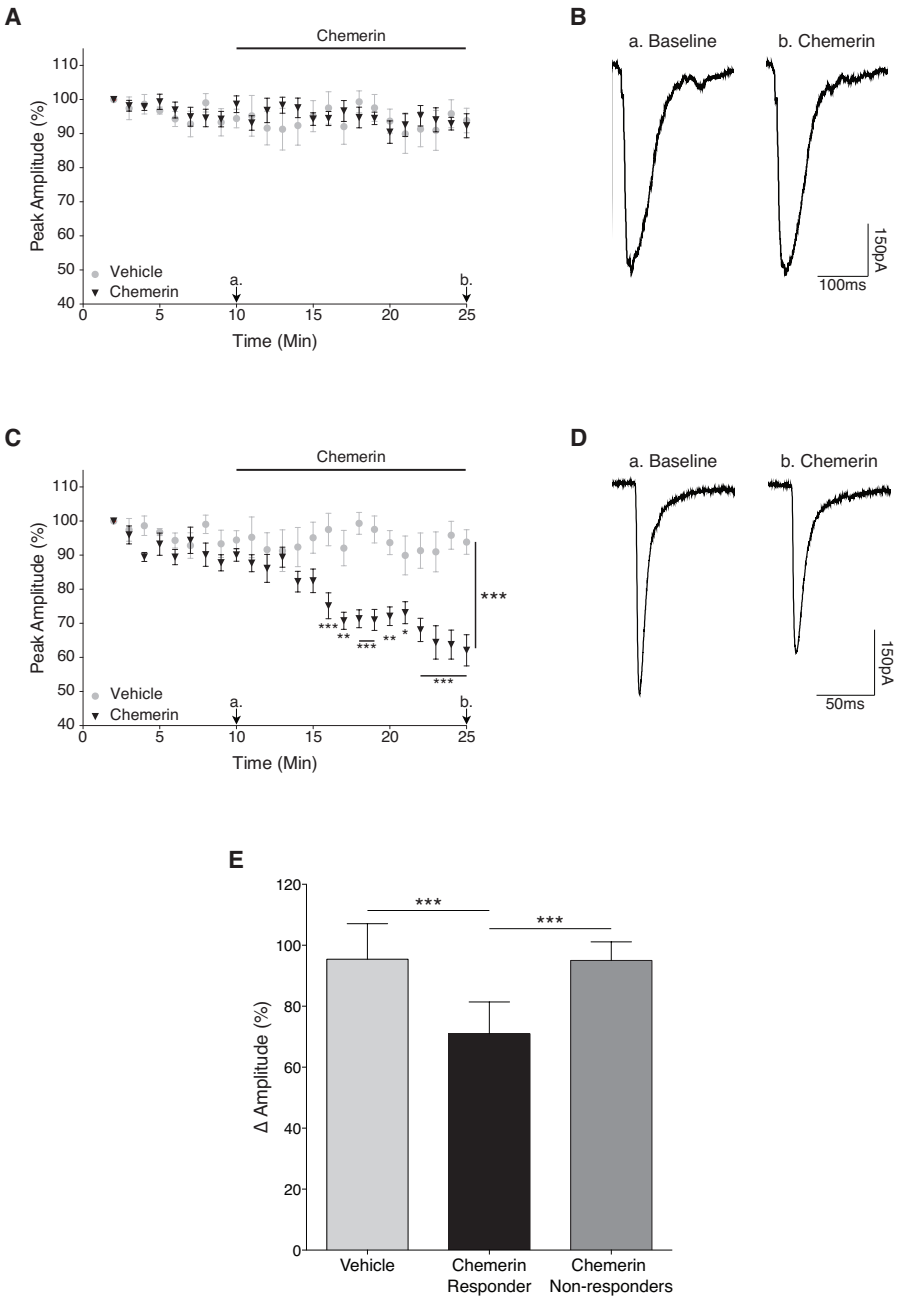

Figure 3.7: Chemerin attenuates C-fibre eEPSC peak amplitude in a subset of lamina I NK1R+ neurons in CFA inflammation. ***A*** Chemerin did not significantly alter C-fibre eEPSC peak amplitude in chemerin non-responders (P=0.802, 2-way repeated measures ANOVA), representative traces shown in ***B***. ***C*** In the subgroup of chemerin responders, chemerin significantly attenuated C-fibre eEPSC amplitude (P=0.001, 2-way repeated measures ANOVA), example traces shown in ***D***. ***E*** The amplitude change was significantly different between groups ($P<0.001$, 1-way ANOVA) and Bonferroni post-tests revealed that the amplitude change was significantly greater in chemerin responders compared to vehicle and chemerin non-responders (P=0.0003 & P=0.0002, respectively), there was no difference between vehicle and chemerin non-responders (P=0.996). $*P<0.05$, $**P<0.01$, $***P<0.001$. Example traces = average of 3 sweeps, baseline and chemerin traces recorded at points 'a.' and 'b.', respectively, denoted by arrows on relevant graphs. Vehicle n=7, chemerin responders n=7, chemerin non-responders n=9. Error bars = SEM.

To determine whether the chemerin attenuation of monosynaptic C-fibre input to a subset of lamina I NK1R+ neurons was pre- or post-synaptically mediated, C-fibre evoked paired-pulse recordings were performed. Figure 3.8A shows example traces of paired-pulse recordings before ('pre-chemerin') and during chemerin ('chemerin') application. These traces demonstrate that in this neuron, paired-pulse stimulation resulted in paired-pulse depression (PPD) of C-fibre eEPSCs and that chemerin increased the PPR / decreased PPD. Overall, there were no significant effects of chemerin or whether a neuron was classified as a responder or not (P=0.069 & P=0.699, respectively, 2-way repeated measures ANOVA), however there was a significant interaction between these factors (P=0.044, 2-way repeated measures ANOVA, figure 3.8B). Post-tests revealed that in the subgroup of neurons classified as chemerin responders, chemerin significantly increased the PPR / decreased PPD (P=0.031, 2-way repeated measures ANOVA, Bonferroni post-tests). This indicates that chemerin presynaptically attenuates C-fibre input to a subset of lamina I NK1R+ neurons in CFA inflammation.

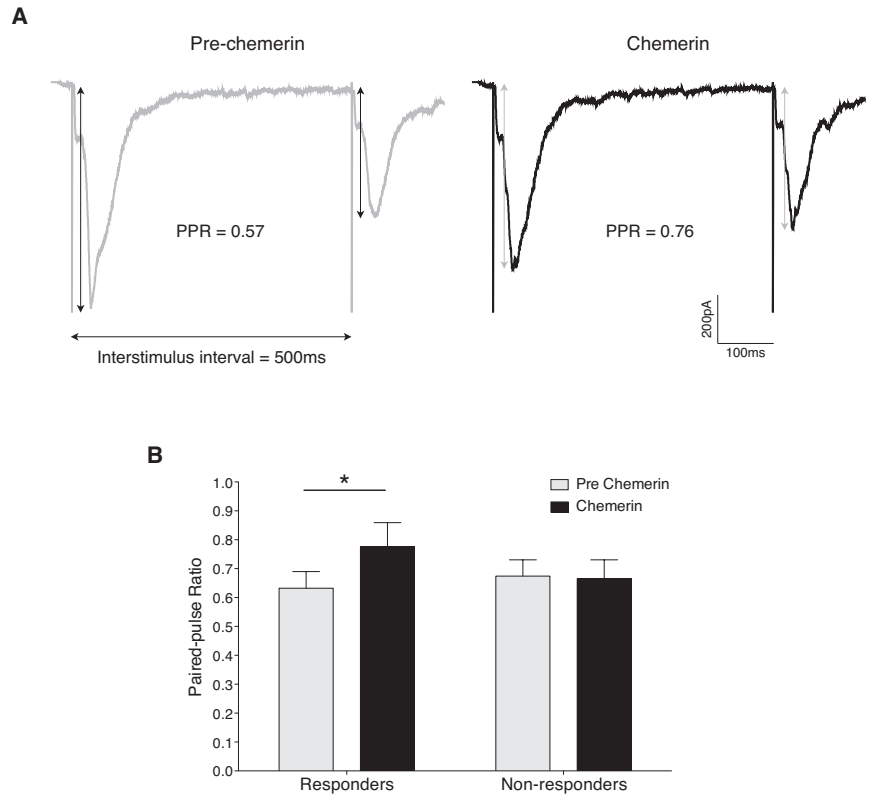

Figure 3.8: Chemerin presynaptically attenuates C-fibre eEPSC peak amplitude to a subset of lamina I NK1R+ neurons. ***A*** Representative traces of C-fibre paired-pulse stimulation in a chemerin responder recorded before (left, grey trace) and during (right, black trace) chemerin application (average of 6 sweeps shown), where chemerin has increased the paired-pulse ratio / decreased paired-pulse depression. ***B*** Paired-pulse ratio was unaltered by chemerin or whether neurons were classified as responders or not (P=0.069 & P=0.699, respectively), however there was a significant interaction between these factors (P=0.044). Post-tests revealed that in neurons classified as responders chemerin significantly increased the paired-pulse ratio / decreased paired-pulse depression (P=0.031). 2-way repeated measures ANOVA followed by Bonferroni post-tests, $*P<0.05$, responders n=5, non-responders n=6. Error bars = SEM.

3.4.4 Chemerin does not alter monosynaptic Aδ-fibre input to lamina I neurokinin 1 receptor expressing neurons

To assess whether ChemR23 activation modulated monosynaptic Aδ-fibre input to lamina I NK1R+ neurons under control or inflamed conditions, Aδ-fibre eEPSCs were recorded in the presence of chemerin or vehicle. In control tissue, there was a significant change in peak eEPSC amplitude over time (P=0.040, 2-way repeat measures ANOVA, figure 3.9A), which was independent of chemerin (P=0.309, 2-way repeat measures ANOVA). Overall there was no difference between chemerin and vehicle groups (P=0.194, 2-way repeat measures ANOVA). In tissue isolated from CFA treated rats, chemerin was without effect on eEPSC peak amplitude (P=0.550, 2-way repeat measures ANOVA, figure 3.9B), there was no significant

effect of time and no interaction between these factors (P=0.891 & P=0.541, respectively, 2-way repeat measures ANOVA). The change in Aδ-fibre eEPSC amplitude was not altered by CFA inflammation or chemerin and there was no significant interaction between these factors (P=0.357, P=0.528 & P=0.225, respectively, 2-way repeat measures ANOVA, figure 3.9C). When the criteria used to categorise neurons as chemerin responders or non-responders was applied (as detailed in section 3.4.3), 1/6 CFA and 2/6 control neurons were classified as responders.

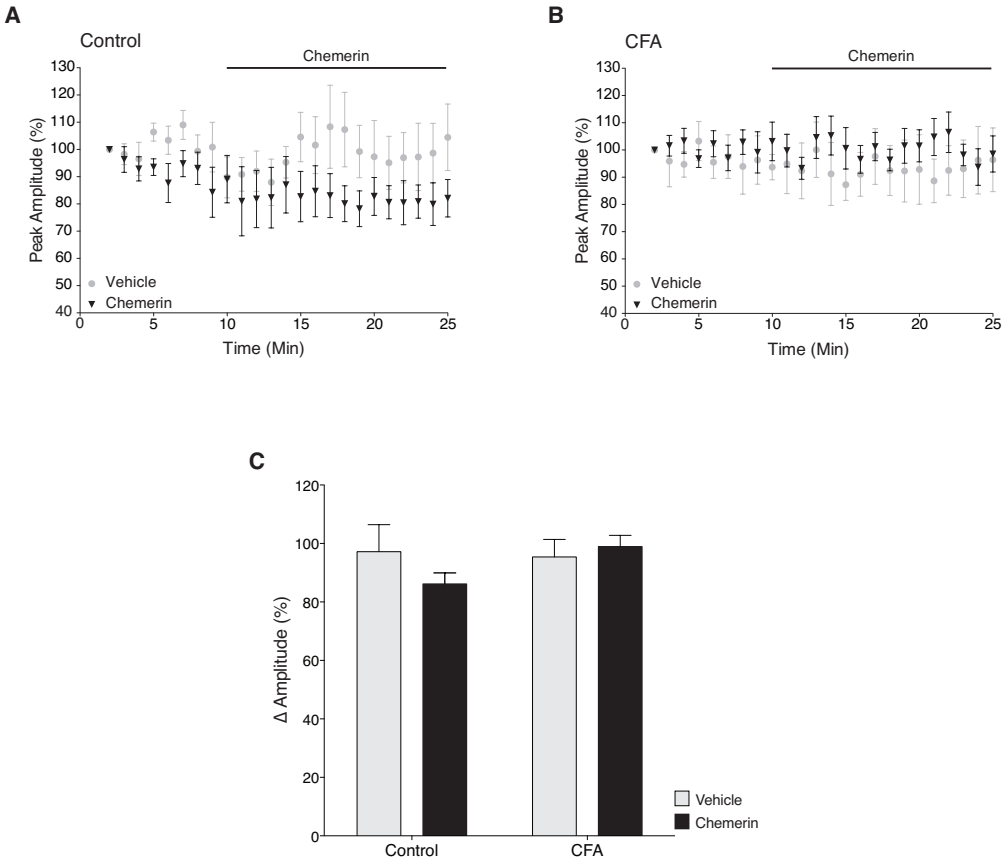

Figure 3.9: Chemerin does not alter monosynaptic Aδ-fibre input to lamina I NK1R+ neurons. ***A*** In control tissue there was no significant difference in eEPSC peak amplitude between neurons which had been treated with chemerin and those treated with vehicle (P=0.194, chemerin n=6, vehicle n=5). ***B*** Chemerin did not alter the peak amplitude of the Aδ-fibre eEPSC response in tissue isolated from CFA treated rats (P=0.550, chemerin n=6, vehicle n=6). ***C*** The amplitude change was not significantly unaltered by chemerin (P=0.528), CFA inflammation (P=0.357) and there was no interaction between chemerin and CFA inflammation (P=0.225). All statistics: 2-way repeated measures ANOVA. Error bars = SEM.

3.4.5 Summary

A summary of the findings of the mEPSC experiments reported in this chapter are presented in table 3.3. In those experiments where mEPSCs were recorded, chemerin

	Frequency	Amplitude	Capsaicin *vs.* Chemerin & Capsaicin	
Chemerin	no change	no change	Frequency	Amplitude
Capsaicin	increase	increase	reduce	no change
Chemerin & Capsaicin	increase	no change		

Table 3.3: Summary table of results from capsaicin potentiation mEPSC experiments.

Fibre	Group	Chemerin response
Aδ	control	no effect
	CFA	no effect
C	control	no effect
	CFA responder	reduction
	CFA non-responder	no effect

Table 3.4: Summary table of results from chemerin eEPSC experiments.

alone was found to be without effect on mEPSC frequency or amplitude in lamina I NK1R+ neurons. When the capsaicin potentiation of mEPSCs and the influence of chemerin upon this potentiation was assessed, it was discovered that while capsaicin applied in the presence and absence of chemerin resulted in a significant increase in mEPSC frequency, when the response in these two groups was compared it was found that chemerin significantly attenuated the capsaicin potentiation. The amplitude of mEPSCs was significantly increased following application of capsaicin alone, but was unaltered when capsaicin was applied in the presence of chemerin. When these effects were compared between groups it was found that chemerin did not significantly attenuate the capsaicin potentiation of mEPSC amplitude.

The findings of eEPSC experiments in which the effects of chemerin on monosynaptic Aδ- and C-fibre input to lamina I NK1R+ neurons, in control and CFA inflammation was investigated, are summarised in table 3.4. In both control and CFA, chemerin did not alter the peak amplitude of monosynaptic Aδ-fibre eEPSCs. Likewise, chemerin was without effect upon the monosynaptic C-fibre input to these neurons in control conditions. In CFA inflammation, chemerin was found to significantly attenuate monosynaptic C-fibre input to a subset of lamina I NK1R+ neurons that were classified as ‘responders’ and which accounted for 44% of all neurons recorded from. Furthermore, the PPR in those neurons classified as responders was significantly increased following the application of chemerin, indicating the chemerin reduction of eEPSC amplitude resulted from a presynaptic inhibition of monosynaptic C-fibre input. Chemerin did not alter monosynaptic C-fibre eEPSCs or PPR in those neurons classified as ‘non-responders’.

3.5 Discussion

3.5.1 Chemerin is without effect in non-potentiated conditions

It has previously been established that activation of ChemR23 does not alter acute pain sensitivity and has no effect on the frequency or amplitude of sEPSCs in unidentified lamina II neurons in non-potentiated conditions (Xu et al. 2010). The data presented here expand on these previous findings and novelly show that the ChemR23 agonist, chemerin, does not alter mEPSC frequency or amplitude in lamina I NK1R+ neurons in basal conditions (figure 3.2). In addition, chemerin does not alter monosynaptic C- or monosynaptic Aδ-fibre inputs to these neurons in tissue isolated from control rats (figures 3.5 & 3.9). It is proposed that activation of ChemR23 reduces inflammatory pain hypersensitivity by a mechanism that involves the normalisation of potentiated spinal cord responses as opposed to a general reduction in sensory transmission (Ji et al. 2011, Xu et al. 2010). The findings presented here can be considered to support this.

3.5.2 Chemerin attenuates the capsaicin potentiation of primary afferent input to lamina I neurokinin 1 receptor expressing neurons

It has previously been established that chemerin prevents the capsaicin mediated potentiation of sEPSC frequency in random lamina II neurons (Xu et al. 2010). Because Xu et al. (2010) recorded sEPSCs in unidentified lamina II neurons it is not clear where in the spinal cord network or upon which neuronal subtypes that the effects of ChemR23 agonists are mediated. The experiments in this thesis improves on these previous findings in that recordings were made from pre-identified lamina I NK1R+ neurons and that mEPSCs were recorded, in which network activity is blocked by application of TTX, which enables the effects upon direct excitatory inputs to be observed. The results presented here demonstrate that application of chemerin significantly attenuates the capsaicin potentiation of mEPSC frequency in lamina I NK1R+ neurons (figure 3.3).

It was previously reported that activation of ChemR23 with chemerin (100ng/ml) or RvE1 (1ng/ml) blocks the capsaicin potentiation of primary afferent input (Xu et al. 2010), however in the data presented here the potentiation is attenuated rather than abolished. This difference could be accounted for by the fact that the capsaicin dose

used here (1μM) was 10 times greater than that used by Xu et al. (2010) and this higher concentration may override the effect of chemerin. This increased dose undoubtably resulted in a greater potentiation, with a ~44-fold increase reported here versus a 2-fold increase (Xu et al. 2010). Using a lower capsaicin concentration of 100nM was investigated, however this did not reliably potentiate primary afferent input to lamina I NK1R+ neurons, as only 1 out of 5 neurons showed a significant change in the inter-event interval distribution and overall 100nM capsaicin did not significantly alter mEPSC frequency (P=0.625, Wilcoxon, data not shown).

Although it is not explicitly stated by Xu et al. (2010), the data presentation would suggest that 100nM capsaicin resulted in potentiation in all recorded neurons. However, as Xu et al. (2010) recorded sEPSCs, where network activity is not blocked, as opposed to mEPSCs in this study, so capsaicin could have had more of a widespread effect. Other groups have demonstrated that 100nM capsaicin can potentiate mEPSC frequency in unidentified lamina I/II neurons (Baccei et al. 2003, Spicarova & Palecek 2009), so it is questionable as to whether the effects of 100nM capsaicin are only detectable in conditions were network activity is not blocked. Another key difference between this study and that of Xu et al. (2010) is that the recordings presented here were made from a defined neuronal subset, as opposed to unidentified neurons. Interestingly, potentiation of mEPSC frequency in lamina I NK1R+ neurons has only been demonstrated at concentrations of 1μM (Labrakakis & MacDermott 2003).

In this study mouse chemerin was used to activate ChemR23 receptors. As these experiments were performed in rat tissue, ideally rat chemerin would have been used, however rat chemerin is not commercially available. While there is a high level of homology between rat and mouse chemerin and evidence for cross-species activity (Busmann et al. 2004) it is possible that the effects of chemerin were not optimal due to species differences. The fact that chemerin did not abolish the capsaicin potentiation of mEPSC frequency could be a reflection of these species differences. However, chemerin significantly reduced this capsaicin potentiation and attenuated C-fibre eEPSCs in a subset of lamina I NK1R+ neurons in subsequent experiments. Therefore, these results clearly show mouse chemerin is active in rat tissue.

It is possible that not every neuron recorded from received ChemR23 expressing (ChemR23+) primary afferent input, which could account for the result that chemerin attenuated rather than abolished the capsaicin potentiation of mEPSC frequency. Most lamina I NK1R+ neurons in this study received input from TRPV1+ afferents, as evidenced by the significant leftward shifts in inter-event interval distributions in

83% of neurons, which confirms previous findings (Labrakakis & MacDermott 2003). While TRPV1 is co-expressed with ChemR23, expression of the former does not assure expression of the latter, as only 61% of TRPV1+ neurons co-express ChemR23 (Xu et al. 2010). The premise that not all lamina I NK1R+ neurons receive ChemR23+ input is supported by additional results reported here, that show chemerin only attenuates monosynaptic C-fibre input to a subset (44%) of lamina I NK1R+ neurons in inflammation (figure 3.7). One way in which a similar subset of chemerin responsive neurons could have been identified in these capsaicin potentiation studies would have been to apply both capsaicin alone and chemerin & capsaicin in individual neurons. However, this approach was not possible due to the fact that mEPSC frequency did not reliably return to baseline after capsaicin application, even following long wash periods (data not shown), as has been described elsewhere (Baccei et al. 2003). Another concern is that capsaicin treatment is known to desensitise nociceptors (O'Neill et al. 2012), which may involve TRPV1 inactivation and/or downregulation (Sanz-Salvador et al. 2012, Szallasi & Blumberg 1999). Furthermore, repeated capsaicin application results in a progressive reduction in the capsaicin mediated increase in mEPSC frequency (Baccei et al. 2003, Sikand & Premkumar 2007). Therefore, this approach would have required careful differentiation of the chemerin effect from the general run-down in the capsaicin response.

This study has for the first time demonstrated that capsaicin alone significantly increases mEPSC amplitude in lamina I NK1R+ neurons, as has been reported in other spinal cord neurons (Baccei et al. 2003, Wrigley et al. 2009). Altered mEPSC amplitude typically indicates a change in the postsynaptic response to neurotransmitters (Engelman & MacDermott 2004). However, as the capsaicin receptor, TRPV1, is likely to be expressed only on primary afferent terminals in the spinal cord (Caterina et al. 1997, Cavanaugh et al. 2011, Tominaga et al. 1998) this effect is most likely to be presynaptic, although this is disputed by Kim et al. (2012) (discussed below). Other groups have reported this dual increase in mEPSC frequency and amplitude in spinal cord neurons as a result of capsaicin applied at $\geq 1\,\mu M$ (Baccei et al. 2003, Wrigley et al. 2009). It has been argued that this apparent increase in amplitude is as a result of the summation of high frequency events (Baccei et al. 2003, Wrigley et al. 2009). The fact that a significant increase in mEPSC amplitude was seen following the application of capsaicin alone, but not capsaicin & chemerin, where the overall mEPSC frequency was lower, could be considered to support this postulate. To study the effects of capsaicin on mEPSC amplitude in isolation from the summation of high frequency events other groups have used a

lower capsaicin concentration of 100nM, which significantly increases mEPSC frequency, but not amplitude (Baccei et al. 2003). However, when capsaicin was applied at a dose of 100nM it did not reliably evoke an increase in mEPSC frequency, as discussed previously (data not shown).

TRPV1 channels are considered to be only expressed on primary afferent terminals in the dorsal horn of the spinal cord (Caterina et al. 1997, Cavanaugh et al. 2011, Tominaga et al. 1998). The strongest evidence for this comes from Cavanaugh et al. (2011) who demonstrated, using a transgenic TRPV1 reporter mouse, that TRPV1 expression in the dorsal horn is unequivocally restricted to the central terminals of primary afferents. Therefore, applying the TRPV1 agonist, capsaicin, can be considered to presynaptically potentiate primary afferent input to dorsal horn neurons (Baccei et al. 2003, Wrigley et al. 2009). Recent evidence has suggested that TRPV1 may also be expressed in a subpopulation of GABAergic interneurons in lamina II (Kim et al. 2012), although this finding conflicts with previous studies (Caterina et al. 1997, Cavanaugh et al. 2011, Tominaga et al. 1998) and has yet to be replicated. The expression of TRPV1 channels on this interneuron population are proposed to be involved in spinal cord disinhibition by mediating long-term depression (LTD) of these inhibitory interneurons (Kim et al. 2012). However, in the experiments reported here, TRPV1 channels if expressed on this subpopulation of inhibitory interneuron would have had a limited effect on the results of capsaicin application. TTX was applied to block action-potential dependent events and inhibition was blocked by holding neurons at E_{anion} and bath applying bicuculline and strychnine. As such, any potential TRPV1 mediated changes in inhibitory tone within the wider spinal cord network or directly upon inhibitory inputs to lamina I NK1R+ neurons would not affect the mEPSCs recorded here.

In this study only a single dose of chemerin (100ng/ml) was used, in both mEPSC and eEPSC experiments. Xu et al. (2010) demonstrated that *i.t.* administration of chemerin at doses ranging from 0.45ng to 100ng resulted in a dose-dependent inhibition of the second phase of the formalin test. Similarly, *i.t.* injection of RvE1 at various concentrations resulted in a dose-dependent attenuation of heat hyperalgesia and mechanical allodynia resulting from CFA injection. However, in their electrophysiological studies, only a single concentration of chemerin (100ng/ml) and RvE1 (1ng/ml) was used, therefore as 100ng/ml of chemerin was previously found to effectively attenuate potentiated spinal cord responses, it was decided that this was an appropriate dose to use. While it would have been interesting to have investigated using different doses of chemerin, time limitations prevented this. Had higher doses

been investigated it may have been possible to identify a concentration that abolished the capsaicin potentiation of mEPSCs, in the manner that Xu et al. (2010) demonstrated 100ng/ml could prevent the capsaicin (100nM) potentiation of sEPSC frequency. Likewise, it would have been of interest in the eEPSC studies to determine whether increased chemerin concentrations result in a greater inhibition of monosynaptic C-fibre input to lamina I NK1R+ neurons and/or a greater proportion of neurons being classified as chemerin responders.

As with different doses of chemerin, it would have been interesting to have studied the effect of applying chemerin for varying lengths of time, particularly as the chemerin attenuation of monosynaptic C-fibre eEPSCs in CFA inflammation, as seen in figure 3.7C, does not appear to have peaked. Xu et al. (2010) fail to state the duration of chemerin / RvE1 application used in each of their electrophysiological experiments. However, from the figure showing example traces recorded during the PTX inhibition of the RvE1 attenuation of capsaicin potentiation it was possible to determine that RvE1 was applied for 8 minutes, with capsaicin applied for 4 minutes of that time, therefore, it seems reasonable to assume that these durations were used across all their experiments. In the mEPSC and eEPSC studies presented here, chemerin was applied for a total of 15 minutes, with, in the case of the mEPSC experiments, capsaicin applied for 5 minutes of that time. While applying chemerin for this duration did significantly attenuate the capsaicin potentiation of mEPSC frequency and the amplitude of monosynaptic C-fibre eEPSCs, in a subset of neurons, future studies could investigate whether altering the duration of application impacts upon the efficacy of chemerin.

3.5.3 Chemerin presynaptically attenuates monosynaptic C-fibre input to a subset of lamina I neurokinin 1 receptor expressing neurons in inflammation

This study presents new evidence that ChemR23 is likely functionally expressed on a subset of monosynaptic C-fibre input to lamina I NK1R+ neurons and that chemerin, presumably by acting upon these receptors can attenuate this monosynaptic C-fibre input in inflammatory pain.

While the data presented here strongly suggests that there is functional expression of ChemR23 on a subset of C-fibres, this cannot be considered conclusive. To confirm that these chemerin effects are mediated via ChemR23, ideally it would have been demonstrated that these effects could be blocked by a ChemR23 antagonist, however

no such ligand is commercially available. PTX, an inhibitor of $G_{\alpha}i$ coupled GPCRs, the receptor family to which ChemR23 belongs, has been used by others to inhibit the RvE1 attenuation of capsaicin potentiated input to lamina II neurons (Xu et al. 2010). While this approach could have been used to provide additional confirmation that the actions of chemerin were mediated by ChemR23, PTX inhibition of the chemerin response would only indicate that chemerin acted via a $G_{\alpha}i$ coupled GPCR and not ChemR23 specifically.

Chemerin has also been shown to bind to chemokine (C-C motif) receptor-like 2 (CCRL2), but the current evidence suggests that this receptor is not involved in cell signalling and may play a functional role in presenting chemerin to ChemR23 (Bondue et al. 2011, Yoshimura & Oppenheim 2010, Zabel et al. 2008). It is therefore possible that altered CCRL2 expression in inflammation could play a role in the chemerin effects reported here. It could be speculated that inflammation induced up-regulation of this receptor in C-fibres could result in a greater accumulation and more efficient presentation of chemerin to ChemR23 that drives an enhanced chemerin response. While it would have been interesting to investigate the role of CCRL2 in the chemerin response, this was not possible due to the lack of commercially available antagonists. There is currently no evidence that CCRL2 is expressed in DRG neurons and while CCRL2 is known to be expressed in the spinal cord and central nervous system (CNS), this is predominantly on microglia (Brouwer et al. 2004) and possibly astrocytes (Hamby et al. 2012). However, it is worth noting that Brouwer et al. (2004) demonstrated spinal cord expression of CCRL2 in cells which did not express the glial marker, glial fibrillary acidic protein (GFAP). It would therefore be important to establish the expression of this receptor in pain pathways before further investigating its role in or its potential as a novel target for the treatment of inflammatory pain.

Chemerin has also been shown to bind to a third receptor, the G protein-coupled receptor 1 (GPR1). Unlike CCRL2, binding of chemerin to GPR1 does result in internalisation and signalling, however signalling is weak (Barnea et al. 2008) and it has been speculated that GPR1 may act as a decoy receptor (Bondue et al. 2011). This raises the prospect that the chemerin response in this study was influenced by this receptor, either directly by cell signalling or indirectly by acting as a decoy to alter chemerin availability to ChemR23. While there is also no evidence for *in vivo* GPR1 mediated activity (Bondue et al. 2011), it was not possible to investigate the role of this receptor in the chemerin responses described here as there are no selective agonists or antagonists available. There is evidence that GPR1 is expressed in the

CNS (Croitoru-Lamoury et al. 2003, Marchese et al. 1994a, Marchese et al. 1994b, Shimizu et al. 1999), however spinal cord and DRG expression has not been investigated. Therefore, as with CCRL2, it would be useful to establish whether GPR1 is expressed in pain pathways before a more comprehensive study into its possible role in modulating C-fibre input to lamina I NK1R+ neurons.

There is some evidence that ChemR23 is expressed on microglia (Connor et al. 2007), however this has not been confirmed in the spinal cord. The activation of spinal microglia is known to be a key feature of neuropathic pain (Coull et al. 2005, Tsuda et al. 2013, 2005) and is also implicated in inflammatory pain (Cao & Zhang 2008, McMahon & Malcangio 2009). It has been argued that ChemR23 agonists acting upon ChemR23 on microglia could prevent their activation and and thus reduce pain (Ji et al. 2011). Interestingly, Xu et al. (2013) have recently demonstrated that pretreatment with RvE1 (100ng for 3 days, delivered *i.t.*) attenuates chronic constriction injury (CCI)-induced mechanical allodynia, but not heat hyperalgesia, in mice. CCI produced a corresponding increase in the levels of mRNA for the microglial marker, ionized calcium binding adaptor molecule 1 (IBA-1) and the expression of TNF-α, in the dorsal horn, with both IBA-1 and TNF-α expression being reduced in RvE1 treated animals. Furthermore, in microglial cultures, RvE1 dose-dependently reduced the number of activated microglia and the microglial release of TNF-α in response to lipopolisaccharide application. These findings suggest that ChemR23 agonists reduce neuropathic pain by an attenuation of microglial activation and signalling. Therefore, it is possible that the RvE1 / chemerin mediated reduction in inflammatory pain hypersensitivity and/or potentiated spinal cord activity may in part be due to an attenuation of microglial activity.

Activation of ChemR23 is proposed to reduce inflammatory pain in part by normalising potentiated spinal cord responses (Ji et al. 2011, Xu et al. 2010). Monosynaptic C-fibre input to a subset of lamina I NK1R+ neurons may be potentiated in inflammatory pain (Ikeda et al. 2006). Therefore, it could be hypothesised that those neurons in this study that were classified as responders belonged to a subgroup that received potentiated input. To test whether this was the case, analysis was performed to ascertain whether there was any correlation between initial C-fibre eEPSC amplitude and the amplitude change resulting from chemerin. If chemerin was acting specifically on potentiated inputs, it could be expected that those neurons with greater initial eEPSC amplitudes (possibly indicating potentiated inputs) would exhibit an enhanced chemerin response and thus there would be a positive correlation between increasing initial amplitude and increasing chemerin response.

However, this was not the case as there was no significant correlation between initial amplitude and the chemerin response (figure 3.6E). The data presented here cannot confirm or refute the hypothesis that chemerin acts only on potentiated inputs. While no potentiation of C-fibre eEPSC amplitude as a result of CFA inflammation was reported here (figure 2.15B), it is impossible to know whether inputs to individual neurons in CFA tissue were potentiated relative to the same inputs in control tissue. It is difficult in comparative population recordings, as used here, to ascertain whether a particular subgroup exhibits potentiation without the means to identify the subgroup in both control and CFA tissue.

3.5.4 Chemerin has no effect on monosynaptic Aδ-fibre input to lamina I neurokinin 1 receptor expressing neurons

The data presented here provide new evidence that chemerin does not effect monosynaptic Aδ-fibre input to lamina I NK1R+ neurons in tissue isolated from control or CFA treated rats. RvE1 was previously shown to attenuate mechanical hypersensitivity associated with CFA inflammation (Xu et al. 2010). Given that Aδ-fibres are known to mediate mechanical hypersensitivity (Fuchs et al. 2000, Magerl et al. 2001, Ziegler et al. 1999) it could be expected that chemerin would exhibit some activity upon Aδ-fibre eEPSCs, particularly in inflammatory conditions, however this is not supported by the data presented here.

One explanation for this apparent lack of effect on Aδ-fibres could be that these inputs do not express ChemR23. As discussed, ChemR23 is expressed on TRPV1+ and SP containing afferents (Xu et al. 2010). Only a small proportion of Aδ-fibres express TRPV1 (Amaya et al. 2003, Kobayashi et al. 2005, Michael & Priestley 1999, Yu et al. 2008) or contain SP (Lawson et al. 1997, 1993, McCarthy & Lawson 1989) which could suggest that ChemR23 expression is limited in these afferents. However, less than half of ChemR23+ DRG neurons co-express TRPV1 and expression of ChemR23 on SP containing afferents has not been quantified (Xu et al. 2010), so a lack of TRPV1 and/or SP expression does not preclude ChemR23 expression on Aδ-fibres. To date no study has investigated the co-expression of ChemR23 with neurofilament 200 (NF200), a typical marker of both Aβ-fibre and Aδ-fibres, with large and small diameter NF200+ fibres typically signifying the former and latter, respectively (Perry et al. 1991), therefore the degree to which ChemR23 is expressed on Aδ-fibres is unclear. This question could be answered by future anatomical studies whereby co-expression of ChemR23 and NF200 in small diameter DRG neurons is

quantified.

Activation of ChemR23 by the agonists RvE1 or chemerin has been proposed to attenuate inflammatory pain hypersensitivity by a mechanism involving the normalising of potentiated spinal cord responses (Ji et al. 2011, Xu et al. 2010). While there is evidence that Aδ-fibre input to lamina I NK1R+ neurons may be potentiated in a subset of neurons in CFA inflammation (Torsney 2011), this was not found to be the case in the present studies (figure 2.15B). Therefore, the lack of chemerin effect on monosynaptic Aδ-fibre input reported here, particularly in recordings from tissue isolated from CFA treated rats, could simply reflect that none of these inputs were potentiated. However, despite finding no evidence for the CFA potentiation of monosynaptic C-fibre input to lamina I NK1R+ neurons (figure 2.15B), chemerin did significantly reduce C-fibre input to a subset of these neurons.

The lack of chemerin effect on Aδ-fibre inputs to lamina I NK1R+ neurons could also be a reflection that the sample size of the Aδ-fibre groups was insufficient to detect any effects. When the criteria used to classify neurons as responders was applied, 1 CFA and 2 control neurons were classified as responders. If the sample size was larger this could have lead to a greater proportion of responders that would have enabled the analysis of subgroups of responders and non-responders, as was done for neurons receiving monosynaptic C-fibre input. Therefore, to fully ascertain whether chemerin alters monosynaptic Aδ-fibre input to these neurons, a larger sample size would be advantageous.

3.5.5 Chemerin receptor 23 agonists as future inflammatory pain treatments

While the evidence presented here and elsewhere (Xu et al. 2010) suggests that drugs which target ChemR23 may be effective in the treatment of inflammatory pain, it is worth stating that chemerin or RvE1 may have limited therapeutic potential given that they are not metabolically stable and are rapidly inactivated *in vivo* (Arita et al. 2006, Shimamura et al. 2009). Interestingly, a stable analogue of RvE1, 19-(p-fluorophenoxy)-RvE1 (19-pf-RvE1), has been shown to effectively reduce thermal hypersensitivity in the CFA inflammatory pain model for an extended time period compared to RvE1 (Xu et al. 2010). Stable chemerin analogues have been developed (Shimamura et al. 2009), however these have not been investigated in models of inflammatory pain. Further research into the use of these or new stable analogues that target ChemR23 could provide additional evidence for the potential

that ChemR23 has as a target for the treatment of inflammatory pain.

3.6 Conclusions

The results presented here have novelly demonstrated that application of the ChemR23 agonist, chemerin, in non-potentiated conditions does not alter primary afferent input, specifically monosynaptic Aδ- and monosynaptic C-fibre input to lamina I NK1R+ neurons (figures 3.2, 3.5 & 3.9). This supports the premise that targeting this receptor with chemerin or RvE1 does not alter basal synaptic transmission in the spinal cord and that the ChemR23-mediated reduction in inflammatory pain is as a result of an anti-hyperalgesic mechanism of action, whereby potentiated spinal cord responses are normalised (Ji et al. 2011, Xu et al. 2010). This lack of effect on normal pain processing and particularly in pain pathways involving these key spinal cord output neurons (Nichols et al. 1999), adds to the evidence that new treatment strategies that target this receptor will have the advantage that the acute protective pain responses are left intact (Ji et al. 2011, Xu et al. 2010).

The data shown here supports previous studies that report ChemR23 activation can attenuate the capsaicin potentiation of spinal cord responses (Xu et al. 2010). In addition, it has been established for the first time that chemerin can specifically attenuate the capsaicin potentiation of primary afferent input onto lamina I NK1R+ neurons (figure 3.3). It is known that TRPV1 channels can be sensitised by a number of inflammatory mediators, which can contribute to the development and maintenance of inflammatory pain hypersensitivity (Basbaum et al. 2009, Palazzo et al. 2012, Pingle et al. 2007). Therefore, the finding that chemerin can attenuate the capsaicin potentiation of primary afferent input to lamina I NK1R+ neurons, that are essential for inflammatory pain (Nichols et al. 1999), strongly suggests that the development of drugs directed towards the ChemR23 receptor could provide new inflammatory pain treatments.

This thesis has presented novel data that strongly suggest ChemR23 is functionally expressed on a subset of monosynaptic C-fibre inputs onto lamina I NK1R+ neurons and that activation of these receptors with chemerin can presynaptically attenuate this C-fibre input in inflammatory pain conditions (figures 3.7 & 3.8). Lamina I NK1R+ neurons are crucial for the manifestation of inflammatory pain (Nichols et al. 1999), which is known to be driven by C-fibre activity (Abrahamsen et al. 2008). Therefore, these data can be considered to lend support to suggestions that agents that target ChemR23, such as RvE1, represent a promising new class of analgesics for the treatment of inflammatory pain (Ji et al. 2011, Lee 2012, Xu et al. 2010).

The findings described here have shown for the first time that chemerin does not alter monosynaptic Aδ-fibre input to lamina I NK1R+ neurons in inflammatory pain (figure 3.9). Mechanical hyperalgesia is know to be mediated by Aδ-fibres (Fuchs et al. 2000, Magerl et al. 2001, Ziegler et al. 1999), therefore treatment strategies that target ChemR23 may be of limited efficacy in combating mechanical hypersensitivity associated with inflammatory pain. However, animal studies show that RvE1 can reduce mechanical hypersensitivity, although with reduced potency compared to its actions against thermal hypersensitivity, in that higher doses are required to produce an effect (Xu et al. 2010). This suggests that the actions of ChemR23 agonists against mechanical hypersensitivity may be mediated elsewhere within pain circuits and further investigations would be needed to establish the site of action.

Chapter 4

Activity-dependent slowing in inflammatory pain

4.1 Introduction

4.1.1 Activity-dependent slowing

Repetitive firing of C-fibres is known to drive central sensitisation (Latremoliere & Woolf 2009, Woolf 1983) and inflammatory pain hypersensitivity has been established to be mediated by C-fibre nociceptors (Abrahamsen et al. 2008). Interestingly, C-fibres display a phenomenon called activity-dependent slowing (ADS), whereby repetitive electrical stimulation at frequencies of 1Hz or above, results in a progressive slowing of action potential conduction velocity, which manifests as a progressive increase in response latency (Gee et al. 1996, Serra et al. 1999, Thalhammer et al. 1994, Weidner et al. 1999). ADS has been demonstrated in unmyelinated fibres from a number of species including; human (Grafe et al. 1997, Hilliges et al. 2002, Jørum et al. 2007, Ørstavik et al. 2003, Schmelz et al. 2000, Serra et al. 1999, Shim et al. 2007, Weidner et al. 1999), rat (de Col et al. 2008, 2012, Gee et al. 1996, Grafe et al. 1997, Nakatsuka et al. 2000, Raymond et al. 1990, Takigawa et al. 1998, Thalhammer et al. 1994), mouse (Mazo et al. 2013, Shim et al. 2007), pig (Obreja et al. 2011a, 2011b), monkey (Ringkamp et al. 2010), rabbit (Zhu et al. 2009) and tortoise (Bliss & Rosenberg 1979). While much of the research describing ADS has investigated this phenomenon in primary sensory neurons (de Col et al. 2008, 2012, Gee et al. 1996, Nakatsuka et al. 2000, Takigawa et al. 1998, Thalhammer et al. 1994, Zhu et al. 2009), ADS has also been reported in unmyelinated axons in the hippocampus (Soleng et al. 2003), olfactory nerve (Bliss & Rosenberg 1979) and

vagus nerve (Grafe et al. 1997, Takigawa et al. 1998).

The underlying physiological role of ADS is unclear. ADS has been proposed to represent a form of self inhibition, that could act to limit nociceptive input to the dorsal horn and therefore could regulate central sensitisation (de Col et al. 2012, Mazo et al. 2013). Interestingly, ADS has been shown to occur in response to natural heat and cold stimuli (Thalhammer et al. 1994), which demonstrates that ADS results from natural stimuli as well as artificial electrical stimulation, which further suggests a physiological role for this phenomenon. The progressive slowing of conduction velocity that arises from ADS can be considered to alter the timing of nociceptive inputs being relayed from peripheral nerves to the spinal cord and could potentially alter spinal pain processing. However, to date there has been no investigation into the influence of ADS on central nociceptive processing. Therefore, this chapter will aim to establish whether ADS is present in monosynaptic C-fibre input to neurokinin 1 receptor expressing (NK1R+) neurons that reside in the lamina I region of the spinal cord. These NK1R+ neurons are essential for the development of inflammatory pain (Nichols et al. 1999), which is mediated by C-fibres (Abrahamsen et al. 2008). It is important to determine if ADS is present in the monosynaptic C-fibre input to these neurons, whether it is regulated in inflammatory pain and whether this ADS provides a novel target with which to modulate the nociceptive input to these key spinal cord neurons.

ADS has been described using electrophysiological recordings, both intracellular and extracellular, from individual sensory neurons only (de Col et al. 2008, 2012, Gee et al. 1996, Grafe et al. 1997, Nakatsuka et al. 2000, Raymond et al. 1990, Takigawa et al. 1998, Thalhammer et al. 1994). The studies presented here will examine whether ADS can be detected using extracellular population recordings from isolated rat dorsal roots. These dorsal root recordings are technically simpler than the methods that will be used to examine ADS in the monosynaptic primary afferent input to lamina I NK1R+ neurons. As such, the ability to detect ADS in primary afferent fibres in these dorsal root recordings will offer a simple means to characterise ADS and to investigate the potential modulation in inflammatory pain and pharmacological manipulations of ADS and ultimately inform the more technically challenging patch-clamp recordings from lamina I NK1R+ neurons.

A number of physiological properties of C-fibres, which could act to modulate the transmission of nociceptive input from peripheral nerves to the spinal cord, have been shown to be correlated with ADS. Increased ADS is known to correspond with higher mechanical activation thresholds (de Col et al. 2012). The ADS that results from

mechanical stimulation is greater in C-fibres that responded with increased magnitude (number of spikes) and peak discharge of spikes (Taguchi et al. 2010). Following repetitive electrical stimulation, in a manner known to evoke ADS, C-fibres have been shown to display a persistent altered response to natural stimuli, in that they fire less action potentials and exhibit longer interspike intervals in response to cold and heat stimuli (Thalhammer et al. 1994). It has also been demonstrated that greater ADS is linked with an increase in the probability that conduction failure will occur (Nakatsuka et al. 2000, Obreja et al. 2011a, Raymond et al. 1990, Zhu et al. 2009). Clearly, failures in the monosynaptic C-fibre input to lamina I NK1R+ neurons, in addition to ADS, will influence the pattern of nociceptive input being relayed to these key spinal cord output neurons and potentially impact upon spinal pain processing. Therefore, failures in the monosynaptic C-fibre input to lamina I NK1R+ neurons during repetitive stimulation and whether this is altered in inflammatory pain, will be quantified.

In addition to C-fibres, there is limited evidence that ADS occurs in A-fibres. Aβ-fibres have been shown to exhibit negligible ADS at frequencies of $\leq$25Hz, but display some ADS when stimulated at frequencies of $\geq$50Hz (Shin et al. 1997). While some authors report that ADS occurs in C- but not Aδ-fibres (Mazo et al. 2013, Nakatsuka et al. 2000), others provide clear evidence that Aδ-fibres display ADS, although typically in response to higher stimulation frequencies than C-fibres (Raymond et al. 1990, Thalhammer et al. 1994, Won et al. 1997). Aδ-fibres are known to mediate mechanical hyperalgesia (Fuchs et al. 2000, Magerl et al. 2001, Ziegler et al. 1999) and it is widely considered that Aβ-fibres mediate allodynia (Latremoliere & Woolf 2009, Treede & Magerl 2000). It is important to establish the presence of ADS in A-fibres and whether this is altered in inflammatory pain, as this could offer insight into whether ADS represents a novel target for the treatment of inflammatory pain. Therefore, this chapter will also aim to characterise ADS in A-fibres in isolated dorsal roots and in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons. ADS in monosynaptic Aβ-fibre input to lamina I NK1R+ neurons will not be investigated, as these neurons have very limited monosynaptic Aβ-fibre input (Torsney 2011, Torsney & MacDermott 2006) (figure 2.14)

It is well established using *in vivo* recordings in a number of species including; human (Obreja et al. 2010, Serra et al. 1999, Weidner et al. 1999), rat (Gee et al. 1996, Raymond et al. 1990, Thalhammer et al. 1994), pig (Obreja et al. 2010) and monkey (Ringkamp et al. 2010), that the ADS profile that results from repetitive electrical stimulation of primary afferent fibres can be used to functionally classify

C-fibres. It has widely been shown that mechano-insensitive C-fibres demonstrate the greatest magnitude of ADS in response to repetitive electrical stimulation while mechano-sensitive C-fibres display minimal ADS (Gee et al. 1996, Obreja et al. 2010, Serra et al. 1999, Weidner et al. 1999). Although these different levels of ADS have been used to identify different functional classes of C-fibres in *in vivo* recordings (George et al. 2007, Hilliges et al. 2002, Jørum et al. 2007, Schmelz et al. 2000), the possibility of using ADS profiles to classify C-fibres of different sensory modalities in *ex vivo* tissue preparations has not been explored. This chapter will therefore aim to establish whether monosynaptic C-fibre input to lamina I NK1R+ neurons can be grouped according to distinct ADS profiles, which could potentially be indicative of mechano-insensitive / mechano-sensitive inputs, as this could provide a way to enable the study of these different sensory modalities in the *ex vivo* spinal cord slice preparation.

The relationship between the initial conduction velocity / latency of C-fibre responses and the magnitude of ADS is unclear. While slower initial conduction velocities / greater initial latencies have been shown to be correlated with greater levels of ADS (Ringkamp et al. 2010, Shim et al. 2007, Swadlow & Waxman 1976, Weidner et al. 1999, Zhu et al. 2009), this is not supported by other groups who report there is no correlation between initial latency and ADS magnitude (Gee et al. 1996, Serra et al. 1999, Taguchi et al. 2010, Thalhammer et al. 1994). Shim et al. (2007), who report that C-fibres with greater initial latency display greater ADS, found that this relationship was not altered following nerve injury, however it is unknown whether this correlation is altered in inflammatory pain. Therefore, whether the initial response latency of monosynaptic C-fibre input to lamina I NK1R+ neurons is predictive of the magnitude of ADS as a result of repetitive stimulation and whether this relationship is altered in inflammatory pain conditions, will be investigated.

4.1.2 Injury-induced changes in activity-dependent slowing

To my knowledge, there have been no investigations into whether ADS in A- or C-fibres is altered in inflammatory pain, however ADS may be altered in neuropathic pain states in both humans (Kleggetveit et al. 2012, Ørstavik et al. 2006, 2003) and animals (Mazo et al. 2013, Shim et al. 2007, Shin et al. 1997, Won et al. 1997). Erythromelalgia patients exhibit significantly greater C-fibre ADS than control subjects (Ørstavik et al. 2003). Patients with painful diabetic neuropathy display enhanced recovery from C-fibre ADS when compared to non-painful diabetic

neuropathy patients and healthy control (Ørstavik et al. 2006). Likewise, polyneuropathy patients who suffer from pain show faster ADS recovery than those without pain (Kleggetveit et al. 2012). In the spinal nerve ligation (SNL) neuropathic pain model, where the L5 dorsal root is lesioned, C-fibre ADS is enhanced in the uninjured L4 dorsal root compared to sham operated rats (Shim et al. 2007). Mazo et al. (2013) have recently reported that C-fibre ADS is reduced following saphenous nerve transection in mice, however they fail to show a direct comparison between ADS in control and axotomised groups and no statistics are provided to support their claim, so it cannot be considered conclusive. In the SNL neuropathic pain model, Aδ-fibres, which display negligible ADS in control animals, show a significant increase in ADS (Won et al. 1997). In Aβ-fibres, nerve injury novelly induces ADS during stimulation at 5 - 25Hz and enhances ADS at $\geq$50Hz (Shin et al. 1997). Application of nerve growth factor (NGF), which is a major mediator in inflammatory pain (Cheng & Ji 2008, Pezet & McMahon 2006), reduces ADS in pig C-fibres (Obreja et al. 2011a, 2011b). Interestingly, NGF can alter the expression and activity of a number of ion channels, including voltage-gated sodium channels (Na_vs) (Diss et al. 2008, Fang et al. 2005, Fjell et al. 1999, Gould et al. 2000), which have been implicated in ADS (Baker & Waxman 2012, de Col et al. 2008, 2012, Obreja et al. 2012). It is therefore hypothesise that ADS could be reduced in inflammatory pain conditions.

4.1.3 Underlying mechanisms of activity-dependent slowing

While the physiological mechanisms underlying ADS remain unclear, a number of hypotheses have been proposed. Repetitive action potential firing is associated with increased Na^+-K^+-ATPase activity, which drives axonal hyperpolarisation (Kobayashi et al. 1997, Morita et al. 1993, Rang & Ritchie 1968) and this hyperpolarisation has been proposed to account for ADS (Gee et al. 1996). However, direct or indirect Na^+-K^+-ATPase inhibition, by ouabain or cyanide, respectively, results in facilitation rather than attenuation of ADS (de Col et al. 2008).

It has been suggested that accumulation of intracellular Na^+ could account for ADS, whereby repetitive action potential firing drives a cumulative decrease in the Na^+ Nernst potential (Bliss & Rosenberg 1979), which is known to result in conduction velocity slowing (Hodgkin & Katz 1949). However, blocking Na_v activity with tetrodotoxin (TTX) to decrease Na^+ entry into the neuron, presumably reducing the intracellular accumulation of Na^+, enhances rather than inhibits ADS (de Col et al.

2008).

Further evidence suggests that the activity of hyperpolarization-activated cyclic nucleotide-gated (HCN) (Grafe et al. 1997, Mazo et al. 2013, Takigawa et al. 1998, Zhu et al. 2009) and/or Na_v (Baker & Waxman 2012, de Col et al. 2008, 2012, Obreja et al. 2012) channels may be involved in mediating ADS. These channels, are regulated and play a crucial role in inflammatory pain (Amir et al. 2006, Cummins et al. 2007, Dib-Hajj et al. 2010, Emery et al. 2012, Papp et al. 2010, Weng et al. 2012).

The role of voltage-gated sodium channels in activity-dependent slowing

One of the key physiological mechanisms underlying ADS that has recently been proposed is an activity-dependent modulation of Na_v channel availability (Baker & Waxman 2012, de Col et al. 2008, 2012, Obreja et al. 2012). In recordings from unmyelinated dural afferents, de Col et al. (2008) have shown that application of low doses of the Na_v blocker, TTX, enhances ADS and increases the initial response latency in a dose dependent manner. They also demonstrated that application of the local anaesthetic, lidocaine, and the anti-convulsant, carbamazepine, which both stabilise the slow-inactivated state of Na_v channels (Cardenas et al. 2006, Sandtner et al. 2004), at low doses increased the initial response latency and enhanced ADS. These findings lead the authors to propose that ADS is caused by an activity-dependent reduction in operational Na_v channel availability, driven by a gradual increase in the number of Na_v channels entering a slow-inactivated state. This hypothesis is supported by findings that show $Na_v1.8$ channels enter a slow-inactivated state following repetitive activation, as evidenced by patch-clamp recordings from C-fibre dorsal root ganglia (DRG) neurons that show repetitive activation leads to a reduction in Na^+ currents (Blair & Bean 2003, Choi et al. 2007) and increased action potential induction time, relating to reduced action potential currents (Snape et al. 2010). Furthermore, $Na_v1.8$ channels have greater susceptibility to activity-dependent inactivation than $Na_v1.7$ (Chevrier et al. 2004) and in the SNL neuropathic pain model, uninjured C-fibres axons show increased $Na_v1.8$ immunoreactivity, resulting from the redistribution of $Na_v1.8$ as opposed to an upregulation of expression (Gold et al. 2003) and display greater ADS (Shim et al. 2007). However, it should be recognised that Obreja et al. (2012) have recently shown that ADS was reduced, rather than increased, in pig C-fibres following the application of lacosamide, which selectively enhances Na_v slow-inactivation (Errington et al.

2008) and the local anaesthetic, lidocaine, which stabilises the slow-inactivated state of Na_v channels (Sandtner et al. 2004). It is also worth noting that Na_v slow-inactivation may also be relevant for ADS in A-fibres, in that application of lidocaine causes a profound increase in ADS in Aβ-fibres, which display negligible ADS in control conditions (Huang et al. 1997).

In inflammatory pain conditions, the expression of Na_v channels in primary sensory neurons is known to be regulated (Amir et al. 2006, Black et al. 2004, Cummins et al. 2007, Dib-Hajj et al. 2010, Gould et al. 2004). Carrageenan inflammation significantly upregulates $Na_v1.3$, 1.7 and 1.8 mRNA and protein and enhances Na^+ currents in small, presumably C-fibre, DRG neurons, while there is no change in the expression of $Na_v1.1$, 1.2, 1.6 or 1.9 (Black et al. 2004, Tanaka et al. 1998). Expression of $Na_v1.6$, 1.7, 1.8 and 1.9 has also been shown to be increased in the complete Freund's adjuvant (CFA) inflammatory pain (Gould et al. 2004, Yu et al. 2011) and joint pain models (Strickland et al. 2008), however this is disputed by others (Okuse et al. 1997). Furthermore, $Na_v1.8$ and 1.9 currents are known to be enhanced in CFA inflammation (Yu et al. 2011). Given the key role that Na_v channels are purported to play in mediating ADS (Baker & Waxman 2012, de Col et al. 2008), this inflammation induced regulation of Na_v channels suggests that ADS could be altered in inflammatory pain. This can be considered to provide support for the hypothesis, that ADS could be altered in inflammatory pain.

The role of HCN channels in activity-dependent slowing

HCN channels, which mediate the hyperpolarisation-activated current (I_h), have also been implicated in ADS (Grafe et al. 1997, Mazo et al. 2013, Takigawa et al. 1998, Zhu et al. 2009). In addition to the hypothesis that ADS is mediated by an activity-dependent reduction in the availability of operational Na_v channels, it has alternatively been suggested that ADS could arise due to prolonged after-hyperpolarisation, mediated by Ca^{2+}-dependent K^+ currents, following repetitive stimulation (Gee et al. 1996, Soleng et al. 2003). It has been suggested that this prolonged after-hyperpolarisation could be counteracted by I_h, based on evidence that blocking I_h with Cs or the non-selective HCN antagonist, ZD7288, enhances ADS in isolated rat vagus (Grafe et al. 1997, Takigawa et al. 1998), human sural (Grafe et al. 1997), rabbit saphenous nerves (Zhu et al. 2009) and unmyelinated hippocampal axons (Soleng et al. 2003), indicating that the activity of HCN channels constrains ADS. Recently, Mazo et al. (2013) have showed that ZD7288 significantly increases

ADS in a subpopulation of ZD7288-sensitive C-fibres and interestingly show that this effect of ZD7288 is enhanced following nerve axotomy, with nerve injury being known to result in increased expression of HCN channels and enhanced I_h (Chaplan et al. 2003, Emery et al. 2012, Jiang et al. 2008).

In recent years a great deal of interest has been expressed in targeting HCN channels for the treatment of inflammatory pain (Emery et al. 2012). HCN2 channels in particular have been shown to play a crucial role in the development of inflammatory pain (Emery et al. 2011). The behavioural hypersensitivity observed during the second phase of the formalin test is significantly reduced by application of the non-specific HCN antagonist, ZD7288, or in knockout mice in which HCN2 has been deleted from $Na_v1.8$ expressing nociceptors ($Na_v1.8$-HCN2$^{-/-}$) (Emery et al. 2011). Similarly, thermal hypersensitivity in response to prostaglandin E_2 (PGE_2) or carrageenan hindpaw injection and mechanical hypersensitivity in response to PGE_2 application was abolished in $Na_v1.8$-HCN2$^{-/-}$ mice, or by application of ZD7288 to wild type mice (Emery et al. 2011). The enhancement of action potential firing in DRG neurons in response to forskolin application, that acts to elevate intracellular cyclic adenosine monophosphate (cAMP), which increases the activity of HCN channels (Biel et al. 2002, Kaupp & Seifert 2001), has been shown to be abolished in DRG neurons isolated from HCN2 global knockout mice (HCN2$^{-/-}$) and by application of ZD7288 (Emery et al. 2011).

Anatomical studies in rat and mouse have shown that HCN2 channels are expressed on the central terminals of C-fibres in the lamina I/II region of the dorsal horn and that these channels are largely expressed on peptidergic afferents (Antal et al. 2004, Hughes et al. 2012). Further evidence reveals that HCN2 channels are expressed on the central terminals of substance P (SP) containing afferents, which form contacts with NK1R+ neurons in lamina I (Papp et al. 2010, 2006, Todd et al. 2002). The expression of HCN2 in lamina I/II and specifically on SP containing afferents is significantly increased during CFA inflammation (Papp et al. 2010). HCN1 and HCN2 expression has also been shown to be increased in trigeminal ganglia (TG) neurons following the application of CFA to the dura mater (Cho et al. 2009). Furthermore, recent evidence has demonstrated that HCN2 is upregulated in C-, but not Aδ-fibres, 7 days after CFA hindpaw injection (Weng et al. 2012). This finding is contrasted by Acosta et al. (2012) in that while HCN2 expression was found to be elevated 1 day following CFA injection in small and medium diameter DRG neurons, presumably C- and Aδ-fibre, respectively, it returned to baseline levels by day 4. Furthermore, CFA injection into the temporomandibular joint does not cause a change

in the intensity of HCN1, 2, 3 or 4 immunoreactivity, or in the number of TG neurons that express these channels (Hatch et al. 2013). Inflammation-induced changes in HCN2 immunoreactivity in large diameter, presumably Aβ-fibre, DRG neurons shows no increase in expression 1 day after, but a reduction 4 days after CFA injection (Acosta et al. 2012). Interestingly, similar reductions in HCN channel expression have been reported in L4/5 DRG neurons following nerve injury, where expression of HCN2, as well as 1, but not 3 or 4, was reported to be reduced 7 days post injury (Chaplan et al. 2003).

In addition to changes in the expression of HCN channels, recent evidence has also shown that I_h is upregulated in primary afferents in both inflammatory (Cho et al. 2009, Weng et al. 2012) and neuropathic (Chaplan et al. 2003, Kitagawa et al. 2006, Tsuboi et al. 2004, Yao et al. 2003) pain models. The increase in I_h in CFA inflammation has been shown to mirror the changes in HCN2 expression, in that I_h is increased in C-, but not Aδ-fibres (Weng et al. 2012). However, large diameter DRG neurons in the SNL neuropathic pain model, in which HCN expression is reduced, also show a potentiation in I_h, which is attributed to to an increase in the open channel probability and possibly an increase in channel conductance (Chaplan et al. 2003).

Given the findings that HCN2 expression and I_h is specifically upregulated in C-fibres following injection of CFA to the hindpaw, it is hypothesised that ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons could be modified in inflammatory pain and that part of the mechanism underlying this change could involve altered HCN channel activity / expression.

ZD7288 has also been shown to significantly increase the rate of synaptic response failures in monosynaptic C-fibre evoked excitatory postsynaptic currents (eEPSCs) recorded in unidentified lamina I/II neurons (Papp et al. 2006). Further evidence from single unit recordings of C-fibre activity report that ZD7288 increases action potential failures in a dose dependent manner (Zhu et al. 2009). As with ADS, changes in the rate of action potential failures will alter the pattern of the transmission of nociceptive inputs to the spinal cord. Therefore, in addition to investigating the role of HCN channels in ADS, it is important to establish whether blocking I_h leads to a change in the number of synaptic response failures in monosynaptic C-fibre input to lamina I NK1R+ neurons, as changes in failure rate could clearly impact upon nociceptive processing by these key spinal cord output neurons.

In unmyelinated cerebellar parallel fibres, ZD7288 has been shown to reduce the amplitude of extracellular compound action potentials (CAPs), which the authors

interpret as resulting from a reduction in the number of fibres contributing to the response, due to a ZD7288-induced increase in conduction failures (Baginskas et al. 2009). Whether ZD7288 causes a similar reduction in response amplitude in C-fibre CAP recordings from isolated dorsal roots will be investigated, which could aid the interpretation of any ZD7288-induced changes in the synaptic response failure rate in the monosynaptic C-fibre input to lamina I NK1R+ neurons, seen during eEPSC recordings.

ZD7288 has been reported to significantly reduce the peak amplitude of monosynaptic C-fibre eEPSCs in unidentified lamina II neurons, with the inhibitory effect of ZD7288 being enhanced in tissue isolated from nerve injured animals (Takasu et al. 2010). The authors also shown that ZD7288 significantly reduces miniature excitatory postsynaptic current (mEPSC) frequency, but not amplitude, in recordings from neuropathic mouse spinal cord slices, which they argue indicates that ZD7288 results in a presynaptic inhibition of excitatory transmission, presumably due to a reduction in the presynaptic release of glutamate. It is unclear whether ZD7288 can similarly inhibit monosynaptic C-fibre input to lamina I NK1R+ neurons, or whether this effect is altered in inflammatory pain. This study will therefore investigate whether ZD7288 can modulate the amplitude of monosynaptic C-fibre eEPSCs in lamina I NK1R+ neurons. Given that these neurons are crucial for the manifestation of inflammatory pain (Nichols et al. 1999), which is mediated by C-fibre nociceptors (Abrahamsen et al. 2008), the ability of ZD7288 to attenuate the monosynaptic C-fibre input to these neurons could provide additional evidence to support the targeting of HCN channels as a novel approach for the alleviation of inflammatory pain (Emery et al. 2012).

Some recent evidence has suggested that ZD7288 acts as an antagonist of Na_v channels, in addition to its known ability to block HCN channels (Wu et al. 2012b). ZD7288, at a concentration of 10μM, has been shown to significantly reduce the conduction velocity / increase the initial latency of Aβ-, but not Aδ- or C-fibre DRG neurons (Hogan & Poroli 2008). Similarly, 20μM ZD7288 has been shown to reduce the conduction velocity of unmyelinated cerebellar parallel fibres (Baginskas et al. 2009). Interestingly, an increase in the initial latency of C-fibre responses can also arise following application of the Na_v channel antagonists; TTX, lidocaine or carbamazepine (de Col et al. 2008, Pinto et al. 2008). Given the key role played by Na_v channels in action potential propagation (Gold & Gebhart 2010, Liu & Wood 2011), the finding that ZD7288 can modulate conduction velocity could be considered to support the finding that ZD7288 acts as an antagonist of Na_v channels. However,

this effect of ZD7288 upon the initial latency / conduction velocity is disputed by others who report that ZD7288 (10μM) does not alter the initial latency of C-fibre responses (Takigawa et al. 1998) and while not explicitly stated, Mazo et al. (2013) imply that the initial response latency is not altered at concentrations as high as 100μM. Therefore, in this study the effect of ZD7288 upon the initial latency of C-fibre responses will be examined, which could give an indication as to whether any ZD7288-induced changes in ADS could involve the modulation of Na_v channels, as well as HCN channels.

4.2 Chapter aims and hypotheses

This chapter has investigated the phenomenon of ADS and sought to determine whether ADS is altered in inflammatory pain. Furthermore, this chapter provides a preliminary investigation into the role of HCN channels in mediating ADS in control and CFA inflammation. The specific aims and hypotheses are presented below.

Aims

1. Investigate whether ADS in Aβ-, Aδ- and C-fibres can be detected in extracellular electrophysiological recordings from isolated rat dorsal roots and if this ADS is altered in inflammatory pain.
2. Determine whether ADS is present in monosynaptic Aδ- and/or monosynaptic C-fibre input to lamina I NK1R+ neurons and ascertain if this ADS is modified in inflammatory pain.
3. Using the repetitive stimulation protocols that are used to elicit ADS, quantify the failure rate of monosynaptic C-fibre activation of lamina I NK1R+ neurons, which is likely to reflect C-fibre conduction failures, that is correlated with ADS, and investigate whether the incidence of failures is altered in inflammatory pain.
4. Investigate whether monosynaptic C-fibre input to lamina I NK1R+ neurons can be categorised into distinct groups based on ADS profile.
5. Undertake a preliminary investigation into the role of HCN channels in C-fibre ADS to establish:
   (a) Whether application of the non-specific HCN antagonist, ZD7288, alters ADS.
   (b) If the effect of ZD7288 on ADS is influenced by inflammatory pain.
   (c) Whether ZD7288 alters the rate of failures in monosynaptic C-fibre input to lamina I NK1R+ neurons.

Hypotheses

1. ADS will be detectable using extracellular recordings from isolated dorsal roots, with ADS being present in the C-fibre, but not Aδ- or Aβ-fibre components and that this C-fibre ADS will be altered in CFA inflammation.

2. ADS will be present in the monosynaptic C-fibre, but not monosynaptic Aδ-fibre input to lamina I NK1R+ neurons and that this C-fibre ADS will be altered in CFA inflammation.

3. Increasing stimulation frequency will result in a greater number of synaptic response failures in monosynaptic C-fibre input to lamina I NK1R+ neurons, which may also be influenced by CFA inflammation.

4. Monosynaptic C-fibre input to lamina I NK1R+ neurons will fall into distinct groups based on their ADS profile, which could be indicative of inputs that detect and convey specific sensory modalities.

5. The non-specific HCN antagonist, ZD7288, will increase C-fibre ADS, with the effect of ZD7288 being enhanced in inflammatory pain.

4.3 Methods

4.3.1 Animals

As described in section 2.3.1.

4.3.2 Complete Freunds's adjuvant inflammation

As described in section 2.3.2.

4.3.3 Activity-dependent slowing in isolated rat dorsal roots

Tissue preparation

Dorsal roots were isolated from control and CFA treated rats as described in section 2.3.3

Compound action potential, activity-dependent slowing recordings

CAP recordings were carried out in the manner described in section 2.3.3. To investigate ADS, isolated dorsal roots were stimulated 16 times at frequencies of 1 and 2Hz, at a stimulation intensity of 500μA and stimulus duration of 0.1ms. An interval of 3 minutes was left between periods of stimulation to allow the fibres to recover from ADS (Shim et al. 2007, Weidner et al. 1999). These stimulation frequencies were chosen as they mimic the spontaneous firing rate of C-fibres observed in inflammatory pain (Djouhri et al. 2006, Puig & Sorkin 1996, Xiao & Bennett 2007). Aβ-, Aδ- and C-fibre components were identified on the basis of activation threshold and conduction velocity, as described previously in section 2.3.3. The latency of the Aβ-, Aδ- and C-fibre responses were measured as the latency between the stimulus artefact and the negative peak of the triphasic response, as shown in figure 4.1A. To assess ADS, these latency values were normalised to the value obtained at stimulus 1, by calculating the latency change between stimulus 1 and all other subsequent stimuli. In addition, the area under the curve (AUC) of latency change was calculated, as described in figure 4.1B. To confirm that the dorsal roots included in control and CFA groups were of comparable length, dorsal root length was measured as the distance between the recording and stimulating electrodes.

In some cases, when the absolute latency values obtained during repetitive stimulation were analysed using linear regression (as described in section 4.3.6), the intercepts of the line of best fit were found to be significantly different, which was suggestive of a CFA effect upon the initial latency / conduction velocity, which was contradictory to the results in chapter 2 (figures 2.9C & 2.15D) and published studies (Baba et al. 1999, Djouhri & Lawson 2001, Nakatsuka et al. 1999, Torsney 2011). The intercept of the line of best fit is an extrapolated value, which is influenced by the response to repetitive stimulation (i.e. the slope of the line of best fit) and as such is not an independent measure of initial latency / conduction velocity. Therefore, to further investigate this potential CFA effect, the actual initial latency, recorded during stimulus 1 of the Aβ-, Aδ- and C-fibre responses, was compared between control and CFA groups. Only the initial latency recorded during 1Hz stimulation was compared, to avoid any potential influence from repetitive stimulation.

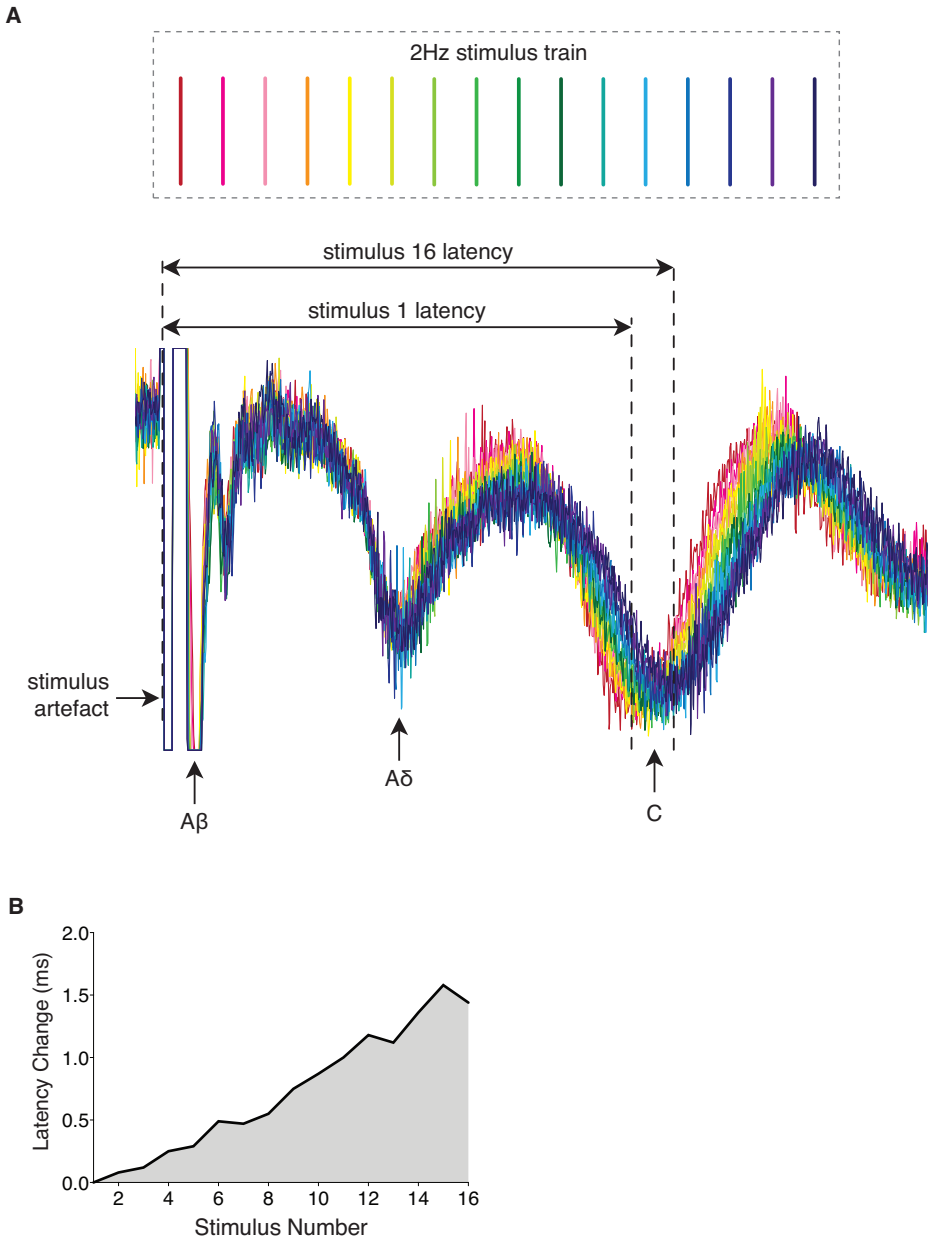

Figure 4.1: Compound action potential recording of activity-dependent slowing. ***A*** Trace demonstrates the responses to repetitive stimulation of a control dorsal root stimulated at C-fibre strength at a frequency of 2Hz. Trace is colour coded to match the stimulus train shown above. The latency of C-fibre responses was measured as the time between the stimulus artefact and the negative peak of the response, as indicated by the arrows and broken lines. ***B*** The area under the curve of latency change recorded during repetitive stimulation was calculated as the grey shaded area, for the dorsal root shown in ***A***.

4.3.4 Activity-dependent slowing in monosynaptic primary afferent input to lamina I neurokinin 1 receptor expressing neurons

To investigate ADS in monosynaptic Aδ- and monosynaptic C-fibre input to lamina I NK1R+ neurons, patch-clamp electrophysiology was used to record eEPSCs from

TMR-SP labelled (TMR-SP+) / NK1R+ neurons while the dorsal root was electrically stimulated to activate these inputs. ADS in monosynaptic Aβ-fibre input was not assessed as there is minimal monosynaptic Aβ-fibre input to these neurons (Torsney 2011, Torsney & MacDermott 2006) (figure 2.14).

Spinal cord slice preparation

Spinal cord slices with dorsal roots attached were prepared from tissue isolated from control and CFA treated rats, as described in section 2.3.4.

Patch-clamp activity-dependent slowing recordings

Monosynaptic Aδ- and monosynaptic C-fibre input to lamina I NK1R+ neurons was identified in the manner described in section 2.3.5. To assess ADS in monosynaptic Aδ- and monosynaptic C-fibre input to lamina I NK1R+ neurons, eEPSCs were recorded in response to dorsal root stimulation. Monosynaptic Aδ-fibre inputs were stimulated 16 times at 2Hz, with a subset also being stimulated at 1 and/or 10Hz, at a stimulus intensity of 100μA and a stimulus duration of 0.1ms. Monosynaptic C-fibre inputs were stimulated 16 times at 1Hz, a subset of which were also stimulated at 2Hz, at an intensity of 500μA and a stimulus duration of 0.1ms. These frequencies were chosen to mimic the spontaneous C-fibre firing rate observed during inflammation (Djouhri et al. 2006, Puig & Sorkin 1996, Xiao & Bennett 2007). To assess C-fibre ADS in response to higher stimulation frequencies, that more closely match the C-fibre evoked firing rate (Thalhammer et al. 1994, Yeomans & Proudfit 1996), combined with longer stimulation trains, the monosynaptic C-fibre input to a subset of lamina I NK1R+ neurons was also stimulated 40 times at frequencies of 2, 5 and 10Hz, at an intensity of 500μA with a stimulus duration of 0.1ms. In an individual lamina I NK1R+ neuron, between periods of repetitive dorsal root stimulation, intervals of 3 minutes, following 16 stimuli and 5 minutes, following 40 stimuli, were left to allow the fibres to recover from ADS (Shim et al. 2007, Weidner et al. 1999). The latency of monosynaptic Aδ- and monosynaptic C-fibre responses were measured as the time between the stimulus artefact and the onset of the monosynaptic response, examples shown in figures 4.8 and 4.11. The data were normalised to the latency recorded at stimulus 1, by calculating the latency change between stimulus 1 and all other subsequent stimuli. Furthermore, the area under the curve of latency change was calculated, as described for the CAP recordings in

figure 4.1B. The dorsal root length in spinal cord slices was measured as the distance between the stimulating electrode and the point at where the dorsal root entered the dorsal horn and compared to confirm that comparable lengths of dorsal root were used in each group, to ensure any ADS differences between groups were not influenced by differences in root length. This method of measuring can only provide a rough estimate of the distance of the conduction pathway, as it cannot account for the section within the spinal cord, however it is still informative.

It is widely reported that C-fibres which convey information relating to different sensory modalities exhibit different ADS profiles, specifically that mechano-insensitive C-fibres display a high level of ADS, while mechano-sensitive C-fibres display minimal ADS (Gee et al. 1996, Obreja et al. 2010, Serra et al. 1999, Weidner et al. 1999). Therefore, for data obtained from C-fibre eEPSC recordings in lamina I NK1R+ neurons, where 16 stimuli were used, frequency histograms of the AUC of latency change were plotted to assess whether the distribution was bimodal (Serra et al. 2004, 1999, Shim et al. 2007), which could be indicative of neurons that receive mechano-sensitive or mechano-insensitive C-fibre input.

There is debate as to whether the initial C-fibre latency is correlated with the magnitude of ADS that results from repetitive stimulation (Gee et al. 1996, Ringkamp et al. 2010, Serra et al. 1999, Shim et al. 2007, Swadlow & Waxman 1976, Taguchi et al. 2010, Thalhammer et al. 1994, Weidner et al. 1999, Zhu et al. 2009). Therefore, to assess whether the initial latency of the monosynaptic C-fibre input to lamina I NK1R+ neurons is predictive of the magnitude of ADS, correlation between the initial C-fibre latency, measured during the first stimulus in the train and the AUC of latency change was calculated.

As with the CAP experiments detailed in 4.3.3, when absolute latency values of the monosynaptic Aδ-fibre / monosynaptic C-fibre responses recorded during repetitive stimulation were analysed, some of the results showed significant differences in the intercept of the lines of best fit between control and CFA groups, which was suggestive of a CFA effect upon the initial latency / conduction velocity. However, as the intercept is influenced by the slope and is an extrapolated value as opposed to an actual value, this potential CFA effect was investigated further by comparing the actual initial latency values obtained during the first stimulation of trains of 16 / 40 sweeps, at frequencies of 1, 2, 5 and 10Hz, in control and CFA tissue.

In the extended stimulation train experiments, the synaptic response failure rate was analysed to ascertain whether CFA inflammation altered the degree of C-fibre

conduction failures. A failure was determined as having occurred when a stimulus did not result in a clear eEPSC, an example of which is shown in figure 4.2. The synaptic response failure rate was determined per stimuli, during dorsal root stimulation at 2, 5 and 10Hz, with control and CFA groups being compared. Additionally, the total failure rate that occurred during these stimulation frequencies was calculated.

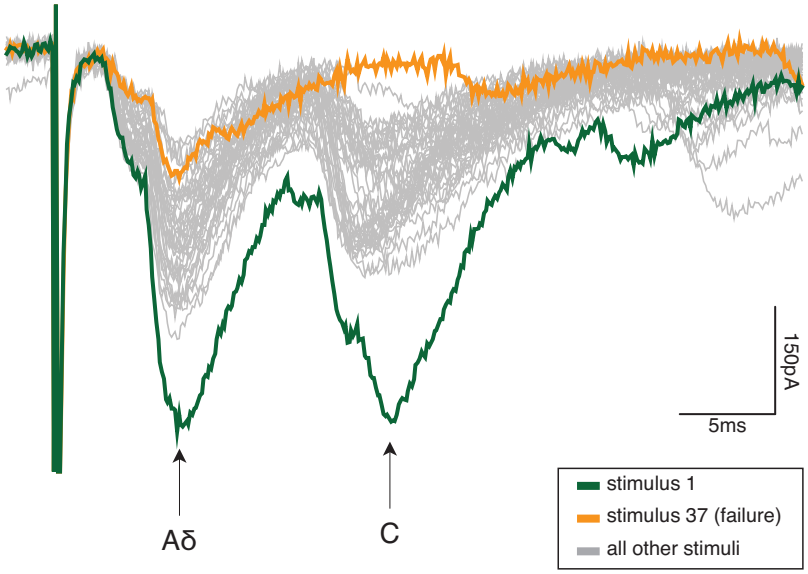

Figure 4.2: Example of a monosynaptic C-fibre failure during repetitive stimulation. Trace shows eEPSCs recorded in a lamina I NK1R+ neurons isolated from a CFA treated rat, in response to C-fibre stimulation at 2Hz (40 stimuli). Stimuli 1 shown in green, while stimuli 37 in orange demonstrates a failure in the monosynaptic C-fibre in which the stimulus has not resulted in a clear eEPSC. Notably, no failure was present in the monosynaptic Aδ-fibre component during this stimulus.

4.3.5 The role of HCN channels on activity-dependent slowing

Influence of HCN channels in activity-dependent slowing in C-fibres in isolated rat dorsal roots

In these experiments, Ms Veny Lukito, a Physiological Society vacation student, performed the electrophysiological recordings and conducted some analysis on the resulting data.

To assess the role of HCN channels in constraining C-fibre ADS in isolated dorsal roots and whether this is altered in CFA inflammation, CAP recordings of C-fibres were made from dorsal roots isolated from control and CFA treated rats, as described in sections 2.3.3 and 4.3.3. Dorsal roots were stimulated 40 times at a frequency of 2Hz, stimulation intensity of 500μA and a stimulus duration of 0.1ms, before

('baseline') and during bath application of the non-selective HCN antagonist, ZD7288 (10μM, Sigma) or vehicle (Krebs only). These two stimulation periods were separated by an interval of 10 minutes, during which ZD7288 was washed into the recording bath. The C-fibre latency was measured for each stimulation as the time between the stimulus artefact and the negative peak of the triphasic response (figure 4.1A). The data were normalised, by calculating the latency change between the response latency at stimulus 1 and all other subsequent stimuli. To assess the degree of facilitation of ADS caused by ZD7288, the latency change recorded at baseline was subtracted from the latency change recorded during ZD7288 application.

There is debate as to whether ZD7288 alters the initial latency of the C-fibre response (Baginskas et al. 2009, Hogan & Poroli 2008, Mazo et al. 2013, Takigawa et al. 1998), which may be indicative of ZD7288s reported actions against Na_v channels (Wu et al. 2012b). Therefore, the effect of ZD7288 application on the initial C-fibre response latency was investigated, by comparing the response latency measured during the first stimuli during baseline and ZD7288 recordings.

ZD7288 has previously been shown to reduce the amplitude of CAPs in unmyelinated cerebellar parallel fibres, which is suggested to indicate an increase in conduction failures (Baginskas et al. 2009). Therefore, to aid interpretation of any ZD7288 effect upon the synaptic response failure rate in monosynaptic C-fibre input to lamina I NK1R+ neurons (detailed below), the effect of ZD7288 on the amplitude of the C-fibre response was examined, by measuring the amplitude of the response, defined as the difference between the positive and negative peak (as described previously in section 2.3.3 and figure 2.6), during the first stimuli delivered during baseline and ZD7288 recordings. Amplitude was only measured during the first stimuli so as to study the effect of ZD7288 in isolation from the effect of repetitive stimulation.

Influence of HCN channels on activity-dependent slowing in monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons

To investigate the capacity of HCN channels to constrain ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons in tissue isolated from control and CFA treated rats, patch-clamp recordings were made from pre-labelled neurons in the manner described above in section 4.3.4. Monosynaptic C-fibre input was stimulated 40 times at 2, 5 and 10Hz, at an intensity of 500μA, with a stimulus duration of 0.1ms, prior to ('baseline') and during application of 10μM ZD7288. ZD7288 was

bath applied and a wash-in period of 10 minutes was allowed between baseline and ZD7288 recordings. A 5 minute period was left between recordings using 2, 5 and 10Hz stimulation to allow the tissue to recover from ADS (de Col et al. 2008, 2012). The latency of monosynaptic C-fibre responses was measured as the time between the stimulus artefact and the onset of the eEPSC (figure 4.11). Data were normalised by calculating the latency change from the first stimuli. To determine the degree of ZD7288 facilitation, the baseline latency change was subtracted from the latency change recorded during ZD7288 application. To determine whether ZD7288 altered the initial latency of the C-fibre response, the latency measured during the first stimuli during baseline and ZD7288 recordings were compared.

ZD7288 has previously been shown to increase the number of failures in saphenous nerve recordings (Zhu et al. 2009) and in monosynaptic C-fibre input to unidentified lamina I/II neurons (Papp et al. 2006). To investigate whether ZD7288 similarly alters the number of synaptic response failures in monosynaptic C-fibre input, which is likely indicative of C-fibre conduction failures, the failure rate in response to 2, 5 and 10Hz stimulation was recorded prior to and during ZD7288 application.

The eEPSC amplitude of monosynaptic C-fibre input to unidentified lamina II neurons is known to be attenuated by ZD7288 (Takasu et al. 2010). To assess whether ZD7288 application altered the amplitude of monosynaptic C-fibre input to lamina I NK1R+ neurons, the peak amplitude of the C-fibre eEPSC during the first stimuli of baseline and ZD7288 trains was measured, as described previously in section 2.3.5 and figure 2.15A. Only the first stimuli was measured to enable the study of the effect of ZD7288 in isolation from any potential effects of repetitive stimulation.

4.3.6 Statistical analysis

To ascertain whether it was appropriate to use parametric or non-parametric statistical tests, or whether data should be transformed prior to performing parametric tests, all data were assessed for normality using D'Agostino & Pearson omnibus normality tests, or Kolmogorov-Smirnov tests if sample sizes were insufficient to use the former.

In CAP recordings from isolated dorsal roots and in eEPSC recordings of monosynaptic Aδ- and monosynaptic C-fibre input to lamina I NK1R+ neurons, the effects of CFA inflammation on the the responses to repetitive stimulation were analysed using linear regression. Lines of best fit were fitted to the absolute latency data and to the normalised ('latency change') data. In the case of normalised data, the

intercept was constrained to X = stimulus 1, Y = 0ms. To determine whether a response was altered by repetitive stimulation and thus exhibited ADS, the slope of the data was compared to a hypothetical slope of 0. To analyse whether CFA inflammation altered the response, the slope of the control group was compared to the slope of the CFA group. When the absolute latency data were plotted, in many cases there appeared to be difference in the intercept of the line of best fit between control and CFA groups, therefore the intercepts were compared in addition to the slopes. In all cases slopes and intercepts were compared using a sum-of-squares F test. To compare the effect of different stimulation frequencies and CFA inflammation on ADS, the AUC of latency change was calculated. In CAP recordings, AUC of latency change was statistically analysed using 2-way repeated measures ANOVA, while in eEPSC data 2-way ANOVA was used. To determine whether the initial latency of responses in CAP and eEPSC recordings was altered by CFA inflammation, the latencies recorded during stimuli 1 were compared using 2-way ANOVA followed by Bonferroni post-tests, in the case of CAP recordings a 2-way repeated measures ANOVA was used. 2-way ANOVA or an unpaired t-test was used to determine whether the length of dorsal root stimulated differed between groups. Linear regression was used, as described above, to analyse the effects CFA inflammation on the C-fibre failure rate during repetitive stimulation. The total C-fibre failure rates during 2, 5 and 10Hz stimulation, in control and CFA groups were compared using 2-way ANOVA followed by Tukey post-tests. To assess whether the distribution of the AUC of latency change was altered by CFA inflammation, a Kolmogorov-Smirnov 2-sample test was used. Pearson's r test was used to determine whether the initial latency of monosynaptic C-fibre input to lamina I NK1R+ neurons was correlated with the AUC of latency change.

In CAP and eEPSC recordings, the effect of ZD7288 (or vehicle in the case of CAP recordings) application on C-fibre ADS was analysed using linear regression. Lines of best fit were fitted to absolute and normalised ('latency change') data, in the case of normalised data the intercept was constrained to X = stimulus 1, Y = 0ms. To determine whether ADS was present, the slopes were compared to a hypothetical slope of 0. To ascertain the effects of ZD7288 (or vehicle), the baseline slope was compared to the ZD7288 slope. Where absolute latency data were analysed, the intercepts of the slope were also compared. To compare the magnitude of ZD7288 facilitation in control and CFA inflammation groups, linear regression was used as described above to compare the slopes of facilitation recorded in tissue isolated from control and CFA treated rats. A sum-of-squares F test was used in all cases to compare slopes and intercepts. 2-way repeated measures ANOVA followed by

Bonferroni post-tests was used to statistically analyse the effect of ZD7288 on the initial response latency and on the C-fibre peak amplitude. The effect of ZD7288 on the synaptic response failure rate in monosynaptic C-fibre input to lamina I NK1R+ neurons was assessed using 2-way repeated measures ANOVA followed by Bonferroni post-tests.

All data are presented as mean $\pm$ standard error of the mean (SEM). All statistical analysis was performed using Prism 6 (Graphpad Software).

4.4 Results

4.4.1 Inflammatory pain attenuates activity-dependent slowing in C-, but not A-fibres in isolated rat dorsal roots

Isolated dorsal roots were stimulated repetitively at frequencies of 1 and 2Hz to assess whether ADS can be detected in extracellular electrophysiological population recordings and whether CFA inflammation alters this phenomenon.

Representative traces of CAP recordings of the Aβ-fibre latency shift during repetitive stimulation at 2Hz, in dorsal roots isolated from control and CFA treated rats are shown in figure 4.3A. Analysis of the absolute latency of Aβ-fibre responses demonstrated that 1 and 2Hz stimulation did not alter the latency, while CFA inflammation did not alter the slope (P=0.953 & P=0.917, respectively, linear regression, figure 4.3B & C, table 4.1) or the intercept (P=0.325 & P=0.172, respectively, linear regression). When the data were normalised, it was shown that repetitive stimulation at 1 and 2Hz caused a significant reduction in the response latency, indicating 'activity-dependent speeding' (all $P<0.0001$, linear regression, figure 4.3D & C, table 4.2). During 1Hz stimulation, no significant difference was detected in the degree of latency change between control and CFA groups (P=0.445, linear regression), however during 2Hz stimulation, there was significantly less speeding in dorsal roots isolated from CFA treated rats (P=0.0002, linear regression, figure 4.3E, table 4.1). When the AUC of latency change was compared, this demonstrated that the Aβ-fibre response to repetitive stimulation was not influenced by stimulation frequency (P=0.536, 2-way repeated measures ANOVA, figure 4.6A), or CFA inflammation (P=0564. 2-way repeated measures ANOVA) and there was no interaction between these factors (P=0.304, 2-way repeated measures ANOVA).

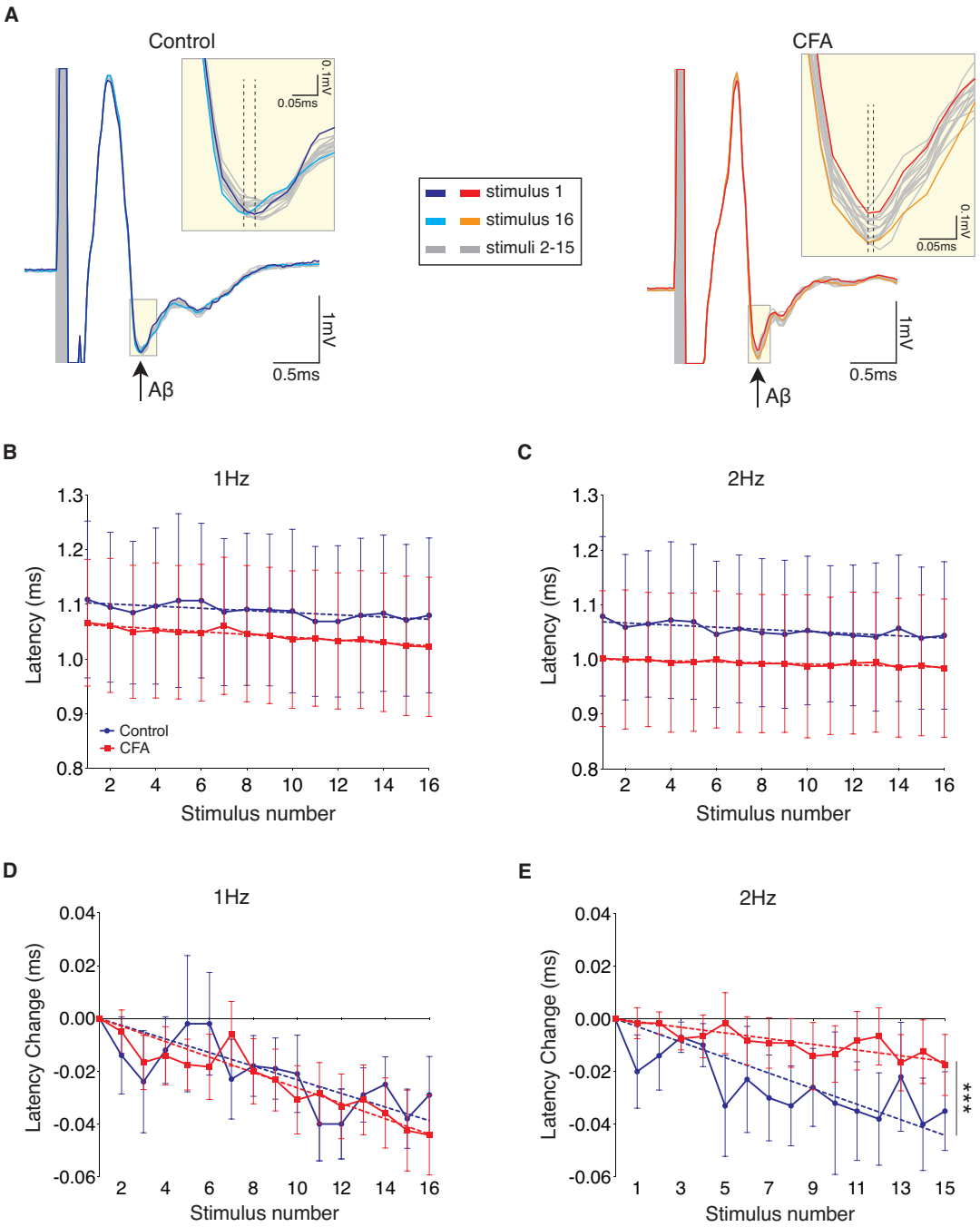

Figure 4.3: Aβ-fibres exhibit activity-dependent speeding. ***A*** Representative traces of Aβ-fibre compound action potentials recorded in dorsal roots isolated from control (left) and CFA (right) treated rats, during 2Hz stimulation. Large yellow box shows magnified portion of the negative peak of the response, dotted lines intersect negative peak of the response. ***B*** & ***C*** show absolute latency values recorded during 1 and 2Hz stimulation, respectively. Stimulation at these frequencies did not significantly alter the absolute latency, in control or CFA roots. CFA inflammation did not significantly alter the slope or intercept of the data recorded during stimulation at 1 or 2Hz. When the data were normalised, it was found that repetitive stimulation at 1 (***D***) and 2Hz (***E***) resulted in a small but significant decrease in Aβ-fibre response latency (all $P<0.0001$). CFA inflammation significantly attenuated the latency change seen during 2Hz ($P=0.0002$) but not 1Hz stimulation ($P=0.445$). Statistics: linear regression. Slope, r^2 and 95% CI values for ***B***/***C*** & ***D***/***E*** are presented in tables 4.1 & 4.2, respectively. Control n=10, CFA n=12. Legends in ***B*** apply to ***C***, ***D*** & ***E***. Error bars indicate SEM. Dashed lines represent line of best fit. $***P<0.001$.

Figure 4.4A shows representative traces of Aδ-fibre CAP recordings during repetitive stimulation at 2Hz, in dorsal roots isolated from control and CFA treated rats. The absolute latency of the Aδ-fibre response was not altered by repetitive stimulation at 1 or 2Hz and there was no difference in the slopes fitted to data obtained from control and CFA groups (P=0.923 & P=0.356, respectively, linear regression, figure 4.4B & C, table 4.1). In dorsal roots stimulated at 1 and 2Hz, the intercept was significantly greater in the CFA group (P=0.0006 & P<0.0001, respectively, linear regression). When the data were normalised, it was found that 1 and 2Hz stimulation resulted in a small but significant progressive increase in response latency (all P<0.0001, figure 4.4D & E, respectively, linear regression, table 4.2). CFA inflammation was without effect during both 1Hz and 2Hz stimulation (P=0.075 & P=0.356, respectively, linear regression). Analysis of the AUC of the latency change confirmed that CFA inflammation did not alter ADS in Aδ-fibres (P=0.533, 2-way repeated measures ANOVA, figure 4.6B) and showed that the magnitude of ADS was not significantly altered by stimulation frequency (P=0.812, 2-way repeated measures ANOVA) and there was no interaction between these variables (P=0.949, 2-way repeated measures ANOVA).

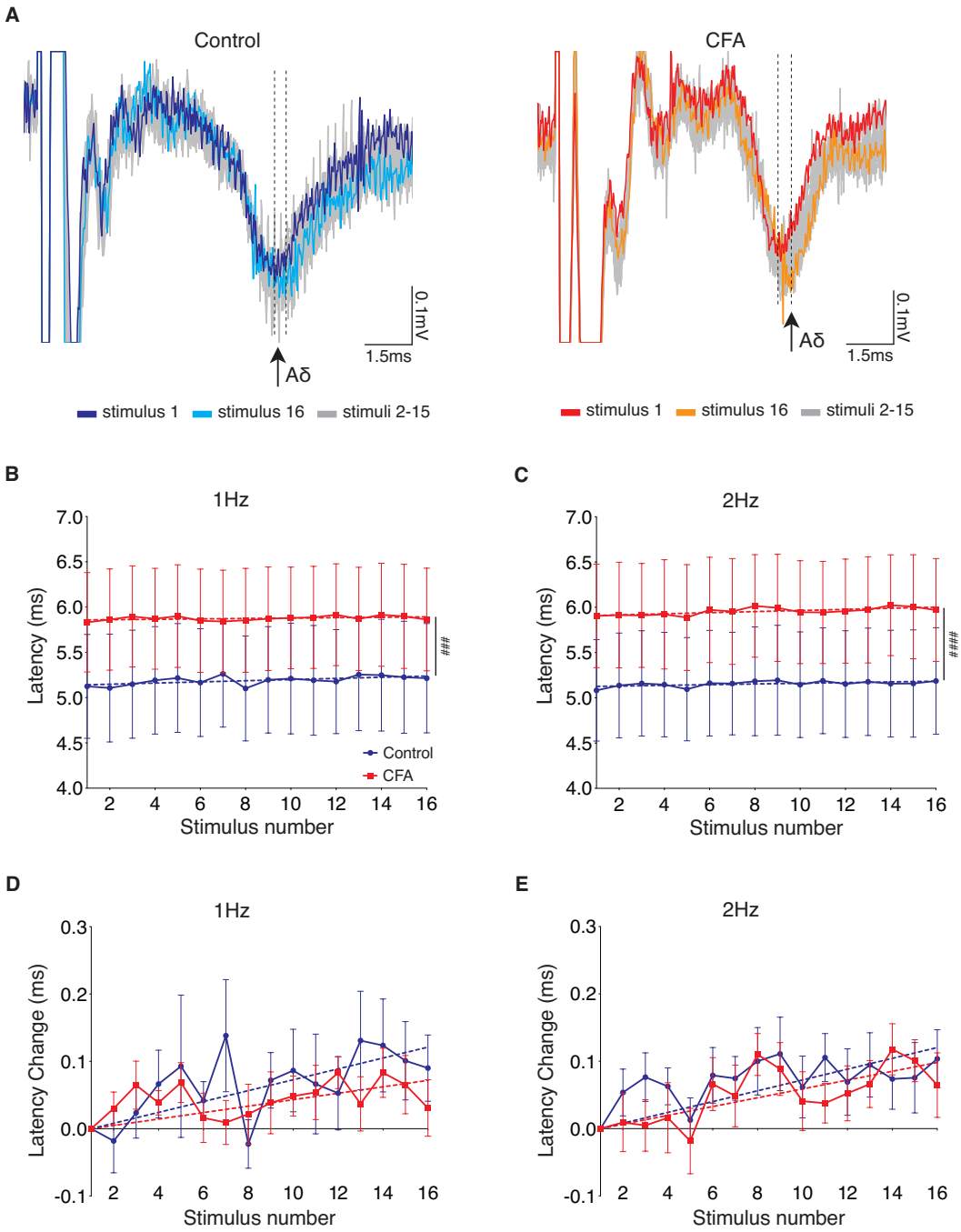

Figure 4.4: Aδ-fibre activity-dependent slowing. ***A*** Representative traces of Aδ-fibre compound action potentials recorded in dorsal roots isolated from control (left) and CFA (right) treated rats, during 2Hz stimulation. Dotted lines intersect negative peak of the response during stimuli 1 and 16. The absolute latency of the Aδ-fibre response did not significantly change during repetitive stimulation at 1 (***B***) or 2Hz (***C***). The intercept of the slope was greater in CFA inflammation, during 1 and 2Hz stimulation (P=0.0006 & P<0.0001, respectively), but CFA did not affect the slope. Analysis of normalised data revealed that repetitive stimulation at 1 (***D***) and 2Hz (***E***) produced a significant progressive increase in response latency (all P<0.0001), however CFA inflammation was without effect (1Hz P=0.075, 2Hz P=0.356). Statistics: linear regression. Slope, r^2 and 95% CI values for ***B***/***C*** & ***D***/***E*** are presented in tables 4.1 & 4.2, respectively. Control n=11, CFA n=13. Legends in ***B*** apply to ***C***, ***D*** & ***E***. Error bars indicate SEM. Dashed lines represent line of best fit. Control vs. CFA, intercept: ### P<0.001, #### P<0.0001.

Representative traces of C-fibre CAP recordings, in dorsal roots isolated from control and CFA treated rats, during repetitive stimulation at 2Hz are shown in figure 4.5A, which clearly demonstrates the shift in latency that arises due to ADS. Analysis of the absolute latency of the C-fibre response revealed that 2Hz, but not 1Hz, stimulation of dorsal roots isolated from control treated rats resulted in a significant increase in latency (P=0.038, linear regression, figure 4.5B, table 4.1), while no significant differences were seen in CFA tissue. CFA inflammation did not alter the slope of the data recorded during 1 or 2Hz stimulation (P=0.957 & P=0.628, respectively, linear regression, figure 4.5A & B, table 4.1). In the CFA group, the intercept was significantly less in data obtained during stimulation at 1 and 2Hz (both $P<0.0001$, linear regression). Analysis of normalised data revealed that dorsal root stimulation at 1 and 2Hz resulted in a significant progressive increase in C-fibre response latency (all $P<0.0001$, linear regression, figure 4.5D & E, respectively, table 4.2). Dorsal roots isolated from CFA treated rats displayed significantly less ADS than control dorsal roots during stimulation at 1 and 2Hz (both $P<0.0001$, linear regression). Comparison of the AUC of latency change confirmed that CFA inflammation significantly reduced C-fibre ADS (P=0.0006, 2-way repeated measures ANOVA, figure 4.6C) and revealed that 2Hz stimulation resulted in significantly greater ADS than 1Hz (P=0.0009, 2-way repeated measures ANOVA). There was no interaction between CFA inflammation and stimulation frequency (P=0.719, 2-way repeated measures ANOVA).

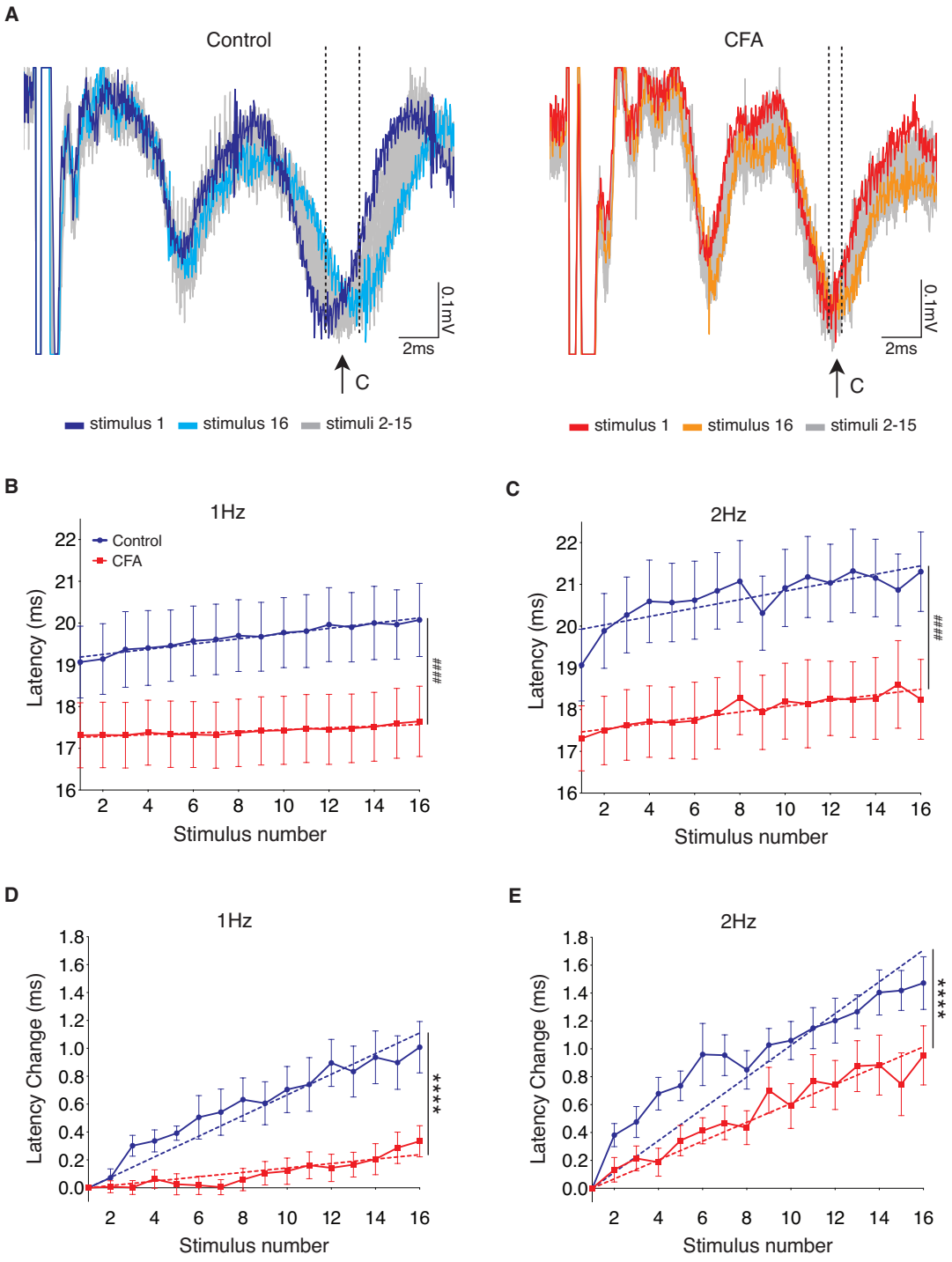

Figure 4.5: CFA inflammation attenuates activity-dependent slowing in C-fibres in isolated dorsal roots. ***A*** Representative traces of C-fibre compound action potentials recorded in dorsal roots isolated from control (left) and CFA (right) treated rats, during 2Hz stimulation, dotted lines transect the negative peak of the components. ***B*** & ***C*** The absolute C-fibre latency was significantly increased in response to 2Hz stimulation, in control dorsal roots only (P=0.038). CFA inflammation did not alter the slope of the fitted line, but significantly reduced the intercept during 1 and 2Hz stimulation (both P$<$0.0001). When the data were normalised, stimulation at 1 (***D***) and 2Hz (***E***) was found to result in a significant increase in the C-fibre response latency (all P$<$0.0001), which was significantly attenuated by CFA inflammation (both P$<$0.0001). Statistics: linear regression. Slope, r^2 and 95% CI values for ***B***/***C*** & ***D***/***E*** are presented in tables 4.1 & 4.2, respectively. Control n=11, CFA n=13. Legends in ***B*** apply to ***C***, ***D*** & ***E***. Error bars indicate SEM. Dashed lines represent line of best fit. Control vs. CFA, slope: ****P$<$0.0001, intercept: #### P$<$0.0001.

Input	Frequency (Hz)	Treatment	Y Intercept	Intercept 95% CI	Control vs. CFA	Slope	Slope 95% CI	Slope vs. Slope = 0	Control vs. CFA	r^2
Aβ	1	control	1.10	0.977 to 1.229	P=0.325	-0.002	-0.016 to 0.012	P=0.784	P=0.953	0.000
		CFA	1.06	0.951 to 1.175		-0.003	-0.015 to 0.010	P=0.692		0.000
	2	control	1.07	0.948 to 1.190	P=0.172	-0.002	-0.016 to 0.012	P=0.780	P=0.917	0.000
		CFA	1.00	0.886 to 1.115		-0.001	-0.014 to 0.012	P=0.886		0.000
Aδ	1	control	5.14	4.605 to 5.678	P=0.0006	0.007	-0.055 to 0.067	P=0.837	P=0.923	0.000
		CFA	5.86	5.348 to 6.369		0.002	-0.056 to 0.060	P=0.939		0.000
	2	control	5.13	4.602 to 5.651	P<0.0001	0.030	-0.056 to 0.063	P=0.900	P=0.356	0.000
		CFA	5.91	5.388 to 6.431		0.006	-0.053 to 0.065	P=0.840		0.000
C	1	control	19.19	18.41 to 19.96	P<0.0001	0.045	-0.025 to 0.150	P=0.164	P=0.957	0.011
		CFA	17.27	16.54 to 18.00		0.020	-0.063 to 0.103	P=0.634		0.001
	2	control	19.93	19.10 to 20.76	P<0.0001	0.048	0.006 to 0.196	P=0.038	P=0.628	0.025
		CFA	17.47	16.66 to 18.27		0.068	-0.024 to 0.160	P=0.148		0.010

Table 4.1: Comparison of the absolute latency in Aβ-, Aδ- and C-fibre CAP recordings.

Input	Frequency (Hz)	Treatment	Slope	r^2	95% CI	Slope vs. Slope = 0	Control vs. CFA
Aβ	1	control	-0.003	0.036	-0.003 to -0.002	P<0.0001	P=0.445
		CFA	-0.003	0.080	-0.004 to -0.002	P<0.0001	
	2	control	-0.003	0.021	-0.004 to -0.002	P<0.0001	P=0.0002
		CFA	-0.001	0.019	-0.002 to -0.001	P<0.0001	
Aδ	1	control	0.008	0.021	0.005 to 0.011	P<0.0001	P=0.075
		CFA	0.005	-0.005	0.003 to 0.007	P<0.0001	
	2	control	0.008	-0.012	0.006 to 0.010	P<0.0001	P=0.356
		CFA	0.007	0.039	0.004 to 0.009	P<0.0001	
C	1	control	0.074	0.258	0.004 to 0.082	P<0.0001	P<0.0001
		CFA	0.016	0.089	0.011 to 0.020	P<0.0001	
	2	control	0.114	0.339	0.106 to 0.122	P<0.0001	P<0.0001
		CFA	0.068	0.220	0.059 to 0.076	P<0.0001	

Table 4.2: Comparison of the latency change in Aβ-, Aδ- and C-fibre CAP recordings.

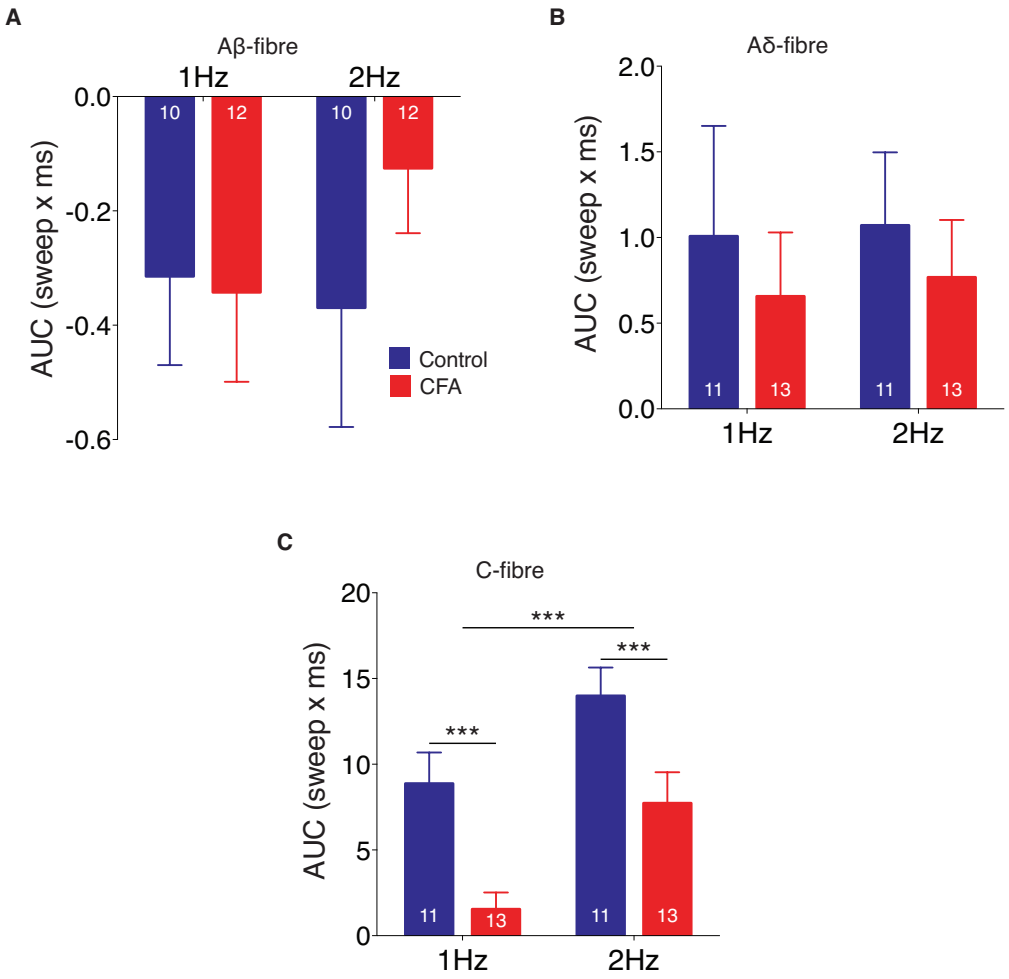

Figure 4.6: Area under the curve analysis of activity-dependent slowing in isolated dorsal roots. ADS was unaffected by CFA inflammation, stimulation frequency and there was no interaction between these factors in Aβ- (***A***, P=0.564, P=0.536 & P=0.304 respectively) or Aδ-fibres (***B***, P=0.533, P=0.812 & P=0.949, respectively). ***C*** In C-fibres, there was significantly greater ADS at 2Hz than 1Hz (P=0.0009), while CFA inflammation significantly reduced the level of ADS (P=0.0006) irrespective of frequency (P=0.719). All statistics: 2-way repeated measures ANOVA. Sample sizes indicated on bars. Legend in ***A*** applies to all. Error bars indicate SEM. $***P<0.001$.

To confirm that the length of dorsal root stimulated was similar between control and CFA groups, the length of dorsal root stimulated was compared. The length of isolated dorsal roots used in these CAP studies did not significantly differ between control and CFA groups (P=0.777, unpaired t-test, figure 4.7A) and so the inflammation induced changes in ADS that were observed are unlikely to have resulted due to differences in the length of dorsal root stimulated.

The initial response latency of the primary afferent components, recorded at stimuli 1 during 1Hz stimulation, was significantly altered by fibre type ($P<0.0001$, 2-way repeated measures ANOVA, figure 4.7B). CFA inflammation did not alter the initial

latency (P=0.458, 2-way repeated measures ANOVA) and there was no interaction between these factors (P=0.108, 2-way repeated measures ANOVA). There is evidence that primary afferents that display greater initial response latencies show increased ADS (Ringkamp et al. 2010, Shim et al. 2007, Swadlow & Waxman 1976, Weidner et al. 1999, Zhu et al. 2009), although this is disputed by others (Gee et al. 1996, Serra et al. 1999, Taguchi et al. 2010, Thalhammer et al. 1994). Therefore, the changes in ADS seen during CFA inflammation reported here are unlikely to have arisen due to alterations in initial latency. While these data do contradict the findings in figures 4.4 and 4.5, that show significant differences in the intercept between control and CFA groups for Aδ- and C-fibre responses, it should be noted that the intercept is extrapolated from all data points and thus subject to influence from the response to repetitive stimulation (i.e. the slope of the line of best fit). Therefore, comparing the initial latency, which is not influenced by ADS, can be considered to be a more accurate measure.

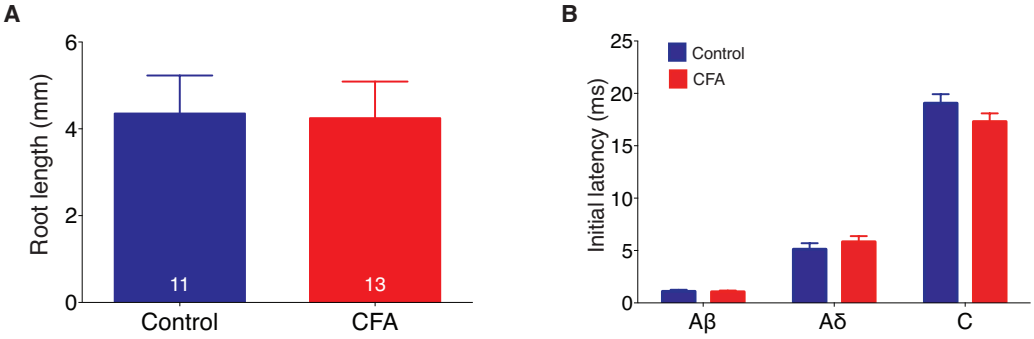

Figure 4.7: Initial latency of Aβ-, Aδ- and C-fibre components in isolated dorsal roots. ***A*** The dorsal root length in control and CFA groups was not significantly different (P=0.777, unpaired t-test). ***B*** The initial latency of the responses was significantly different between the different primary afferent components (P<0.0001, 2-way repeated measures ANOVA), however CFA inflammation was without effect and there was no interaction between these factors (P=0.458 & P=0.108, respectively, 2-way repeated measures ANOVA). Sample size for ***A*** indicated on bars, ***B***: Aδ & C; control n=11, CFA n=13, Aβ; control n=10, CFA n=12. Error bars indicate SEM.

4.4.2 Activity-dependent slowing in monosynaptic Aδ-fibre input to lamina I neurokinin 1 receptor expressing neurons

To investigate ADS in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons, Aδ-fibre eEPSCs were recorded while stimulating the dorsal root at frequencies of 1, 2 and 10Hz. Figure 4.8 shows representative traces of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons, in tissue isolated from control and CFA treated rats, during repetitive stimulation at 2Hz, which demonstrates a negligible latency change. The

absolute latency of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons was not altered by repetitive stimulation at 1, 2 or 10Hz. CFA inflammation did not alter the slope fitted to the data recorded during any of these three stimulation frequencies (P=0.935, P=0.995 & P=0.994, respectively, linear regression, figure 4.9A, B & C, table 4.3). The intercept was significantly less in the CFA group during stimulation at 1 and 2Hz (P<0.0001 & P=0.006, respectively, linear regression), but not 10Hz (P=0.239, linear regression). However, when the initial latency of the monosynaptic Aδ-fibre response was compared this did not support the above data, in that the initial Aδ-fibre latency was not altered by CFA inflammation (P=0.631, 2-way ANOVA on rank transformed data, figure 4.9D) and did not differ between groups stimulated at 1, 2 or 10Hz (P=0.658, 2-way ANOVA on rank transformed data). There was no significant interaction between these factors (P=0.600, 2-way ANOVA on rank transformed data). The length of dorsal root stimulated did not differ between groups (P=0.305, frequency P=0.497, interaction P=0.976, 2-way ANOVA, figure 4.9E).

Analysis of the normalised data demonstrated that stimulation of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons at frequencies of 1, 2 and 10Hz, in tissue isolated from control and CFA treated rats, resulted in a small but significant progressive increase in response latency (all P<0.0001, linear regression, figure 4.10A, B & C, table 4.4). The degree of ADS elicited by 1 or 2Hz stimulation was not affected by CFA inflammation (P=0.946 & P=0.277, respectively, linear regression, figure 4.10A, & B, table 4.4). The ADS elicited by Aδ-fibre stimulation at 10Hz, was significantly reduced in tissue isolated from CFA treated rats (P=0.010, linear regression, figure 4.10C, table 4.4). AUC analysis showed that the magnitude of ADS was significantly altered by stimulation frequency (P=0.003, 2-way ANOVA, figure 4.10D). CFA inflammation was without effect (P=0.465, 2-way ANOVA) and there was no interaction between these variables (P=0.641, 2-way ANOVA).

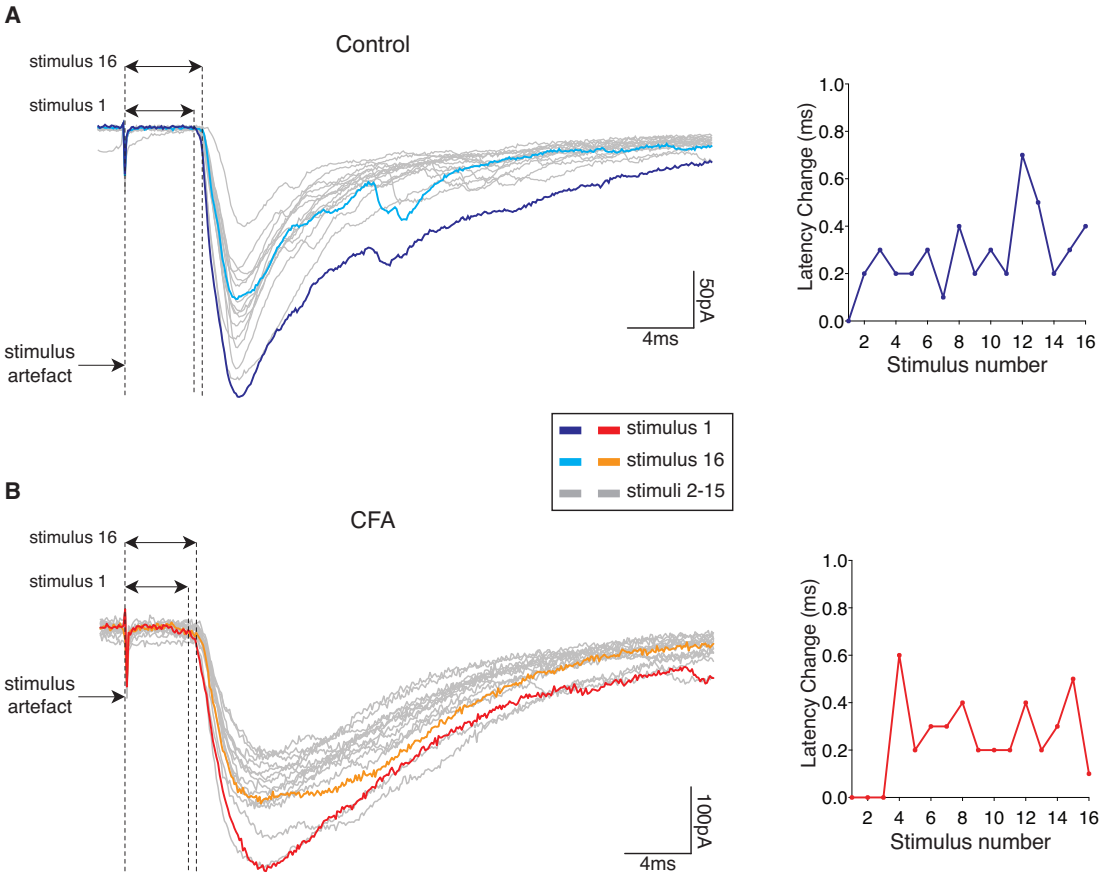

Figure 4.8: Representative traces of Aδ-fibre activity-dependent slowing. Traces (left) show Aδ-fibre eEPSCs recorded in tissue isolated from control (***A***) and CFA treated (***B***) rats, during 2Hz stimulation. Latency was measured as the time between the stimulus artefact and the monosynaptic response, indicated by the broken lines and arrows. The latency change calculated from each example is shown to the right of the trace.

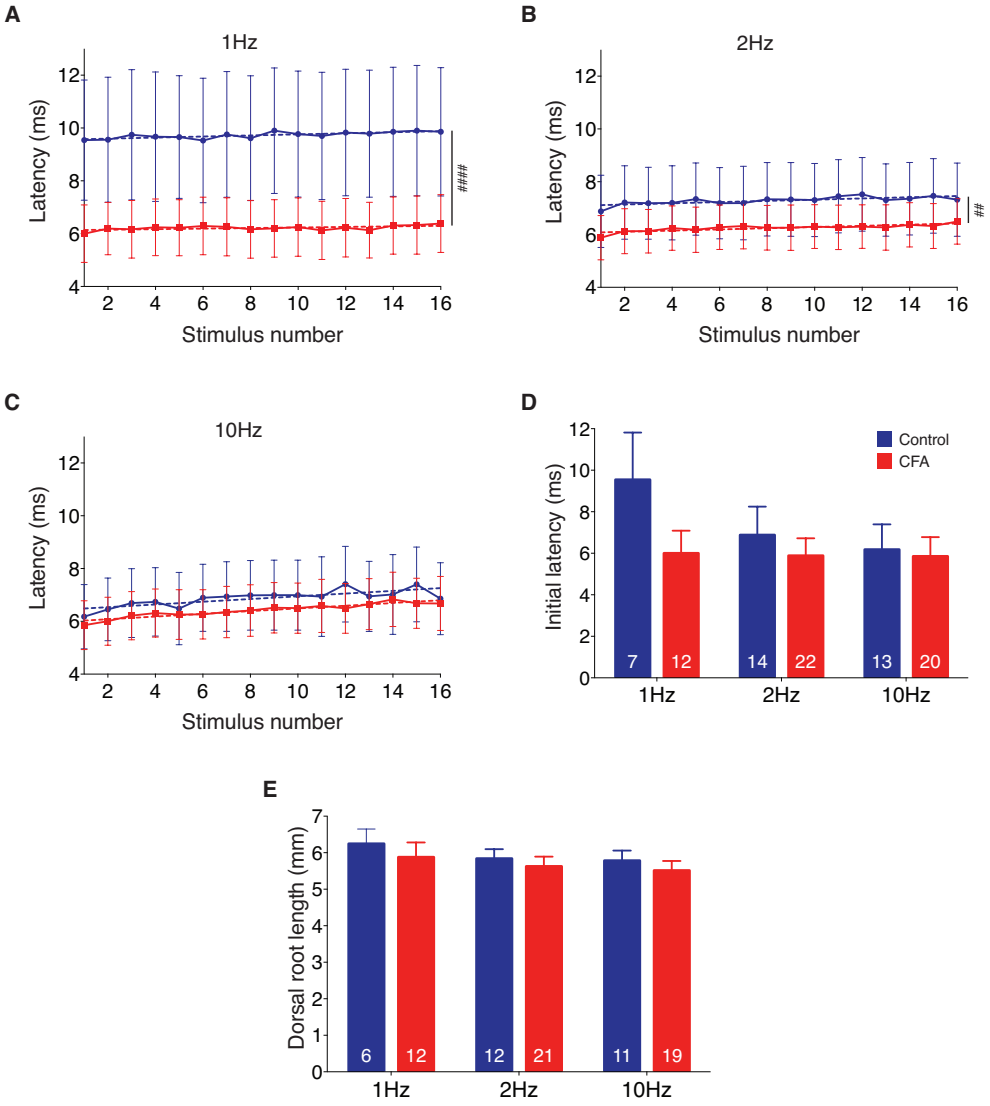

Figure 4.9: ***A*** , ***B*** & ***C*** show the absolute latency of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons recorded during repetitive stimulation at 1, 2 and 10Hz, respectively. In all cases the slope of absolute latency was unaltered by repetitive stimulation, while CFA inflammation was also without effect (1Hz P=0.935, 2Hz P=0.995, 10Hz P=0.994). CFA inflammation significantly reduced the intercept in the groups stimulated at 1 and 2Hz ($P<0.0001$ & P=0.006, respectively), but not 10Hz (P=0.239). ***D*** The initial latency of the Aδ-fibre responses did not differ between control and CFA groups (P=0.631) or between stimulation frequencies (P=0.658) and there was no interaction between these factors (P=0.600). ***E*** The length of dorsal root stimulated did not differ between control and CFA groups (P=0.305) or between the different stimulation frequencies (P=0.497) and there was no significant interaction (P=0.976). Statistics (***A***–***C***): linear regression. Slope, r^2 and 95% CI values for ***A***–***C*** are presented in table 4.3. Statistics (D): 2-way ANOVA on rank transformed data. Statistics (E): 2-way ANOVA. Sample sizes for ***A***–***D*** indicated on bars in ***D***. Sample sizes for ***E*** indicated on bars. Legend in ***A*** applies to ***B*** & ***C***. Legend in ***D*** applies to ***E***. Error bars indicate SEM. Dashed lines represent line of best fit. Control vs. CFA intercept: ## $P<0.01$, #### $P<0.0001$.

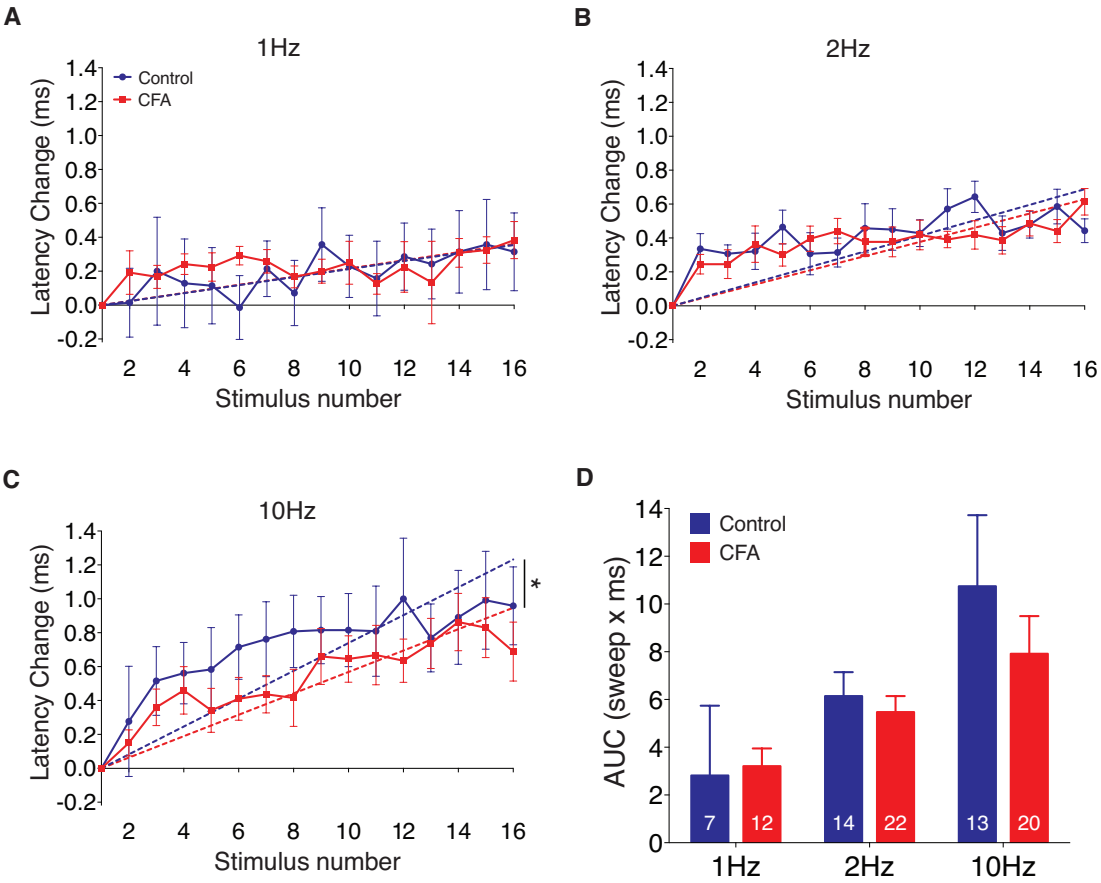

Figure 4.10: Activity-dependent slowing in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons. Stimulation at 1 (***A***), 2Hz (***B***) and 10Hz (***C***) resulted in a small but significant increase in response latency (all $P<0.0001$). CFA inflammation was without effect when monosynaptic Aδ-fibre input to lamina I NK1R+ neurons was stimulated at 1 or 2Hz ($P=0.945$ & $P=0.277$, respectively). However, during 10Hz stimulation there was a small but significant reduction in the latency change in the CFA inflammation group ($P=0.010$). ***D*** Area under the curve analysis of latency change demonstrated that the degree of ADS was significantly altered by stimulation frequency ($P=0.003$), but CFA inflammation was without effect ($P=0.465$) and there was no interaction ($P=0.641$). Statistics (***A***–***C***): linear regression. Slope, r^2 and 95% CI values are presented in table 4.4. Statistics (***D***): 2-way ANOVA. Sample sizes indicated on bars in ***D***. Legend in ***A*** applies to ***B*** & ***C***. Error bars indicate SEM. Dashed lines represent line of best fit. $*P<0.05$.

Frequency (Hz)	Treatment	Y Intercept	Intercept 95% CI	Control vs. CFA	Slope	Slope 95% CI	Slope vs. Slope = 0	Control vs. CFA	r^2
1	control	9.577	7.459 to 11.69	P<0.0001	0.020	-0.220 to 0.261	P=0.867	P=0.935	0.000
	CFA	6.138	5.163 to 7.114		0.011	-0.100 to 0.121	P=0.851		0.000
2	control	7.114	5.861 to 8.368	P=0.006	0.023	-0.119 to 0.165	P=0.752	P=0.995	0.000
	CFA	6.081	5.306 to 6.857		0.023	-0.066 to 0.111	P=0.616		0.000
10	control	6.485	5.290 to 7.679	P=0.239	0.052	-0.086 to 0.189	P=0.464	P=0.994	0.003
	CFA	6.029	5.160 to 6.897		0.051	-0.048 to 0.151	P=0.313		0.003

Table 4.3: Comparison of absolute latency in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons during stimulation at 1, 2 and 10Hz.

Frequency (Hz)	Treatment	Slope	r^2	95% CI	Slope vs. Slope = 0	Control vs. CFA
1	control	0.024	0.029	0.012 to 0.040	P<0.0001	P=0.946
	CFA	0.024	-0.022	0.018 to 0.030	P<0.0001	
2	control	0.046	-0.028	0.040 to 0.052	P<0.0001	P=0.277
	CFA	0.042	-0.001	0.037 to 0.046	P<0.0001	
10	control	0.821	0.028	0.069 to 0.095	P<0.0001	P=0.010
	CFA	0.063	0.091	0.055 to 0.071	P<0.0001	

Table 4.4: Comparison of latency change in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons, during stimulation at 1, 2 and 10Hz.

4.4.3 Activity-dependent slowing in monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons is reduced in inflammatory pain

To assess ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons, C-fibre eEPSCs were recorded in response to dorsal root stimulation at 1 and 2Hz. Representative traces of C-fibre eEPSCs recorded during 1Hz stimulation, in tissue isolated from control and CFA treated rats, are shown in figure 4.11, which demonstrates the progressive increase in response latency. The absolute latency of monosynaptic C-fibre input to lamina I NK1R+ neurons, recorded in tissue isolated from control rats, during stimulation at 1Hz, showed a significant progressive increase (P=0.044, linear regression, figure 4.12A, table 4.5). The absolute latency was not altered in CFA tissue during 1 or 2Hz stimulation, or in control tissue during 2Hz stimulation. CFA inflammation did not alter the slope of the data (1Hz P=0.533, 2Hz P=0.631, linear regression, figure 4.12A & B, table 4.5). The data recorded in tissue isolated from CFA treated rats, during 1 and 2Hz stimulation, displayed a significantly reduced intercept compared to control (both $P<0.0001$, linear regression). However, this finding was not supported by the analysis of the initial C-fibre latency, where CFA inflammation was found to be without effect (P=0.085, 2-way ANOVA, figure 4.13A). The initial latency was significantly greater in those neurons stimulated at 2Hz (P=0.020, 2-way ANOVA), without influence from CFA (P=0.590, 2-way ANOVA). This was paralleled by the finding that the length of dorsal root stimulated was significantly longer in the group stimulated at 2Hz (P=0.037, 2-way ANOVA, figure 4.13B). No significant differences in root length were detected between control and CFA inflammation groups (P=0.675, 2-way ANOVA) and there was no interaction between stimulation frequency and CFA inflammation (P=0.475, 2-way ANOVA).

When the ADS latency data were normalised, it was found that repetitive stimulation of monosynaptic C-fibre input to lamina I NK1R+ neurons, at frequencies of 1 and 2Hz resulted in a significant progressive increase in response latency, in both control and CFA groups (all $P<0.0001$, linear regression, figure 4.12C & D, table 4.6). In tissue isolated from CFA treated rats, the magnitude of ADS seen during 1 and 2Hz C-fibre stimulation was significantly reduced (both $P<0.0001$, linear regression). AUC analysis of latency change confirmed that CFA inflammation significantly reduced ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons ($P<0.0001$, 2-way ANOVA, figure 4.12E). There was a trend towards greater ADS during 2Hz stimulation, however this was not significant (P=0.060, 2-way ANOVA). There was no interaction between CFA inflammation and stimulation frequency (P=0.204, 2-way

ANOVA).

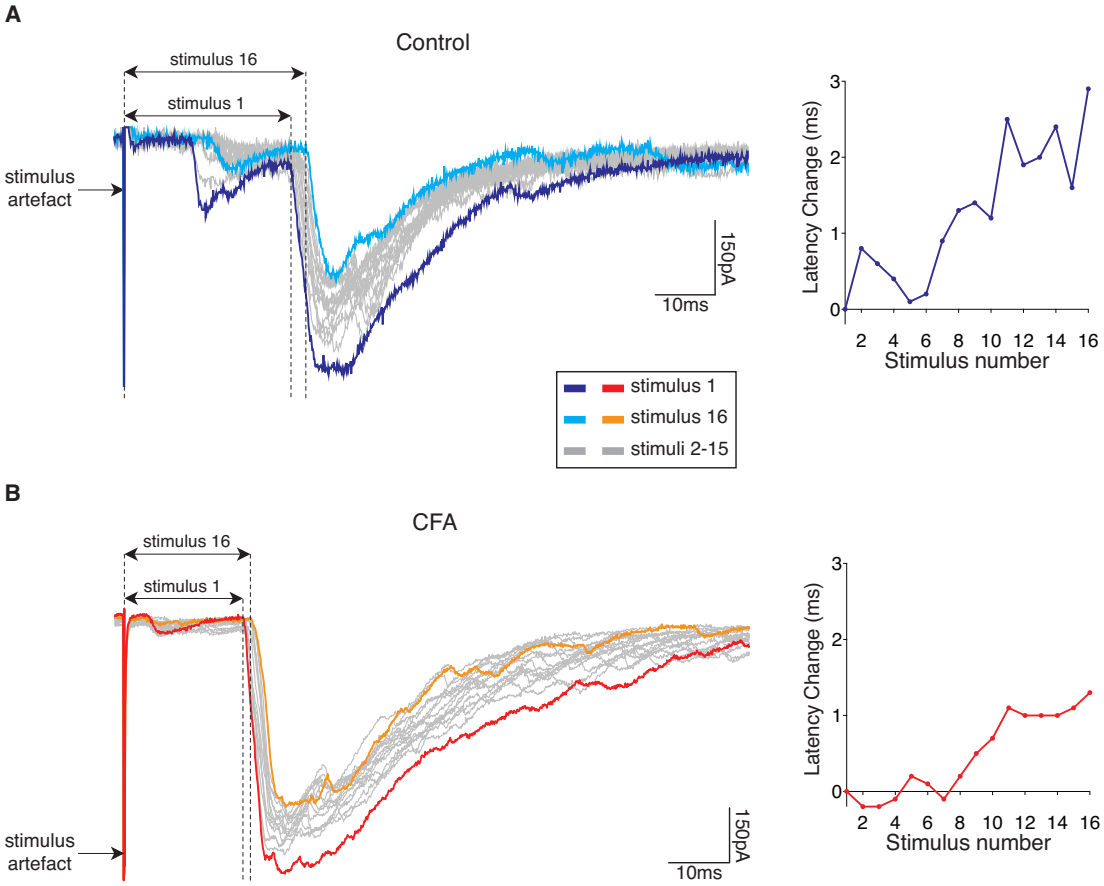

Figure 4.11: Representative traces of C-fibre activity-dependent slowing. Traces (left) show C-fibre eEPSCs recorded in tissue isolated from control (***A***) and CFA treated (***B***) rats, during 1Hz stimulation. Latency was measured as the time between the stimulus artefact and the onset of the response, indicated by the broken lines and arrows. The latency change measured from the example traces are shown to the right. Traces in ***A*** & ***B*** displayed with same X scale.

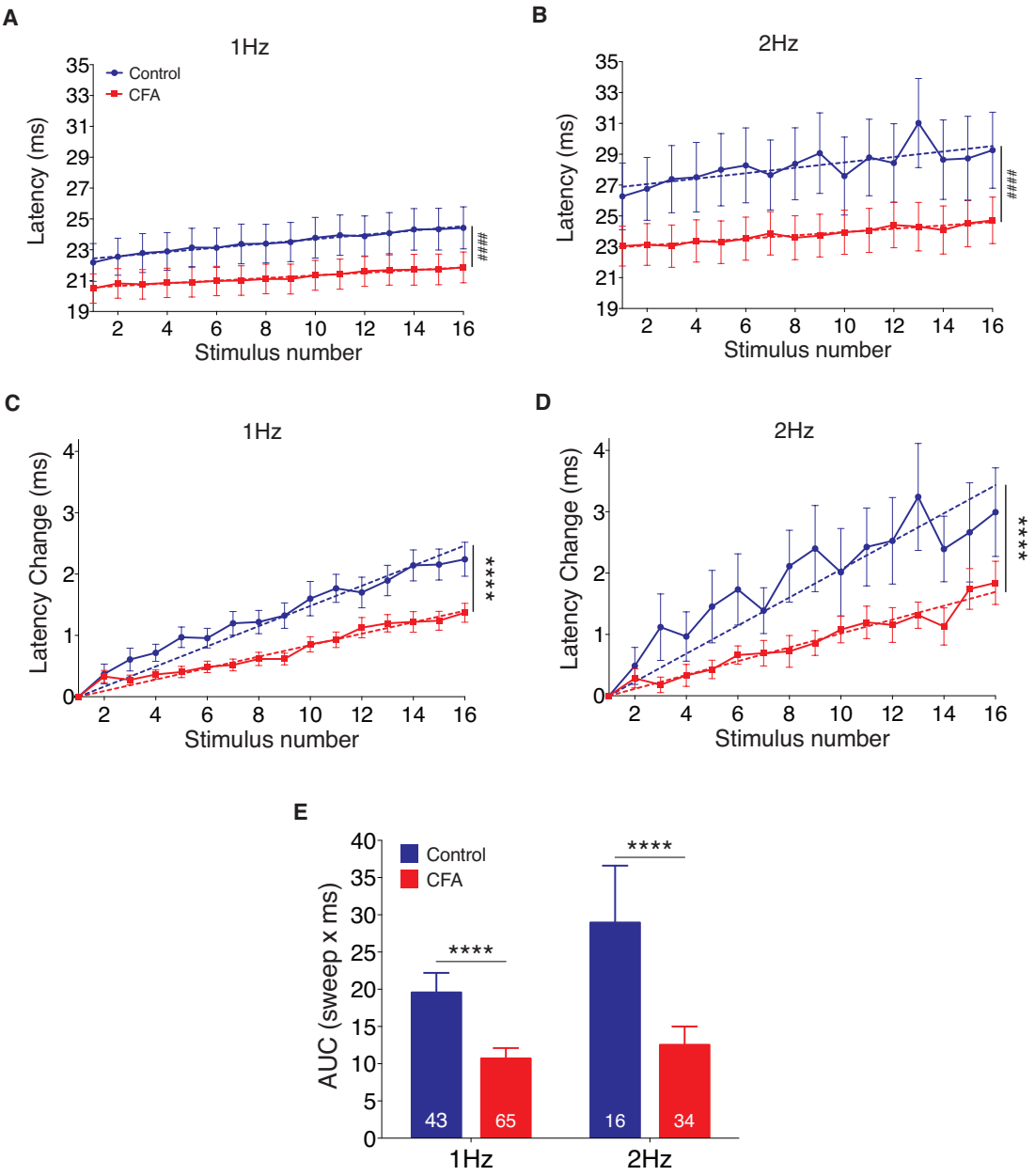

Figure 4.12: Activity-dependent slowing in monosynaptic C-fibre input to lamina I NK1R+ neurons is attenuated by CFA inflammation. C-fibre stimulation at 1 (***A***) or 2Hz (***B***) did not alter the absolute latency of the response, except in the control group during 1Hz stimulation (P=0.044). CFA inflammation significantly reduced the intercept of the line calculated from data obtained during 1 and 2Hz stimulation (both P$<$0.0001), but did not alter the slope. When the data were normalised, stimulation at 1 (***C***) and 2Hz (***D***) was shown to cause a significant progressive increase in response latency (all P$<$0.0001). The latency change seen during stimulation at these frequencies was significantly reduced by CFA inflammation (both P$<$0.0001). ***E*** Area under the curve analysis of latency change confirmed that CFA inflammation significantly reduced ADS (P$<$0.0001). There was a trend towards greater ADS at 2Hz, however this effect was not significant (P=0.060). There was no interaction between CFA inflammation and stimulation frequency (P=0.204). Statistics (***A***–***D***): linear regression. Slope, r^2 and 95% CI values for ***A***/***B*** and ***C***/***D*** are presented in tables 4.5 and 4.6, respectively. Statistics (***E***): 2-way ANOVA. Sample sizes indicated on bars in ***E***. Legend in ***A*** applies to ***B***–***D***. Error bars indicate SEM. Dashed lines represent line of best fit. Control vs. CFA slope: ****P$<$0.0001, intercept: #### P$<$0.0001

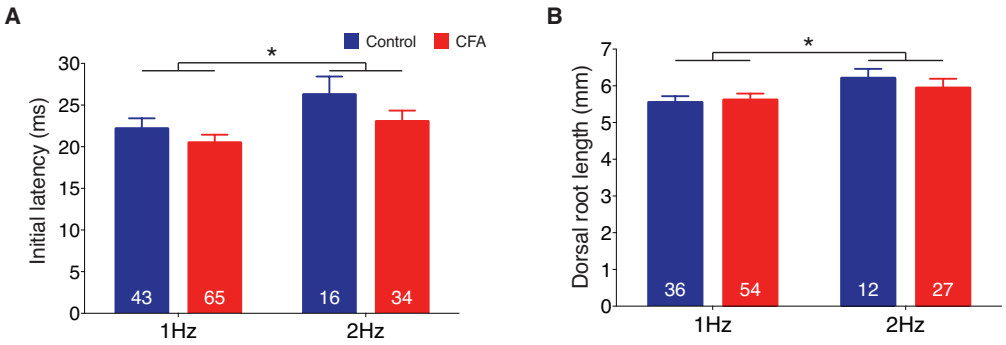

Figure 4.13: Dorsal root length and initial C-fibre eEPSC latency. ***A*** The initial latency in the 2Hz stimulation group was significantly greater than in the 1Hz group (P=0.020), however CFA was without effect (P=0.085) and there was no interaction between these factors (P=0.590). ***B*** The length of dorsal root stimulated was significantly greater in the group stimulated at 2Hz (P=0.037), but did not differ between control and CFA groups (P=0.675) and there was no interaction (P=0.475). Statistics: 2-way ANOVA. Legend in ***A*** applies to ***B***. Samples sizes indicated on bars. Error bars indicate SEM. $*P<0.05$.

Frequency (Hz)	Treatment	Y Intercept	Intercept 95% CI	Control vs. CFA	Slope	Slope 95% CI	Slope vs. Slope = 0	Control vs. CFA	r^2
1	control	22.44	21.25 to 23.63	P<0.0001	0.139	0.005 to 0.275	P=0.044	P=0.533	0.006
	CFA	20.57	19.67 to 21.47		0.086	-0.016 to 0.189	P=0.099		0.003
2	control	26.89	24.73 to 29.05	P<0.0001	0.176	-0.072 to 0.424	P=0.165	P=0.631	0.008
	CFA	22.98	21.68 to 24.28		0.664	-0.042 to 0.258	P=0.158		0.004

Table 4.5: Comparison of absolute latency in monosynaptic C-fibre input to lamina I NK1R+ neurons, during stimulation at 1 and 2Hz.

Frequency (Hz)	Treatment	Slope	r^2	95% CI	Slope vs. Slope = 0	Control vs. CFA
1	control	0.164	0.173	0.153 to 0.176	P<0.0001	P<0.0001
	CFA	0.93	0.148	0.087 to 0.100	P<0.0001	
2	control	0.229	0.106	0.196 to 0.262	P<0.0001	P<0.0001
	CFA	0.113	0.138	0.100 to 0.126	P<0.0001	

Table 4.6: Comparison of latency change in monosynaptic C-fibre input to lamina I NK1R+ neurons, during stimulation at 1 and 2Hz.

Monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons cannot be classified on the basis of activity-dependent slowing profiles

It has previously been established that C-fibres which convey different sensory modalities display different levels of ADS, namely that mechano-insensitive C-fibres exhibit a large degree of ADS, while mechano-sensitive C-fibres display minimal ADS (Gee et al. 1996, Obreja et al. 2010, Serra et al. 1999, Weidner et al. 1999). To assess whether it was possible to detect two distinct populations of neurons that displayed high or low levels of ADS, which may be indicative of those receiving input from mechano-insensitive or mechano-sensitive C-fibres, respectively, frequency histograms of the AUC of latency change were plotted. The AUC of latency change recorded during 1 and 2Hz stimulation, in tissue isolated from control and CFA treated rats, displayed a unimodal distribution in all cases. CFA inflammation caused a significant leftwards shift in the distribution, indicating less ADS, during 1 and 2Hz stimulation (P=0.012 & P=0.025, respectively, figure 4.14A & B, Kolmogorov-Smirnov 2-sample test).

Correlation between the initial latency of monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons and the magnitude of activity-dependent slowing

To determine whether the initial latency of the C-fibre eEPSC was predictive of the magnitude of ADS exhibited in monosynaptic C-fibre input to lamina I NK1R+ neurons, correlation between the initial C-fibre latency, recorded during stimulus 1, and the AUC of the latency change was assessed. During 1Hz stimulation, in tissue isolated from control and CFA treated rats, there was no significant correlation between initial latency and the AUC of the latency change (P=0.107 & P=0.358, respectively, figure 4.14C, Pearson's r test). During 2Hz stimulation, there was a significant positive correlation between the initial C-fibre latency and the AUC of latency change in tissue isolated from CFA treated (P=0.013, Pearson's r test, figure 4.14D), but not control rats (P=0.655, Pearson's r test).

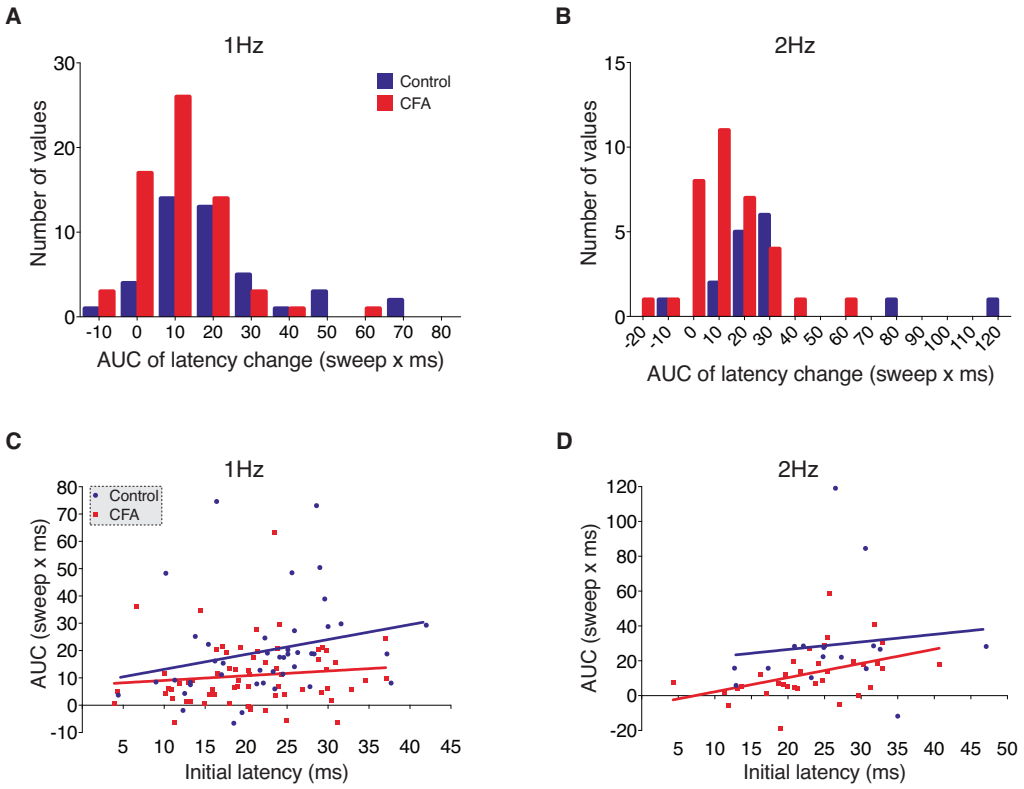

Figure 4.14: Distribution of the magnitude of activity-dependent slowing and its correlation with initial latency. The distribution of the AUC of latency change between control and CFA groups, obtained during C-fibre stimulation at 1 (***A***) and 2Hz (***B***), was significantly different (P=0.012 & P=0.025, Kolmogorov-Smirnov 2-sample test). Correlation between the initial latency and the AUC of latency change was calculated to determine whether the initial C-fibre latency was predictive of the magnitude of ADS that would be observed in monosynaptic C-fibre input to lamina I NK1R+ neurons. ***C*** There was no significant correlation between the initial C-fibre latency and the AUC of latency change during 1Hz stimulation, recorded in tissue isolated from control or CFA treated rats (P=0.107 & P=0.358, respectively, Pearson's r test). ***D*** In the CFA group, during 2Hz stimulation, there was a significant positive correlation between initial C-fibre latency and the resulting AUC of latency change (P=0.013, Pearson's r test), while no such correlation was seen in the control group (P=0.655, Pearson's r test). Legend in ***A*** applies to ***B*** . Lines in ***C*** & ***D*** indicate line of best fit. Legend in ***C*** applies to ***D*** . Control: 1Hz n=43, 2Hz n=16. CFA: 1Hz n=65, 2Hz n=34.

Activity-dependent slowing in monosynaptic C-fibre input to lamina I neurokin 1 receptor expressing neurons, in response to longer and higher frequency stimulus trains, is attenuated in inflammatory pain

To determine the effect of higher stimulation frequencies combined with longer stimulus trains on ADS in monosynaptic C-fibre inputs to lamina I NK1R+ neurons, C-fibre eEPSCs were recorded in response to trains of 40 stimuli at 2, 5 and 10Hz. C-fibre stimulation at 2Hz resulted in a significant increase in the absolute latency of the eEPSC, in tissue isolated from control and CFA treated rats (P=0.009 &

P=0.0003, respectively, linear regression, figure 4.15A, table 4.7). CFA inflammation did not alter the slope (P=0.415, linear regression), but the intercept in the CFA group was significantly reduced (P<0.0001, linear regression). During 5Hz and 10Hz stimulation, a significant increase in the absolute latency was found in the CFA group (P=0.0002 & P=0.002, respectively, linear regression, figure 4.15B & C, table 4.7), while in the control group latency was not significantly altered (P=0.209 & P=0.673, respectively, linear regression). The intercept of the data obtained during 5 and 10Hz stimulation was significantly lower in the CFA group (both P<0.0001, linear regression), while the slope was unaffected (P=0.788 & P=0.404, respectively, linear regression). The data demonstrating the reduction in the intercept of the line of best fit in CFA inflammation was not supported by the finding that the initial latency of monosynaptic C-fibre input to lamina I NK1R+ neurons, measured from the first stimuli during 2Hz stimulation trains, was not significantly altered by CFA inflammation (P=0.102, unpaired t-test, figure 4.15D). The length of dorsal root stimulated was significantly shorter in the CFA groups (P=0.009, unpaired t-test, figure 4.15E).

Analysis of normalised data revealed that repetitive stimulation of monosynaptic C-fibre input to lamina I NK1R+ neurons at 2, 5 and 10Hz resulted in a significant progressive increase in response latency (all P<0.0001, linear regression, figure 4.16A, B & C, table 4.8). Consistent with the results presented in the previous section, CFA inflammation significantly reduced ADS at all stimulation frequencies (all P<0.0001, linear regression). AUC analysis confirmed that CFA inflammation significantly reduced the ADS evoked by 2, 5 and 10Hz stimulation of C-fibres (P=0.015, 2-way ANOVA, figure 4.16D), while the magnitude of ADS was unaffected by stimulation frequency and there was no interaction between these factors (P=0.267 & P=0.759, respectively, 2-way ANOVA).

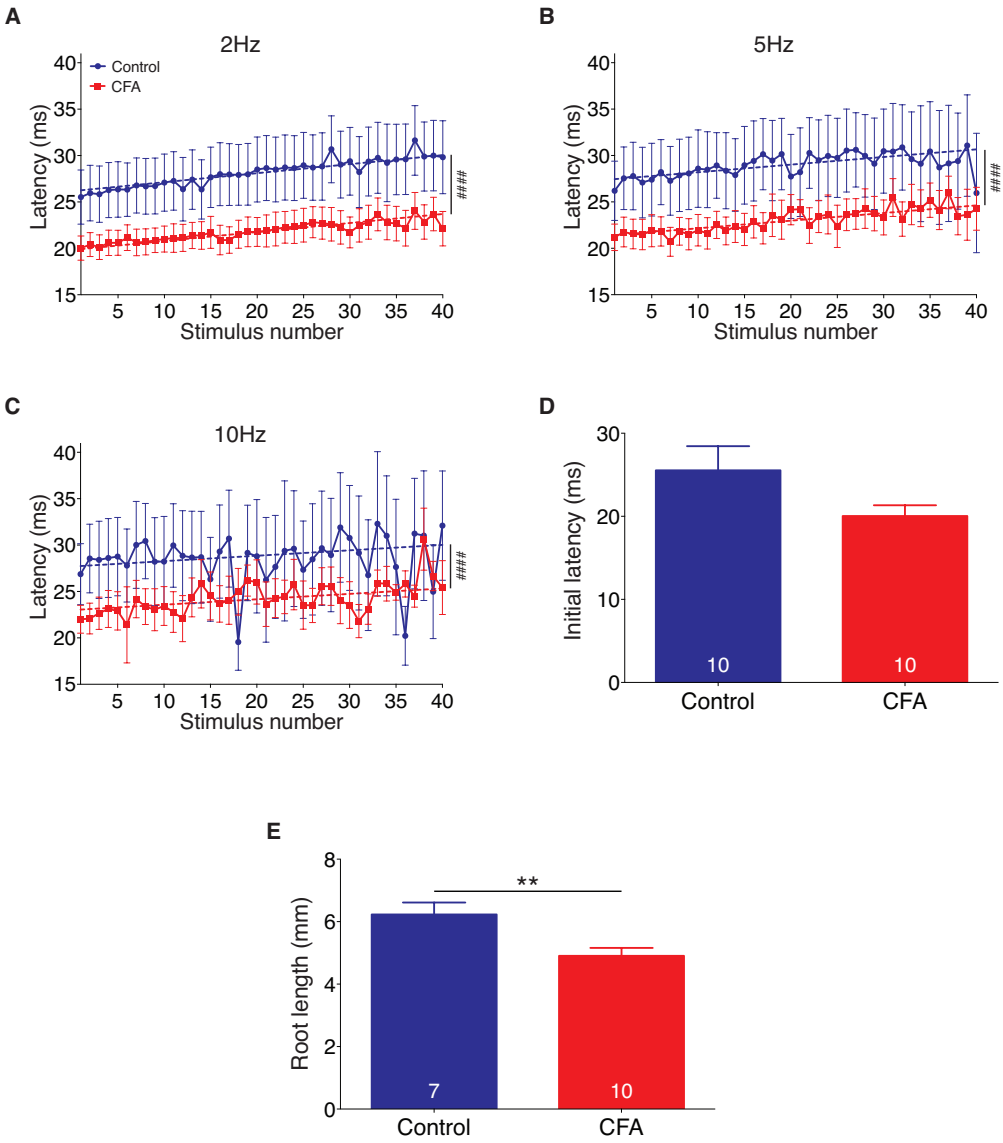

Figure 4.15: C-fibre eEPSC absolute latency in response to increased stimulation frequency / longer stimulus trains. ***A*** 40 stimuli delivered at 2Hz resulted in a significant increase in latency in both control and CFA groups (P=0.009 & P=0.0003, respectively). The intercept of the line of best fit in the CFA group was significantly less that the control (P<0.0001), but there was no difference in the slopes (P=0.415). Stimulation at 5 (***B***) and 10Hz (***C***) resulted in a significant progressive increase in latency in CFA groups only (5Hz P=0.0002, 10Hz P=0.002, Control: 5Hz P=0.209, 10Hz P=0.673). CFA inflammation significantly reduced the intercept of the line fitted to data recorded during 5 and 10Hz stimulation (both P<0.0001), but did not alter the slope (P=0.742 & P=0.404, respectively). ***D*** The initial latency of the C-fibre response, recorded during stimuli 1 of 2Hz stimulation trains, was not altered by CFA inflammation (P=0.102). ***E*** The length of dorsal root stimulated was significantly less in the CFA inflammation group (P=0.009). Statistics (***A***–***C***): linear regression. Slope, r^2 and 95% CI values are presented in table 4.7. ***D*** & ***E*** analysed using unpaired t-test. Samples sizes for A-***C***, both control & CFA; 2Hz n=10, 5 & 10Hz n=9. Sample sizes for ***D*** & ***E*** indicated on bars. Legend in ***A*** applies to ***B*** & ***C***. Error bars indicate SEM. Dashed lines represent line of best fit. Control vs. CFA slope: ∗P<0.05, ∗∗∗∗P<0.0001; intercept: #### P<0.0001.

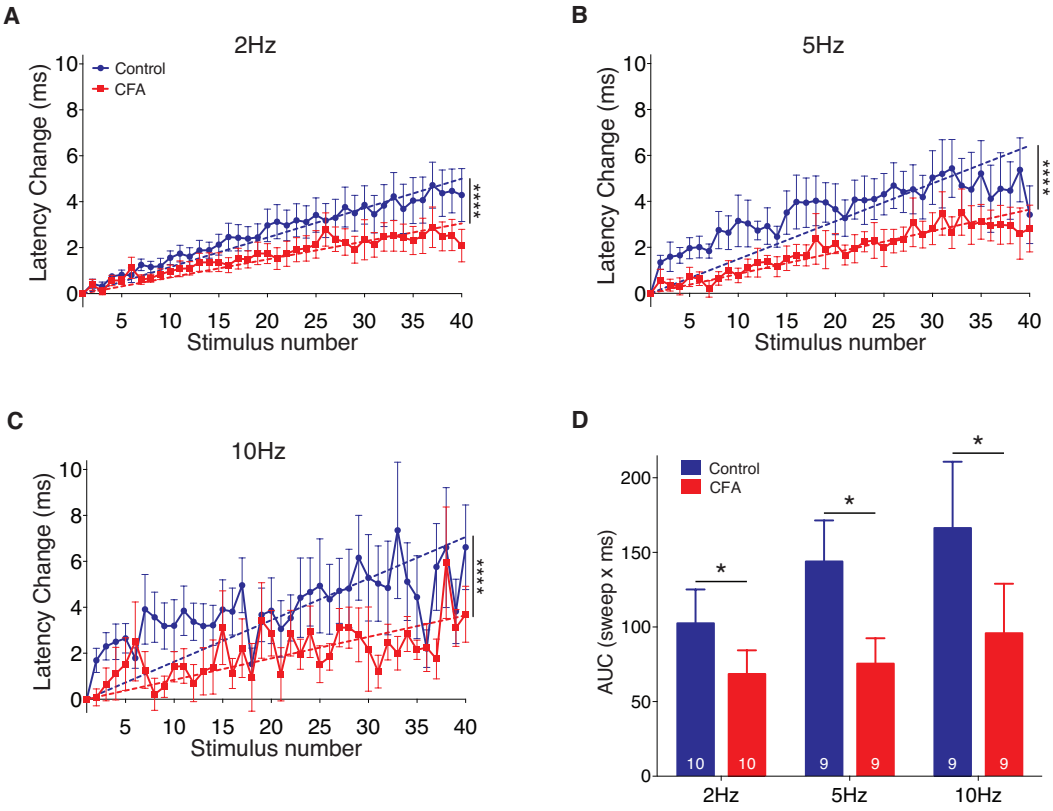

Figure 4.16: Activity-dependent slowing in monosynaptic C-fibre input to lamina I NK1R+ neurons in response to increased stimulus frequency and longer stimulus trains. Extended periods of repetitive stimulation of monosynaptic C-fibre input to lamina I NK1R+ neurons at frequencies of 2 (***A***), 5 (***B***) and 10Hz (***C***) resulted in a significant increase in response latency (all $P<0.0001$). In all cases, CFA inflammation significantly reduced the degree of the latency increase (all $P<0.0001$). ***D*** Area under the curve analysis of latency change confirms that CFA inflammation significantly reduced ADS in C-fibre input to lamina I NK1R+ neurons ($P=0.015$) and demonstrates that ADS was not influenced by stimulation frequency ($P=0.267$). There was no interaction between these factors ($P=0.759$). Statistics (***A***–***C***): linear regression. Slope, r^2 and 95% CI values are presented in table 4.8. Statistics (***D***) 2-way ANOVA. Sample sizes indicated on bars in ***D*** . Legend in ***A*** applies to ***B*** & ***C***. Error bars indicate SEM. Dashed lines represent line of best fit. $*P<0.05$, $****P<0.0001$.

Frequency (Hz)	Treatment	Y Intercept	Intercept 95% CI	Control vs. CFA	Slope	Slope 95% CI	Slope vs. Slope = 0	Control vs. CFA	r^2
2	control	25.87	23.90 to 27.85	P<0.0001	0.117	0.030 to 0.205	P=0.009	P=0.415	0.017
	CFA	20.32	19.40 to 21.23		0.076	0.035 to 0.117	P=0.0003		0.034
5	control	27.65	25.22 to 30.08	P<0.0001	0.072	-0.040 to 0.184	P=0.209	P=0.742	0.005
	CFA	21.23	20.21 to 22.26		0.092	0.045 to 0.139	P=0.0002		0.044
10	control	28.29	25.48 to 31.10	P<0.0001	0.028	-0.102 to 0.159	P=0.673	P=0.404	0.001
	CFA	22.49	21.27 to 23.70		0.090	0.033 to 0.146	P=0.002		0.037

Table 4.7: Comparison of absolute latency in monosynaptic C-fibre inputs to lamina I NK1R+ neurons during increased stimulation frequency / longer stimulus trains.

Frequency (Hz)	Treatment	Slope	r^2	95% CI	Slope vs. Slope = 0	Control vs. CFA
2	control	0.128	0.269	0.119 to 0.137	P<0.0001	P<0.0001
	CFA	0.078	0.182	0.072 to 0.085	P<0.0001	
5	control	0.165	0.064	0.151 to 0.179	P<0.0001	P<0.0001
	CFA	0.093	0.223	0.085 to 0.102	P<0.0001	
10	control	0.181	0.015	0.161 to 0.201	P<0.0001	P<0.0001
	CFA	0.094	0.067	0.079 to 0.108	P<0.0001	

Table 4.8: Comparison of latency change in monosynaptic C-fibre inputs to lamina I NK1R+ neurons during increased stimulation frequency / longer stimulus trains.

Synaptic response failure rate in monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons

To determine whether CFA inflammation potentially alters the synaptic response failure rate in monosynaptic C-fibre input to lamina I NK1R+ neurons, when stimulated at frequencies of 2Hz or above, the rate of synaptic response failures during repetitive stimulation was analysed. C-fibre stimulation at 2, 5 and 10Hz resulted in a progressive increase in synaptic response failures (all $P<0.0001$, except 2Hz control $P=0.005$, linear regression, figure 4.17A, B & C, table 4.9). During 2Hz stimulation, tissue isolated from CFA treated rats exhibited a significantly greater degree of failures ($P<0.0001$, linear regression, figure 4.17A table 4.9), while CFA inflammation was without effect during 5 and 10Hz stimulation ($P=0.303$ & $P=0.604$, respectively, linear regression, figure 4.17 B & C, table 4.9). Analysis of the total failure rate revealed that CFA inflammation did not significantly alter the synaptic response failure rate in monosynaptic C-fibre input to lamina I NK1R+ neurons ($P=0.733$, 2-way ANOVA, figure 4.17D). Stimulation frequency significantly affected the total failure rate ($P=0.0002$, 2-way ANOVA), but there was no interaction between CFA inflammation and stimulation frequency ($P=0.855$, 2-way ANOVA). Post-hoc analysis showed that there was significantly more failures during 10Hz stimulation than 2Hz ($P=0.002$, 2-way ANOVA followed by Tukey post-tests). There was a trend towards a greater failure rate during 10Hz stimulation than 5Hz, however this was not significant ($P=0.058$, 2-way ANOVA followed by Tukey post-tests), while there was no difference in the failure rate between 2 and 5Hz stimulation ($P=0.428$, 2-way ANOVA followed by Tukey post-tests).

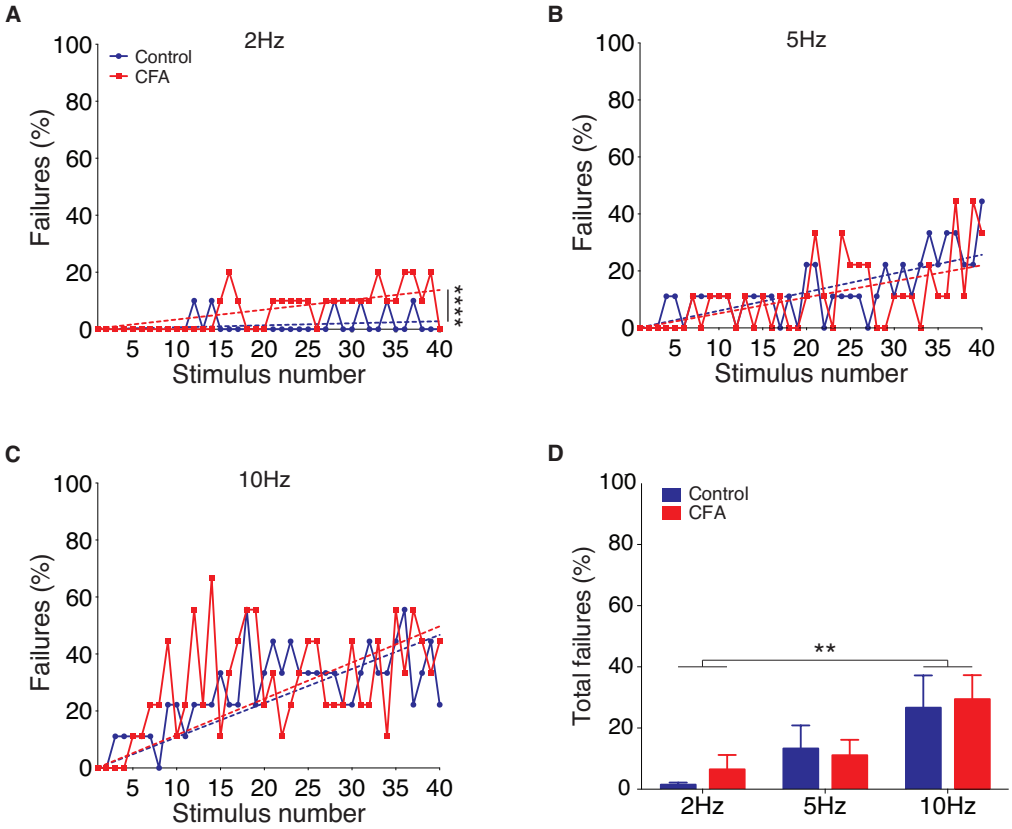

Figure 4.17: Failures in monosynaptic C-fibre input to lamina I NK1R+ neurons during repetitive stimulation. ***A***, ***B*** and ***C*** show the percentage of failures that occur for each stimuli at 2, 5 and 10Hz, respectively. In all cases the failure rate significantly increased with increasing stimulus number (all $P<0.0001$, except 2Hz control where $P=0.005$). CFA inflammation significantly increased the failure rate when monosynaptic C-fibre input was stimulated at 2Hz ($P<0.0001$), but was without effect when stimulated at 5 or 10Hz ($P=0.303$ & $P=0.604$, respectively). ***D*** Analysis of the total percentage of failures demonstrates that failures in monosynaptic C-fibre input to lamina I NK1R+ neurons is not influenced by CFA inflammation ($P=0.733$), but that increased stimulation frequency results in a greater level of failures ($P=0.002$), although there were no interactions between these factors ($P=0.855$). Post-hoc analysis revealed that 10Hz stimulation resulted in significantly more failures that 2Hz ($P=0.002$). Statistics (***A***–***C***): linear regression. Slope, r^2 and 95% CI values are presented in table 4.9. Statistics (***D***): 2-way ANOVA, followed by Tukey post-tests. Legend in ***A*** applies to ***B*** & ***C*** . Error bars indicate SEM. Control & CFA: 2Hz n=10, 5Hz n=9, 10Hz n=9. $****P<0.0001$.

Frequency (Hz)	Treatment	Slope	r^2	95% CI	Slope vs. Slope = 0	Control vs. CFA
2	control	0.073	0.038	0.023 to 0.123	P=0.005	P<0.0001
	CFA	0.354	0.462	0.282 to 0.426	P<0.0001	
5	control	0.054	0.484	0.534 to 0.781	P<0.0001	P=0.303
	CFA	0.073	0.313	0.431 to 0.726	P<0.0001	
10	control	0.079	0.336	1.068 to 1.389	P<0.0001	P=0.604
	CFA	0.125	0.025	1.053 to 1.557	P<0.0001	

Table 4.9: Comparison of failures in monosynaptic C-fibre eepsc recorded in lamina I NK1R+ neurons during stimulation at 2, 5 and 10Hz.

4.4.4 The role of HCN channels in C-fibre activity-dependent slowing

ZD7288 enhances activity-dependent slowing in C-fibres in isolated dorsal roots

The data presented in this section were collected by and some analysis was performed by Ms Veny Lukito, a Physiological Society vacation student.

To investigate the role of HCN channels in constraining C-fibre ADS, CAPs were recorded from dorsal roots isolated from control and CFA treated rats, in the absence and presence of the non-selective HCN channel antagonist ZD7288 or vehicle. Representative traces of C-fibre CAP recordings conducted prior to ('baseline') and during application of ZD7288, from dorsal roots isolated from control and CFA treated rats are shown in figure 4.18A and B, respectively. The slope fitted to the absolute latency of C-fibre responses recorded during 2Hz stimulation was not altered by application of ZD7288 or vehicle, in either control or CFA groups (figure 4.19A & B, table 4.10). In the CFA group, ZD7288 resulted in a small but significant increase in the intercept of the line of best fit (P=0.003, linear regression), while in control tissue there was a trend towards an increased intercept, which was not significant (P=0.058, linear regression). Vehicle application did not significantly alter the intercept in control or CFA groups (P=0.431 & P=0.860, respectively, linear regression).

When these data were normalised, it was found that in control and CFA groups, ZD7288 significantly enhanced ADS (both $P<0.0001$, linear regression, figures 4.20A & B, table 4.11), however vehicle was without effect (P=0.952 & P=0.231, respectively, linear regression). To determine the extent to which ZD7288 enhanced ADS, the latency change measured at baseline was subtracted from the latency change in the presence of ZD7288 / vehicle. This analysis revealed that the facilitatory effect of ZD7288 was significantly greater in tissue isolated from CFA treated rats ($P<0.0001$, linear regression, figure 4.20C (left), table 4.12). Vehicle caused no facilitation of C-fibre ADS in dorsal roots isolated from control treated rats (P=0.318, linear regression, figure 4.20C (right), table 4.12). In dorsal roots isolated from CFA treated rats, vehicle had no significant effect upon ADS (P=0.231, linear regression), however when the baseline subtracted values were compared, there was a significant difference between control and CFA, with a small but significant depression of ADS in the CFA group ($P<0.0001$, linear regression).

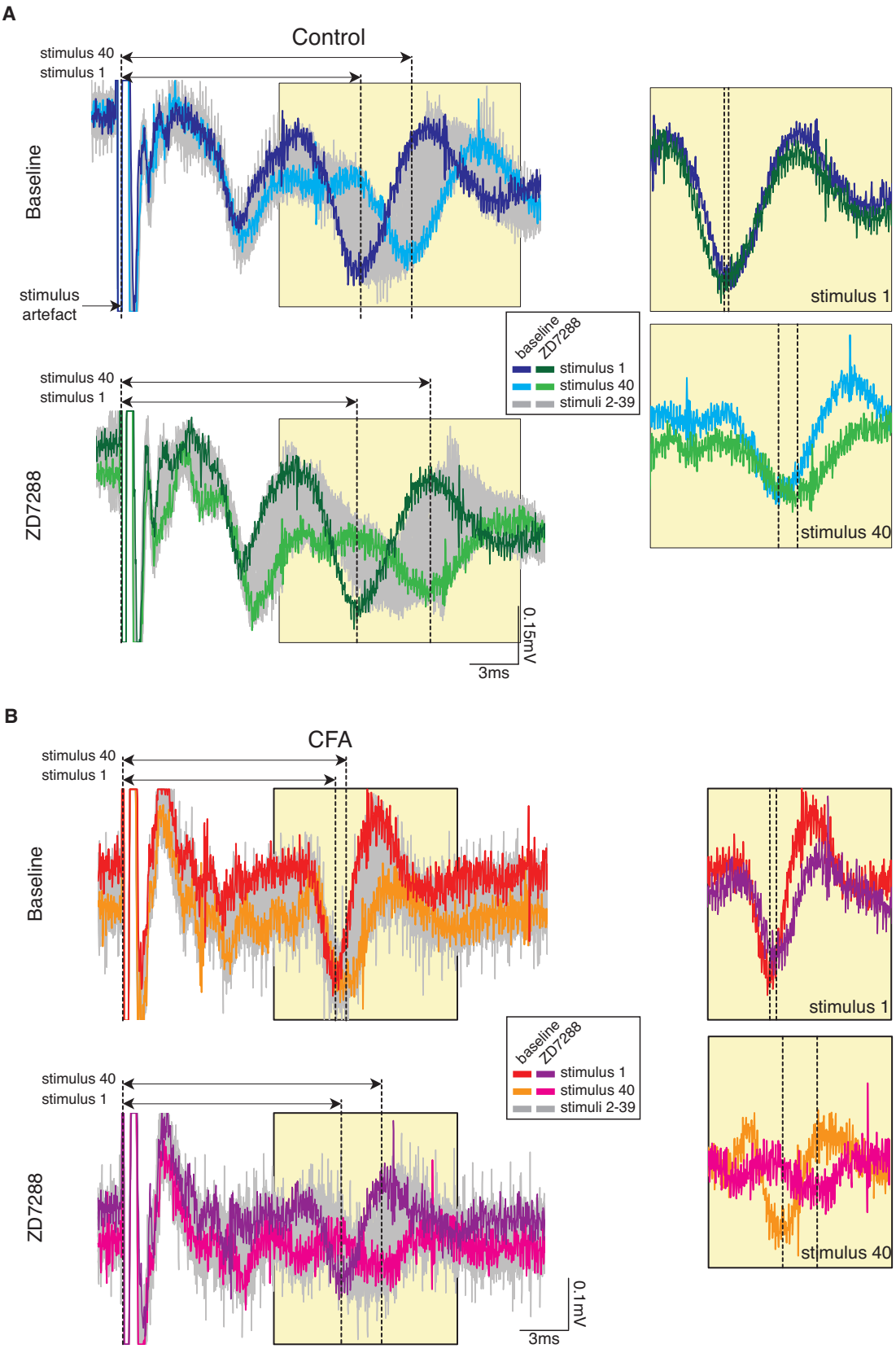

Figure 4.18: Representative traces of C-fibre activity-dependent slowing in the presence of ZD7288, in dorsal roots isolated from control (***A***) and CFA (***B***) treated rats. Traces demonstrate ADS in response to 40 stimuli delivered at 2Hz, prior to ('baseline', top left) and during 10μM ZD7288 application (bottom left). Response latency was measured as the difference between the stimulus artefact and the negative peak of the C-fibre component (indicated by broken lines). The top right insert shows baseline sweep 1 and ZD7288 sweep 1 overlaid, bottom right shows the baseline sweep 40 and ZD7288 sweep 40 traces overlaid. Inserts taken from area on main traces indicated by yellow box.

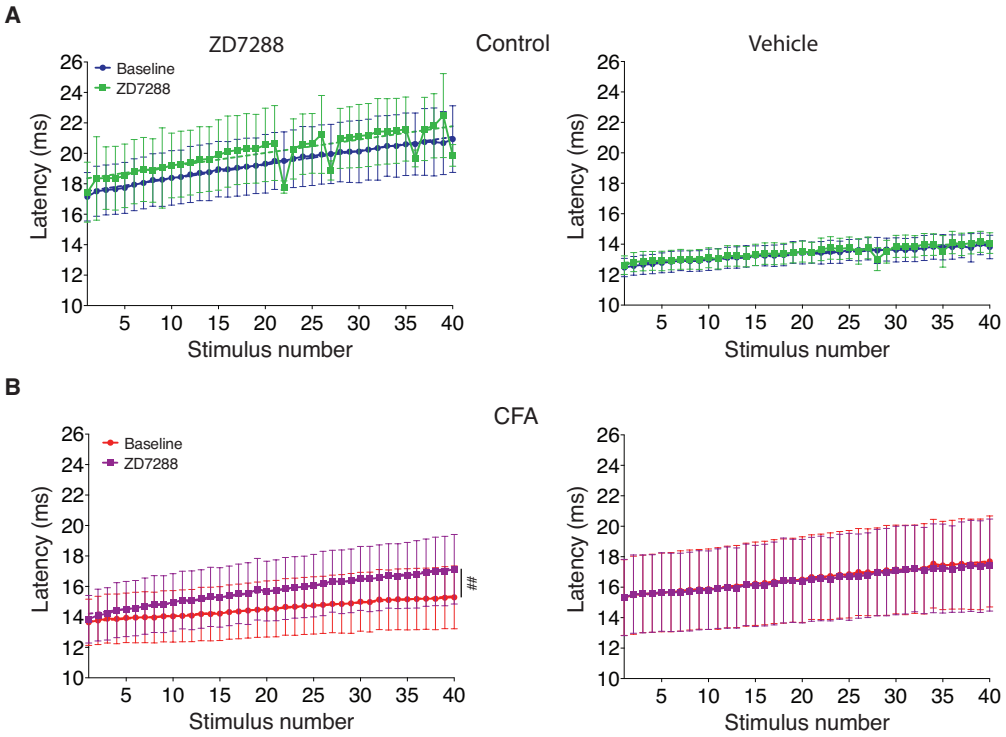

Figure 4.19: Effect of ZD7288 application on the absolute latency of C-fibre responses during repetitive stimulation of isolated dorsal roots. ***A*** In dorsal roots isolated from control rats, application of 10μM ZD7288 (left) or vehicle (right) did not alter the slope (P=0.919 & P=0.985, respectively) or intercept (P=0.058 & P=0.431, respectively) of the C-fibre response latency recorded during stimulation at 2Hz . ***B*** In CAP recordings from dorsal roots isolated from CFA treated rats, application of ZD7288 (left) or vehicle (right) had no effect on the slope of the C-fibre response latency (P=0.313 & P=0.919, respectively), however ZD7288 caused a small but significant increase in the intercept (P=0.003). Statistics: linear regression. Slope, R^2 and 95% CI values are presented in table 4.10. Control: ZD7288 n=5, vehicle n=6. CFA: ZD7288 n=6, vehicle n=5. Error bars indicate SEM. Dashed lines represent line of best fit. Control vs. CFA intercept: ## $P<0.01$.

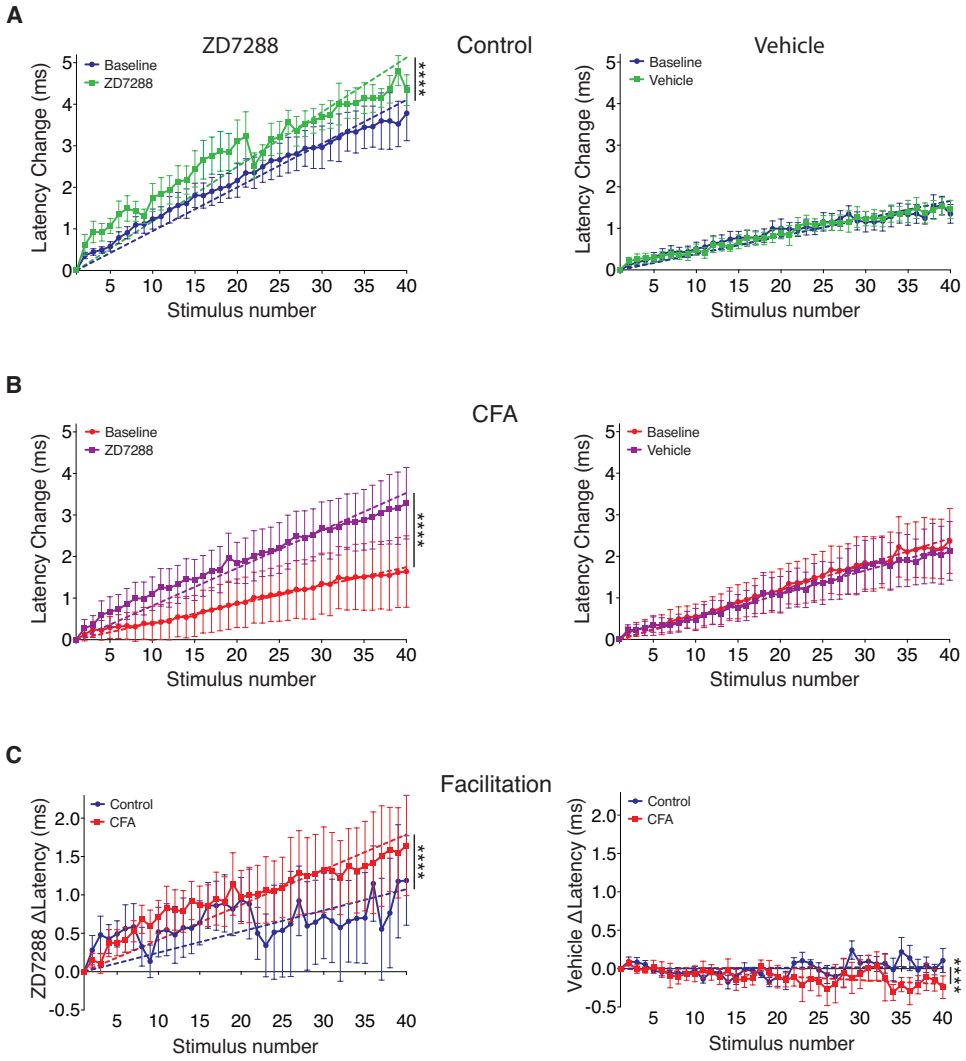

Figure 4.20: ZD7288 enhances C-fibre activity-dependent slowing in isolated rat dorsal roots. ***A*** In dorsal roots isolated from control rats, 2Hz stimulation prior to ('baseline') and during application of 10μM ZD7288 (left) or vehicle (right) resulted in a significant increase in the latency change (all $P<0.0001$). ZD7288 significantly enhanced this latency change ($P<0.0001$), while vehicle was without effect ($P=0.952$). ***B*** Stimulation of dorsal roots isolated from CFA treated rats resulted in a significant increase in response latency in all conditions (all $P<0.0001$). ZD7288 application significantly elevated this latency increase ($P<0.0001$, left), while vehicle had no effect ($P=0.231$, right). To determine whether the ZD7288 facilitation of ADS was altered in CFA inflammation, the latency change recorded at baseline was subtracted from that recorded during ZD7288 / vehicle application. ***C*** The facilitatory effect of ZD7288 on ADS was significantly greater in dorsal roots isolated from CFA treated rats ($P<0.0001$, left). When the baseline subtracted values obtained during vehicle application were compared, it was found that there was a small but significant depression of ADS in CFA tissue compared to control ($P<0.0001$, right). Statistics: linear regression. Slope, r^2 and 95% CI values for ***A***/***B*** & ***C*** are presented in tables 4.11 & 4.12, respectively. Control: ZD7288 n=5, vehicle n=6. CFA: ZD7288 n=6, vehicle n=5. Error bars indicate SEM. Dashed lines represent line of best fit. $****P<0.0001$.

Treatment	Recording	Y Intercept	Intercept 95% CI	Control vs. CFA	Slope	Slope 95% CI	Slope vs. Slope = 0	Baseline vs. ZD7288	r^2
Control	baseline	17.52	16.48 to 18.56	P=0.058	0.091	0.045 to 0.137	P=0.0001	P=0.920	0.071
	ZD7288	18.36	17.19 to 19.53		0.088	0.036 to 0.140	P=0.001		0.054
	baseline	12.67	12.27 to 13.08	P=0.431	0.034	0.016 to 0.052	P=0.0002	P=0.985	0.057
	vehicle	12.79	12.40 to 13.17		0.196	0.017 to 0.052	P=0.0001		0.063
CFA	baseline	13.71	12.68 to 14.73	P=0.003	0.042	-0.003 to 0.087	P=0.067	P=0.313	0.014
	ZD7288	14.21	13.09 to 15.33		0.077	0.027 to 0.126	P=0.003		0.038
	baseline	15.40	13.87 to 16.92	P=0.860	0.059	-0.008 to 0.127	P=0.085	P=0.919	0.015
	vehicle	15.39	13.89 to 16.90		0.054	-0.012 to 0.121	P=0.109		0.013

Table 4.10: Comparison of the effect of ZD7288 / vehicle on the absolute latency of C-fibre responses, during repetitive stimulation, in isolated dorsal roots.

Treatment	Drug	Recording	Slope	r^2	95% CI	Slope vs. Slope = 0	Baseline vs. Drug
Control	ZD7288	baseline	0.105	0.615	0.100 to 0.110	P<0.0001	P<0.0001
		drug	0.131	0.633	0.126 to 0.137	P<0.0001	
	Vehicle	baseline	0.043	0.446	0.040 to 0.045	P<0.0001	P=0.952
		drug	0.043	0.526	0.040 to 0.045	P<0.0001	
CFA	ZD7288	baseline	0.045	0.117	0.037 to 0.052	P<0.0001	P<0.0001
		drug	0.091	0.310	0.083 to 0.098	P<0.0001	
	Vehicle	baseline	0.062	0.308	0.056 to 0.069	P<0.0001	P=0.231
		drug	0.057	0.323	0.052 to 0.063	P<0.0001	

Table 4.11: Comparison of the effect of ZD7288 / vehicle on C-fibre ADS in isolated dorsal roots.

Drug	Treatment	Slope	r^2	95% CI	Slope vs. Slope = 0	Control vs. CFA
ZD7288	control	0.028	-0.021	0.022 to 0.033	P<0.0001	P<0.0001
	CFA	0.046	0.134	0.041 to 0.051	P<0.0001	
Vehicle	control	0.001	0.003	-0.001 to 0.002	P=0.381	P<0.0001
	CFA	-0.005	0.034	-0.007 to -0.003	P<0.0001	

Table 4.12: Comparison of the degree of ZD7288 / vehicle modulation of C-fibre ADS in isolated dorsal roots.

The length of dorsal roots recorded from did not differ between control and CFA groups (P=0.787, 2-way ANOVA, figure 4.21A) or between those treated with ZD7288 or vehicle (P=0.312, 2-way ANOVA) and there was no interaction between these factors (P=0.206, 2-way ANOVA). The initial latency, recorded during the first stimuli of ZD7288 baseline or vehicle baseline, did not differ between groups treated with ZD7288 or vehicle (P=0.362, 2-way ANOVA, figure 4.21B) or between dorsal roots isolated from control or CFA treated rats (P=0.841, 2-way ANOVA). There were no interactions between these variables (P=0.064, 2-way ANOVA). Application of ZD7288 or vehicle did not significantly alter the initial C-fibre latency (P=0.295 & P=0.210, respectively, 2-way repeated measures ANOVA, figure 4.21C & D). In both cases CFA inflammation was without effect (P=0.162 & P=0.268, respectively, 2-way repeated measures ANOVA) and there was no interaction between ZD7288 / vehicle application and CFA inflammation (P=0.835 & P=0.232, respectively, 2-way repeated measures ANOVA). When the change in initial latency, calculated by subtracting the baseline initial latency from the initial latency recorded during ZD7288 / vehicle application, was compared, it was discovered that there was no difference in the change in initial latency between ZD7288 and vehicle groups (P=0.473, 2-way ANOVA, figure 4.21E). CFA inflammation did not alter the change in initial latency (P=0.601, 2-way ANOVA) and there was no interaction between these factors (P=0.908).

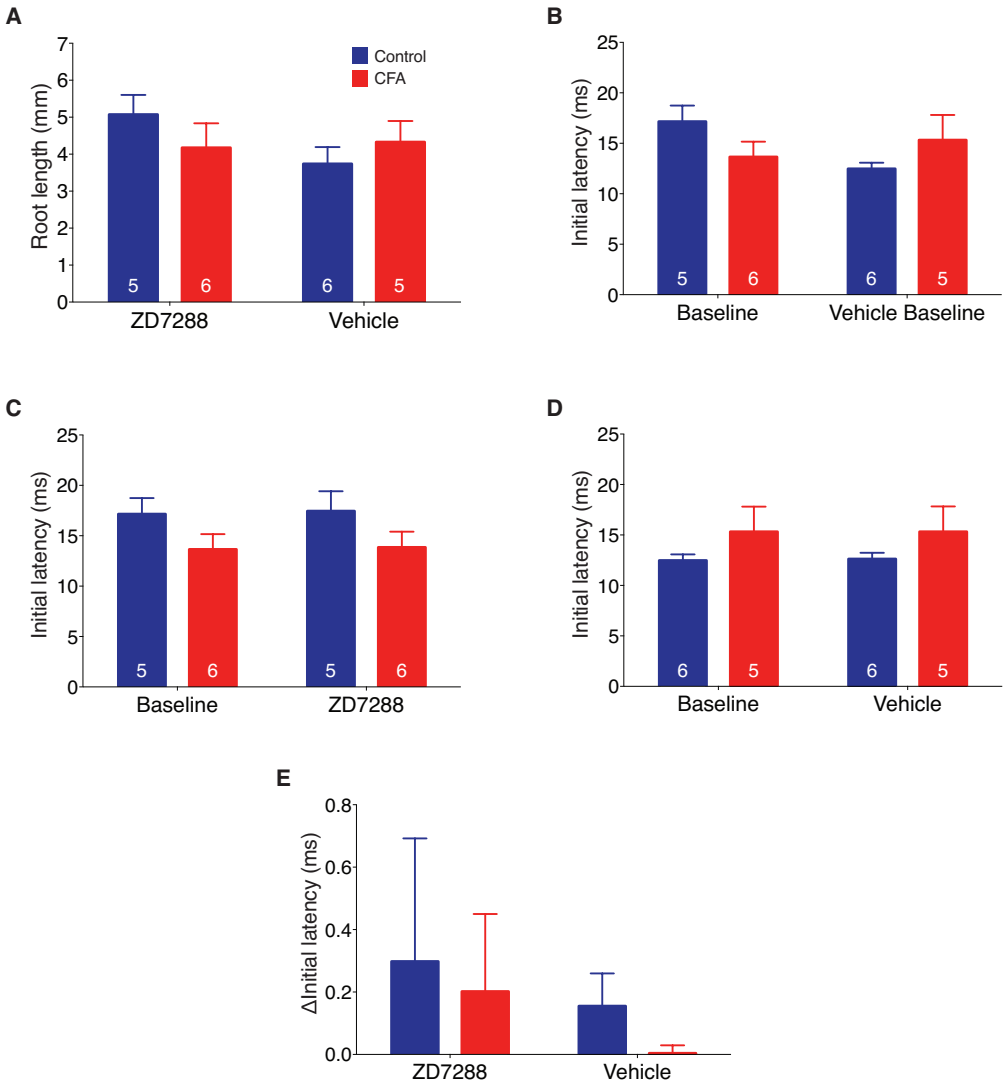

Figure 4.21: ZD7288 does not alter the initial latency of the C-fibre response in compound action potential recordings from isolated dorsal roots. ***A*** The length of isolated dorsal roots used in these experiments did not significantly differ between groups (CFA vs. control P=0.787, ZD7388 / vehicle P=0.312, interaction P=0.206). ***B*** The initial C-fibre response latency, in CAP recordings from isolated dorsal roots, prior to ZD7288 (10μM, 'baseline') or vehicle ('vehicle baseline'), was no different between these groups (P=0.362) and was unaltered by CFA inflammation (P=0.841). There was no significant interaction between these factors (P=0.064). ***C*** Application of ZD7288 did not significantly change the initial C-fibre latency (P=0.295), while CFA inflammation was without effect (P=0.162) and there was no interaction (P=0.835). ***D*** Similarly, vehicle application did not alter the initial latency (P=0.210) and there was no effect of CFA (P=0.268) and no interaction (P=0.232). ***E*** When the change in initial latency that resulted from ZD7288 / vehicle application was compared, it was found that the change in initial response latency was not significantly affected by CFA inflammation (P=0.601) and did not differ between application of ZD7288 or vehicle (P=0.473). There was no interaction between these factors (P=0.908). Statistics: ***A***, ***B*** & ***E*** 2-way ANOVA; ***C*** & ***D*** , 2-way repeated measures ANOVA. Samples sizes indicated on bars, except ***E*** , where sample sizes are shown on ***A***. Error bars indicate SEM.

Application of ZD7288 significantly reduced the peak amplitude of the C-fibre response (P=0.006, 2-way repeated measures ANOVA, figure 4.22A), while CFA inflammation was without effect (P=0.994, 2-way repeated measures ANOVA) and there was no interaction between these factors (P=0.720, 2-way repeated measures ANOVA). Application of vehicle did not alter the peak amplitude of the C-fibre response (P=0.228, 2-way repeated measures ANOVA, figure 4.22B). CFA inflammation did not alter the peak amplitude of the C-fibre response in the vehicle group (P=0.876, 2-way repeated measures ANOVA) and there was no interaction between CFA inflammation and vehicle application (P=0.677, 2-way repeated measures ANOVA).

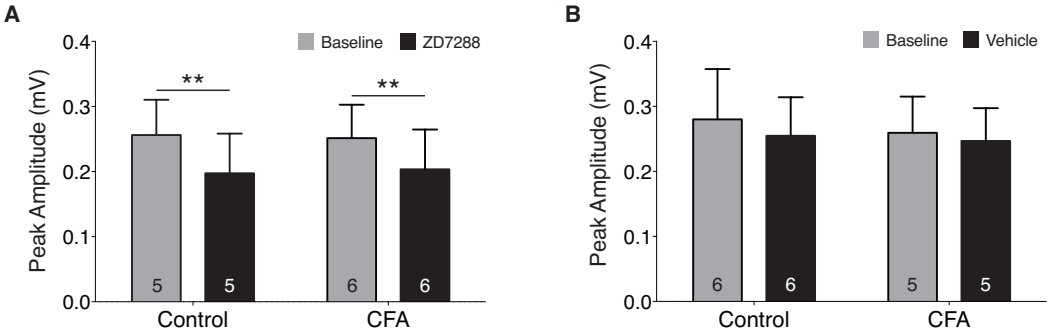

Figure 4.22: ZD7288 reduces C-fibre amplitude in isolated rat dorsal roots. ***A*** In compound action potential recordings from isolated rat dorsal roots, 10μM ZD7288 significantly reduced the peak amplitude of the C-fibre response (P=0.006), while CFA inflammation was without effect (P=0.994) and there was no interaction between these factors (P=0.720). ***B*** The peak amplitude of the C-fibre response was unaffected by vehicle application (P=0.228), CFA inflammation (P=0.876) and there was no interaction between these variables (P=0.677). Statistics: 2-way repeated measures ANOVA. Sample sizes indicated on bars. Error bars indicate SEM. $**P<0.01$.

ZD7288 enhances activity-dependent slowing in monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons

A preliminary investigation into the role of HCN channels in limiting ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons was conducted by recording C-fibre eEPSCs in response to dorsal root stimulation at 2, 5 and 10Hz, prior to ('baseline') and during application of 10μM ZD7288. In control tissue, ZD7288 did not alter the slope or intercept of lines fitted to the absolute latency data obtained during C-fibre stimulation at 2, 5 or 10Hz (figure 4.23A, B & C, respectively, table 4.13). In CFA inflammation, the effect of ZD7288 upon the absolute latency of C-fibre eEPSCs was dependent upon the stimulation frequency used. During stimulation at 2 and 5Hz, ZD7288 significantly increased the intercept (P=0.037 &

P=0.0008, respectively, linear regression, 4.23A & B, table 4.13), but did not alter the slope of the line fitted to the absolute latency data (P=0.243 & P=0.569, linear regression). Conversely, during 10Hz stimulation, ZD7288 was found to alter the slope of the line fitted to the data (P=0.003, linear regression, 4.23C, table 4.13), however because the slopes were significantly different it was not possible to accurately compare intercepts.

When the data assessing the effect of ZD7288 on ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons was normalised, the result of ZD7288 application was found to be inconsistent, with responses differing depending upon the stimulation frequency used and/or whether the tissue was isolated from control or CFA treated rats. In both control and CFA treated rats during 2Hz stimulation, ZD7288 significantly enhanced ADS (both $P<0.0001$, linear regression, figure 4.24A & B, table 4.14). When the facilitatory effect of ZD7288 was compared between these control and CFA groups, it was found that the ZD7288 enhancement of ADS was not influenced by CFA inflammation (P=0.125, linear regression, figure 4.24C, table 4.15). Unlike 2Hz stimulation, the ADS elicited by 5 and 10Hz stimulation in control tissue was unaffected by ZD7288 (P=0.849 & P=0.137, respectively, linear regression, figures 4.25A & 4.26A, table 4.14). However, ZD7288 significantly increased ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons in CFA tissue during both 5 and 10Hz stimulation (both $P<0.0001$, linear regression, figures 4.25B & 4.26B, table 4.14). In contrast to the results obtained during 2Hz stimulation, the facilitatory effect of ZD7288 upon ADS recorded during both 5 and 10Hz was found to be significantly greater in CFA inflammation (P=0.001 & $P<0.0001$, respectively, linear regression, figures 4.25C & 4.26C, table 4.15).

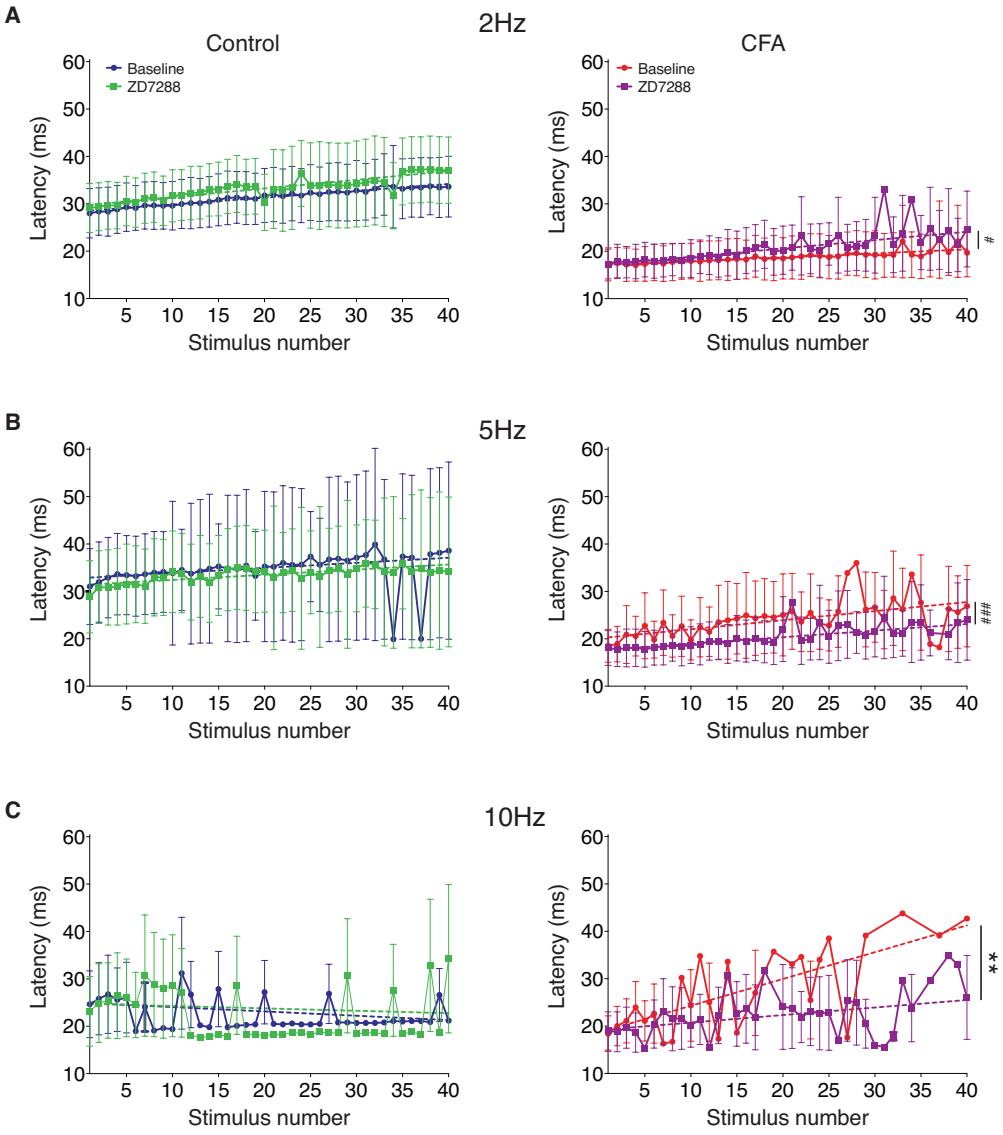

Figure 4.23: Effect of ZD7288 on the absolute latency of monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons, during stimulation at 2, 5 and 10Hz. In eEPSC recordings from control tissue, application of 10μM ZD7288 during stimulation at 2 (***A***), 5 (***B***) or 10Hz (***C***) did not alter the absolute latency of the C-fibre response. In tissue isolated from CFA treated rats, ZD7288 caused a small but significant increase in the intercept of the line fitted to data recorded during stimulation at 2 and 5Hz (P=0.037 & P=0.0008, respectively), but did not alter the slope (P=0.243 & P=0.569, respectively). During 10Hz stimulation, ZD7288 significantly increased the latency of monosynaptic C-fibre input to lamina I NK1R+ neurons (P=0.003), because the slopes were significantly different it was not possible to test for differences in intercepts. Statistics: linear regression. Slope, r^2 and 95% CI values are presented in table 4.13. Control: 2Hz n=4, 5 & 10Hz n=3. CFA: all n=3. Legend in ***A*** applies to all. Error bars indicate SEM. Dashed lines represent line of best fit. Baseline vs. ZD7288 slope: $**$P$<$0.01; intercept: # P$<$0.05, ### P$<$0.001.

Frequency (Hz)	Treatment	Recording	Y Intercept	Intercept 95% CI	Control vs. CFA	Slope	Slope 95% CI	Slope vs. Slope = 0	Baseline vs. ZD7288	r^2
2	control	baseline	28.55	25.53 to 31.57	P=0.105	0.068	0.011 to 0.278	P=0.036	P=0.709	0.028
		ZD7288	29.76	26.41 to 33.11		0.183	0.032 to 0.333	P=0.019		0.038
	CFA	baseline	17.09	14.82 to 19.36	P=0.037	0.052	-0.017 to 0.187	P=0.103	P=0.243	0.023
		ZD7288	17.25	14.73 to 19.77		0.176	0.059 to 0.293	P=0.004		0.077
5	control	baseline	31.45	26.71 to 36.18	P=0.468	0.109	-0.112 to 0.329	P=0.330	P=0.994	0.009
		ZD7288	32.91	27.19 to 38.63		0.108	-0.164 to 0.379	P=0.433		0.007
	CFA	baseline	17.69	15.25 to 20.13	P=0.0008	0.139	0.026 to 0.253	P=0.017	P=0.569	0.054
		ZD7288	20.30	17.21 to 23.39		0.191	0.047 to 0.336	P=0.010		0.078
10	control	baseline	24.74	20.21 to 29.26	P=0.711	-0.050	-0.261 to 0.160	P=0.633	P=0.751	0.004
		ZD7288	24.88	21.86 to 27.90		-0.092	-0.234 to 0.050	P=0.200		0.033
	CFA	baseline	19.31	16.24 to 22.39	n/a	0.159	0.006 to 0.313	P=0.042	P=0.003	0.055
		ZD7288	19.21	15.73 to 22.69		0.565	0.346 to 0.784	P<0.0001		0.411

Table 4.13: Comparison of the effect of ZD7288 application on the absolute latency of monosynaptic C-fibre input to lamina I NK1R+ neurons, during stimulation at 2, 5 and 10Hz.

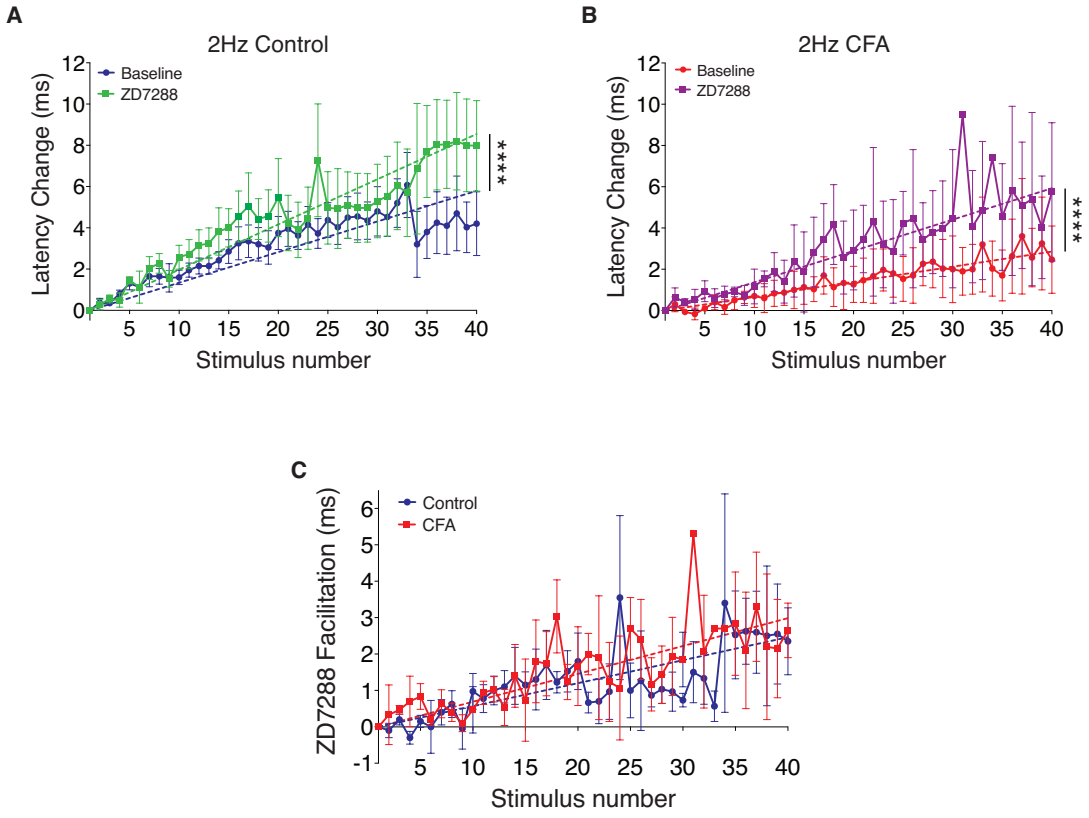

Figure 4.24: ZD7288 enhances activity-dependent slowing, in response to stimulation at 2Hz, in monosynaptic C-fibre input to lamina I NK1R+ neurons. Application of 10μM ZD7288 significantly enhanced the progressive increase in C-fibre response latency during 2Hz stimulation, in tissue isolated from control (***A***, $P<0.0001$) and CFA treated rats (***B***, $P<0.0001$). To assess whether the facilitatory effect of ZD7288 was different between control and CFA groups, the baseline latency change was subtracted from that recorded in the presence of ZD7288. ***C*** The ZD7288 enhancement of latency change in response to 2Hz stimulation was not altered by CFA inflammation ($P=0.125$). Statistics: linear regression. Slope, R^2 and 95% CI values for ***A***/***B*** & ***C*** are presented in tables 4.14 & 4.15, respectively. Control n=4, CFA n=3. Error bars indicate SEM. Dashed lines represent line of best fit. $****P<0.0001$.

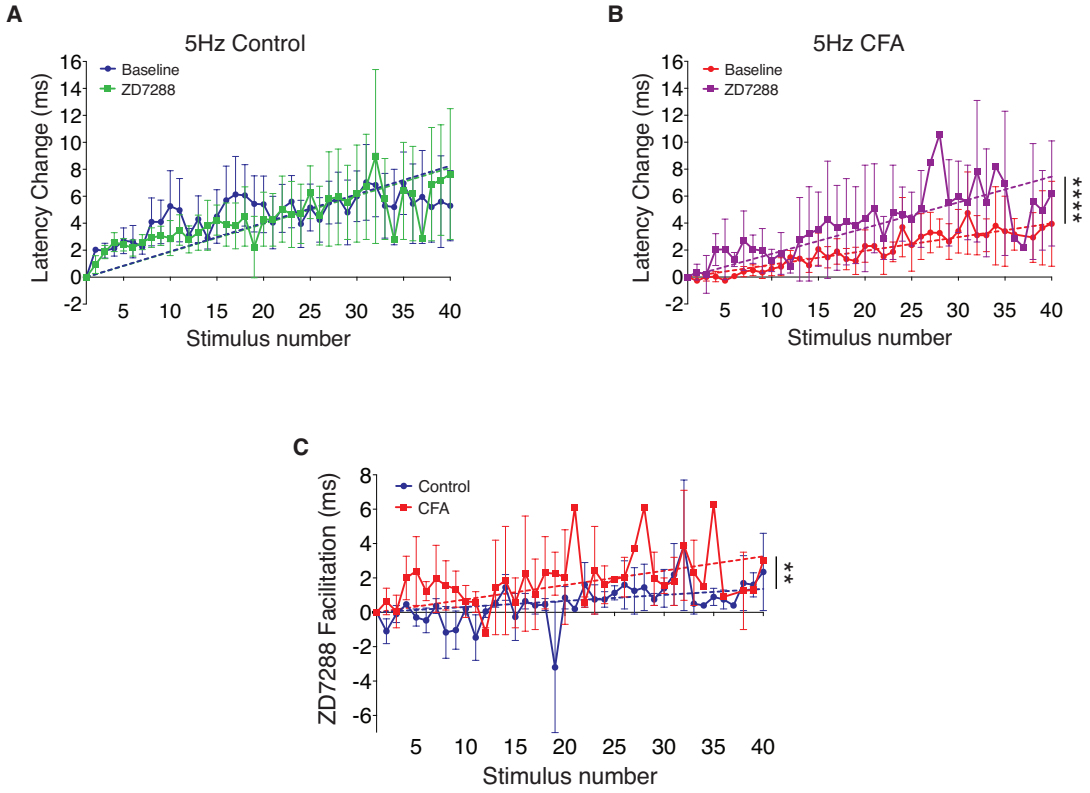

Figure 4.25: ZD7288 enhances activity-dependent slowing, in response to stimulation at 5Hz, in monosynaptic C-fibre input to lamina I NK1R+ neurons. ***A*** In tissue isolated from control treated rats, ZD7288 (10μM) did not significantly influence the latency change during 2Hz stimulation (P=0.849). ***B*** ZD7288 significantly facilitated the progressive latency increase in monosynaptic C-fibre seen during 5Hz stimulation, in the CFA group ($P<0.0001$). ***C*** When the ability of ZD7288 to enhance ADS was compared between control and CFA, it was revealed that ZD7288 had a significantly greater effect during CFA inflammation (P=0.001). Statistics: linear regression. Slope, R^2 and 95% CI values for ***A***/***B*** & ***C*** are presented in tables 4.14 & 4.15, respectively. Control n=3, CFA n=3. Error bars indicate SEM. Dashed lines represent line of best fit. $**P<0.01$, $****P<0.0001$.

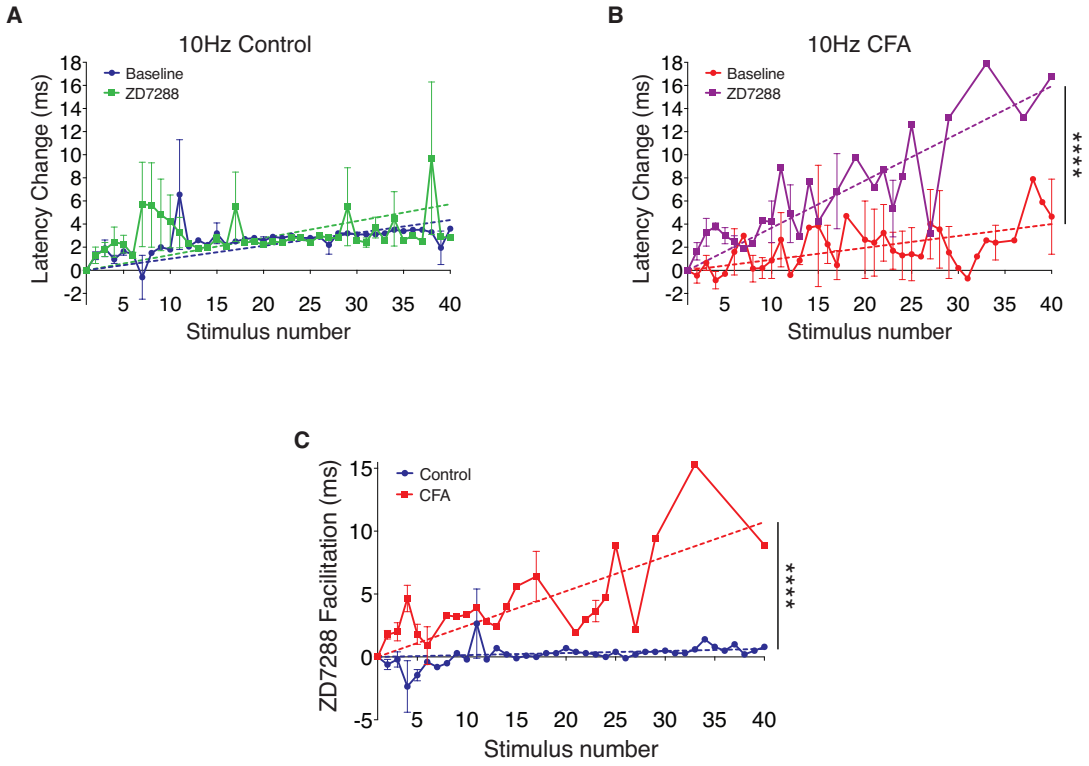

Figure 4.26: ZD7288 enhances activity-dependent slowing, in response to stimulation at 10Hz, in monosynaptic C-fibre input to lamina I NK1R+ neurons. ***A*** 10μM ZD7288 had no effect on the progressive latency increase in control tissue, during 10Hz stimulation (P=0.137). ***B*** In tissue isolated from CFA treated rats, ZD7288 significantly enhanced the progressive increase in C-fibre response latency ($P<0.0001$). ***C*** The facilitatory effect of ZD7288 was significantly greater in CFA inflammation conditions ($P<0.0001$). Statistics: linear regression. Slope, R^2 and 95% CI values for ***A***/***B*** & ***C*** are presented in tables 4.14 & 4.15, respectively. Control n=3, CFA n=2. Error bars indicate SEM. Dashed lines represent line of best fit. $****P<0.0001$.

Frequency (Hz)	Treatment	Recording	Slope	r^2	95% CI	Slope vs. Slope = 0	Baseline vs. ZD7288
2	control	baseline	0.149	0.330	0.136 to 0.161	P<0.0001	P<0.0001
		ZD7288	0.219	0.434	0.200 to 0.238	P<0.0001	
	CFA	baseline	0.073	0.234	0.061 to 0.086	P<0.0001	P<0.0001
		ZD7288	0.152	0.273	0.129 to 0.175	P<0.0001	
5	control	baseline	0.212	-0.030	0.185 to 0.239	P<0.0001	P=0.849
		ZD7288	0.208	0.219	0.180 to 0.236	P<0.0001	
	CFA	baseline	0.102	0.365	0.087 to 0.117	P<0.0001	P<0.0001
		ZD7288	0.191	0.246	0.159 to 0.223	P<0.0001	
10	control	baseline	0.112	-0.077	0.088 to 0.135	P<0.0001	P=0.137
		ZD7288	0.147	-0.159	0.109 to 0.185	P<0.0001	
	CFA	baseline	0.103	0.155	0.072 to 0.133	P<0.0001	P<0.0001
		ZD7288	0.409	0.655	0.355 to 0.462	P<0.0001	

Table 4.14: Comparison of the ZD7288 enhancement of ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons.

Frequency (Hz)	Treatment	Slope	r^2	95% CI	Slope vs. Slope = 0	Control vs. CFA
2	control	0.063	0.178	0.052 to 0.075	P<0.0001	P=0.125
	CFA	0.077	0.231	0.064 to 0.089	P<0.0001	
5	control	0.035	0.098	0.016 to 0.054	P=0.0004	P=0.001
	CFA	0.083	0.017	0.060 to 0.107	P<0.0001	
10	control	0.016	0.077	0.000 to 0.032	P=0.045	P<0.0001
	CFA	0.275	0.450	0.223 to 0.327	P<0.0001	

Table 4.15: Comparison of the magnitude of ZD7288 facilitation of ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons.

The initial latency of monosynaptic C-fibre eEPSCs, recorded in lamina I NK1R+ neurons was significantly shorter in tissue isolated from CFA treated rats (P=0.018, 2-way ANOVA on rank transformed data, figure 4.27A), although the initial latency did not differ between recordings employing 2, 5 or 10Hz stimulation (P=0.888, 2-way ANOVA on rank transformed data). There was no significant interaction between CFA inflammation and stimulation frequency (P=0.696, 2-way ANOVA on rank transformed data). Similarly, the length of dorsal root stimulated in C-fibre eEPSC ZD7288 recordings was significantly shorter in CFA groups ($P<0.0001$, 2-way ANOVA on rank transformed data, figure 4.27B), but did not differ between stimulation frequencies (P=0.911, 2-way ANOVA on rank transformed data) and there was no interaction (P=0.911, 2-way ANOVA on rank transformed data). The effect of ZD7288 on the initial latency of monosynaptic C-fibre input to lamina I NK1R+ neurons was assessed by comparing the initial latency recorded during baseline recordings with the initial latency during ZD7288 recordings. In tissue isolated from control rats, ZD7288 caused a small but significant increase in the initial latency of the C-fibre response (P=0.0004, 2-way repeated measures ANOVA, on rank transformed data, figure 4.27C), while stimulation frequency was without effect (P=0.924, 2-way repeated measures ANOVA, on rank transformed data) and there was no interaction (P=0.149, 2-way repeated measures ANOVA, on rank transformed data). However, in tissue isolated from CFA treated rats, ZD7288 had no effect on the initial C-fibre latency (P=0.431, 2-way repeated measures ANOVA, on rank transformed data, figure 4.27D). Stimulation frequency was also without effect (P=0.552, 2-way repeated measures ANOVA, on rank transformed data) and there was no interaction between these factors (P=0939, 2-way repeated measures ANOVA, on rank transformed data). The change in the initial latency produced by ZD7288 application was calculated by subtracting the baseline initial latency from the ZD7288 initial latency. It was found that ZD7288 caused a significantly greater change in initial latency in tissue isolated from control rats (P=0.003, 2-way ANOVA, on rank transformed data, figure 4.27E), while stimulation frequency had no significant effect (P=0.293, 2-way ANOVA, on rank transformed data) and there was no interaction between these variables (P=0.810, 2-way ANOVA, on rank transformed data).

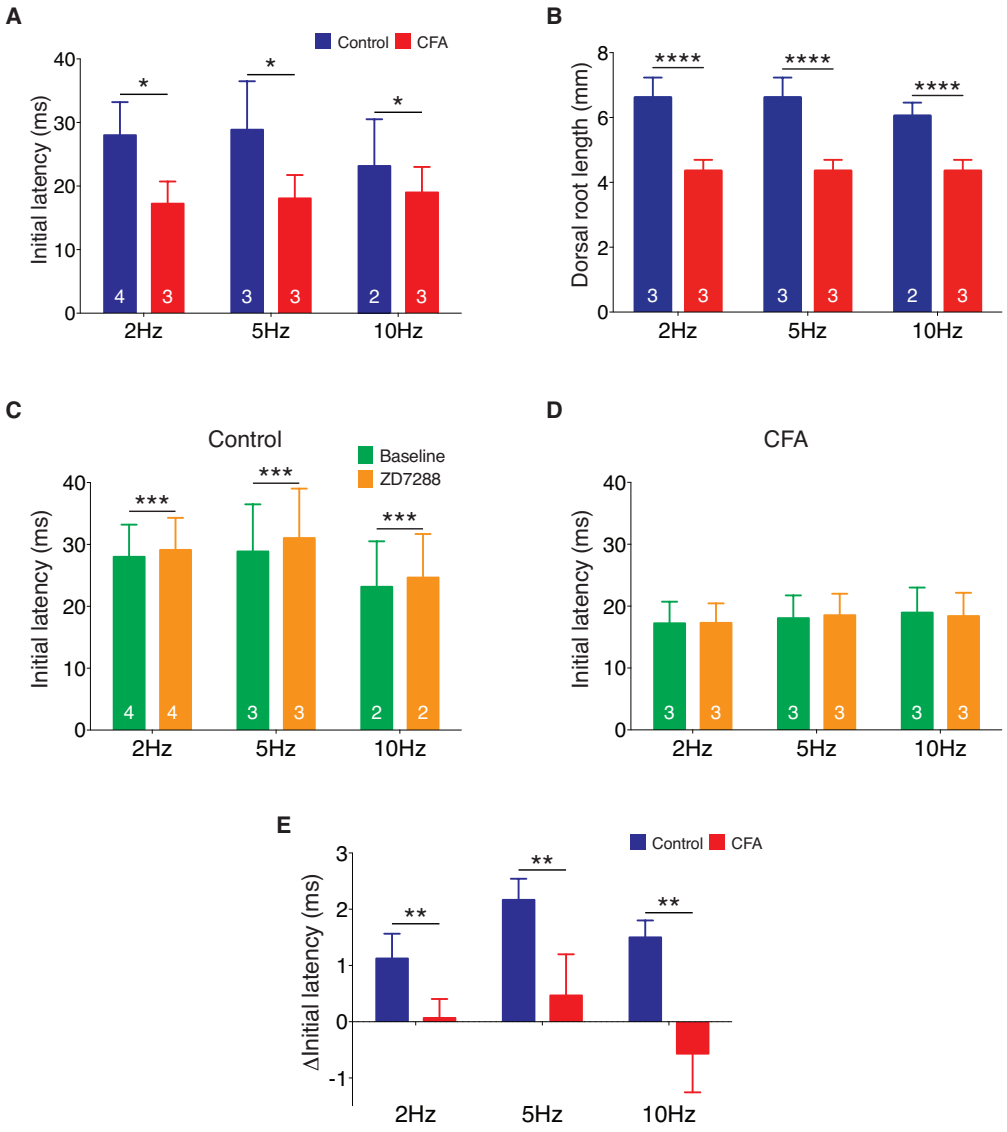

Figure 4.27: ZD7288 increases the initial latency of monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons. ***A*** The initial latency of monosynaptic C-fibre input to lamina I NK1R+ neurons was significantly shorter in tissue isolated from CFA treated rats (P=0.018), however stimulation frequency was without effect (P=0.888) and there was no interaction between these variables (P=0.696). ***B*** The length of dorsal root stimulated was significantly different between control and CFA groups (P$<$0.0001), but did not differ between stimulation frequencies (P=0.911) and there was no interaction (P=0.911). ***C*** In control tissue, application of 10μM ZD7288 produced a small but significant increase in the initial latency of C-fibre eEPSCs (P=0.0004), while stimulation frequency was without effect (P=0.924) and there was no interaction (P=0.149). ***D*** In the CFA group, ZD7288 did not alter the initial C-fibre latency (P=0.431) and there was no effect of stimulation frequency (P=0.552) and no interaction (P=0.939). ***E*** When the change in the initial C-fibre response latency was compared between control and CFA groups, it was discovered that ZD7288 resulted in a significant increase in initial latency in tissue isolated from control rats (P=0.003). Stimulation frequency did not alter the ZD7288 change in initial latency (P=0.293) and there was no significant interaction between these variables (P=0.810). Statistics (***A***, ***B*** & ***E***): 2-way ANOVA on rank transformed data. Statistics (***C*** & ***D***): 2-way repeated measures ANOVA on rank transformed data. Sample sizes indicated on graphs, except ***E*** , where sample sizes are the same as ***A***. Legend in ***A*** applies to ***B***. Legend in ***C*** applies to ***D***. Error bars indicate SEM. $*$P$<$0.05, $**$P$<$0.01, $***$P$<$0.001.

To determine whether application of ZD7288 altered the number of synaptic response failures in monosynaptic C-fibre input to lamina I NK1R+ neurons, a preliminary investigation into the synaptic response failure rate during repetitive stimulation in the absence and presence of ZD7288 was conducted. During 2Hz stimulation, the synaptic response failure rate was not altered by ZD7288, CFA inflammation and there was no interaction between these factors (P=0.238, P=0.826 & P=0.900, respectively, 2-way repeated measures ANOVA, figure 4.28A). Similarly, ZD7288 and CFA inflammation was without effect during C-fibre stimulation at 5Hz and there was no interaction between these variables (P=0.221, P=0.779 & P=0.642, respectively, 2-way repeated measures ANOVA, figure 4.28B). During 10Hz stimulation, ZD7288 significantly altered the monosynaptic C-fibre synaptic response failure rate (P=0.016, 2-way repeated measures ANOVA, figure 4.28C). This effect was influenced by CFA inflammation, but CFA alone had no effect (P=0.026 & P=0.599 respectively, 2-way repeated measures ANOVA). Post-hoc analysis revealed that ZD7288 significantly increased the synaptic response failure rate seen during 10Hz stimulation, in tissue isolated from CFA treated rats (P=0.011, 2-way ANOVA followed by Bonferroni post-tests).

To examine whether ZD7288 altered the amplitude of monosynaptic C-fibre input to lamina I NK1R+ neurons, the peak C-fibre amplitude recorded prior to and during ZD7288 application, in tissue isolated from control and CFA treated tissue, was compared. The results of this preliminary study showed that ZD7288 did not significantly alter the peak amplitude of monosynaptic C-fibre input to lamina I NK1R+ neurons (P=0.944, 2-way repeated measures ANOVA on rank transformed data, figure 4.29). CFA inflammation was similarly without effect on C-fibre peak amplitude (P>0.999, 2-way repeated measures ANOVA on rank transformed data) and there was no interaction between these factors (P=0.220, 2-way repeated measures ANOVA on rank transformed data).

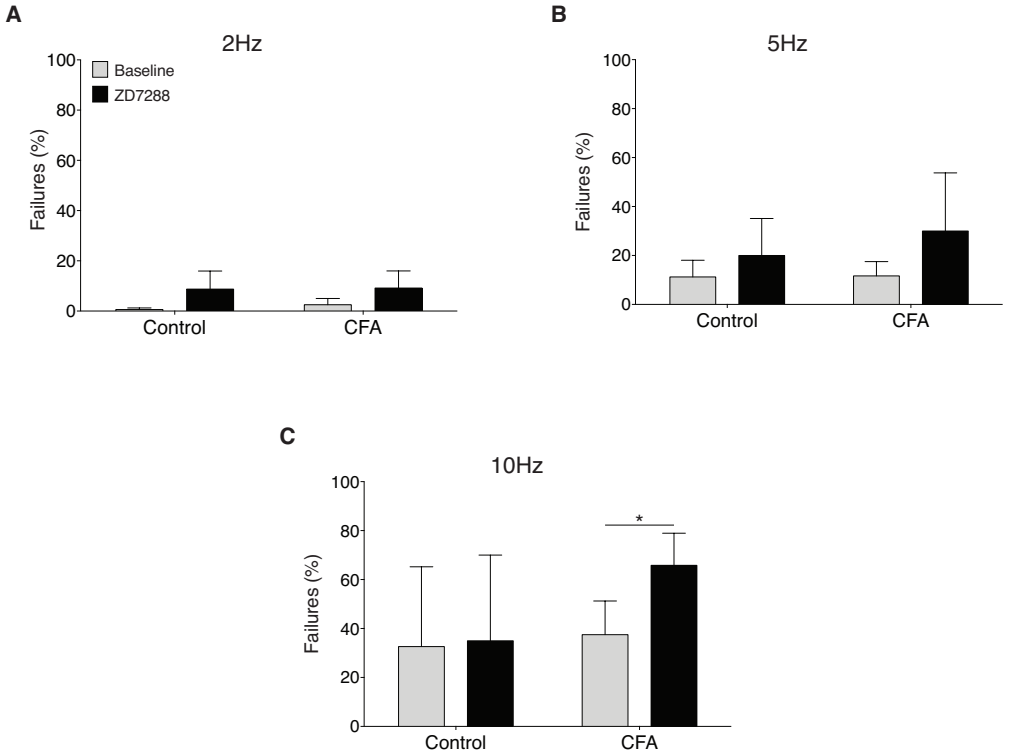

Figure 4.28: Synaptic response failures in monosynaptic C-fibre input to lamina I NK1R+ neurons during ZD7288 application. Although application of ZD7288 typically resulted in a greater number of failures in monosynaptic C-fibre input to lamina I NK1R+ neurons during stimulation at frequencies of 2 and 5Hz (***A*** & ***B*** , respectively), this effect was not significant (P=0.238 & P=0.221, respectively). CFA inflammation was also without effect (P=0.826 & P=0.779, respectively) and there was no interaction between these factors (P=0.900 & P=0.642, respectively). ***C*** During 10Hz stimulation, ZD7288 significantly altered the failure rate (P=0.016) and this effect was influenced by CFA inflammation (P=0.026). CFA inflammation by itself was without effect (P=0.599). However, post-tests revealed that ZD7288 significantly increased synaptic response failures in CFA inflammation (P=0.011). All statistics: 2-way repeated measures ANOVA followed by Bonferroni post-tests. Legend in ***A*** applies to all. Error bars indicate SEM. Control: 2Hz n=4, 5Hz n=3, 10Hz n=2. CFA: all n=3. $*P<0.05$.

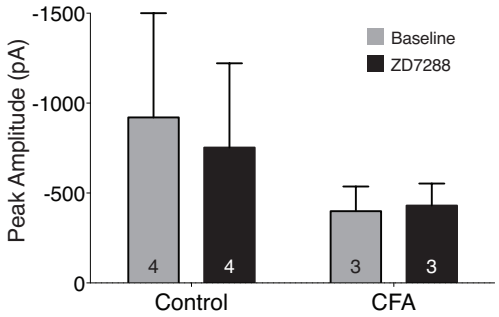

Figure 4.29: ZD7288 does not alter the peak amplitude of monosynaptic C-fibre input to lamina I neurokinin 1 receptor expressing neurons. Application of ZD7288 did not alter the peak amplitude of monosynaptic C-fibre input to lamina I NK1R+ neurons (P=0.944). CFA inflammation was similarly without effect ($P>0.999$) and there was no interaction between these factors (P=0.220). Statistics: 2-way repeated measures ANOVA on rank transformed data. Sample sizes indicated on graph. Error bars indicate SEM.

4.4.5 Summary

A summary of the key findings from this chapter, based on the analysis performed on normalised data, are presented in tables 4.16, 4.17 and 4.18. C-fibre ADS in CAP recordings from isolated dorsal roots and in monosynaptic C-fibre input to lamina I NK1R+ neurons, elicited by repetitive stimulation at frequencies of 1, 2, 5 or 10Hz, using stimulus trains of either 16 or 40 stimuli, was in all cases found to be significantly reduced in CFA inflammation (table 4.16). Aδ-fibre ADS elicited by stimulation at frequencies of 1 and 2Hz, in isolated dorsal roots or in the monosynaptic Aδ-fibre input to lamina I NK1R+ neurons, was not altered in CFA inflammation, however when monosynaptic Aδ-fibre input was stimulated at 10Hz, the ADS recorded in CFA was found to be significantly reduced compared to control (table 4.17). A preliminary investigation into the effect of ZD7288 on ADS was conducted, where in almost all conditions it was found that ZD7288 significantly enhanced ADS in CFA inflammation. In control tissue, ZD7288 increased ADS during 2Hz stimulation, in both CAP and monosynaptic C-fibre eEPSC recordings, but was without effect in eEPSC recordings when stimulated at 5 and 10Hz. In all cases, except ADS elicited in monosynaptic C-fibre input during 2Hz stimulation, the facilitatory effect of ZD7288 was significantly enhanced in tissue isolated from CFA treated rats (table 4.18).

Stimulation frequency	Stimulus N^o	Recording	CFA effect
1Hz	16	CAP	↓ ADS
		eEPSC	↓ ADS
2Hz	16	CAP	↓ ADS
		eEPSC	↓ ADS
	40	eEPSC	↓ ADS
5Hz	40	eEPSC	↓ ADS
10Hz	40	eEPSC	↓ ADS

Table 4.16: Summary of results from C-fibre ADS recordings.

Stimulation frequency	Stimulus N^o	Recording	CFA effect
1Hz	16	CAP	no effect
		eEPSC	no effect
2Hz	16	CAP	no effect
		eEPSC	no effect
10Hz	16	eEPSC	↓ ADS

Table 4.17: Summary of results from Aδ-fibre ADS recordings.

Stimulation frequency	Stimulus N^o	Recording	Treatment	ZD7288 effect	Facilitation
2Hz	40	CAP	control	↑ ADS	>CFA
			CFA	↑ ADS	
		eEPSC	control	↑ ADS	no difference
			CFA	↑ ADS	
5Hz	40	eEPSC	control	no effect	>CFA
			CFA	↑ ADS	
10Hz	40	eEPSC	control	no effect	>CFA
			CFA	↑ ADS	

Table 4.18: Summary of the effect of ZD7288 on C-fibre ADS recordings.

4.5 Discussion

The findings of this chapter have confirmed the following:

1. C-fibres display a progressive slowing of conduction velocity / increase in response latency, during repetitive stimulation at frequencies of 1Hz or above (figures 4.5 & 4.6, table 4.2).

In addition these studies have revealed for the first time that:

1. ADS can be observed in extracellular population electrophysiological recordings from isolated dorsal roots (figures 4.4 & 4.5).
2. Aβ-fibres, in isolated dorsal roots show a progressive reduction in response latency during repetitive stimulation, which is altered by CFA inflammation at 2, but not 1Hz stimulation (figures 4.3 & 4.6, table 4.2).
3. Aδ-fibres in isolated dorsal roots and monosynaptic Aδ-fibre input to lamina I NK1R+ neurons exhibit ADS, the latter of which which is reduced by CFA inflammation, during stimulation at 10Hz (figures 4.4, 4.6 & 4.10, tables 4.2 & 4.4).
4. C-fibre ADS in isolated dorsal roots is significantly reduced in CFA inflammation (figures 4.5 & 4.6, table 4.2).
5. Monosynaptic C-fibre input to lamina I NK1R+ neurons exhibits ADS in response to repetitive stimulation, which is significantly attenuated in CFA inflammation (figures 4.12 & 4.16, tables 4.6 & 4.8).
6. The non-selective HCN antagonist, ZD7288, significantly enhances C-fibre ADS in isolated dorsal roots and monosynaptic C-fibre input to lamina I NK1R+ neurons, where the facilitatory effect is significantly greater in tissue isolated from CFA treated rats (figures 4.20, 4.24, 4.25 & 4.26, tables 4.11, 4.12, 4.14 & 4.15).

4.5.1 Aβ-fibres display 'activity-dependent speeding' in response to repetitive stimulation

This study has shown that Aβ-fibres, in isolated rat dorsal roots, display a small but significant reduction in response latency during stimulation at 1 and 2Hz, indicating

an activity-dependent speeding rather than slowing (figures 4.3, 4.6 and table 4.2). Furthermore, the speeding that occurred during 2Hz stimulation was significantly reduced in dorsal roots isolated from CFA treated rats.

There is limited evidence that repetitive stimulation of Aβ-fibres results in an activity-dependent change in conduction velocity / response latency. *In vivo* single unit recordings from dorsal roots has shown that Aβ-fibres display minimal ADS at stimulation frequencies of $\leq$25Hz, which is increased in a frequency dependent manner at frequencies of $\geq$50Hz (Shin et al. 1997). This study examined changes in Aβ-fibre response latency during a maximum stimulation frequency of 2Hz, while Shin et al. (1997) employed frequencies up to 200Hz. It would therefore be of interest to examine the changes in Aβ-fibres in response to increased stimulation frequencies to determine whether Aβ-fibres display enhanced speeding or ADS.

The results presented here shown that the progressive reduction in Aβ-fibre response latency observed during 2Hz stimulation is significantly attenuated by CFA inflammation. Previous studies have shown that Aβ-fibre ADS is enhanced following nerve injury (Shin et al. 1997), however this is the first evidence to suggest that the activity-dependent changes in response latency of Aβ-fibres may be altered in inflammatory pain.

The functional implications of Aβ-fibre activity-dependent speeding and its subsequent attenuation in CFA inflammation, are unclear. Given that Aβ-fibres are considered to mediate allodynia (Latremoliere & Woolf 2009, Treede & Magerl 2000), it could be speculated that any change in Aβ-fibre responses in inflammatory pain conditions could have implications for the development / maintenance of allodynia. However, the activity-dependent speeding reported here would lead a reduction in the time between impulses arriving at the dorsal horn and arguably a more efficient transmission of somatosensory information to the dorsal horn. Whereas, the attenuation of this speeding in CFA inflammation would result in less efficient transmission, so it is questionable whether this mechanism would play a prominent role in inflammatory pain allodynia. As the CFA reduction in Aβ-fibre speeding was seen at 2, but not 1Hz it may be more relevant to ascertain the influence of inflammation upon activity-dependent speeding at higher frequencies, that are more consistent with the evoked Aβ-fibre firing rate.

An important question with regards to these activity-dependent changes in Aβ-fibres, is whether these changes in isolated dorsal roots are reflected in monosynaptic Aβ-fibre input to lamina I NK1R+ neurons, which are crucial for the manifestation of

inflammatory pain (Nichols et al. 1999). This was not explored in this current study due to the fact that lamina I NK1R+ neurons receive limited monosynaptic Aβ-fibre input (figure 2.14) (Torsney 2011, Torsney & MacDermott 2006). This thesis has presented data that show a trend towards increased monosynaptic Aβ-fibre input to lamina I NK1R+ neurons in CFA inflammation (figure 2.14), therefore it would be important to establish whether this potential increase in the incidence of monosynaptic Aβ-fibre input is likely to be accompanied by altered patterns of Aβ-fibre input during inflammatory pain.

4.5.2 Aδ-fibres display activity-dependent slowing, which at higher stimulation frequencies is attenuated in inflammatory pain

This study has established that Aδ-fibres in isolated dorsal roots (figures 4.4D & E, 4.6B, table 4.2) and monosynaptic Aδ-fibre input to lamina I NK1R+ neurons (figure 4.10, table 4.4) exhibit small but significant levels of ADS in response to repetitive stimulation at 1Hz or above. Furthermore, CFA inflammation significantly attenuated the ADS in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons, elicited during 10Hz stimulation (figure 4.10C).

It has previously been reported that ADS in primary afferent fibres occurs in C-, but not Aδ-fibres (Mazo et al. 2013, Nakatsuka et al. 2000), which the findings of this study do not support. Although it should be recognised that several other studies do provide evidence that Aδ-fibres exhibit ADS in response to repetitive stimulation (Raymond et al. 1990, Thalhammer et al. 1994, Won et al. 1997). While Nakatsuka et al. (2000) report that ADS does not occur in Aδ-fibres, it should be noted that they use a maximum stimulation frequency of 10Hz, a frequency at which others report minimal Aδ-fibre ADS (Won et al. 1997). This does raise the possibility that Aδ-fibre ADS may only reveal itself during high-frequency stimulation, however this is unlikely given that the findings of this study demonstrates Aδ-fibre ADS during stimulation at 1Hz.

Aδ-fibre ADS has been shown to be frequency dependent, with increased stimulation frequency being correlated with greater ADS (Raymond et al. 1990, Thalhammer et al. 1994, Won et al. 1997). This is supported by the findings of the eEPSC experiments reported here, where it was demonstrated that stimulation frequency significantly alters the magnitude of monosynaptic Aδ-fibre ADS (figure 4.10D). However, the Aδ-fibre ADS recorded in CAP studies did not display a frequency dependence (figure 4.4B). This contradiction could suggest that while monosynaptic

Aδ-fibre input to lamina I NK1R+ neurons displays frequency dependent ADS, not all Aδ-fibres exhibit ADS and so any frequency dependent effect in the population CAP recordings could be diluted. This hypothesis could be tested by comparing the ADS profile of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons with that of other dorsal horn neurons that receive monosynaptic Aδ-fibre input.

This study has found that CFA inflammation significantly attenuates ADS in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons, during repetitive stimulation at 10, but not 1 or 2Hz (figure 4.10, table 4.4). It has previously been shown that Aδ-fibre ADS is enhanced in nerve injured animals, but only when dorsal roots are stimulated at 5Hz or above (Won et al. 1997).

CFA was found to significantly alter the intercept of the line of best fit that was fitted to the absolute latency of Aδ-fibre responses in CAP and eEPSC recordings during 1 and 2Hz stimulation. However, these results are contradictory, in that the CFA inflammation group exhibited an increased intercept in CAP recordings (figure 4.4B & C, table 4.1), but a decreased intercept in eEPSC recordings (figure 4.10A & B, table 4.3). While these findings could suggest that the conduction velocity of Aδ-fibres is altered in inflammatory pain conditions, this postulate is not supported by other data. When the initial latencies of the Aδ-fibre responses were compared, no significant differences were discovered between control and CFA groups, in CAP (figure 4.7C) or eEPSC recordings (figure 4.9D). Arguably, comparing initial latencies is a more accurate way to determine the effects of CFA inflammation upon the initial latency of the Aδ-fibre response, as the intercept is influenced by the slope of the line of best fit and is an extrapolated value, whereas the initial latency is an actual measured value that is independent from the response to repetitive stimulation. The results presented in chapter 2 also show that CFA inflammation does not alter the conduction velocity of Aδ-fibres in isolated dorsal roots (figure 2.9C) or the estimated conduction velocity of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons (figure 2.15D). Numerous other studies have also reported that CFA inflammation does not significantly alter the conduction velocity of Aδ-fibres (Baba et al. 1999, Nakatsuka et al. 1999, Torsney 2011) and importantly this has been shown to be the case in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons (Torsney 2011). However, the conduction velocity of Aδ-fibre CAPs has been shown to be increased in guinea pig dorsal roots following CFA inflammation (Djouhri & Lawson 2001). There were no significant differences in the length of dorsal root stimulated between control and CFA groups in CAP (figure 4.7A) or eEPSC studies (figure 4.9E), so this is unlikely to account for the differences in the intercepts.

The difference in intercept in the eEPSC data appears to be due to an increase in the initial latency of the control group, in that the initial latency recorded in tissue isolated from CFA treated rats remains comparable between different stimulation frequencies, while in the control group the initial latency gradually declined between 1, 2 and 10Hz stimulation periods (figure 4.9). Furthermore, the intercepts of the absolute latency recorded during 1, 2 and 10Hz stimulation were significantly different in tissue isolated from control ($P<0.0001$, linear regression, data not shown), but not CFA treated rats ($P=0.821$, linear regression, data not shown). In these Aδ-fibre eEPSC studies, dorsal roots were stimulated at 1Hz followed by 2Hz followed by 10Hz, although only a subset of Aδ-fibre inputs were stimulated at 1 and/or 10Hz. This raises the question of whether the reduction in Aδ-fibre latency, in control tissue, between these different stimulation periods is an effect of the previous stimulation train. It is possible that the period of time left between stimulus trains was not sufficient to allow full recovery from ADS. However, if these monosynaptic Aδ-fibre inputs had not fully recovered from the ADS elicited by the previous stimulation train, you would expect the latency to increase, rather than decrease. Future studies should therefore examine monosynaptic Aδ-fibre inputs to lamina I NK1R+ neurons in the period following repetitive stimulation at 1Hz or above, to determine the time course of recovery from ADS.

4.5.3 Activity-dependent slowing in C-fibres is altered in inflammatory pain

The results of this study have novelly revealed that monosynaptic C-fibre input to lamina I NK1R+ neurons display ADS in response to repetitive stimulation at 1Hz or above (figures 4.12 & 4.16, tables 4.6 & 4.8). Furthermore, ADS in these C-fibre inputs, as well as in isolated dorsal roots (figure 4.5, table 4.2), was significantly reduced in tissue isolated from CFA treated rats.

It has been argued that ADS acts as a protective mechanism to limit nociceptive input to the spinal cord and thus may act to regulate central sensitisation (de Col et al. 2012, Mazo et al. 2013), which is known to arise from repetitive firing of C-fibres (Latremoliere & Woolf 2009, Woolf 1983). Lamina I NK1R+ neurons are know to be essential for the development of inflammatory pain (Nichols et al. 1999), which is driven by activity in C-fibre nociceptors (Abrahamsen et al. 2008). Therefore, the finding that ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons is significantly reduced in CFA inflammation suggests that the transmission of

nociceptive information to these key spinal cord output neurons could be enhanced in inflammatory pain conditions, which may drive inflammatory pain spinal plasticity.

This thesis has revealed that ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons is significantly reduced in inflammatory pain conditions. This is consistent with previous evidence that application of NGF, which is a key mediator in inflammatory pain (Cheng & Ji 2008, Pezet & McMahon 2006), can modify ADS (Obreja et al. 2011a, 2011b). Specifically, *in vivo* electrophysiological recordings from pig saphenous nerve have shown that pre-treatment with NGF, 4–7 days or 3 weeks prior to recording, significantly reduces the degree of ADS seen in mechano-insensitive C-fibre nociceptors, but not mechano-sensitive C-fibres or sympathetic efferents (Obreja et al. 2011a, 2011b).

ADS in C-fibres has previously been shown to be altered in neuropathic pain states in both humans (Kleggetveit et al. 2012, Ørstavik et al. 2006, 2003) and rats (Shim et al. 2007), although in these cases ADS was found to be enhanced. In the SNL neuropathic pain model, electrophysiological recordings from isolated uninjured L4 dorsal roots demonstrated that ADS was elevated in nerve injured rats compared to sham operated controls (Shim et al. 2007). While in humans, erythromyalgia patients have been found to express greater ADS that controls (Ørstavik et al. 2003). Mazo et al. (2013) report that following axotomy, ADS is reduced in mouse C-fibres, however while ADS was shown to be reduced following nerve injury, the authors do not present a direct comparison of ADS in control and axotomy groups and no statistics are provided to support their claims. Therefore, it appears that inflammatory and neuropathic pain may results in contrasting effects upon ADS.

The results presented here show a progressive increase in the latency of C-fibre, as well as Aδ-fibre, eEPSCs that is considered to result from a gradual slowing of conduction velocity. However, it should be recognised that this result could equally arise from a gradual increase in the action potential initiation time. An underlying mechanism of ADS that has been proposed is an activity-dependent reduction in the availability of operational Na_v channels, driven by an accumulation of Na_v channels entering the slow-inactivated state (Baker & Waxman 2012, de Col et al. 2008). In patch-clamp recordings from C-fibre DRG neurons, slow-inactivation of Na_v channels driven by repetitive stimulation has been shown to lead to a progressive increase in action potential initiation time (Snape et al. 2010). It is unclear whether the total latency change reported in this thesis can be attributed solely to changes in action potential initiation time. The change in action potential initiation time after 16 stimuli at 2Hz was shown to be around 1 – 1.5ms (Snape et al. 2010), which is comparable

with the latency change seen in C-fibre CAP recordings but approximately half of the latency change seen in monosynaptic C-fibre inputs to lamina I NK1R+ expressing neurons in this study. Furthermore, if the latency changes seen during repetitive stimulation were entirely due to changes in action potential initiation duration, it would be expected that the latency changes recorded would not be influenced by the length of the conduction pathway. However, in eEPSC recordings, where a greater proportion of the conduction pathways is stimulated, the degree of latency change is larger than in CAP recordings, where the length of conduction pathway is shorter. The contribution of changes in action potential initiation time to ADS could be addressed by performing CAP recordings from isolated dorsal roots where two recording electrodes are employed, rather than the single recording electrode used here. This would enable the latency to be determined as the time taken for the response to travel between the two recording electrodes, where action potential initiation has already occurred and so enable the study of changes in conduction only.

In CAP recordings there are several possibilities that could account for the reduced C-fibre ADS seen in CFA inflammation, as opposed to a simple reduction in ADS. It has been established that there is a positive correlation between the probability of conduction failure and the magnitude of ADS in C-fibres (Obreja et al. 2011a, Zhu et al. 2009). Therefore, if CFA inflammation increased ADS in C-fibres in isolated dorsal roots, an increase in the proportion of C-fibres that display conduction failures would be expected. This could lead to a reduced contribution from those C-fibres that display the greatest ADS in the extracellular population CAP recordings, which in turn could lead to an apparent reduction in ADS. Interestingly, in eEPSC recordings the progressive increase in synaptic response failure rate was significantly enhanced in the CFA group during stimulation at 2Hz (figure 4.17A), although the overall percentage of failures was not changed (figure 4.17D). The proposal that reduced ADS is counterintuitively the result of increased ADS resulting in increased failures is however unlikely, given that the CFA reduction in C-fibre ADS seen in CAP recordings is mirrored in eEPSC recordings. If there was an increase in ADS / failures in CFA inflammation, then arguably the criteria used to classify C-fibre input to lamina I NK1R+ neurons as monosynaptic, a lack of failures when stimulated 20 times at 1Hz (section 2.3.5) (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006), could have unintentionally excluded those neurons that received inputs displaying the highest levels of ADS / failures, as failures would have resulted in misidentification as polysynaptic C-fibre input. However, this scenario is not considered likely given that the incidence of monosynaptic C-fibre input reported here (figure 2.14) and by others (Torsney 2011) is unaltered by CFA inflammation.

Likewise, if monosynaptic C-fibre inputs were misidentified as polysynaptic in CFA inflammation, a concurrent increase in the incidence of polysynaptic input would be expected, but this is not the case (figure 2.14) (Torsney 2011).

Repetitive stimulation has previously been shown to increase activation thresholds in response to natural stimulation (de Col et al. 2012, Thalhammer et al. 1994). As such, an alternative explanation for the reduction in ADS seen in C-fibres in dorsal roots isolated from CFA treated rats, could be that CFA increased ADS / electrical activation thresholds, preventing action potential initiation in those fibres that displayed high levels of ADS. If during CFA inflammation fibres displayed increased ADS / activation threshold and were not activated by the stimulation intensity used in CAP recordings, this could have lead to an apparent decrease in ADS. However, results in chapter 2 demonstrate that the electrical activation thresholds of primary afferent fibres are not altered by CFA inflammation (figure 2.9), a finding which is supported by several published studies (Baba et al. 1999, Nakatsuka et al. 1999, Torsney 2011). It is also worth noting that suprathreshold intensities were used to activate primary afferents in this study. Furthermore, repetitive stimulation has been shown to have no effect on the electrical activation threshold of C-fibres (Obreja et al. 2012).

Previous studies report that increasing stimulation frequencies are correlated with a greater number of failures in the C-fibre response during repetitive stimulation (Nakatsuka et al. 2000, Raymond et al. 1990, Zhu et al. 2009), however it was unknown whether this is altered in inflammatory pain. While reduced ADS in inflammatory pain has the potential to transform the temporal pattern of synaptic input to lamina I NK1R+ neurons, an altered number of failures could clearly fundamentally alter the synaptic drive to these neurons. To investigate this, the number of synaptic response failures in the monosynaptic C-fibre input to lamina I NK1R+ neurons was measured during repetitive stimulation at 2, 5 and 10Hz, in both control and CFA tissue. In both control and CFA tissue, at all stimulation frequencies, there was a progressive increase in the number of failures (figure 4.17A, B & C). During 2Hz stimulation, it was discovered that there was significantly more failures in the CFA group, however this was not supported by the findings obtained during 5 and 10Hz, where the number of failures was not altered by CFA inflammation. Likewise, when the total percentage of failures was compared, it was found that CFA was without effect (figure 4.17D). The total percentage of failures was however altered by stimulation frequency, specifically 10Hz stimulation was found to result in significantly more failures than 2Hz, which is consistent with previous data

(Nakatsuka et al. 2000, Raymond et al. 1990, Zhu et al. 2009). Given that CFA inflammation, in general, has no effect upon the number of failures in the monosynaptic C-fibre input to lamina I NK1R+ neurons, it is unlikely that the pattern of synaptic input to these neurons will be influenced by differing degrees of synaptic response failures between control and inflammation conditions.

It has been established in numerous studies that different functional classes of C-fibres display different ADS profiles, whereby mechano-insensitive C-fibres demonstrate the greatest degree of ADS, while minimal ADS is seen in mechano-sensitive C-fibres (Gee et al. 1996, Obreja et al. 2010, Serra et al. 1999, Weidner et al. 1999). With this in mind, it could be argued that the significantly reduced C-fibre ADS seen in CFA inflammation in the data presented here, could result from an expansion in mechano-sensitive C-fibre input to lamina I NK1R+ neurons during inflammation, which could manifest as an apparent reduction in ADS. To examine whether it was possible to identify two distinct populations of lamina I NK1R+ neuron that received monosynaptic C-fibre input that exhibited high or low levels of ADS, which could be indicative of mechano-insensitive or mechano-sensitive input, respectively, frequency histograms of the AUC of latency change, in response to 16 stimuli at 1 and 2Hz were plotted. The results demonstrated that while CFA inflammation caused a significant leftwards shift in the distribution of data obtained during 1 and 2Hz stimulation (figure 4.14A & B), the distribution was unimodal and did not identify two distinct populations of neuron based on ADS profile. However, had this analysis identified two separate populations it should be recognised that the electrophysiological recording techniques employed in these experiments do not enable the activation of primary afferent inputs with natural stimulation, therefore further studies would have been required to validate that the two populations did in fact consist of mechano-sensitive and mechano-insensitive C-fibres.

There is conflicting evidence as to whether the initial C-fibre conduction velocity / latency is related to the degree of ADS that is expressed in response to repetitive stimulation. Recordings from rats (Gee et al. 1996, Taguchi et al. 2010, Thalhammer et al. 1994) and humans (Serra et al. 1999) have found no correlation between initial conduction velocity and ADS. However, others report that slower initial conduction velocities / greater latencies are correlated with greater levels of ADS, in C-fibre recordings from rat (Shim et al. 2007), human (Weidner et al. 1999), rabbit (Swadlow & Waxman 1976, Zhu et al. 2009) and monkey (Ringkamp et al. 2010). While nerve injury has been shown to have no effect on the correlation between initial conduction velocity and ADS (Shim et al. 2007), it was not known whether inflammatory pain

altered this relationship. To investigate this, the initial response latency of monosynaptic C-fibre input to lamina I NK1R+ neurons was plotted against the degree of ADS, as measured by the AUC of latency change. The results of this analysis revealed that in the responses recorded during 1Hz stimulation, there was no significant correlation between initial latency and ADS in either control or CFA groups (figure 4.14C). During 2Hz stimulation there was no significant correlation in the control group, however there was a significant positive correlation in the CFA group (figure 4.14D), indicating that C-fibres which display a greater initial latency display greater ADS.

C-fibre ADS in isolated dorsal roots demonstrated a frequency dependent effect, whereby stimulation at 2Hz resulted in significantly greater ADS than 1Hz (figure 4.6C). While in monosynaptic C-fibre input to lamina I NK1R+ neurons there was a trend towards greater ADS at 2Hz than 1Hz, although this was not significant (P=0.060, figure 4.12D). There is a precedent for the magnitude of ADS to be frequency dependent. Electrophysiological recordings from isolated rat DRG neurons (Nakatsuka et al. 2000) and *in vivo* single unit recordings in the rat (Raymond et al. 1990, Thalhammer et al. 1994) have shown that over a range of stimulation frequencies, increasing frequency is correlated with an increase in the magnitude of ADS.

In eEPSC studies, where monosynaptic C-fibre input to lamina I NK1R+ neurons was stimulated 16 times at frequencies of 1 and 2Hz, the initial latency of the response was significantly elevated at the onset of 2Hz stimulation compared to 1Hz (figure 4.13A). However, this is likely to be accounted for by the fact that the dorsal root length in the 2Hz group is significantly greater (figure 4.13B). Incidentally, if the initial latency values are converted into estimated conduction velocity, this significant difference between 1 and 2Hz is no longer apparent (P=0.368, 2-way ANOVA, data not shown).

During monosynaptic C-fibre eEPSC recordings, the intercept of the line of best fit was significantly reduced in the CFA group, during stimulation with 16 stimuli at 1 and 2Hz (figure 4.12) and 40 stimuli at 2, 5 and 10Hz (figure 4.15). These results could be interpreted as showing that CFA inflammation significantly reduces the conduction velocity of monosynaptic C-fibre input to lamina I NK1R+ neurons. However, this is most likely not the case. As the intercept of the line of best fit is extrapolated from all data points, it is influenced by the response to repetitive stimulation. Comparing the actual initial latencies, which are recorded during the first stimuli and as such are independent from any effect of repetitive stimulation, can therefore be considered to be a more accurate way to evaluate the effect of CFA

inflammation upon the initial C-fibre latency / conduction velocity. When the initial latencies recored during these trains were compared, CFA was without effect. A similar difference in intercept but not initial latency was found in Aδ- and C-fibre CAP recordings (figures 4.4, 4.5 and 4.7) and Aδ-fibre eEPSC recordings (figure 4.9) and can be explained by the same reasoning.

4.5.4 ZD7288 enhances C-fibre activity-dependent slowing

The results presented here demonstrate that the non-selective HCN antagonist, ZD7288, significantly enhances C-fibre ADS in CAP recordings from isolated dorsal roots (figure 4.20) and eEPSC recordings from lamina I NK1R+ neurons (figures 4.24, 4.25 & 4.26), which suggests that HCN channels play a crucial role in constraining ADS. This finding confirms previous reports that HCN channels are involved in constraining ADS (Grafe et al. 1997, Mazo et al. 2013, Takigawa et al. 1998, Zhu et al. 2009).

An important point that should be considered when interpreting the data presented here and elsewhere on the role of HCN channels in ADS, is the specificity of ZD7288. While ZD7288 has long been considered a non-selective HCN antagonist, a recent paper published by Wu et al. (2012b), following the completion of the ZD7288 recordings in this thesis, calls this into question. The authors provide evidence that ZD7288, in addition to HCN channels, may act upon Na_v channels, in that ZD7288 inhibited Na^+ currents in DRG neurons (IC_{50}1.17μM) and in HEK cells expressing Na_v1.4, where 30μM ZD7288 virtually blocked the current (Wu et al. 2012b).

As discussed, Na_v channels are thought to play a key role in mediating ADS (Baker & Waxman 2012, de Col et al. 2008, 2012), so it is possible that the ZD7288 enhancement of ADS reported here and by others (Grafe et al. 1997, Mazo et al. 2013, Takigawa et al. 1998, Zhu et al. 2009) could be driven by an inhibition of Na_v channel activity. Mazo et al. (2013) report that the basal properties of C- and A-fibre CAPs were unaffected by 100μM ZD7288, but that CAPs were blocked by 1μM TTX, which they argue provides evidence that ZD7288 has no effect upon Na_v channels. If true, this could suggest that the Na_v channel activity of ZD7288 in the results presented here was potentially negligible, particularly given that this study used ZD7288 at a concentration of 10μM. However, Mazo et al. (2013) do not show their data, provide a limited description of what aspects of basal CAPs they measured and give no details of statistical analysis to support their claims, so it is unclear how robust these findings are.

In CAP recordings from unmyelinated cerebellar parallel fibres, Baginskas et al. (2009) have demonstrated that ZD7288 reduces conduction velocity. As Na_v channels play a crucial role in mediating action potential propagation (Gold & Gebhart 2010, Liu & Wood 2011) and Na_v channel antagonists have been shown to reduce conduction velocity / increase response latency in C-fibres (de Col et al. 2008, Pinto et al. 2008), the finding that ZD7288 modulates C-fibre conduction velocity could be seen as supporting evidence for a ZD7288 effect upon Na_v channels. However, the results of the CAP recordings presented here found that ZD7288 (10μM) does not alter the initial latency of C-fibre responses in either control or CFA tissue (figure 4.21C). This finding is supported by previous studies which also show that 10μM ZD7288 does not alter the initial latency / conduction velocity of C-fibres (Hogan & Poroli 2008, Takigawa et al. 1998).

In C-fibre eEPSC recordings, ZD7288 resulted in a small but significant increase in the initial latency of monosynaptic C-fibre input to lamina I NK1R+ neurons in control, but not CFA tissue (figure 4.27C & D), with the ZD7288 effect being significantly greater in control than CFA (figure 4.27E). However, if these results are due to ZD7288-altered Na_v channel activity, then these findings can be considered counterintuitive, given that the expression of Na_v channels and Na^+ currents are known to be enhanced in inflammatory pain (Black et al. 2004, Dib-Hajj et al. 2010, Tanaka et al. 1998) and in particular in the CFA inflammatory pain model (Gould et al. 2004). Therefore, ZD7288 could be predicted to have a greater effect in CFA inflammation. Although, if ZD7288 preferentially inhibits $Na_v1.1$, 1.2, 1.6 or 1.9 channels, the expression of which has been shown to be unaltered in inflammatory pain (Black et al. 2004, Tanaka et al. 1998), then the known upregulation of $Na_v1.3$, 1.7 and 1.8 channels in inflammatory pain (Black et al. 2004, Gould et al. 2004, Tanaka et al. 1998) may have compensated against a ZD7822-induced change in initial latency.

In this study the facilitatory effect of ZD7288 on ADS was significantly greater in tissue isolated from CFA treated rats. This could be considered to support previous findings that report HCN2 expression and I_h is increased in C-fibres following CFA inflammation (Weng et al. 2012). While the fact that the effect of ZD7288 on ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons was greater in CFA, arguably could support previous data showing HCN2 channels are expressed on the central terminals of SP containing afferents which form contacts with NK1R+ neurons in lamina I (Papp et al. 2010, 2006) and that HCN2 expression is increased specifically on SP containing afferents during CFA inflammation (Papp et al. 2010). However,

given the questions over the specificity of ZD7288, with its reported activity against Na_v channels (Wu et al. 2012b), it is not possible to make these conclusions. Given that the ZD7288 enhancement of ADS is greater in inflammatory pain conditions, this could suggest that altered expression / activity of Na_v channels is responsible for the reduction in ADS seen in CFA inflammation. It is already known that Na_v channels are regulated in inflammatory pain and that expression of $Na_v1.3$, 1.7 and 1.8 channels specifically are upregulated in inflammatory pain models (Amir et al. 2006, Black et al. 2004, Cummins et al. 2007, Dib-Hajj et al. 2010, Gould et al. 2004). ADS may be mediated by an activity-dependent reduction in the number of operational Na_v channels, resulting from an increase in the number of Na_v channels entering a slow-inactivated state (Baker & Waxman 2012, de Col et al. 2008, 2012, Obreja et al. 2012). Therefore, it is possible that the changes in Na_v channel expression that occur in inflammatory pain could impact upon ADS.

To overcome the limited pharmacological tools available to probe the role of HCN channels in ADS, future studies could utilise genetic manipulation strategies. One such way would be to investigate whether ADS and the impact of inflammation upon ADS is altered in $Na_v1.8$-HCN2$^{-/-}$ mice, which have been used in previous studies into the importance of HCN channels in inflammatory pain (Emery et al. 2011). Not only would such an approach negate the issues relating to the non-specificity of ZD7288 (Wu et al. 2012b) it would provide insight into the specific role of HCN2 channels in ADS. If, for example, the inflammation-induced change in ADS was not seen in these $Na_v1.8$-HCN2$^{-/-}$ mice, then that would provide strong evidence that the altered ADS in CFA inflammation that is described here, was mediated by altered activity / expression of HCN2 channels. Given recent evidence suggesting a key role for HCN2 channels in inflammatory pain (Emery et al. 2011, 2012, Weng et al. 2012), such studies could provide additional evidence that HCN2 channels represent a promising inflammatory pain target.

The data presented here show that ZD7288 application results in a significant increase in synaptic response failures, that likely represent C-fibre action potential failures, in monosynaptic C-fibre input to lamina I NK1R+ neurons, in tissue isolated from CFA treated rats, when stimulated at 10Hz (figure 4.28). This finding is in agreement with previous reports that ZD7288 increases action potential failures following C-fibre stimulation at frequencies of 0.2 (Papp et al. 2006) and 20Hz (Zhu et al. 2009). While Papp et al. (2006) report ZD7288 increased the failure rate in monosynaptic C-fibre input to unidentified lamina I/II neurons, it is questionable as to whether these inputs were actually monosynaptic. They report that in control conditions, during 0.2Hz

stimulation there is a 34% failure rate, while other groups consider the presence of failures when C-fibres are stimulated at 1Hz to indicate polysynaptic C-fibre input (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006). However, this classification criteria is typically in response to 20 stimuli (Nakatsuka et al. 2000, Torsney 2011, Torsney & MacDermott 2006), whereas the failure rate these authors report was in response to 360 stimuli (Papp et al. 2006), which could account for the discrepancy.

This study has demonstrated that application of ZD7288 significantly reduces the amplitude of C-fibre CAP recordings (figure 4.22), which confirms previous reports from population CAP recordings from unmyelinated hippocampal axons (Soleng et al. 2003) and unmyelinated cerebellar parallel fibres (Baginskas et al. 2009). Baginskas et al. (2009) argue that this ZD7288-induced reduction in CAP amplitude most likely arises due to an increase in the number of conduction failures, resulting in a reduction in the number of fibres contributing to the response. Therefore, it is possible that the reduction of C-fibre amplitude in the dorsal root recordings described here, results from a ZD7288-driven increase in conduction failures. As such, this finding could be seen to provide an explanation for the increase in synaptic response failures in monosynaptic C-fibre input to lamina I NK1R+ neurons during 10Hz stimulation in the presence of ZD7288, as discussed above. However, the ZD7288-induced increase in failures in these eEPSC recordings was only seen in CFA tissue, while the CAP amplitude effect was seen in both control and CFA groups. In the eEPSC recordings, ZD7288 did produce more failures in the control and CFA group, when stimulated at 2 and 5Hz (and 10Hz in the control group), but this was not significant. Although, it should be recognised that the sample sizes involved are small, ranging from n=2 to n=4, so if these were increased a significant effect may become evident.

It has previously been shown that application of ZD7288 significantly attenuates the eEPSC amplitude of monosynaptic C-fibre input to unidentified lamina II neurons, with this effect being enhanced following nerve injury (Takasu et al. 2010). The authors suggest that this ZD7288 reduction of eEPSC amplitude is a presynaptic effect, given that ZD7288 reduced mEPSC frequency but not amplitude, although a postsynaptic effect cannot be discounted as HCN channels are known to be expressed on postsynaptic spinal cord neurons (Hughes et al. 2013, 2012, Milligan et al. 2006, Santoro et al. 2000). In this chapter I conducted a preliminary investigation into whether monosynaptic C-fibre input to lamina I NK1R+ neurons, with some of this C-fibre input being known to express HCN channels (Papp et al. 2010, 2006), could similarly be inhibited by ZD7288. The results presented demonstrate that ZD7288 did

not alter the peak eEPSC amplitude of monosynaptic C-fibre input to lamina I NK1R+ neurons, in either control or CFA tissue (figure 4.29). However, small sample sizes, n=3 and n=4, were used in this experiment so it cannot be considered conclusive.

4.6 Conclusions

The results described in this chapter have novelly shown that monosynaptic C-fibre input to lamina I NK1R+ neurons display ADS in response to repetitive stimulation and that the magnitude of this ADS is significantly reduced in the CFA inflammatory pain model (figures 4.12 & 4.16, tables 4.6 & 4.8). Lamina I NK1R+ neurons are essential for the manifestation of inflammatory pain (Nichols et al. 1999) and C-fibres have been established to drive inflammatory pain (Abrahamsen et al. 2008). Therefore, it is possible that the altered C-fibre ADS during inflammation plays a role in the development of inflammatory pain, by enhancing the transmission of nociceptive signals from the periphery to these key spinal cord output neurons, which could drive inflammatory pain spinal plasticity. Further investigation is required to fully elucidate the functional impact of altered ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons. However, it is possible that novel treatment strategies that restore the diminished ADS seen during inflammation, or which amplify ADS could prove efficacious in the management of inflammatory pain.

It has been shown that monosynaptic Aδ-fibre input to lamina I NK1R+ neurons displays a small but significant degree of ADS during repetitive stimulation, which was frequency dependent (figure 4.10). Furthermore, for the fist time it has been shown that Aδ-fibre ADS, elicited at 10, but not 1 or 2Hz, is significantly attenuated in tissue isolated from CFA treated rats. As with C-fibre ADS, this suggests that Aδ-fibre input to these key spinal cord output neurons could be intensified in inflammatory pain conditions. Given the evidence that Aδ-fibres play a key role in mediating mechanical hyperalgesia (Fuchs et al. 2000, Magerl et al. 2001, Ziegler et al. 1999), this reduction of ADS could be involved in mechanical hyperalgesia mechanisms, while treatments that restore or enhance Aδ-fibre ADS could represent a novel approach to treat inflammatory hyperalgesia.

This study has demonstrated that application of the non-selective HCN antagonist, ZD7288, significantly enhances C-fibre ADS in isolated dorsal roots and in monosynaptic C-fibre input to lamina I NK1R+ neurons and that the facilitatory effect of ZD7288 is significantly greater in CFA inflammation (figures 4.20, 4.24, 4.25 & 4.26, tables 4.11, 4.12, 4.14 & 4.15). This confirms previous studies that demonstrate ZD7288 enhances C-fibre ADS and suggests a key role for HCN channels in constraining ADS (Grafe et al. 1997, Mazo et al. 2013, Takigawa et al. 1998, Zhu et al. 2009). The enhanced effect of ZD7288 in CFA provides support to published studies that show expression of HCN channels are upregulated and I_h is increased in

C-fibres in CFA inflammation (Papp et al. 2010, 2006, Weng et al. 2012). Given recent evidence that HCN2 channels play a crucial role in inflammatory pain, whereby pharmacological or genetic manipulation of these channels attenuates inflammatory pain (Emery et al. 2011), it is possible that the mechanism by which HCN channels regulate pain could involve an enhancement of ADS. However, caution should be exercised in interpreting these results due to the likelihood that ZD7288 also acts upon Na_v channels (Wu et al. 2012b), which could equally explain these results, given that a reduction in the availability of operational Na_v channels is also thought to play a key role in ADS (Baker & Waxman 2012, de Col et al. 2008), while Na_v channels are also critically implicated in inflammatory pain (Amir et al. 2006, Black et al. 2004, Cummins et al. 2007, Dib-Hajj et al. 2010, Gould et al. 2004). Therefore, future studies should investigate whether the altered ADS seen in inflammatory pain are associated with altered Na_v channel activity.

Chapter 5

Summary and conclusions

The aim of this thesis was to study inflammatory pain spinal plasticity mechanisms by investigating the synaptic input to lamina I neurokinin 1 receptor expressing (NK1R+) neurons, which are known to be essential for the manifestation of inflammatory pain (Nichols et al. 1999). Inflammation-induced changes in and pharmacological manipulation of the primary afferent drive to these lamina I NK1R+ neurons was assessed.

Findings presented in chapter 2 show that complete Freund's adjuvant (CFA) inflammation does not alter the relative distribution of the type of primary afferent input that lamina I NK1R+ neurons receive. These results did not replicate previous work from within my laboratory that showed the incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons is significantly increased and that a subset of this monosynaptic Aδ-fibre input is potentiated in CFA inflammation (Torsney 2011). This previously reported facilitation of Aδ-fibre input is however consistent with published functional-anatomical studies that show activation of Aδ-fibres, some of which express substance P (SP) (Lawson et al. 1997), with noxious mechanical or electrical stimuli, can drive the internalisation of neurokinin 1 receptors (NK1Rs) in lamina I NK1R+ neurons, with the number of neurons displaying NK1R internalisation being significantly increased following CFA inflammation (Abbadie et al. 1997, Allen et al. 1999, Honor et al. 1999). The results in this chapter did however report a trend towards an increase in the incidence of monosynaptic Aβ-fibre input to lamina I NK1R+ neurons in CFA inflammation, although this did not reach statistical significance. Given that Aβ-fibres are considered to mediate allodynia (Latremoliere & Woolf 2009, Treede & Magerl 2000), an increase in Aβ-fibre input to lamina I NK1R+ neurons in inflammatory pain conditions could play a role in the development of allodynia, by recruiting low threshold / touch input to these

nociceptive specific pathways. Further studies will need to be conducted to validate this increase in Aβ-fibre input and to elucidate the potential mechanisms by which these novel Aβ-fibre inputs are unmasked.

The peak amplitude of monosynaptic C-fibre input to lamina I NK1R+ neurons was not altered following CFA inflammation. This finding confirms previous reports that the long-lasting potentiation of monosynaptic C-fibre input to these neurons, as has been elicited by electrical stimulation of these C-fibre inputs (Ikeda et al. 2003, 2006), does not appear to be implicated in CFA inflammation (Torsney 2011).

The spontaneous excitatory drive to lamina I NK1R+ neurons and the influence of inflammatory pain on this input had not previously been studied. Results presented here demonstrate that CFA inflammation does not alter the frequency or amplitude of spontaneous excitatory postsynaptic currents (sEPSCs) in lamina I NK1R+ neurons, either in the entire population or when neuronal subpopulations that receive monosynaptic input from Aδ- only, Aδ- & C- or C-fibre only are examined independently. The sEPSC frequency was significantly elevated in those neurons that received monosynaptic Aδ-fibre only input compared to neurons that received monosynaptic C-fibre input only. This suggests that the previously reported increase in the prevalence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons in inflammatory pain (Torsney 2011) could be associated with an increase in the spontaneous excitatory drive to these neurons. However, as the findings in this thesis did not replicate the previously described increase in the incidence of monosynaptic Aδ-fibre input to these neurons in inflammatory pain (Torsney 2011), the findings presented here can neither confirm or refute this hypothesis.

Chapter 3 has presented an investigation into the capacity of chemerin, an agonist of the chemerin receptor 23 (ChemR23), which is a potential new inflammatory pain target (Ji et al. 2011, Xu et al. 2010), to pharmacologically modulate primary afferent input to lamina I NK1R+ neurons. It was found that chemerin was without effect in non-potentiated conditions, in that chemerin alone does not alter the frequency or amplitude of miniature excitatory postsynaptic currents (mEPSCs) in lamina I NK1R+ neurons and that chemerin does not affect the peak amplitude of monosynaptic Aδ- or monosynaptic C-fibre evoked excitatory postsynaptic currents (eEPSCs) in these neurons in control animals. However, chemerin significantly reduces the capsaicin potentiation of excitatory input to lamina I NK1R+ neurons, where chemerin attenuates the increase in mEPSC frequency resulting from capsaicin application. Furthermore, the data presented here novelly show that chemerin presynaptically attenuates monosynaptic C-fibre input to a subset of lamina I NK1R+

neurons in inflammatory pain. Chemerin was observed to significantly reduce the peak amplitude of monosynaptic C-fibre eEPSCs in a subset of neurons compared to vehicle and significantly increased the paired-pulse ratio (PPR) / decreased paired-pulse depression (PPD), indicating the chemerin effect was presynaptic, rather than postsynaptic. The finding that chemerin acts only upon a subset of monosynaptic C-fibre input to lamina I NK1R+ neurons is consistent with published expression data that shows ChemR23 is expressed on a subset of transient receptor potential subtype vanilloid 1 (TRPV1) expressing dorsal root ganglia (DRG) neurons and SP containing primary afferent terminals in lamina I of the dorsal horn (Xu et al. 2010). Importantly, lamina I NK1R+ neurons are known to receive inputs from both TRPV1 expressing (Hwang et al. 2003, Labrakakis & MacDermott 2003, Tong & MacDermott 2006) and SP containing afferents (Hwang et al. 2003, Todd et al. 2002).

The lack of chemerin effect in non-potentiated conditions supports previous findings that ChemR23 agonists, such as chemerin and resolvin E1 (RvE1), do not impact upon acute nociceptive processing (Xu et al. 2010), which suggests inflammatory pain treatments that target ChemR23 will not alter protective acute pain responses. Sensitisation of TRPV1 channels is known to contribute to inflammatory pain hypersensitivity (Basbaum et al. 2009, Palazzo et al. 2012, Pingle et al. 2007), while lamina I NK1R+ neurons are recognised to be essential for the manifestation of inflammatory pain (Nichols et al. 1999), which is mediated by C-fibres (Abrahamsen et al. 2008). Therefore, the ability of chemerin to reduce the capsaicin potentiation of excitatory input to lamina I NK1R+ neurons and to presynaptically attenuate monosynaptic C-fibre input to a subset of these neurons in inflammatory pain, adds to the growing evidence that ChemR23 offers a promising target for the development of novel inflammatory pain treatments (Ji et al. 2011, Lee 2012, Xu et al. 2010).

The phenomenon of activity-dependent slowing (ADS), whereby repetitive electrical stimulation of C-fibres, at frequencies of 1Hz or above, results in a progressive reduction in action potential conduction velocity, which manifests as a progressive increase in response latency (Gee et al. 1996, Serra et al. 1999, Thalhammer et al. 1994, Weidner et al. 1999), was investigated in chapter 4. The data presented demonstrate for the first time that C-fibre ADS can be observed in extracellular population recordings from isolated dorsal roots during stimulation at 1 and 2Hz. This C-fibre ADS was shown to be frequency dependent, with 2Hz stimulation resulting in significantly greater ADS than 1Hz. Furthermore, the results novelly show that the ADS elicited by dorsal root stimulation at 1 and 2Hz is significantly reduced in tissue isolated from CFA treated rats.

ADS in the monosynaptic C-fibre input to lamina I NK1R+ neurons was also investigated. This thesis has novelly shown that ADS is present in monosynaptic C-fibre input to lamina I NK1R+ neurons, as evidenced by a significant progressive increase in response latency resulting from 16 stimuli delivered at 1 and 2Hz and 40 stimuli delivered at 2, 5 and 10Hz. There was a trend towards greater ADS during 2Hz stimulation in experiments where 16 stimuli were delivered, however this did not reach significance. As with the results from the dorsal root recordings, CFA inflammation was found to consistently result in a significantly reduction of ADS during all stimulation protocols, compared to control.

The physiological role of ADS is unclear, however it has been argued that it represents a form of self-inhibition that limits nociceptive input to the spinal cord, which could reduce central sensitisation (de Col et al. 2012, Mazo et al. 2013). This thesis has shown that altered ADS in CFA changes the timing of the transmission of nociceptive input from peripheral C-fibres to lamina I NK1R+ neurons, which could have implications for the temporal summation of nociceptive input to these neurons and for spinal cord inflammatory pain plasticity. Importantly, excitatory postsynaptic potentials (EPSPs) in these neurons have been shown to have a long duration (mean duration 3.1s) (Cheunsuang et al. 2002), so there is ample scope for summation of repetitive inputs. While the ADS reported here results in small latency changes ($\sim$1–7ms) in the timing of monosynaptic C-fibre input arriving at lamina I NK1R+ neurons, it should be noted that in these experiments only a small portion of the conduction pathway was stimulated. With this in mind the sciatic nerve and its peripheral and central branches were traced and measured in a number of rats to gain an estimate of the total length of the conduction pathway. It was found that the total estimated length of the conduction pathway, from the hindpaw to the dorsal root entry zone, was 81.4 $\pm$ 2.0mm. In eEPSC ADS experiments the group mean length of dorsal roots stimulated ranged between 5.5 – 6.0mm (data not shown), which accounts for $\sim$7% of the estimated total conduction pathway. If the complete conduction pathway is accounted for then the predicted latency changes would be $\sim$40–100ms, which arguably could limit the central summation of monosynaptic C-fibre input, particularly during stimulation at frequencies that more closely mimic the evoked C-fibre firing rate ($\sim$5 – 40Hz) (Thalhammer et al. 1994, Yeomans & Proudfit 1996), where for example the interstimulus interval during 10Hz stimulation is only 100ms. Notably, CFA inflammation reduces ADS by $\sim$45% which could promote the temporal summation of C-fibre input and drive action potential firing in these neurons.

To investigate whether the inflammation-reduced ADS impacts upon the function of lamina I NK1R+ neurons, monosynaptic C-fibre evoked excitatory postsynaptic potentials (eEPSPs) and action potential firing in these neurons could be recorded, in current-clamp configuration, while the afferent input is electrically stimulated with estimated stimulation trains, that consider ADS in the entire conduction path in control and inflammatory pain conditions. Given that there are numerous factors that could alter the activity of lamina I NK1R+ neurons in inflammatory pain (Latremoliere & Woolf 2009) undertaking these studies in control tissue only would enable the direct study of the effect of changes in ADS upon the function of these neurons without confounding influences. However, it should be noted that the suggested method of scaling up the latency changes lacks precision and does not take into account the fact that conduction velocity (Waddell et al. 1989) and/or ADS (Won et al. 1997) may be altered along the length of the conduction pathway. While the main interest concerns ADS in monosynaptic C-fibre input to lamina I NK1R+ neurons, it would be technically demanding and perhaps not feasible to record eEPSPs in lamina I NK1R+ neurons in spinal cord slices with the central and peripheral branches of the nerve intact. However, it may be feasible to conduct compound action potential (CAP) recordings from isolated nerves, with central and peripheral branches intact, to provide insight into ADS along the entire conduction path and improve the accuracy of the estimated stimulation trains.

Results in chapter 4 also demonstrate that monosynaptic Aδ-fibre input to lamina I NK1R+ neurons exhibits a small, frequency dependent degree of ADS in response to repetitive stimulation at frequencies of 1, 2 and 10Hz. Furthermore, the ADS elicited in monosynaptic Aδ-fibre input to lamina I NK1R+ neurons during 10Hz, but not 1 or 2Hz, stimulation is significantly reduced in CFA inflammation. As with C-fibre ADS, this Aδ-fibre ADS could act to limit nociceptive input to lamina I NK1R+ neurons, with the inflammation-induced reduction in ADS facilitating nociceptive drive. The incidence of monosynaptic Aδ-fibre input to lamina I NK1R+ neurons has previously been show to be increased in CFA inflammation (Torsney 2011). The findings presented here suggest that in inflammatory pain conditions, the timing of monosynaptic Aδ-fibre input arriving at lamina I NK1R+ neurons may also be altered, which could act to increase the excitability of these neurons and potentially drive plasticity. Given that Aδ-fibres are known to mediate mechanical hyperalgesia (Fuchs et al. 2000, Magerl et al. 2001, Ziegler et al. 1999), this reduced ADS could be implicated in inflammatory hyperalgesia. As this inflammation induced change in Aδ-fibre ADS only revels itself at higher stimulation frequencies, that more closely relate to the Aδ-fibre evoked firing rate (Yeomans & Proudfit 1996), it may be

relevant for future studies to investigate the effects of inflammation upon ADS in response to stimulation at frequencies greater than 10Hz.

In summary, this thesis has investigated inflammatory pain spinal plasticity mechanisms by studying the synaptic input to lamina I NK1R+ neurons and has presented new evidence for ways in which this synaptic input may be altered and pharmacologically manipulated in inflammatory pain. The spontaneous excitatory drive to those lamina I NK1R+ neurons that receive monosynaptic input from Aδ-fibres only was found to be elevated. Given previous evidence has shown that the monosynaptic Aδ-fibre input to these neurons is increased in CFA inflammation (Torsney 2011), this finding suggests there could be a corresponding increase in the excitatory drive to these neurons in inflammatory pain. This thesis has investigated the modulation of primary afferent input to lamina I NK1R+ neurons with chemerin, an agonist of the novel inflammatory pain target, ChemR23 (Ji et al. 2011, Xu et al. 2010). Chemerin was shown to reduce the capsaicin potentiation of excitatory input to lamina I NK1R+ neurons and presynaptically attenuate monosynaptic C-fibre input to a subset of these neurons in CFA inflammation, but was without effect in control conditions. These findings provide further evidence that ChemR23 offers a potential target for the development of novel inflammatory pain treatments. The data presented here have shown for the first time that ADS is present in monosynaptic C-fibre input to lamina I NK1R+ neurons. Furthermore, C-fibre ADS was found to be significantly reduced in CFA inflammation, in extracellular population recordings from isolated dorsal roots and specifically in monosynaptic C-fibre input to lamina I NK1R+ neurons. While the physiological role of ADS is unclear, it is thought to limit nociceptive input to the spinal cord and could thus act to regulate central sensitisation (de Col et al. 2012, Mazo et al. 2013). Therefore the reduced ADS seen during inflammation could facilitate nociceptive input to and significantly impact upon nociceptive processing in these key spinal cord output neurons. This raises the possibility that treatments which restore or enhance ADS could provide a novel approach to the treatment of inflammatory pain.

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Appendix A

Publications and presented abstracts

A.1 Oral Communications

Dickie, A.C. (2012), 'Chemerin attenuation of potentiated C-fibre input to the lamina I pain pathway', *The Physiological Society: Young Physiologists' Symposium.* 2^{nd} July 2012, Edinburgh, UK.

Dickie, A.C. (2012), 'Chemerin attenuates potentiated primary afferent input to the lamina I pain pathway', *Annual Scientific Meeting of the Scottish Pain Research Community.* 29^{th} March 2012, Dundee, UK.

A.2 Poster Communications

Dickie, A.C., Lukito, V., and Torsney, C. (2013), 'Hindpaw inflammation reduces C-fibre activity-dependent slowing in isolated rat dorsal roots', *Annual Scientific Meeting of the Scottish Pain Research Community.* 28^{th} Match 2013, Dundee, UK.

Dickie, A.C., Lukito, V., and Torsney, C. (2013), 'Hindpaw inflammation reduces C-fibre activity-dependent slowing in isolated rat dorsal roots', *University of Edinburgh - Neuroscience Day.* 20^{th} March 2013, Edinburgh, UK

Lukito, V., Dickie, A.C., and Torsney, C. (2013), 'Investigation of the role of HCN channels in C-fibre activity-dependent slowing in inflammatory pain', *1^{st} National 'Neuroscience to Neurology' Undergraduate Conference.* 26^{th} January 2013, Edinburgh, UK.

Dickie, A.C., and Torsney, C. (2012), 'Chemerin attenuated potentiated primary afferent input to lamina I neurokinin 1 receptor expressing rat spinal cord neurons', *14^{th} World Congress on Pain PT277.* 27^{th} – 31^{st} August 2012, Milan, Italy.

Dickie, A., and Torsney, C. (2012), 'Chemerin attenuation of potentiated C-fibre input to the lamina I pain pathway', *Physiology 2012.* 2^{nd} – 5^{th} July 2012, Edinburgh, UK.

Dickie, A.C., and Torsney, C. (2012), 'Chemerin attenuates potentiated primary afferent input to the lamina I pain pathway', *University of Edinburgh - Neuroscience Day.* 21^{st} March 2012, Edinburgh, UK.

Dickie, A.C., and Torsney, C. (2011), 'Modulation of potentiated primary afferent input to

lamina I neurokinin 1 receptor expressing rat spinal cord neurons by chemerin receptor 23 activation', *British Pain Society & Canadian Pain Society Joint Annual Scientific Meeting.* 21^{st} – 24^{th} June 2011, Edinburgh, UK.

Dickie, A.C., and Torsney, C. (2011), 'Chemerin modulation of potentiated primary afferent input to lamina I neurokinin 1 receptor expressing rat spinal cord neurons', *Annual Scientific Meeting of the Scottish Pain Research Community.* 31^{st} Match 2011, Dundee, UK.

Dickie, A.C., and Torsney, C. (2011), 'Chemerin modulation of potentiated primary afferent input to lamina I neurokinin 1 receptor expressing rat spinal cord neurons', *University of Edinburgh - Neuroscience Day.* 16^{th} March 2011, Edinburgh, UK.

Dickie, A.C., and Torsney, C. (2010), 'Hindpaw inflammation does not alter electrically evoked responses of peripheral sensory fibres in the isolated rat dorsal root', *The 7^{th} Annual Scottish Neuroscience Group Meeting.* 27^{th} August 2010, Glasgow, UK.

Dickie, A.C., and Torsney, C. (2010), 'Hindpaw inflammation does not alter electrically evoked responses of peripheral sensory fibres in the isolated rat dorsal root', *University of Edinburgh - Neuroscience Day.* 29^{th} March 2010, Edinburgh, UK.