AN INVESTIGATION OF PAIN MECHANISMS IN A MODEL OF OSTEOARTHRITIS:

MODULATION BY THE ENDOCANNABINOID RECEPTOR SYSTEM

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Abstract

Osteoarthritis (OA) is expected to become the fourth leading cause of disability worldwide by 2020. There is no cure, and joint replacement surgery becomes a final resort. Chronic pain associated with OA is poorly controlled by current treatments, and often involve chronic use of non-steroidal anti-inflammatory drugs (NSAIDs), which is associated with serious side-effects. OA is associated with alterations in endocannabinoid (EC), an attractive target for the control of pain. ECs are rapidly degraded by a number of enzymes, including cyclooxygenase-2 (COX-2), the major target of NSAIDs. However, the role of COX-2 in EC-mediated effects on nociceptive transmission is not fully understood. The aims of this thesis were to investigate peripheral and spinal pain responses in a model of OA pain, understand the role of COX-2 inhibition on neuronal responses and the potential role of ECs in mediating these effects, and to establish the functional effects of the EC system in a model of OA pain.

Effects of spinal and peripheral administration of the COX-2 inhibitor nimesulide (1-100μg in 50μL) on mechanically evoked responses of dorsal horn neurones in the naive, anaesthetised rat were measured, and the contribution of the CB_1 receptor was determined with the antagonist AM251 (1μg in 50μL). Effects of nimesulide on spinal levels of ECs and related compounds were quantified using liquid chromatography-tandem mass spectrometry. Spinal, but not peripheral, injection of nimesulide significantly reduced mechanically evoked responses of dorsal horn neurones. Inhibitory effects of spinal nimesulide were blocked by the CB_1 receptor antagonist AM251, but spinal EC levels were not elevated. Indeed, both anandamide and N-oleoylethanolamide were significantly decreased by nimesulide, highlighting a putative role for other oxidative enzymes of ECs in the generation of CB_1-active metabolites.

The monosodium-iodoacetate (MIA) model of OA pain has recently received much interest, but is not yet fully defined. Work in this thesis sought to further characterise this model. Cytokine levels in synovial fluid, spinal cord and hindpaw skin at early time-points post- intra-articular injection (1mg MIA in 50μL, P.O. 3-24hr) were measured, and the later (P.O. day 28-31) effects on neuronal responses and pain behaviour were determined. Intra-articular injection of 1mg MIA produced stable and robust changes in two measures of pain relevant to clinical OA, and evidence for the presence of central sensitisation was demonstrated. It was also demonstrated that early-stage painful responses in this model are not associated with changes in cytokines in the joint.

Effects of spinal and systemic administration of nimesulide (3-100μg in 50μL) on mechanically evoked and post-stimulus responses of dorsal horn neurones in MIA-treated rats were also measured, as were the effects of spinal cannabinoid receptor antagonism with AM251 (0.1-10μg in 50μL) and the CB_2 receptor antagonist SR144528 (0.001-0.1μg in 50μL). Spinal and systemic COX-2 inhibition in the MIA model attenuated spinal neuronal responses to both noxious and innocuous stimuli, demonstrating the importance of both spinal and peripheral COX-2 products in mediating neuronal responses in this model. Antagonism of the spinal cannabinoid receptors resulted in elevated spinal neuronal responses in MIA-treated rats, demonstrating a functional role for spinal EC-mediated modulation of nociceptive transmission in the MIA model, expanding on work in this lab which showed elevated spinal ECs in the MIA model of OA pain. This work therefore demonstrates that the central EC system may be an important target for the treatment of OA pain.

Publications and Presentations

Publications

- **L.E. Staniaszek**, L.M. Norris, D.A. Kendall, D.A. Barrett and V. Chapman. Effects of COX-2 inhibition on spinal nociception: the role of endocannabinoids. (2010) *Br. J. Pharmacol.* 160(3):669-676

- **L.E. Staniaszek**, D.A. Kendall, P. Mander and V. Chapman. A role for Endocannabinoids in NSAID-mediated analgesia? (2007) *Proceedings of the British Pharmacological Society* at www.pa2online.org/abstracts/Vol5Issue2abst115P.pdf *Also presented orally at the BPS winter meeting, Brighton 2007*

In Press

- D.R. Sagar, **L.E. Staniaszek**, B.N. Okine, S. Woodhams, L.M. Norris, R.G. Pearson, M.J. Garle, S.P.H. Alexander, A.J. Bennett, D.A. Barrett, D.A. Kendall, B.E. Scammell and V. Chapman. Tonic modulation of spinal hyperexcitability by the endocannabinoid receptor system in a model of osteoarthritic pain (2010) *Arthritis and Rheumatism.*

Presentations (oral and poster)

- **L.E. Staniaszek**, D. Barrett, D. Kendall, V. Chapman. 2008. A role for endocannabinoids in NSAID-mediated antinociception? A study in naïve rats. EFIC (European Federation of IASP Chapters) European Pain School – Hyperexcitable neurons as pain generators. Siena, Italy, June 2008. *Oral presentation.*

- D.R. Sagar, R.G. Pearson, **L.E. Staniaszek**, J.C. Martindale, B. Scammell, D.A. Kendall, V. Chapman. 2008. Characterisation of neuronal responses of wide dynamic range neurones in a rat model of osteoarthritis pain. IASP (International Association for the Study of Pain) 12th World Congress on Pain – Glasgow, August 2008.

- **L.E. Staniaszek**, D. Kendall, P. Mander and V. Chapman. 2007. A role for Endocannabinoids in NSAID-mediated analgesia? BPS (British Pharmacological Society) Winter Meeting in Brighton, December 2007.

- **L.E. Staniaszek**, D. Barrett and P. Mander (2007), COX-2 inhibition induces neuronal firing in rat Dorsal Horn. In-house research forum at the Institute of Neuroscience, University of Nottingham, November 2006.

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I thank my parents, who supported me fully, even through their doubts,
and my brother, Adam, who never doubted me

"It was a hilarious, hilarious moment in a very bleak, bleak time of my life"
Flight of the Conchords

Contents

Chapter 1 General Introduction

1.1. Pain

Arthritic disorders, such as osteoarthritis (OA) are a leading cause of work-related disability in men and women, aged 16-72, and is expected to affect 59.4 million (18.2%) Americans by the year 2020 (Lawrence *et al.*, 1998). It is expected that over half of individuals over the age of 85 will suffer symptomatic knee OA (Murphy *et al.*, 2008). In the U.K., OA currently affects approximately 8.5 million people, and with no cure available, symptomatic treatment is of vital importance. OA occurrence may be secondary to an underlying joint problem such as physical trauma, crystal deposition disorders such as gout, and rheumatoid arthritis (RA), or it may be idiopathic. In idiopathic cases, while the exact cause is unknown, it is thought to be a disease of cartilage or subchondral bone, with widespread disruption of normal turnover systems reported (see section 1.2.1). Prevalence is higher in those over 40, particularly women, and those who are overweight (for review, see Das & Farooqi, 2008). There is also a genetic trait, with presence of OA within the family increasing individual risk.

Symptoms of OA include stiff, swollen and possibly unstable joints which may creak (crepitus), with pain that may worsen following exercise, or inactivity. Levels of pain and stiffness may vary with seasonal weather changes, and may vary throughout the day, with many patients reporting symptoms to be worse in the morning (after inactivity). In joints other than the knee and hip, OA can cause additional problematic symptoms, e.g. OA in the neck and back (spondylosis) can cause outgrowths from the vertebrae and joints with pain and numbness spreading down the arm, while in the foot OA may affect the big toe causing problems with walking. The most troublesome aspect of OA producing a reduction in quality of life for patients is the associated pain (for reviews see Brandt, 2002; Curatolo & Bogduk, 2001).

Pain is defined by the International Association for the Study of Pain (IASP) as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage." Pain is therefore not only a physical event but importantly, comprises an emotional component, and as a result the perception of pain varies from person to person. Nociception comprises the

physical aspect of pain, and is the result of a complex interplay between specialised primary afferents in the periphery, the dorsal horn of the spinal cord, and spinal pathways that ascend to, and descend from, higher pain centres in the brain.

1.1.1. Nociceptive primary afferents

Primary afferents which respond preferentially to stimuli that can, or do cause, tissue damage are termed "nociceptors" (Sherrington, 1906), and may be myelinated by Schwann cells surrounding their axons (A fibres) or unmyelinated (C fibres). Fibres arise from cell bodies in the trigeminal and dorsal root ganglia, terminate in the spinal cord (see later), and are classified into 3 subgroups: Aβ-, Aδ- and C-fibres, typically existing in ratios of 20%:10%:70% in the skin (for review, see Millan 1999). Aβ-fibres are of large diameter (>10μm) and are thickly myelinated, classically thought to transmit signals only from non-noxious stimuli such as touch, vibration and pressure (Millan *et al.*, 1999) with a fast conduction velocity of 30-100m/sec. Some nociceptors have been shown to conduct in the Aβ-fibre conduction velocity (CV) range (Djouhri & Lawson, 2004), and so the status of Aβ-fibres as solely non-noxious mediators is undergoing scrutiny. Aβ-fibres may be particularly important in neuropathic pain-related allodynia, where normally innocuous stimuli are perceived as painful. Aδ-fibres are of medium diameter (2-6μm), thinly myelinated and respond to nociceptive mechanical, thermal and chemical stimuli with an intermediate conduction velocity of 12-30m/sec. Aδ-fibres are thought to be responsible for the initial phase of nociception, producing a sharp, pricking sensation (Meyer *et al.*, 2006; Milan *et al.*, 1999), and may be subdivided into two subgroups, type I and type II. Type I Aδ-fibres respond to mechanical stimuli and intense heat (~53°C; Willis & Coggeshall, 2004), while type II Aδ-fibres are mechanically insensitive but have a lower thermal threshold (~43°C; Treede *et al.*, 1998). C-fibres are of small diameter (0.4-1.2μm), unmyelinated and slow (0.5-2m/sec) conducting, and are responsible for the dull aching, second slow wave of pain following noxious stimulation (Meyer *et al.*, 2005; Millan *et al.*, 1999). C-fibres respond to both innocuous and noxious stimuli and may be subdivided into CH - heat sensitive; CM - mechano-sensitive; CMH - those sensitive to both mechanical and heat stimuli (and almost always also to chemical

stimuli: "Polymodal"; Davis *et al.*, 1993), and "silent nociceptors" which are unresponsive to (or responsive only to intense) mechanical and heat stimuli under normal conditions. Of particular importance in neuropathic pain are polymodal and silent nociceptors. Polymodal fibres are thought to trigger sensitisation of spinal neurones (see later, and review, Millan, 1999), while silent nociceptors may become sensitive to mechanical and thermal stimuli after sensitisation, for example by irritant chemical application such as capsaicin or mustard oil (Schmidt *et al.*, 1995). Silent nociceptors are thought to become active in cases of nerve damage or after inflammation, contributing to hyperalgesia in these cases (Schmidt *et al.*, 1995; Millan *et al.*, 1999).

Receptors and neurotransmitters associated with nociceptive transmission

The most prevalent neurotransmitter used in primary afferent fibres (both non-nociceptive and nociceptive) is glutamate, allowing rapid transmission of excitatory, pronociceptive neurotransmission in the spinal cord (Coderre & Melzack, 1992; Malmberg & Yaksh, 1992; Kontinen & Meert, 2002). Glutamate and glycine, another important excitatory amino acid, act at the ionotropic glutamate receptors AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate), kainate and NMDA (N-methyl-D-aspartic) receptors, which couple cation channels, as well as metabotropic glutamate receptors (mGluRs) - G-protein coupled receptors linked to second messenger systems (see Mayer & Armstrong, 2004).

AMPA receptors are densely located in the superficial dorsal horn, and transmit signals between primary afferent fibres and dorsal horn neurones (Yoshimura & Jessell, 1990; Randic *et al.*, 1993; Seagrove *et al.*, 2004). Activation results in increased sodium conductance and is involved in the transmission of acute noxious and thermal stimuli (Dougherty *et al.*, 1992; King & Lopez-Garcia, 1993).

Kainate receptors are found both pre- and post-synaptically in the dorsal horn and modulate and transmit nociceptive information from primary afferent fibres to the

spinal cord in both acute and chronic pain states (Advocat & Rutherford, 1995; Szekely *et al.*, 1997; Okano *et al.*, 1998). Pre-synaptically they modulate glutamate release (Frerking & Nicoll, 2000; Kerchner *et al.*, 2001), and may also modulate substance P release as in the trigeminal dorsal horn they are found post-synaptically to substance P-containing primary afferents (Hegarty *et al.*, 2007).

NMDA receptors are present both pre- and post-synaptically at the junctions between primary afferent fibres and spinal dorsal horn neurones (Coggeshall & Carlton, 1997; Lu *et al.*, 2003). They are quiescent under normal conditions, requiring both glycine binding and alleviation of Mg^{2+} block for activation. Pre-synaptically, activation results in excitatory amino acid and substance P release from primary afferent fibres. Post-synaptically, activation results in large neuronal depolarisations that greatly increase neuronal excitability, a phenomena known as wind-up. (Heppenstall & Fleetwood-Walker, 1997; Kleckner *et al.*, 1988; Urch *et al.*, 2001).

There are 8 subtypes of metabotropic glutamate receptors, falling into 3 groups. Group I mGluRs include $mGlu_{1+5}$, positively coupled to PLC and possibly NO synthase, and elicit pronociceptive effects, while Group II ($mGlu_{2+3}$) and Group III ($mGlu_{1,6,7+8}$) mGluRs are negatively coupled to AC and act to inhibit Ca^{2+} currents and enhance K^{+} currents (Nakanishi, 1994; Millan, 1999).

Different primary afferent fibres are associated with a range of varying neurotransmitters involved in the conduction of information. C fibres are associated with substance P/Neurokinin A (NKA), calcitonin gene related peptide (CGRP) and other excitatory amino acids, the receptors $NK_{1/2}$, $CGRP_{1/2}$, NMDA/AMPA and metabotropic glutamate receptors (mGlu). Aβ fibres are most typically associated with excitatory amino acids and the AMPA receptor (see review, Millan *et al.*, 1999). In reality there is a plethora of substances synthesised in addition to those mentioned above, which are involved in the central transmission and modulation of nociceptive information, including adenosine triphosphate (ATP), nitric oxide (NO), phospholipid metabolites, prostaglandins (PGs), neurotrophins (growth factors), and a range of other neuropeptides, lectins and enzymes. These substances colocalise in a variety

of ways in different tissue types such as skin, muscle, joints and viscera; various tissue states i.e. physiological versus pathophysiological; between different fibre types, e.g. C versus Aβ fibres; and even within fibre types, for example the smaller primary afferent neurones may be classified as peptidergic or non-peptidergic. Peptidergic neurones express peptides such as substance P, CGRP, and somatostatin (Averill *et al.*, 1995; Molliver *et al.*, 1997; Willis *et al.*, 2004), as well as the TrkA receptor, a high affinity receptor for nerve growth factor (NGF – Molliver *et al.*, 1997). Peptidergic neurones require NGF for development, as well as survival (Averill *et al.*, 1995; Bennett *et al.*, 1998). In the first 3 weeks of development, around half of the neurones cease to express TrkA, and become non-peptidergic (Molliver *et al.*, 1997). Non-peptidergic neurones express growth factor receptor $GFR\alpha_{1-4}$ receptors and so become sensitive to glial cell line derived neurotrophic factor (GDNF – Molliver *et al.*, 1997; Bennett *et al.*, 1998; Willis *et al.*, 2004). They also bind lectin IB-4, and express the adenosine triphosphate (ATP) receptor, $P2X_3$ (Millan *et al.*, 1999; Meyer *et al.*, 2006).

Actions of pro-nociceptive mediators

Nitric oxide and prostaglandins play a large role in mediating nociception. NO is not only constitutively active but is also enhanced by an increase in intracellular Ca^{2+} current, brought about by NMDA receptor activation, as well as cytokine, neurotrophin and prostaglandin release (for review, see Millan, 1999). The actions of NO include enhancement of the release of pronociceptive mediators such as glutamate, substance P and CGRP from primary afferent fibre terminals, as well as enhancement of prostaglandin and cytokine synthesis, positively feeding back into its own activation. NO also has more long-term effects, activating protein kinase G (PKG), thus resulting in neuronal sensitisation through cyclic guanosine monophosphate (cGMP)-mediated phosphorylation of GABAergic (gamma Aminobutyric acid) and glycinergic receptors. Like NO, prostaglandin synthesis is accelerated via an increase in intracellular Ca^{2+} current following NMDA receptor activation (Beiche *et al.*, 1996; Willingale *et al.*, 1997).

PGs are formed by the actions of cyclooxygenases (COX) on arachidonic acid. The COX-2 isoform is of particular importance as it has been shown to be constitutively active in the spinal cord (Ghilardi *et al.*, 2004) and is induced peripherally following injury and/or inflammation. In the spinal cord, COX-2 is found in laminae I, II and X, which receive nociceptive input (see later), and in primary afferent fibre dorsal root ganglia (DRGs - Beiche *et al.*, 1996; Willingale *et al.*, 1997). It is unclear how prostaglandins induce nociception, but may involve enhanced glutamate and substance P release from primary afferent neurones (Ferreira and Lorenzetti, 1996; Minami *et al.*, 1997a) following enhanced cAMP and Ca^{2+}/Na^{+} conductance (White, 1996; Minami *et al.*, 1997a; 1997b), as well as direct activation of several G-protein coupled prostanoid receptors. The prostanoid receptors include prostaglandin D_2 (PGD_2) and PGI_2, positively coupled to AC, PGF_2, coupled to PLC and resulting in increased intracellular Ca^{2+} currents, and PGE_2 (Taiwo and Levine, 1986; Coleman *et al.*, 1994; Mnich *et al.*, 1995). PGE_2 is thought to be the most important pro-nociceptive prostaglandin in the periphery, with actions on 4 types of EP receptors with differential actions on intracellular Ca^{2+} currents, AC, and phosphoinositol (PI) production (Coleman *et al.*, 1994; Boie *et al.*, 1997). EP_2 mRNA is found mostly in laminae I and II while mRNA for EP_1 and EP_3 are found on sensory neurones (Sugimoto *et al.*, 1994; Kawamura *et al.*, 1997). It has been suggested that EP_1 activation is involved with allodynia while activation of EP_{2+3} is involved with hyperalgesia (Minami *et al.*, 1994).

The pronociceptive tachykinins, substance P and NKA, act on NK_{1+2} receptors, positively coupled to phospholipase C (PLC) (Catalioto *et al.*, 1993; Fukuhara *et al.*, 1998), while pronociceptive neuropeptides $CGRP_{\alpha+\beta}$ act on $CGRP_{1+2}$ receptors, positively coupled to adenylyl cyclise (AC) (see van Rossum *et al.*, 1997, Millan *et al.*, 1999). Their activity contributes significantly to nociceptive transmission in both physiological and pathophysiological conditions, where they may be abnormally expressed. Pronociceptive substances act synergistically to elicit excitatory post-synaptic potentials (EPSPs) in dorsal horn (DH) neurones, initially proposed to be caused by AMPA receptor activation which brings about a rapid and transient (lasting only a few milliseconds) EPSP (see Millan, 1999). These are then sustained for tens

of seconds by NMDA, Group I mGlu, NK_{1+2} and CGRP receptor activation. These actions are enhanced by ATP, NO and prostaglandins. ATP acts on P_{2x} receptors, resulting in glutamate release (Inoue *et al.*, 1995), and P_{2y} receptors, positively coupled to PLC, resulting in enhanced excitatory amino acid (EAA) release (Vaziri & Downes, 1992). ATP may also exert antinociceptive effects as it is metabolised to adenosine, which interacts with both opioids and monoamines to decrease nociceptive transmission through negative coupling to AC (Reeve and Dickenson, 1995; Cui *et al.*, 1997).

1.1.2. The dorsal horn of the spinal cord

The grey matter of the spinal cord was first described in terms of 10 laminae, based on their size and packing density, by Rexed in 1952 (cited in Willis *et al.*, 2004). Laminae I-VI comprise the dorsal horn, lamina VII the intermediate grey matter, laminae VIII and IX the medial and ventral horn, while lamina X surrounds the central canal (Millan, 1999) (Figure 1.1).

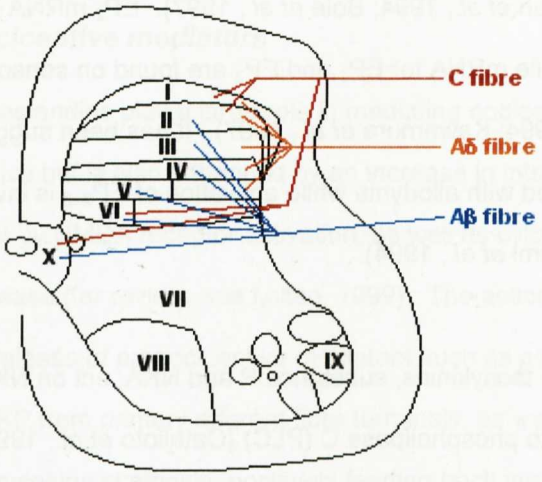


Figure 1.1 Arrangement of the Rexed laminae within the grey matter of the spinal cord. Laminae I-VI comprise the dorsal horn, laminae VII-IX comprise the ventral horn, and lamina X surrounds the central canal. Laminae I-VI and X process nociceptive information with input from **Aβ-(blue)**, **Aδ-(orange)**, and **C-fibres (red)**.

Motor neurones typically originate in laminae VII, VIII and IX, while nociceptive neurones predominantly terminate in the dorsal horn, with Aβ fibres terminating in laminae II-VI and X, Aδ fibres terminating in laminae I-V, and C fibres terminating in laminae I, II, VI and X (Todd & Koerber, 2006). Laminae I, V, VI, X and the outer part of lamina II (II_o) are associated with the processing of nociceptive information.

Within the dorsal horn, three types of neurone are associated with sensory processing: non-nociceptive, nociceptive-specific (NS), and wide dynamic range (WDR). Non-nociceptive neurones are found in laminae II, III and VI, receive input from Aβ fibre primary afferents, and are activated by innocuous stimuli. NS neurones receive input from Aδ and C fibre primary afferents, and are activated solely by high intensity, noxious input (Willis, 2004). NS neurones are found predominantly in laminae I and II_o, with some also being found in laminae V and VI. WDR neurones receive input from all primary afferent fibre types and respond in a graded fashion to both noxious and non-noxious stimuli (Millan, 1999). They are found mostly in laminae IV, V and X, and some in I and II_o (Figure 1.1). Based on their output, these neurones may be classified as interneurones, propriospinal neurones or projection neurones. Interneurones modulate (in both an inhibitory and excitatory manner) input from primary afferent fibres both intra- and inter-lamina. Propriospinal neurones communicate between the two sides of the dorsal horn. Projection neurones are found mostly in laminae I, V and VI and some in laminae II and X, and are involved in nociceptive processing and project to the higher centres.

1.1.3. Ascending pathways of pain

Many ascending pathways are thought to be involved in the relay of pain. Those projecting directly to the brain-stem and thalamus, and those projecting to the forebrain via the brainstem are thought to be the most important. Pathways may be polysynaptic, with synapses in the dorsal column nuclei and the lateral cervical nucleus (Willis *et al.*, 2004), or monosynaptic. Monosynaptic pathways include the much-studied spinothalamic tract (STT), spinoparabrachial (SPBT), spinomesencephalic (SMT), spinoreticular (SRT) and spinohypothalamic (SHT) tracts. Polysynaptic tracts include the spinocervicalthalamic tract (SCT) and the postsynaptic dorsal column (PSDC) (Millan 1999; Willis *et al.*, 2004; Dostrovsky & Craig, 2006). The pathways considered to be the most important are the spinothalamic and spinoparabrachial (within the spinobulbar projections) tracts, with lesions in the STT shown to cause contralateral loss of sensation (White and Sweet, 1969; Craig *et al.*, 2002). Nociceptive input from the PSDC and SCT has also been recognised.

The STT originates from three regions in the spinal grey matter, with approximately 50% of cells in primates coming from lamina I, 25% from laminae IV-V and 25% from laminae VII-VIII (Dostrovsky *et al*, 2006). A total of approximately 10,000 STT cells project to the thalamus from one side. Axons cross in dorsal and ventral spinal commisures to the white matter of the contralateral spinal cord within 1-2 segments rostral of their point of origin, in the lateral and anterior funiculi. Lateral STT neurones originate in lamina I and encode pain and thermosensation, and anterior STT neurones originate in laminae V and VI and encoding crude touch and movement. The STT projects directly to areas of the thalamus such as the ventral posteriomedial / posteriolateral nuclei, and then onto the cerebral cortex (Figure 1.2). Most of the neurones in the STT are nociceptive, with some neurones receiving input from WDR neurones, and others from NS neurones (for review, see Willis and Coggeshall, 2004).

The spinobulbar projections convey nociceptive information to the forebrain via the brainstem. Spinobulbar projections contain many tracts including the spinoparabrachial tract, and have origins in laminae I, V and VII, similar to STT cells. It has been suggested that that spinobulbar projections and STT cells could originate from the same cells. They terminate in the brainstem in four distinct areas; regions of catecholamine cell groups, parabrachial nucleus, periaqueductal grey (PAG), and brain stem reticular formation, from where they project to the hypothalamus, amygdala, medial thalamus and reticular formation (Mantyh *et al.*, 1983, Basbaum & Fields 1978) (Figure 1.2). Their input is thought to encode largely for the motivational-affective component of pain rather than the sensory component (Dostrovsky & Craig, 2006).

The PSDC and SCT originate primarily from laminae IV-VI, and also lamina X. While the majority of input comes from low-threshold, non-nociceptive mechanoreceptors, some input is from nociceptive neurones. PSDC axons transmit nociceptive information to the dorsal column nuclei (DCN) in the rat, and possibly primates and humans (Al-Chaer *et al.*, 1998), areas involved in nociception. The DCN, consisting of gracile and cuneate nuclei, contain GABAergic interneurones and so may be involved in inhibitory action on mechanoreceptive relay cells (Dostrovsky & Craig, 2006).

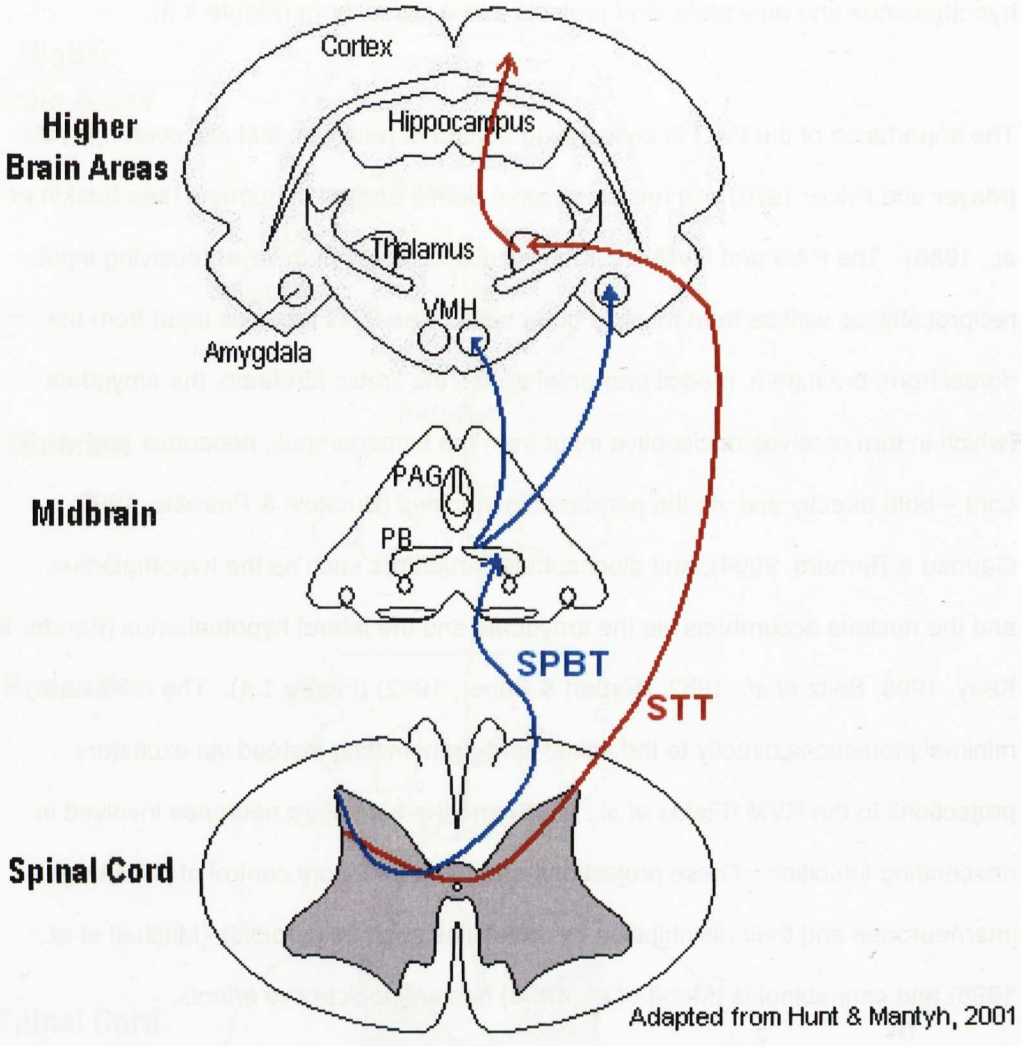


Figure 1.2 The spinothalamic tract (STT, red) and the spinoparabrachial tract (SPBT, blue) are the main ascending nociceptive pathways. The STT originates in the deep dorsal horn and projects to the cortex through the thalamus, while the SPBT originates in the superficial dorsal horn and projects to the parabrachial nucleus (PB) from where they project to the ventromedial hypothalamus (VMH) and the amygdala. RVM, rostroventral medulla; PAG, periaqueductal grey.

1.1.4. Descending control of pain

The brain not only receives and interprets nociceptive signals from the spinal cord, but has a vital role in their modulation, both positively and negatively, through numerous descending pathways. Actions may be on DH neurones or on primary afferent fibre terminals, where neurotransmitter release can be inhibited or facilitated (Millan, 1999). Critically important areas of the brain in downstream modulation of nociception include the periaqueductal grey (PAG) and rostroventral medulla (RVM) (Fields *et al.*, 2006,

1991; Millan, 2002), forming an important descending pathway which originates in the hypothalamus and amygdala, and projects to the dorsal horn (Figure 1.3).

The importance of the PAG in descending control of pain was first discovered in rats (Mayer and Price, 1976) and has since been demonstrated in humans (see Baskin *et al.*, 1986). The PAG and RVM are integrated in complex pathways, receiving input reciprocally, as well as from multiple other sites. The PAG receives input from the dorsal horn, brainstem, medial prefrontal areas, the limbic forebrain, the amygdala (which in turn receives nociceptive input from the hippocampus, neocortex and spinal cord – both directly and via the parabrachial nucleus (Burstein & Potrebic, 1993; Gauriau & Bernard, 2004), and diencephalic structures such as the hypothalamus, and the nucleus accumbens via the amygdala and the lateral hypothalamus (Bander & Keay, 1996; Beitz *et al.*, 1982; Herbert & Saper, 1992) (Figure 1.4). The PAG has minimal projections directly to the spinal cord, transmitting instead via excitatory projections to the RVM (Fields *et al.*, 2006) and the brainstem neurones involved in descending inhibition. These projections are under inhibitory control of GABAergic interneurones and their disinhibition by molecules such as μ opioids (Mitchell *et al.*, 1998) and cannabinoids (Meng *et al.*, 1998) has antinociceptive effects.

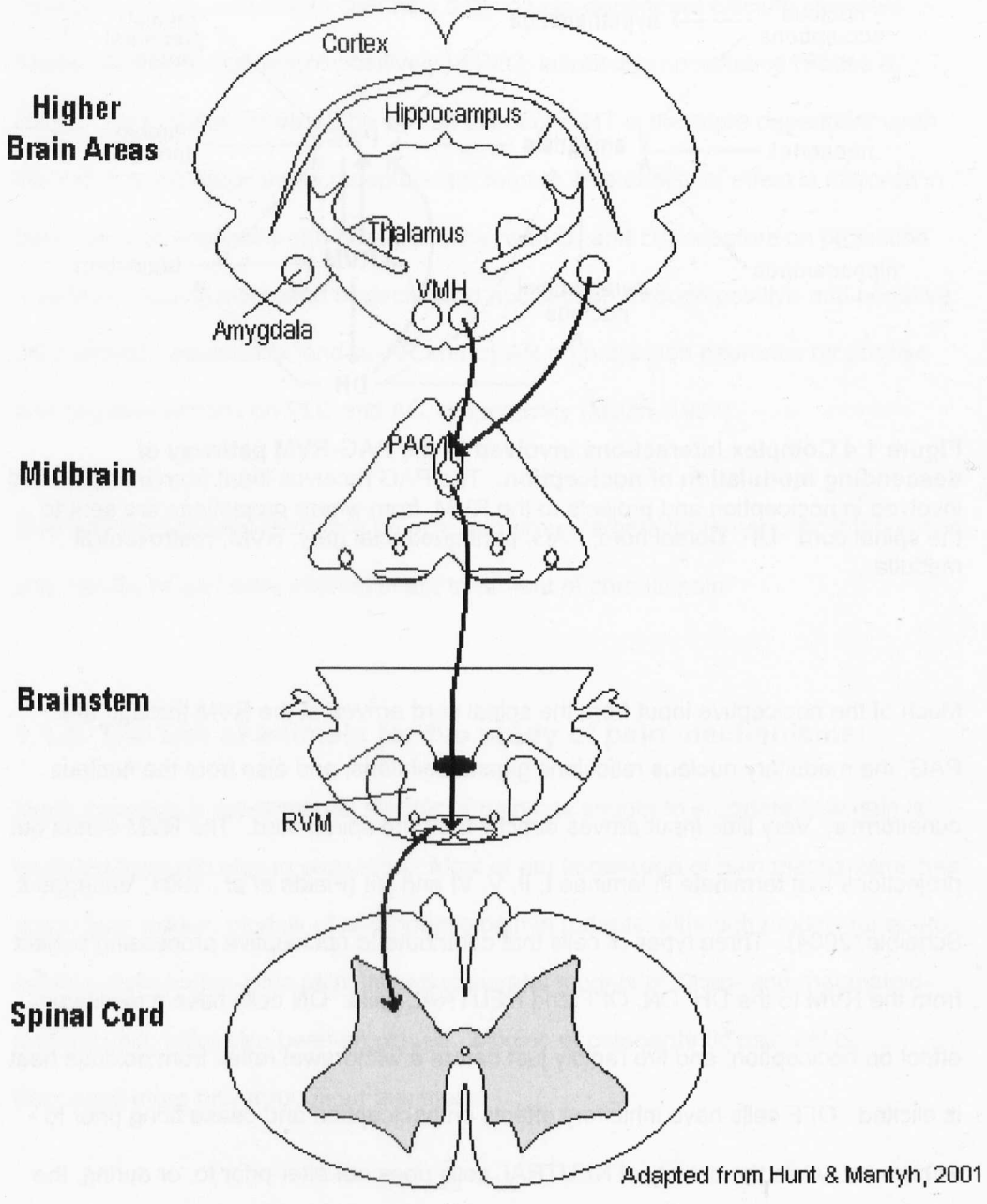


Figure 1.3 The main descending pathway in nociceptive processing. Input from the amygdala and hypothalamus project to the periaqueductal grey, and on to the rostroventral medulla which projects to the dorsal horn. PAG, periaqueductal grey; RVM, rostroventral medulla; VMH, ventral medial nucleus of the hypothalamus.

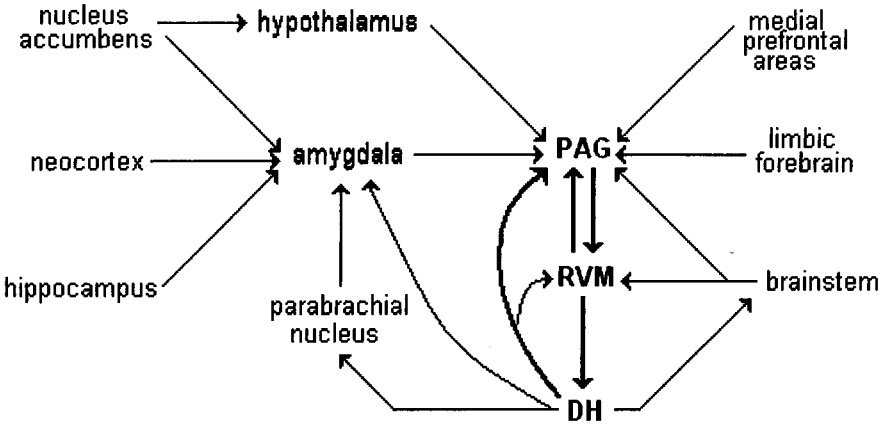


Figure 1.4 Complex interactions involved in the PAG-RVM pathway of descending modulation of nociception. The PAG receives input from multiple sites involved in nociception and projects to the RVM, from where projections are sent to the spinal cord. DH. Dorsal horn; PAG, periaqueductal grey; RVM, rostroventral medulla.

Much of the nociceptive input from the spinal cord arrives at the RVM through the PAG, the medullary nucleus reticularis gigantocellularis, and also from the nucleus cuneiformis. Very little input arrives directly from the spinal cord. The RVM sends out projections that terminate in laminae I, II, V, VI and VII (Fields *et al.*, 1991; Vanegas & Schaible, 2004). Three types of cells that contribute to nociceptive processing project from the RVM to the DH: ON, OFF and NEUTRAL cells. ON cells have a facilitatory effect on nociception, and fire rapidly just before a withdrawal reflex from noxious heat is elicited. OFF cells have inhibitory effects on nociception and cease firing prior to withdrawal, while the activity of NEUTRAL cells does not alter prior to, or during, the withdrawal reflex (Fields *et al.*, 2006).

Many neurotransmitters are involved in the inhibitory and facilitatory effects of the RVM upon the spinal cord, including serotonin (5-HT), noradrenaline, substance P, cholecystokinin endogenous opioids and endocannabinoids (Fields, 2006; Millan, 1999; 2002,), with one neurotransmitter capable of producing both anti- and pro-nociceptive effects. For example, serotonin exerts its actions on 5-HT receptors, of which there are several subtypes. 5-HT_{1A} receptor activation results in opening of K^+ channels and so causes cellular hyperpolarisation. These receptors are also negatively coupled to AC and so decrease nociception in this way. 5-HT_{2+3} receptor activation results in cellular depolarisation through closure of K^+ channels, as well as

opening of cation permeable channels and voltage-dependent calcium channels. These receptors also couple positively to PLC, increasing nociception (Boess & Martin, 1994; Millan, 1995). The overall effect of 5-HT is therefore dependent upon the cell type on which these receptors are found. This duality of effect is mirrored in the actions of dopamine and noradrenaline, with D_1 and D_2 receptors on projection neurones causing increased or decreased nociception through positive and negative AC coupling respectively, and α_1-AR and α_2-AR on projection neurones by positive and negative actions on PLC and AC, respectively (Millan, 1997).

Descending pathways therefore provide numerous targets for therapeutic intervention, and may be of particular interest in the treatment of chronic pain.

1.1.5. The use of animals for the study of pain mechanisms

Much research in experimental models of pain has sought to elucidate how pain is encoded from stimulus to sensation. Most of our knowledge of pain mechanisms has come from work in models of neuropathic pain in rodents, although models for acute, inflammatory and chronic pain, including several models of osteo- and rheumatoid-arthritic pain, have also been employed. Models of osteoarthritic pain will be discussed more fully throughout this thesis.

1.1.6. Central sensitisation

Following peripheral inflammation, responses of primary afferent fibres may become altered due to an increase in release of inflammatory mediators. This may result in nerve injury, and chronic pain, thought to occur due to a combination of sensitisation of peripheral neurones by inflammatory mediators, and sensitisation of spinal neurones and involvement of higher areas, known as "central sensitisation". Central sensitisation is characterised by both hyperalgesia and allodynia, and develops following a primary afferent barrage, producing a sustained activation of WDR neurones in the spinal cord. Following this barrage, dorsal horn neurones in the spinal cord become sensitised, and input from primary afferent neurones is no longer

necessary to elicit responses from dorsal horn neurones. This was demonstrated by the block of mechanical hyperalgesia following intradermal capsaicin injection by a pre-treatment of local anaesthetic (La Motte *et al.*, 1991). However, if anaesthetic injection was delayed until after the onset of hyperalgesia, it could not be reversed, thus demonstrating that input from the periphery is necessary for onset but not maintenance of hyperalgesia.

Central sensitisation has been shown to be due to a number of mechanisms and involves both presynaptic increases in activity from primary afferent neurones, postsynaptic increases in activity of dorsal horn neurones, and loss of inhibitory interneurones. Several receptor systems are involved, including GABA, VGCC (voltage-gated calcium channel), NMDA, AMPA, 5-HT, and chemokine.

GABAergic interneurones are involved in both pre- and post-synaptic inhibition of responses of spinal DH neurones. Blocking GABAergic interneurone function caused symptoms of central sensitisation to occur in naïve rats (Malan *et al.*, 2002), which began to display mechanical allodynia and thermal hyperalgesia following intrathecal administration of $GABA_{A/B}$ receptor antagonists. The converse was seen with $GABA_{A/B}$ receptor agonists in the spinal nerve ligation (SNL) model (Kim & Chung, 1992) of neuropathic pain, with previously present mechanical allodynia and thermal hyperalgesia being abolished (Malan *et al.*, 2002). Changes in VGCC may play a role in the effects of GABAergic neuronal function. In the monosodium-iodoacetate (MIA) model of osteoarthritic pain, with which this thesis is concerned, $\alpha_2\delta$-1 subunit mRNA levels were upregulated in L3-6 DRG (Rahmann *et al.*, 2009). Upregulation was associated with peripheral nerve injury-associated pain (Luo *et al.*, 2001; Li *et al.*, 2004), and the analgesic effects of gabapentinoids (Luo *et al.*, 2002; Maneuf *et al.*, 2006). The loss of inhibitory interneurones causes disinhibition of spinal DH neurones, thus increasing neurotransmission and contributing to central sensitisation.

Pre-synaptically, the involvement of increased C-fibre activation and phenotypic changes and recruitment of Aβ-fibres has been implicated in the development of central sensitisation. Spontaneous activation of primary afferent fibres in both injured

and non-injured nerves may occur (Wu *et al.*, 2001), and C fibres insensitive to heat or mechanical provocation under baseline (non-inflamed, uninjured) conditions (CMiHi, also known as "silent nociceptors"), may become responsive to both mechanical and heat stimuli, adding to the primary afferent barrage (Schmidt R *et al.*, 1995). Altered release of pro-inflammatory substances from primary afferent fibres may occur, for example, Aβ-fibres have been shown to start releasing excitatory amino acids and the neuropeptide substance P, in models of neuropathic pain (Noguchi *et al.*, 1995; Malcangio *et al.*, 2000). Substance P release is normally associated with C-fibres, and results in activation of post-synaptic NK1 receptors. NK1 receptor activation results in downstream activation of intracellular signalling pathways such as that of mitogen-activated protein kinase (MAPK) and extracellular signal-related kinase (ERK). Ultimately this results in activation of NMDA receptors and contributes to hyperexcitability of DH neurones (Woolf & Salter, 2000). The importance of NMDA receptor activation in the development of central sensitisation is further supported by reports that NMDA receptor phosphorylation coincides with the development of mechanical allodynia (Gao *et al.*, 2005), while the NMDA receptor antagonist ketamine inhibits painful symptoms in post-herpetic neuralgia (Eide *et al.*, 1994). Nerve injury also causes the release of other excitatory substances such as glutamate, ATP, NO and prostaglandins. Together with substance P, these substances cause glial cell activation. Glial cell activation has been shown to be crucial in the development of hyperalgesia (Meller *et al.*, 1994) and central sensitisation (Marchand *et al.*, 2005). Upregulation of expression of the fractalkine receptor CX3CR1 has been shown to mediate glial activation in the complete Freund's adjuvant (CFA) inflammatory model of arthritis (Sun *et al.*, 2007), in which a CX3CR1-neutralising antibody not only reversed established pain facilitation but delayed the development of mechanical allodynia and thermal hyperalgesia.

AMPA receptor trafficking to the cell surface has been implicated in the development of central sensitisation, with the time course of trafficking coinciding with the development of mechanical and thermal hyperalgesia in a model of neuropathic pain, peaking at 14 days and decreasing from day 35 onwards (Harris *et al.*, 1996). Finally, descending serotonergic facilitation from the brainstem may also be involved in central

sensitisation, and is a key mechanism underlying some chronic pain states (see Rahman *et al.*, 2009). It has also been shown to play a role in mediating neuronal responses to non-noxious stimuli in the MIA model (Rahman *et al.*, 2009), where $5HT_3$ subunit mRNA is upregulated.

1.2. Osteoarthritis

1.2.1. Pathophysiology of osteoarthritis

Osteoarthritis can affect any synovial joint in the body but is most commonly seen in the knee. In terms of gross morphology, OA of the knee can be thought of as a "wear and tear" process, affecting the cartilage and other knee components (Figure 1.5). Imaging is commonplace in the diagnosis of OA, and in magnetic resonance imaging (MRI) analysis, changes to joint structures including the cartilage, synovium, and the bones themselves, are commonly visible (Guermazi *et al.*, 2005).

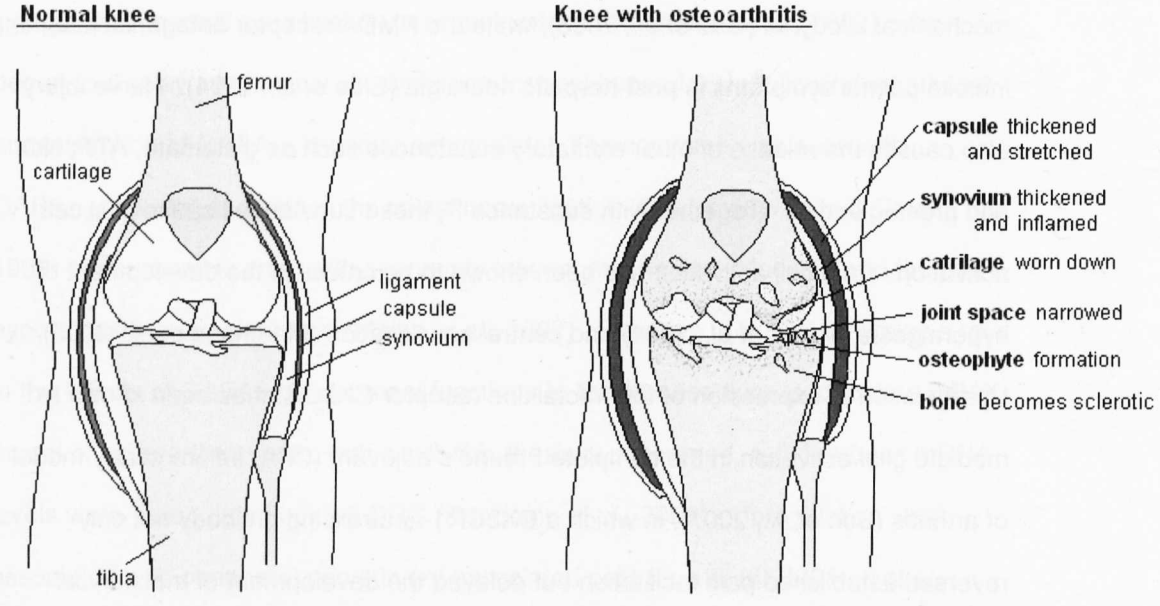


Figure 1.5 Gross morphological changes in the osteoarthritic knee. Osteoarthritis of the knee causes thinning of the cartilage and underlying bone, bony outgrowths (osteophytes), narrowing of the joint space, inflammation and thickening of the synovium and thickening and stretching of the joint capsule.

The importance of cartilage in normal joint function is well known, and its destruction is a key feature in OA. Articular (hyaline) cartilage covers the ends of the bones and functions to allow smooth gliding of the two joint surfaces, with the highly viscous synovial fluid reducing friction and minimising wear (Figure 1.6 A). In addition, a layer

of fibro-cartilage (the meniscus) is present in the knee joint and serves to distribute load across bone surfaces. In OA, articular cartilage degenerates, roughens, thins dramatically and is lost, exposing the underlying bone (Figure 1.6 B, C). Osteophytes (bony outgrowths) and subchondral bone sclerosis occur (Figure 1.6 C) In addition, meniscal tears occur in approximately 50% of patients with knee OA, confounding these issues (Figure 1.6 D). Articular cartilage is avascular, aneural and alymphatic, and does not heal (Felson *et al.*, 2001).

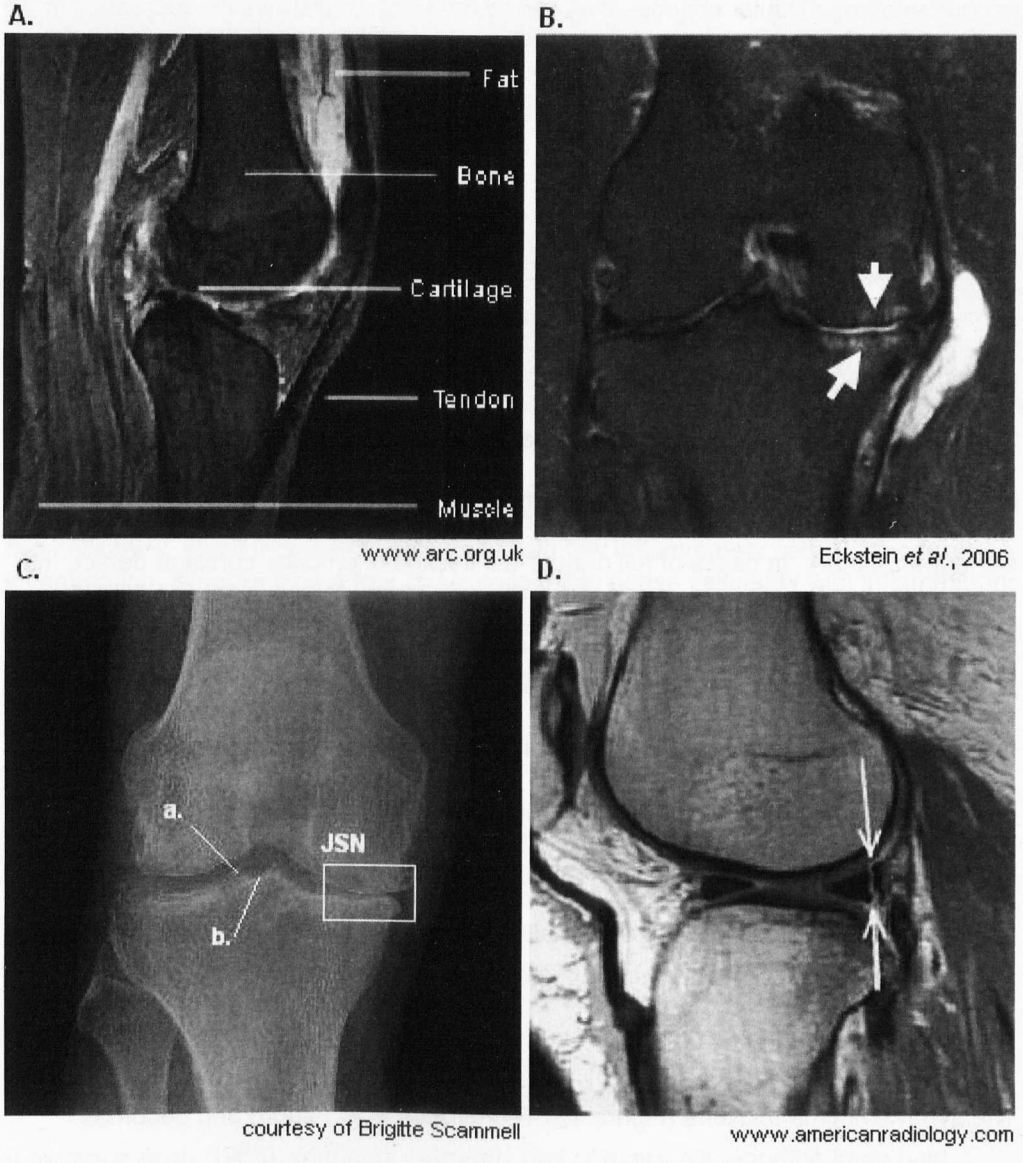


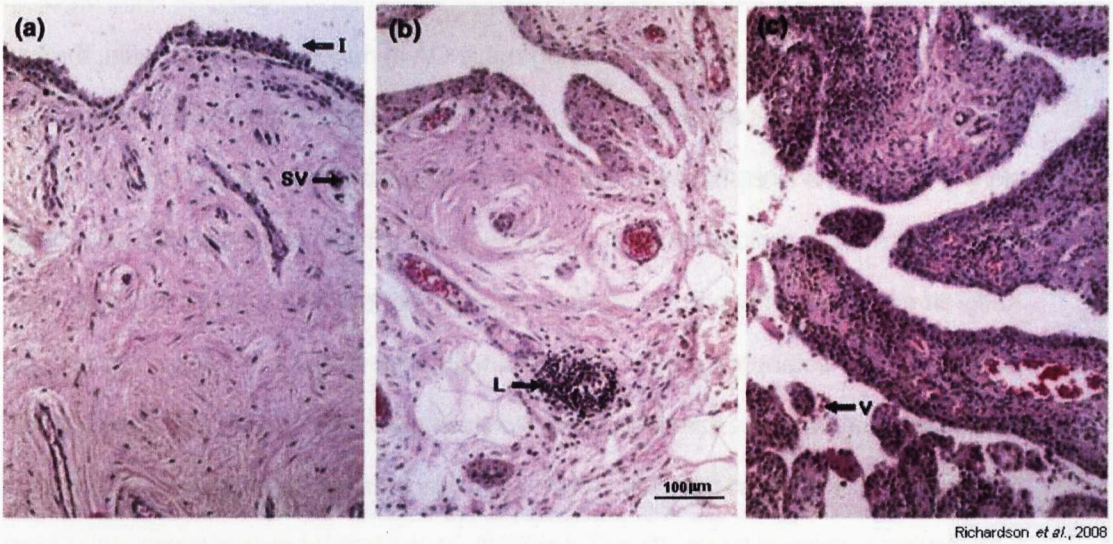
Figure 1.6 MRI (A, B and D) and X-ray (C) images of the knee. A, healthy knee; B, knee from a patient with severe OA; cartilage loss can be seen together with subchondral bone marrow lesions (arrows), adjacent to the joint a synovial cyst (white mass) is visible; C, antero-posterior (AP) view of right knee, showing bilateral medial joint space narrowing (JSN), marginal osteophytes (a) and subchondral bone sclerosis (b); D, a vertical peripheral tear (arrows) in the posterior horn of the lateral meniscus

Cartilage degeneration in OA is partly due to increased synthetic activity of articular chondrocytes, which results in rapid enzymatic degradation of articular cartilage tissue. The rate of synthesis of matrix components by remaining cartilage cells is increased, presumably as a reparative response, but cannot match the rate of cartilage loss (Mankin *et al.,* 1981). At the cellular level, visually intact OA cartilage is similar to non-OA cartilage. Cartilage water content, proteoglycan composition and structure, sulphate incorporation, glycosaminoglycan synthesis rates and cell content remain unchanged (for review, see Brocklehurst *et al.,* 1984). However, following serious damage, cellular changes become evident. Changes include decreases in glycosaminoglycan content, chondroitin sulphate chain length and keratan sulphate content (Bayliss *et al.,* 2001; Bollet *et al.*, 1963; Brocklehurst *et al.*, 1984).

In addition to changes in cartilage morphology, changes to the underlying bone contribute to OA pathophysiology. Articular bone surfaces may flatten and depress (attrition), most common in cases of severe OA where cartilage erosion is extensive. In such areas, physical stress on the bone results in inflammation and oedema of the bone (Bollet, 2001), as well as bone marrow lesions (for review, see Wenham & Conaghan, 2009). In cases of full or near-full thickness articular cartilage defect, the subchondral bone thickens and grows upwards with bony outgrowths (osteophytes) both in marginal and non-marginal (central) locations (McCauley *et al.,* 2001). A reduction in space between the joints is observed following osteophyte formation. Sub-articular cysts may form, either fluid- or fibrous tissue-filled, with a sclerotic margin. In addition to sub-articular cysts, intra-articular periarticular cysts, may form, most of which are small. These may have haemorrage, loose bodies and debris present within them (Janzen *et al.,* 1994).

The synovial intima thickens (Figure 1.7, Richardson *et al.*, 2008) and becomes inflamed in approximately 73% of cases (Fernandez-Madrid *et al.*, 1995). This inflammation of the synovium affects its ability to control diffusion, ingest debris and secrete a number of substances such as hyaluronate, immunoglobulins, and lubricating glycoproteins which act to reduce friction in the joint. As a result, joint effusion, or "fluid on the knee" can occur. Moderate to large effusions are associated

with well-progressed, severe forms of OA (Fernandez-Madrid *et al.*, 1994). The joint capsule is also altered. It normally functions to seal the joint space and provide stability not only by limiting movement but also through the presence of proprioceptive nerve endings within. In OA the joint capsule thickens and stretches, resulting in joint instability and leakage of synovial fluid, diminishing cushioning and lubrication within the joint.



Richardson *et al.*, 2008

Figure 1.7 Haematoxylin and eosin micrographs of synovium biopsies taken at osteoarthritis total knee arthroplasty. A, mild inflammation: synovial intima three to five cells thick, slight increase in cellularity with few inflammatory cells; B, moderate inflammation: synovial intima four to six cells thick, dense cellularity with inflammatory cells, may exhibit as small lymphoid aggregates; C, severe inflammation: synovial intima five to seven or more cells thick, dense cellularity with inflammatory cells, containing many or large perivascular lymphoid aggregates. I, synovium intima; L, lymphoid body; SV, small vessel; V, villus.

1.2.2. Origins of pain in OA

Although pain is the most troublesome complaint for sufferers of OA, the causes of OA-related pain are still unclear. It has been shown that radiographically observed damage to the knee joint does not correlate with the pain experienced by sufferers (Lawrence *et al.*, 1966), with approximately half of over-55's reporting knee pain lacking signs of radiographic OA (McAllindon *et al.*, 1992). Studying the structure-pain relationship in OA is, however, complicated. The subjective nature of pain results in a wide range of pain severity being reported, and is particularly complicated where there is a co-morbidity of events such as depression. Its episodic nature adds difficulty to

the assessment of the structure-pain relationship. The techniques commonly used to assess OA also have their faults (see below), making it difficult to assess structural abnormalities. Many conventional radiographic studies image only the tibiofemoral joint and not the patellofemoral joint (as cited by Wenham & Conaghan, 2009), decreasing the likelihood that structural changes will be detected. One report suggested that the number of views assessed radiographically increases the likelihood of a diagnosis of OA, with the use of just the postero anterior view alone identifying only approximately half of cases of radiographic OA in patients with knee pain, the use of two views increasing the rate to 87%, and all three views (postero anterior, supine skyline and supine lateral) raising the rate of identification to 98% (Duncan *et al.*, 2006). In addition, radiographic OA pathology is most commonly quantified using the Kellgren-Lawrence (K/L) grading system (Lawrence *et al.*, 1966), which assesses only the presence of osteophytes, joint space width and bone sclerosis. While the presence of osteophytes has been shown to correlate to the occurrence of knee pain (Lanyon *et al.*, 1998; Spector *et al*, 1993), joint-space narrowing has proved to be an inconsistent indicator of pain. This is due partly to the lack of a clear threshold of joint-space loss beyond which OA pain incidence increases (Lanyon *et al.*, 1998), and large intra- and inter-observer variation in joint space measurement, bringing into question the reproducibility of such a measure (Spector *et al.*, 1993). MRI analysis is improving, becoming more accurate and reliable with regards to measurements of cartilage volume and thickness (Eckstein & Glaser, 2004), and work into validation of a number of semi-quantitative scores is currently underway (Hunter *et al.*, 2008).

Although the causes of OA-related pain are unclear, the integrity of structures surrounding the knee have been found to be of importance (Hill *et al.*, 2003; Bajaj *et al.*, 2001). Within the joint structure itself, candidate sources of OA pain have been proposed, and include the subchondral bone (Gronblad *et al.*, 1984) and the synovium (Smith *et al.*, 1997). As mentioned previously, the presence of osteophytes correlates with the occurrence of knee pain. In addition, in subjects diagnosed with knee OA, bone marrow lesions were found more commonly in painful knees than non-painful knees (77.5% and 30% respectively), with large lesions almost exclusively in painful knees (35.9% of painful knees versus just 2% of non-painful knees – Felson *et al.*,

2001). The thickness of the synovium was correlated to pain severity in OA sufferers (Hill *et al.*, 2001, Fernandez-Madrid *et al.*, 1994), particularly when synovitis was found to occur in the infrapatellar fat pad (Hill *et al.*, 2007). While synovitis extent did not correlate to cartilage loss (Hill *et al.*, 2007), the cartilage has been identified as a possible source of pain. Healthy cartilage is aneural, however it has been suggested that in damaged areas of OA cartilage, nerve and vascular ingrowth occurs (Ashraf & Walsh, 2008).

Beyond peripheral mechanisms, it has been suggested that central mechanisms contribute to OA pain propagation (Farrell *et al.*, 2000a). Following increased input to the spinal cord from the periphery owing to structural abnormalities, central sensitisation occurs, magnifying nociceptive transmission and sensations of pain. In OA of the hand, patients with persistent pain displayed decreases in thermal and mechanical thresholds in the thumb compared to the forearm, while patients who did not have pain, or in those suffering only from incident-related pain, no difference in thresholds were observed (Farrell *et al.*, 2000b). The decrease in mechanical and thermal thresholds correlated to variance in ratings of movement pain, further supporting the proposed involvement of central mechanisms. In the MIA model of OA pain in the mouse (see Chapter 4 for details), increases in responses of A- and C-fibres following electrical stimulation was observed (Harvey & Dickenson, 2009) together with hyperalgesic responses to mechanical, but not thermal stimuli. Aβ-fibres have been implicated in the alteration of stimulus response characteristic of OA sufferers, with a hyperalgesic state abolished by the tying of a ribbon around the affected wrist (Farrell 2000a, 2000b). The endocannabinoid system has also been found to be altered in OA and may play a role in mediating painful responses (Richardson *et al.*, 2008). This will be described in more detail in section 1.3.5.

1.2.3. Experimental models of OA

Models commonly used for the study of OA (reviewed by Pritzker, 1994, Jouzeau *et al.*, 2000) are classified into two main groups; those brought about by structural alteration of the joint by physical or enzymatic means; and those brought about by a

disturbance of chondrocyte metabolism. Examples for physical structural alteration include patellectomy, ligament transection, meniscectomy, myectomy, denervation, and displaced loading such as by osteotomy and immobilisation, local abrasion or external contusion. Enzymatic structural alteration examples include intra-articular injection of papain or bacterial collagenase, and models that act by disruption of chondrocyte metabolism include intra-articular injection of vitamin A. Models employing spontaneous and structural alteration of the joint have the advantage of mimicking the causes of human OA more closely than chemical or enzymatic intervention, but the disadvantage that unlike chemical intervention, it is difficult or impossible to influence their onset and course in terms of time required for the model to develop and severity of damage (Burton-Wurster *et al.,* 1982, Evans *et al.,* 1994, Smale *et al.,* 1995). Models currently available are good in terms of histological appearance and progression of disease, but there are a variety of problems with their use, including difficulties in reproducibility and slow onset times (spontaneous degeneration models in guinea pigs take 3-18 months to set up - Bendele and Hulman, 1988). In addition, these models tend to focus on structural damage, and pain paradigms in these models are poorly studied. Clinically, while the extent of damage often correlates with pain, pain also occurs when there is no radiographically detectable damage, and in some cases pain does *not* occur even if there is severe damage (Lawrence *et al.*, 1966, McAllindon *et al.*, 1992), highlighting a complex relationship between OA and its associated pain. Therefore, in order to be clinically relevant, models used to study OA must not rely solely on OA-like histology but must also take pain into account. In addition, it must be easily reproducible and have controllable severity. At present, no such model exists, making the search for novel analgesics for use in OA difficult. A model of OA pain that could be quickly implemented and controlled would therefore be of great benefit in the search for novel treatments. The possibility of such a model in the intra-articular injection of monosodium iodoacetate (MIA) has been widely studied and looks promising (see Chapter 4).

1.3. The endocannabinoid system

1.3.1. History

Cannabis sativa has been used medicinally for thousands of years for its treatment of rheumatic pain, malaria and constipation (Felder *et al.*, 1998). The first active constituent of the *Cannabis sativa* plant to be identified and purified was Δ^9-tetrahydrocannabinol (Δ^9-THC, Gaoni and Mechoulam, 1971), although it was several years before a binding site was identified for it in the central nervous system (Devane *et al.*, 1988). This receptor was designated cannabinoid-1 (CB_1), and the discovery of endogenous substances that act at the same receptors followed shortly after (Devane *et al.*, 1992). A second site of action was discovered shortly thereafter in myeloid cells and was designated the cannabinoid-2 (CB_2) receptor (Munro *et al.*, 1993). The orphan G protein-coupled receptor GPR55 which is linked to G_{12} proteins, negatively coupled to adenylyl cyclase, activates rhodopsin A and mobilises intracellular calcium, is another, albeit controversial CB receptor candidate (for review, see Ross, 2009).

1.3.2. Cannabinoid synthesis

The most widely studied endocannabinoids (ECs) are the *N*-acylethanolamine anandamide (AEA - Devane *et al.*, 1992), and 2-acylglycerol (2-AG, Mechoulam *et al.*, 1995, Sugiura *et al.*, 1995). They are derivatives of arachidonic acid conjugated with ethanolamine or glycerol, respectively, with differing mechanisms of synthesis. Other putative EC compounds include N-arachidonolyglycerol (noladin ether), N-arachidonoyl dopamine (NADA), O-arachidonoyl ethanolamine (virodhamine), N-dihomo-γ-lionlenoyl ethanolamine, N-docosatetraenoyl ethanolamine, oleamide, N-oleoyldopamine (OLDA). The EC system could also be said to include *N*-acylethanolamines such as oleoylethanolamide (OEA) and palmitoylethanolamide (PEA) which do not bind cannabinoid (CB) receptors, but exert EC-like effects, and are termed endocannabinoid-like compounds (ECLs, Pertwee, 2006). The ECs and ECLs with which this thesis is most concerned are 2-AG, and the *N*-acylethanolamines AEA, OEA and PEA (Figure 1.8).

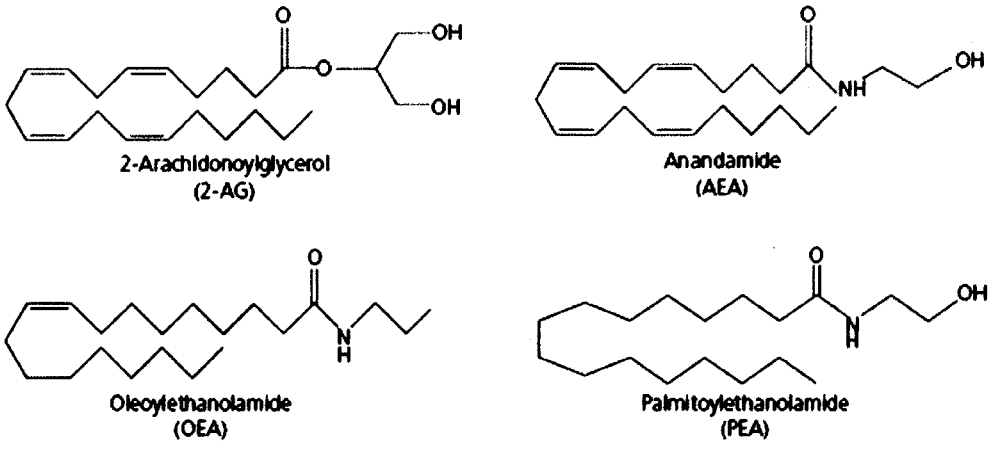
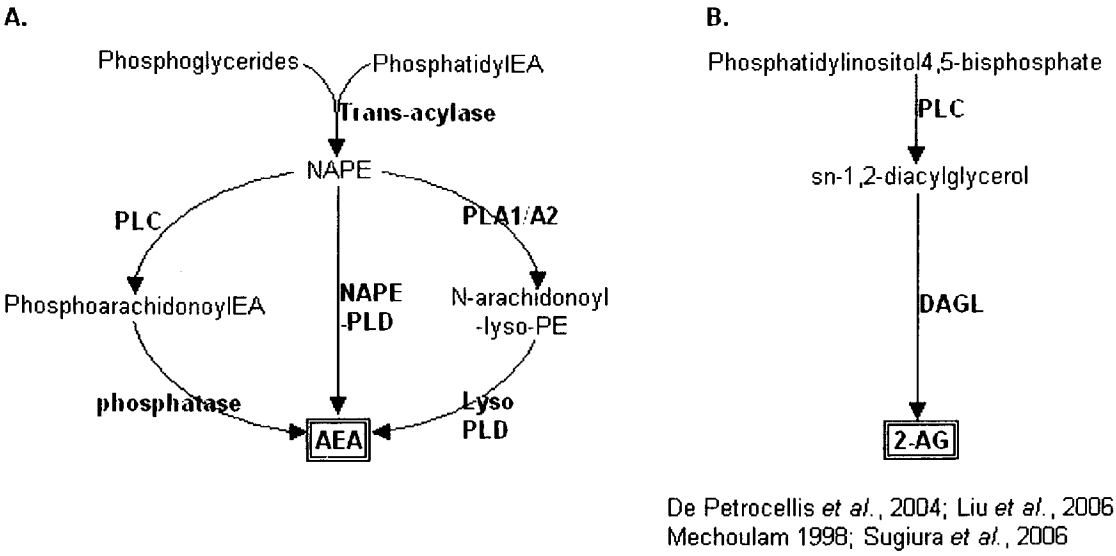


Figure 1.8 The structure of commonly studied molecules in the endocannabinoid system. Top left, 2-arachidonoylglycerol (2-AG); top right, bottom left and bottom right, the N-acylethanolamines anandamide (AEA), oleoylethanolamide (OEA) and palmitoylethanolamide (PEA).

AEA and other *N*-acylethanolamines (NAEs) are widely believed to be produced on demand from the cleavage of membrane-bound precursors in response to specific signals such as raised intracellular calcium concentration and membrane depolarisation (Figure 1.9A). AEA is cleaved from its membrane phospholipid precursor *N*-arachidonoyl-phosphatidylethanolamine (NAPE) by phospholipase D (PLD, Okamoto *et al.*, 2004) in a cAMP- and calcium-dependent manner (Cadas *et al.*, 1996; Piomelli *et al.*, 2003). PLD activity is, therefore, potentially regulated by activation of a number of neurotransmitter receptors, such as dopamine, glutamate and acetylcholine receptors (Stella and Piomelli, 2001; Piomelli *et al.*, 2003; Giuffrida *et al.*, 1999; Varma *et al.*, 2001; Kim *et al.*, 2002). Synthesis of 2-AG may occur via a number of mechanisms (Figure 1.9B). Phospholipase A_1 (PLA_1)-mediated hydrolysis of membrane phosphatidylinositol produces lyso-phosphoinositol (lysoPI), which is hydrolysed by phospholipase C (PLC) to 2-AG (cited by Sugiura and Waku, 2000). PLC may also hydrolyse inositol phospholipids in a calcium-dependent process to diacylglycerol (DAG), which is then metabolised to 2-AG by *sn*-1-DAG lipase (Stella *et al.*, 1997; Bisogno *et al.*, 2003; and for review, see Piomelli *et al.*, 2003).



De Petrocellis *et al.*, 2004; Liu *et al.*, 2006; Mechoulam 1998; Sugiura *et al.*, 2006

Figure 1.9 A simplified schematic of endocannabinoid synthesis. A, routes of synthesis of AEA; **B**, synthesis of 2-AG. AEA is synthesised from NAPE by the actions of PLC and other phosphatases, NAPE-PLD or PLA1PLA2 and LysoPLD. 2-AG is synthesised from its precursor phosphatidylinositol 4,5-bisphosphate by PLC and DAGL. 2-AG, 2-arachidonoylglycerol; AEA, anandamide; DAGL, diacylglycerol lipase; EA, ethanolamine; LysoPLD, lyso-phospholipase D; NAPE, N-acylphosphatidylethanolamine; NAPE-PLD, N-acylphosphatidylethanolamine-phospholipase D; PE, phosphatidylethanolamine; PLA, phospholipase A; PLC, phospholipase C.

In the brain, 2-AG is present in approximately 200-fold higher concentration than AEA (Sugiura *et al.*, 1995; Stella *et al.*, 1997). In unstimulated tissue, levels of AEA are also typically 10 fold less than those of classical neurotransmitters. However, some studies have shown AEA levels to increase 5-12 -fold after depolarisation or receptor stimulation. Levels also increase *post-mortem* in the rat, and molluscs, and *in vitro* in rat tissues (Schmid *et al.*, 1995; Felder *et al.*, 1996; Kempe *et al.*, 1996; Sepe *et al.*, 1998; Kim *et al.*, 2002). 2-AG levels also increase rapidly *post-mortem* (Sugiura *et al.*, 2001), supporting the theory that ECs are produced on demand. N-(2-Hydroxyethyl)hexadecanamide (Palmitoylethanolamide, PEA) is a naturally occurring, shorter chain, fully saturated structural analogue of AEA. It is found in soya bean lecithin, egg yolk, peanut meal (cited in Lambert *et al.*, 2002), marine species and the leech (Sepe *et al.*, 1998, Bisogno *et al.*, 1997, Matias *et al.*, 2001) and most mammalian tissues (Bachur *et al.*, 1965, Schmid *et al.*, 1997, Calignano *et al.*, 1998, Kondo *et al.*, 1998, Baker *et al.*, 2001). Its production is again thought to be activity-dependent, as for AEA and 2-AG (DiMarzo *et al.*, 1998; Cadas *et al.*, 1997; Hansen *et al.*, 1997), and it has been found to be co-synthesised with AEA in leukocytes and

RBL-2H3 cells (Bisogno *et al* 1997, Lambert & Di Marzo, 1999). OEA is naturally present in low concentrations (<2μg/g) in foods such as cocoa powder, oatmeal and nuts (Astarita *et al.*, 2006; Di Marzo *et al.*, 1998). A role for OEA in pain has been suggested, having both pro- and anti-nociceptive effects via action at different receptors (see section 1.3.5) (Wang *et al.*, 2005; Suardiaz *et al.*, 2007). Both PEA and OEA are synthesised by NAPE-PLD, and over-expression of NAPE-PLD *in vitro* results in elevated NAE levels (Okamoto *et al.*, 2005). NAPE-PLD-null mice have decreased levels of OEA and PEA, but not AEA in the brain (Leung *et al.*, 2006), highlighting the importance of this synthetic route for OEA and PEA, but suggesting other routes predominate in the synthesis of AEA. PEA can also be formed by condensation of ethanolamine and palmitic acid in an ATP and coenzyme A-independent fashion (Schmid PC *et al.,* 1985), and OEA may also be formed from oleic acid and phosphatidylethanolamine (see Thabuis *et al.*, 2008).

1.3.3. Cannabinoid action and effect

Cannabinoids act primarily on CB_1 and CB_2 receptors. CB_1 is present presynaptically on axons and terminals of neurones centrally and peripherally, with little or no expression on dendrites or soma (Egertova & Elphick, 2000; Herkenham *et al.,* 1991a; 1991b; Mailleux *et al.*, 1992; Tsou *et al.*, 1998). Here, it is ideally located for pre-synaptic modulation of neurotransmitter release, by retrograde release of ECs from post-synaptic terminals, where NAPE-PLD is located. The CB_1 receptor is also expressed by glial cells, cells in the reproductive system, some endocrine glands, and the microcirculation (Devane *et al.,* 1988; Howlett *et al.,* 1990, Wagner *et al.,* 1997; Batkai *et al.,* 2001). In the brain, the CB_1 receptor is the most abundant G-protein-coupled receptor (Herkenham *et al.*, 1991c). Receptor density is particularly high in the striatum, cerebellum, basal ganglia, cerebral cortex and hippocampus (Herkenham *et al.*, 1990; Herkenham *et al.*, 1991c), and is moderate to high in areas involved in pain processing such as the thalamus, PAG, RVM, amygdala, superficial laminae of the spinal cord, and dorsal root ganglion (DRG) neurones (Herkenham *et al.*, 1991, 1990; Mailleux *et al.*, 1992; Tsou *et al.*, 1998). Its wide distribution is consistent with cannabinoid effects on nociception, learning and memory, satiety, mood and anxiety,

and psychotropic effects. The CB_2 receptor was originally thought to be expressed only in immune cells (Facci *et al.*, 1995; Munro *et al.*, 1993) and is found in lymphoid organs and microglial cells (Munro *et al.,* 1993, Galiegue *et al.,* 1995; Piomelli *et al.,* 2003). However, its expression has also been shown in the CNS, with mRNA expression demonstrated in the spinal cord in a model of neuropathic pain (Beltramo *et al.*, 2006; Zhang *et al.*, 2003), the brain stem (Van Sickle *et al.*, 2005), cortex, striatum, thalamus, PAG, hippocampus and amygdala (Gong *et al.*, 2006). Its function in the CNS remains unclear. AEA is a partial agonist at both receptors with a 4-30 fold preference for CB_1 compared with CB_2. 2-AG is a full agonist at both receptors but with lower affinity than AEA (Stella *et al.,* 1997; Hillard *et al.,* 1999; Howlett *et al.,* 2002).

The CB_1 and CB_2 receptors are coupled negatively to AC through $G_{i/o}$ proteins, resulting in decreased levels of cAMP, and positively to mitogen-activated protein kinase (MAPK) (Howlett *et al.*, 2002; Howlett, 2005). In addition, CB_1 receptors may also activate Gs proteins which are positively coupled to AC and A type inward rectifying potassium channels, and negatively coupled to N type and P/Q type calcium channels (Pertwee, 1997; Howlett *et al.*, 2002; Howlett *et al.*, 2005; Mackie & Hille, 1992; Twitchell *et al.*, 1997). This results in neuronal hyperpolarisation, pre-synaptic inhibition of the release of neurotransmitters such as GABA, glutamate, acetylcholine and noradrenaline (Schlicker and Kathman, 2001; Piomelli *et al.*, 2003) and neuropeptides such as corticotrophin releasing factor and cholesystokinin (Rodriguez de Fonseca *et al.,* 1997; Beinfeld and Connolly 2001). Post-synaptically, effects counteract excitatory inputs from neurotransmitters, preventing the propagation of action potentials (Felder *et al.,* 1998; Rodriguez de Fonseca *et al.,* 1998; Giuffrida *et al.,* 1999).

The inhibitory effects of cannabinoid receptor activation on GABA and glutamate release in the hippocampus cause phenomena termed depolarisation-induced suppression of inhibition (DSI - Wilson and Nicoll 2001) and depolarisation-induced suppression of excitation (DSE -Diana and Marty, 2004), respectively. DSI and DSE are short-term forms of synaptic inhibition and excitation (Freund *et al.*, 2003), and act

oppositely to modulate neurotransmission. While this mechanism has not been demonstrated in the spinal cord, it is likely to contribute to antinociceptive properties of ECs. Through DSI and DSE, CBs have been shown to prevent the induction of long-term potentiation (LTP - Stella *et al.,* 1997) and to facilitate long-term depression (LTD - Gerdeman *et al.,* 2002, Robbe *et al.,* 2002), including that of inhibitory GABAergic neurones. Thus, activation of presynaptic CB_1 receptors may cause neuronal excitation, although effects are mostly inhibitory.

Cannabinoids also act on other receptor systems, including the transient receptor potential vanilloid type-1 ($TRPV_1$) cation channel, the nuclear peroxisome-proliferator activated receptors (PPAR), and "CB-like" receptors (for reviews, see Ross, 2003; Burstein, 2005; Kreitzer & Stella, 2009). $TRPV_1$ is a non-selective ligand-gated cationic channel at which capsaicin exerts its effects (for review, see Palazzo *et al.*, 2008). $TRPV_1$ activation causes excitation of C-fibres and results in nociceptive behaviour (Potenzieri *et al.*, 2009). While the concentration of AEA required for activation of $TRPV_1$ receptors is greater than that required to activate CB receptors, inflammatory mediators such as bradykinin increase the sensitivity of $TRPV_1$ receptors to AEA (Singh-Tahim *et al.*, 2005). Activity of endocannabinoids and related compounds at PPARs, which have roles in inflammatory and pain processes, and neuroprotective properties have been widely studied (Cuzzocrea *et al.*, 2006; Rockwell *et al.*, 2006; Sun *et al.*, 2007; O'Sullivan & Kendall, 2009). Antinociceptive effects of PPAR activation by cannabinoids and related compounds has been observed in models of inflammatory pain (Jhaveri *et al.*, 2008; Sagar *et al.*, 2008). The synergistic effects of the PEA and AEA (see later) are thought to be partly mediated via PPARs (Russo *et al*, 2007; Costa *et al.*, 2008). Residual effects of cannabinoids in the presence of $CB_{1/2}$ antagonists and in CB receptor knockout mice has led to the proposal of additional receptors (for review, see Howlett *et al.*, 2002). These "CB-like" receptors have been described in the vasculature and the terminals of glutamatergic axons in the hippocampus, and are activated by both thermal and chemical stimuli (Hajos *et al.,* 2001, Kunos *et al.,* 2002).

PEA was initially thought to act as a selective CB_2 receptor agonist (Facci *et al.,* 1995; Sheskin *et al.,* 1997). It is now accepted that PEA is inactive at the cannabinoid receptors at concentrations $\leq 10\mu M$. Above this, PEA is a weak ligand at the CB_2 receptor (Lambert *et al.*, 2002). PEA inhibits lipopolysaccharide-induced nitric oxide production independently of CB_2R activation (Ross *et al.,* 2000) and some evidence exists to suggest that PEA is a ligand for GPR55 (Calignano *et al.,* 2001; Griffin *et al.,* 2000). The antinociceptive effects of PEA have been demonstrated to be mediated via the PPARα receptor (LoVerme *et al.*, 2006).

There is also evidence that PEA acts as an "entourage" endocannabinoid, indirectly enhancing the effects of AEA (supported by the finding of co-synthesis in some cells), at the CB_1R (Lambert and DiMarzo, 1999). In Human Embryonic Kidney cells over-expressing $TRPV_1$, PEA enhances the effect of AEA effect on $TRPV_1$ mediated increase in intracellular calcium concentration. The mechanism by which this occurs is unclear, however inhibition of AEA hydrolysis or binding to non-specific sites has been ruled out (De Petrocellis *et al.,* 2001).

OEA does not act at classical CB receptors, but it does have agonist actions at PPARα (Fu *et al.*, 2003), and the orphan receptor GPR119 (Cluny *et al.*, 2009; Overton *et al.*, 2006). GPR 119 is a 335 amino acid protein encoded on chromosome X (Fredriksson *et al.*, 2003; Takeda *et al.*, 2002), and is distributed predominantly in intestinal and pancreatic tissues. It is also found in brain regions such as the substantia nigra in rodents (Overton *et al.*, 2008; Soga *et al.*, 2005; Chu *et al.*, 2007; Lauffer *et al.*, 2009; Lan *et al.*, 2009). Its activation results in increases in intracellular cAMP (Chu *et al.*, 2007), implying its coupling to G_s. Through its actions on PPARα, OEA has an important role to play in satiety and limiting food intake, and it is a possible target in the treatment of obesity (Fu *et al.*, 2003, 2008; Lan *et al.*, 2009). Through actions on PPARα, OEA also has roles in stimulating lipolysis and protection of dopaminergic neurones in the substantia nigra (Guzman *et al.*, 2004; Sun *et al.*, 2007; Galan-Rodriguez *et al.*, 2009), and it has antinociceptive effects through PPARα-independent mechanisms (Suardiaz *et al.*, 2007). It has been suggested that altered glutamatergic transmission may be involved in the antinociceptive effects of

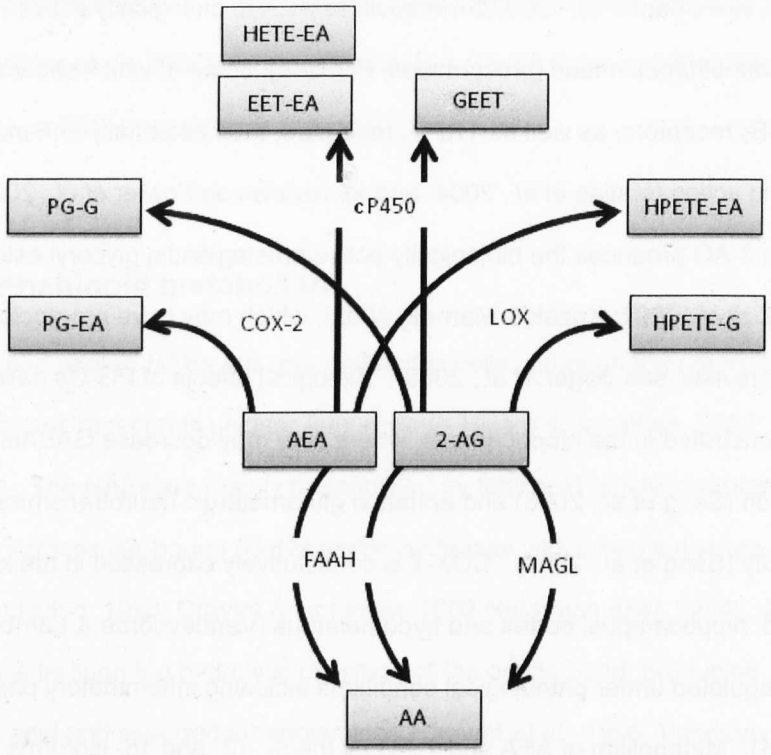
OEA (Suardiaz *et al.*, 2007). OEA has also been suggested have action at $TRPV_1$ receptors (Wang *et al.*, 2005), with pronociceptive effects that can be inhibited by capsazepine, a $TRPV_1$ receptor antagonist.

1.3.4. Cannabinoid metabolism

2-AG, AEA and related NAEs are accumulated in cells, although the nature of this trans-membrane transport is unclear (see reviews Hillard & Jarrahian, 2003; Hermann *et al.*, 2006). The NAEs are mainly metabolised by fatty acid amide hydrolase (FAAH), an intracellular-bound 63kDa serine hydrolase with broad substrate specificity (Deutsch and Chin, 1993; Cravatt & Lichtman, 2002 Natarajan *et al*, 1984). NAE metabolism is through the hydrolytic cleavage of the amide bond, producing arachidonic acid and assorted ethanolamines (Cravatt *et al.*, 1996; Vandevoorde & Lambert, 2007; Deutsch & Chin, 1993). FAAH acts on PEA at a much lower rate than on AEA, due to FAAH having higher catalytic efficiency on shorter molecules with a less saturated fatty acid chain (Bisogno *et al.,* 1997, Desarnaud *et al.,* 1995, Ueda *et al.,* 1995, Matsuda *et al.,* 1997, Katayama *et al.,* 1999). FAAH has also been implicated in 2-AG inactivation (Goparaju *et al.*, 1998; Di Marzo *et al.*, 1998), producing arachidonic acid and glycerol (for review, see DiMarzo, 1999). But approximately 50% of 2-AG metabolism is under the control of monoacylglycerol lipase (MAGL), a 33kDa serine hydrolase which metabolises 2-AG to arachidonic acid and glycerol (Dinh *et al.*, 2002a; Karlsson *et al.*, 1997; Tornqvist & Belfrage, 1976) (Figure 1.10).

AEA and 2-AG also undergo oxidation by the cyclooxygenases COX-1 and COX-2 (Kozak *et al.*, 2004; Yu *et al.*, 1997), lipoxygenases (LOX - Edgemond *et al.*, 1998; Hampson *et al.*, 1995; Ueda *et al.*, 1995a) and cytochrome p450s (cP450 – Bornheim *et al.*, 1993; Snider *et al.*, 2008), resulting in the production of a number of biologically active substrates such as prostaglandins, leukotrienes and thromboxanes, as well as prostaglandin ethanolamides (prostamides) (Figure 1.10). The importance of EC inactivation by these oxidative pathways is beginning to be understood and has potential implications for the antinociceptive effects of the endocannabinoid system

(for details, see Chapter 3). COX-2 metabolises AEA to biologically active prostaglandin ethanolamides (prostamides, PG-EAs), some of which show activity at CB_1 and CB_2 receptors, as well as $TRPV_1$ receptors, thus potentially enhancing cannabinoid action (Matias *et al.*, 2004; and for review see Fowler *et al.*, 2007). COX-2 action on 2-AG produces the biologically active prostaglandin glyceryl esters (PG-Gs) (Kozak *et al.*, 2001; Kozak & Marnett, 2002), which may have pro-nociceptive effects (for review, see Sagar *et al.*, 2009). Biological effects of PG-Gs have also been demonstrated in the hippocampus, where they may decrease GABAergic transmission (Sang *et al.*, 2006) and enhance glutamatergic neurotransmission and neurotoxicity (Sang *et al.*, 2007). COX-2 is constitutively expressed in the kidney, spinal cord, hippocampus, cortex and hypothalamus (Vandevoorde & Lambert, 2007) and is upregulated under pathological conditions including inflammatory pain (Samad *et al.*, 2001). Metabolism of AEA and 2-AG by the 5-, 12- and 15- isoforms of LOX produces hydroxyperoxyeicosa-5,8,10,14-tetraenoic acid ethanolamides (HPETE-EAs) and hydroxyperoxyeicosa-5,8,10,14-tetraenoic acid glycerol esters (HPETE-Gs), respectively. Of these, the 12-LOX metabolite of AEA has activity at the CB_1 receptor (Hampson *et al.*, 1995; Edgemond *et al.*, 1998). Several isoforms of cP450 are capable of metabolising ECs, AEA to hydroxyeicosatetraenoic acid ethanolamide (HETE-EA) and epoxyeicosatrienol ethanolamide (EET-EA), and 2-AG to glycerated epoxyeicosatrienoic acid (GEET) (Chen *et al.*, 2008; Awumey *et al.*, 2008), several forms of which have activity at CB_1 and CB_2 receptors (see Chapter 3)(Figure 1.10).



Sagar *et al.*, 2009

Figure 1.10 Metabolic pathways of endocannabinoids. AEA metabolism is largely under the control of FAAH, however, oxidative metabolism by cytochrome P450, COX-2 and LOX produces biologically active metabolites which may be important in contributing to antinociceptive effects of endocannabinoids. MAGL is the main enzyme responsible for 2-AG metabolism, however, FAAH and the oxidative enzymes also have a role to play. 2-AG, 2-arachidonoyl glycerol; AA, arachidonic acid; AEA, anandamide; COX-2, cyclooxygenase-2; cP450, cytochrome P450; EA, ethanolamide; EET, epoxyeicosatetraenol; FAAH, fatty acid amide hydrolase; HETE, hydroxyeicosatetraenoic acid; HPETE-G, hydroxyperoxyeicosa-5,8,10,14-tetraenoic acid glycerol ester; GEET, glycerated epoxyeicosatrienoic acid; LOX, lipoxygenase; MAGL, monoacyl glycerol lipase; PG-G, prostaglandin glyceryl esters.

1.3.5. Cannabinoid-mediated antinociception

The endocannabinoid system is altered by nociceptive stimuli. Noxious-evoked endocannabinoid release was first reported in the rat peri-aqueductal gray (PAG), a key pain-processing structure in the midbrain (see Walker *et al.*, 1999). In animal models of chronic pain (CCI and SNL in the rat), endocannabinoid (particularly AEA) levels have been shown to be elevated spinally, and supraspinally in several brain regions including the PAG and rostroventral medulla (RVM) (Pallazo *et al.*, 2006; Petrosino *et al.*, 2007). Changes in endocannabinoid levels in human disease states have also been reported, with levels of AEA and 2-AG undetectable in healthy (non-inflamed) synovial fluid but present in the synovial fluid of subjects with rheumatoid

and osteoarthritis (see below) (Richardson *et al.*, 2008). The antinociceptive effects of AEA and 2-AG have been well described in animal models of acute and chronic pain (for reviews, see Iversen *et al.*, 2002; Pertwee, 2001; Walker & Huang, 2002), as well as in humans (Ammar, 1998, Wade *et al.*, 2003), and involve experiments modulating CB receptors and experiments changing EC levels.

Modulating cannabinoid receptors

Activation of the cannabinoid receptors has antinociceptive effects in both behavioural and electrophysiological paradigms. Activation of supraspinal CB receptors was shown to diminish second phase formalin-evoked inflammatory nociceptive behaviour, with the use of HU210, a non-selective cannabinoid receptor agonist, injected directly into the PAG (Finn *et al* 2003). This also diminished spinal and thalamic neuronal responses following noxious stimuli (Buxbaum *et al.*, 1972, Hohman *et al.*, 1995, Martin *et al.*, 1996). In addition, the synthetic cannabinoid WIN55,212-2 produced CB_1- and CB_2-mediated antinociception when given as a pre-treatment in the SNL model of neuropathic pain (Guindon *et al.*, 2007). The importance of endocannabinoids in nociception has also been shown by blockade of the CB_1 receptor with the antagonist rimonabant, which results in enhanced nocifensive responses to formalin injection (Strangman *et al.*, 1998). However, these results are contradictory to data obtained in mice, in which significant increases in nocifensive behaviour or tissue endocannabinoid levels following CB_1 (rimonabant) or CB_2 (SR144528) receptor antagonist application was not observed (Beaulieu *et al.*, 2000).

Effects of modulating endocannabinoid levels

Modulation of endocannabinoid levels by injection of exogenous endocannabinoids or by blocking uptake and/or metabolism of endogenous endocannabinoids have been investigated in models of pain. In the carrageenan model of acute inflammation in the rat, injection of AEA, either alone or with PEA into the hindpaw, decreased thermal hyperalgesia and nociceptive behaviours, an effect shown to be through actions on the peripheral CB_1 receptor (Richardson *et al.*, 1998, Calignano *et al.*, 1998).

Inhibitory effects on the inflammatory responses of oedema, plasma extravasation and neuropeptide release were also observed in inflammatory pain models using formalin and carrageenan (Calignano *et al.,* 1998, Richardson *et al.*, 1998). PEA alone produces similar antinociceptive effects, although in this case they are mediated via a peripheral, CB_2-like receptor (Calignano *et al.,* 1998; Malan *et al.,* 2001). Application of exogenous ECs also inhibited mechanically evoked responses in chronic inflammatory models (Sokal *et al.*, 2003).

Recently, there has been much interest in targeting the metabolism of endocannabinoids *in vivo* as a mechanism of achieving analgesia without the psychotropic side effects seen with direct agonist-mediated CB_1 receptor activation which limits the use of endocannabinoids in the clinic (for review, see Jhaveri *et al.*, 2007). The analgesic potential of preventing the catabolism of endocannabinoids has been studied by blocking EC uptake, and by inhibiting FAAH pharmacologically or by gene deletion. Blocking AEA transport with the anandamide membrane transporter (AMT) blocker AM404, and its analogues UCM707 and LY2183240, dose-dependently reduces nociceptive behaviour following thermal and mechanical stimuli. This is mediated by the CB_1 receptor in models of acute, inflammatory and chronic pain, using formalin, CFA, and surgical methods of SNL and CCI, respectively (LaRana *et al.,* 2006).

Endocannabinoid breakdown may also be prevented by FAAH knockout and by use of specific inhibitors. In FAAH knockout mice, this prevention of endocannabinoid breakdown in the brain results in local increases (15-fold) in levels of AEA in the hippocampus, and displays a trend to do so in the cortex and cerebellum. Levels of PEA and OEA were also greatly elevated in these regions, although this did not reach statistical significance (Clement *et al.*, 2003). FAAH knockouts are associated with CB_1-mediated antinociceptive behaviour in the tail-flick and hot-plate tests of nociception, in addition to other CB_1 receptor mediated effects such as hypothermia, catalepsy and hypomotility (Cravatt *et al* 2001, Lichtman *et al* 2004a). Systemic pharmacological inhibition of FAAH with URB597, OL-92 and OL-139 raised EC levels and produced antinociceptive effects in models of chronic pain in rats, as well as acute

pain in mice (Jhaveri *et al.*, 2006; Lichtman *et al.*, 2004b). Application of the most selective FAAH inhibitors α-ketoheterocytes OL-92 and OL-135 result in anxiolytic and antinociceptive effects in rodents in thermal and formalin-induced paradigms of pain. This too is mediated via the CB_1 receptor and is accompanied by an increase in levels of AEA, PEA and OEA throughout the CNS (Kathuria *et al.*, 2003, Lichtman *et al.*, 2004b). At therapeutic doses, most NSAIDs have been shown to inhibit FAAH activity (Fowler *et al.*, 1997, 1999) and their use is associated with increased levels of AEA, OEA and PEA (Guindon *et al.*, 2006b).

Antinociceptive effects of PEA and OEA

In models of acute pain, PEA acts synergistically with AEA to attenuate pain behaviour with 100 times more potency than with each compound alone, in both phases of formalin and turpentine-induced C fibre activation, sensory fibre inflammation and central sensitisation (Calignano *et al.*, 1998). AEA alone attenuated pain behaviour in the first, but not the second phase, perhaps as a result of its rapid inactivation (Cravatt *et al* 1996, Beltramo *et al* 1997). Co-administration of AEA together with PEA results in a longer duration of antinociceptive effect, with PEA inhibiting FAAH activity (Di Marzo *et al.*, 2001). The long-lasting effects of PEA alone and in combination with AEA make it an attractive subject for further study. In chronic pain states, PEA levels have been shown to be altered in a mouse model of diabetic neuropathic pain and in human patients with migraine and probable analgesic overuse headache (Sarchielli *et al.*, 2007), ulcerative colitis, chronic lower back pain after osteopathic manipulative treatment (Reviewed by Darmani *et al.*, 2005). However, in the chronic constriction injury model of the rat, AEA and 2-AG levels are increased spinally and supra-spinally, while levels of PEA are decreased (Petrosino *et al.*, 2007). Administration of PEA in this model leads to antinociceptive responses (Helyes *et al.*, 2003). This discrepancy between PEA increase and decrease may be explained by the observation that PEA and other EC levels change in degenerating tissues and cells in a specific manner, depending on the physiological stimuli / pathological conditions (Epps *et al.*, 1979; Natarajan *et al*,. 1986; Hansen *et al.*, 1995; Schmid *et al.*, 1995; Kondo *et al.*, 1998; Giuffrida *et al.*, 1999; Berdyshev *et al.*, 2000; Hansen *et al.*, 2000; Franklin *et al.*,

2003). In humans, the analgesic properties of PEA have been demonstrated when given as an epidural injection to relieve postoperative pain (Ammar, 1998).

In addition to its putative entourage effects, PEA has distinct antinociceptive properties in inflammatory, acute, and chronic pain states. Its effects on inflammatory pain are mostly due to its anti-inflammatory properties. PEA results in decreased substance P-induced mast cell degranulation *in vivo* (Aloe *et al.,* 1993), although this is disputed by *in vitro* work in human mast cells (Maccarrone *et al.,* 2000). PEA also results in attenuated oedema and associated inflammatory hyperalgesia by reducing mast cell activation (Mazzari *et al.,* 1996), and it inhibits lipopolysaccharide-induced nitric oxide generation in macrophages (Ross *et al.,* 2000). PEA also decreases neutrophil accumulation in cells (Farquhar-Smith & Rice, 2003), the cause of NGF-induced inflammatory pain (Bennett *et al.,* 1998, Shu and Mendell, 1999). In animals, PEA inhibits inflammation and the sensitising effects of inflammatory products on the nociceptive processes via autacoid local inflammation antagonism (ALIA) - local antagonism by PEA on inflammation via control of mast cell activity and reduction of mast cell degranulation (Aloe *et al.,* 1993; Levi-Montalcini *et al.,* 1996; Mazzari *et al.,* 1996). This is mediated by PPAR-α activation (LoVerme *et al.,* 2005). PEA has been shown act as a PPAR- α ligand, and decreased nocifensive behaviour in formalin and magnesium sulphate-injected mice is PPAR- α mediated (Lo Verme *et al.,* 2005). OEA also has roles in antinociception in visceral and inflammatory pain models, abolishing antinociceptive behaviours following acetic acid, or formalin administration in the rat (Suardiaz *et al.*, 2007).

Involvement of the endocannabinoid system in clinical pain

While CB receptor activation by HU210 has been shown to decrease acute capsaicin-evoked pain in humans (Rukwied *et al.,* 2003), clinical trials activating cannabinoid receptors with the use of cannabis cigarettes, oral-mucosal sprays and rectal suppositories in acute, post-operative and neuropathic pain states, have shown the greatest antinociceptive efficacy of cannabinoids to be in neuropathic pain conditions (as reviewed by Rahn and Hohmann, 2009). Cannabinoid efficacy in suppressing

pain has been shown in a number of clinical conditions, including HIV associated neuropathy (Abrams *et al.*, 2007, Ellis *et al.*, 2009), MS-induced neuropathic pain (Svendsen *et al.*, 2004, Rog *et al.*, 2005), brachial plexus avulsion (Berman *et al.*, 2004) and mixed neuropathies (Wilsey *et al.*, 2008). In arthritic conditions, current trials with Sativex, an oral 1:1 Δ^9-THC and cannabidiol compound, has shown positive results in the treatment of rheumatoid arthritis pain (Blake *et al.*, 2006). Illicit self-administration of cannabis has self-reported beneficial effects in the treatment of pain associated with both rheumatoid- and osteo-arthritis (Wright *et al.*, 2006). In a study of 21 patients with generalized neuropathic pain, displaying symptoms of hyperalgesia and allodynia, the cannabinoid receptor agonist CT-3 decreased perceived pain (Karst *et al.*, 2003). Cannabinoid-based medicines have also been used in the treatment of cancer pain, with mixtures of THC and CBD showing positive effects on pain management but a worsening of associated nausea and vomiting (Johnson *et al.*, 2009). These negative effects contribute to the primary cannabinoid side effect of psychoactivity, restricting the use of cannabinoid-based medicines in the clinic. As a result, they are usually given after opioid treatments have failed (Turcotte *et al.*, 2010).

The endocannabinoid system has been found to be altered in chronic pain conditions. In a study by Richardson *et al.* (2008), AEA and 2-AG were found in the synovial fluid of 32 OA and 13 RA patients undergoing total knee arthroplasty, but not in that of healthy volunteers. Synovia of these patients were found to contain both CB_1 and CB_2 receptor protein and RNA, as well as active FAAH. Interestingly, these findings were associated with a decrease in synovial fluid levels OEA and PEA compared to that of healthy volunteers, further supporting the proposed role of PEA and OEA as "entourage" compounds to ECs.

1.4. Aims of thesis

The aim of this thesis was to further investigate the usefulness of the MIA model of OA joint pathology as a model of pain associated with OA, and to determine the potential role(s) of the endocannabinoids in the modulation of these responses. The objectives of this thesis were to: 1, investigate peripheral and spinal pain responses, both in terms of behaviour and neuronal responses, in a model of OA pain. To this end the effect of MIA on the release of cytokines in the joint, and neuronal activity in the spinal cord was determined (see Chapter 4); 2, to determine the effects of COX-2 inhibition on neuronal responses and the potential role of endocannabinoids in mediating these effects (see Chapters 3 and 5); 3, to investigate whether the endocannabinoid system is upregulated in the spinal cord in a model of OA pain, and the functional effects of such upregulation (see Chapter 6).

Chapter 2 Materials and Methods

2.1. *In vivo* electrophysiology

All experiments were carried out on male Sprague-Dawley rats (Charles River U.K.) weighing 200-220g for studies in naïve rats, 160-190g at the time of injection for studies on the MIA model of OA pain. Rats were group-housed in a temperature controlled (20-22°C) environment with a 12hr light/dark cycle (lights on at 7am) with *ad libitum* access to food and water. Experimental procedures were carried out under Personal Home Office Licence 40/8536, and project licences 40/2564, and 40/3124. All experimental procedures were carried out in accordance with the Animals (Scientific Procedures) Act 1986 and International Association for the Study of Pain (IASP) guidelines.

2.1.1. Anaesthetic system

The anaesthetic system used throughout electrophysiological studies presented herein consisted of the inhalation anaesthetic isoflurane (Abbott, Kent, U.K.), delivered in a mixture of 33% oxygen (200cm^3/mm) and 67% nitrous oxide (300cm^3/mm) (both BOC gases, U.K.). A Vapotech series 3 vapouriser was used, delivered to the rat using silicone rubber tubing (5mm inner diameter, 7mm outer diameter) and a Y-connector. Expelled gases were removed via a Cardiff aldasorber (Datesand Ltd, Manchester, U.K.). Anaesthesia was induced by placing the rats in an induction chamber with 3% isoflurane in 33%: 66% oxygen: nitrous oxide. Once the righting reflex was lost, the rat was removed from the induction chamber, placed on its front, and a nose cone used to supply anaesthetic and gases at 2% isoflurane.

2.1.2. Surgical procedures

Once areflexia (no withdrawal reflex to hindpaw toe pinch) was achieved, the rat was placed on its back and tracheal cannulation was performed. Skin on the underside of the neck was lifted and cut to expose the underlying muscle layers, which were teased apart bilaterally to expose the trachea. Two lengths of suture (Pearsall's Sutures Ltd., U.K.) were passed underneath the trachea and tied loosely. An incision was made in

the trachea between two cartilage rings, and a bevelled-edged 5cm piece of polythene tubing (inner diameter 1.57mm, outer diameter 2.08mm) was inserted approximately 5mm and secured in place above and below the point of insertion with the suture. Anaesthetic was then delivered directly to the cannula through the Y-connector for the duration of the study.

The rat was placed on its front on a stereotaxic frame (University of Nottingham, Medical Faculty Workshop) and placed into ear bars to maintain a fixed head position throughout the study. Core body temperature was maintained at 36.5-37.5°C via a heating blanket receiving feedback from a rectal thermal probe. A midline incision approximately 5cm in length was made through the skin overlying the spine from approximately 2cm above the base of the ribs. Longitudinal incisions were made close to the bone into the muscle on either side of the vertebral column, and a clamp inserted and tightened around the vertebral column on the rostral side of the incisions. A tear-drop shaped incision was made approximately 1.5-2cm in length, into the connective tissue overlying the vertebral column, such that the centre of the incision lay over the base of the ribs, the approximate location of spinal segments L4-L5. Connective tissue was removed to expose lumbar vertebrae L1-L3, creating a well for spinal applications of saline, and where applicable, drug or vehicle solutions throughout the course of the experiment. Rongeurs were then inserted gently into the gap between vertebrae, which were removed to expose spinal segments L4-L5 through an opening approximately 1-2mm either side of the central vessel. The *dura mater* was carefully removed using pointed forceps, leaving the *pia mater* intact, and a second clamp secured around the vertebral column rostral to the incision, holding the spinal cord in a fixed position throughout the study. The well was filled with sterile saline to maintain the exposed spinal cord in a moist environment, and the skin was pulled taut and held together with a crocodile clip throughout the study, minimising dehydration of the rat. Anaesthesia was lowered to approximately 1.5% isoflurane, and areflexia (lack of blink reflex and response to hindpaw toe pinch) maintained throughout the study. At the end of the study, anaesthetic levels were increased to 5% until the rat stopped breathing and no pulse was felt. The neck was then broken to ensure termination. For tissue collection killing procedures, see section 2.3.1.

2.1.3. Electrophysiological recordings

Spike discrimination and audio monitoring was conducted with a Neurolog system (Digitimer, Welwyn Garden City, U.K.), using glass-coated tungsten electrodes (see later). The system was grounded through the stereotaxic frame. Differential recordings between the electrode, and an indifferent electrode (crocodile clip) attached to the skin of the rat, were made (Neurolog headstage NL100AK in A-B position). The voltage signal was amplified (Neurolog NL104 x 2K, Neurolog 106 x 80) and filtered through low and high-pass filters (Neurolog NL125, low frequency cut-off at 300Hz, high frequency cut-off of 5KHz). The signal was displayed on a Tektronix TDS 210 digital oscilloscope allowing visual discrimination of spikes, and audio monitoring was achieved via an audio amp module (Neurolog NL120) and loudspeaker. Analogue spike signals were converted to a digital signal by the spike trigger (Neurolog NL201) with variable threshold, allowing action potentials above certain amplitudes only to be counted, thereby ensuring that only responses from the neurone of interest were being counted and analysed. A typical signal:noise ratio of 5:1 was used. Signals were digitised and analysed using a CED micro1401 interface, a Pentium II PC and Spike 2 data acquisition software (Cambridge Electronic Design, Cambridge, UK).

The electrode was lowered into the spinal cord by hand and input from the ipsilateral hindpaw was confirmed by tapping the toes. The electrode was slowly withdrawn to the surface of the cord (signalled by cessation of input from tapping of the toes) and lowered in 10μm steps using a SCAT-01 microdrive and Epson HX210 Stepper (Digitimer) to a depth of 500μm from the surface. The electrode was then lowered whilst stimulating the toes (brush and pinch stimuli to identify neuronal response to Aβ-, Aδ- and C-fibre input) to a depth of 1200μm. Candidate WDR neurones for further characterisation were selected on the basis of response to both brush and pinch stimuli, as well as continued firing after stimulus cessation. The receptive field was located by the use of von Frey monofilaments ranging from 8-60g, and marked using a fine permanent marker pen to allow accurate stimulation of the identified area throughout the course of the experiment. Candidate WDR neurones were

characterised by electrical stimulation, through two metal pins (26 gauge syringe needles) inserted into the toe around the identified receptive field, ensuring there was no contact between the pins. Pins were connected to a stimulator isolator and single pulses were given, starting at 1mA and increasing by 0.1mA per pulse to a maximum of 3.0mA, until response with C-fibre evoked latency (90-300ms after stimulus) was obtained. If a 1mA pulse generated such a response, the starting input stimulus was reset to 0.5mA. Once the threshold for C-fibre-evoked response was determined, a train of 16 pulses at 3x the C-fibre threshold was delivered using the period generator at a frequency of 0.5Hz in order to stimulate neuronal wind-up. The response of each primary afferent fibre type was determined by the number of responses recorded within each fibres conduction velocity range (Aβ-fibres, 0-20ms; Aδ-fibres, 20-90ms; C-fibres, 90-300ms post-stimulus). The number of post-discharge responses (latencies between 300-800ms post-stimulus), which arises from C-fibre hyperexcitability following repetitive stimulation (i.e. wind-up) were also determined.

Mechanically evoked responses of neurones to punctate stimuli were characterised using von Frey monofilaments (Semmes-Weinstein monofilaments, North Coast Medical Inc, USA, via Linton Instrumentation, Norfolk, UK) applied to the centre of the receptive field on the toes of the hindpaw in ascending (8, 10, 15, 26 and 60g) bending force order, representing both non-noxious (8 and 10g) and noxious (15, 26 and 60g) stimuli (Chaplan *et al.*, 1994). Monofilaments were applied every 10 minutes for 10 seconds with 10 seconds between each monofilament, to the centre of the receptive field. WDR neurones exhibit a graded response to ascending bending-force von Frey filaments (Willis *et al.*, 2004). Stable responses (<10% variation between stimuli) were obtained before drug administration, and quantified by the compilation of stimulus-evoked histograms and analysis of the mean firing rate during application.

2.1.4. Drugs

Nimesulide, AM251 and SR144528 (Tocris Bioscience, Bristol, UK), were dissolved and kept in a stock solution in 100% ethanol at -20°C. For spinal or peripheral administration, nimesulide, AM251, and SR144528 were dried and reconstituted in 3%

polyethylene glycol sorbitan monooleate (Tween 80; Sigma-Alrdich, Dorset, UK) in physiological saline (together constituting the vehicle) on the day of use. For studies involving subcutaneous administration, nimesulide was dissolved directly in 3% Tween 80, without first being made up to an ethanol stock solution, on account of the larger amount of drug used.

All drug preparations were pipetted vigorously, vortexed and ultrasonicated to ensure dissolution. Prepared solutions were kept at 4°C until use.

The effects of drug administration on mechanically evoked responses of WDR neurones were measured as a percentage change of firing rates compared to pre-drug control values. For spinal administration studies, drugs were administered directly onto the exposed spinal cord using a 50µL Hamilton syringe (Hamilton-Bonaduz, Bonaduz, Switzerland). Each dose was studied for between 40-60 minutes and followed by a higher dose after careful removal of fluid from the well using tissue paper, up to a maximum of 4 doses per rat. For peripheral administration studies, drugs were injected under the skin of the hindpaw, directly above the receptive field, in a 50µL volume using a 25 gauge needle (BD Microlance, Drogheda, Ireland). For systemic administration studied, drugs were injected in a 250µL volume under the scruff of the neck using a 25 gauge needle (BD Microlance, Drogheda, Ireland).

2.1.5. Glass-coated tungsten electrodes

Glass-coated tungsten microelectrodes were produced in a 3-stage process involving etching, glass coating, and removal of glass tip from the coated electrode (Bullock *et al.*, 1988).

Etching

A perspex jig (75 x 50mm, University of Nottingham, Medical Faculty Workshop) was loaded with 30 lengths of tungsten wire (Harvard Apparatus, Kent, UK) and brushed with 100% acetone to remove dirt. The brass barrel used for etching was cleaned thoroughly with sandpaper to ensure good contact between the barrel and electrodes.

The electrodes were transferred from the jig to the barrel using a length of sellotape equivalent to the barrel circumference, taped over half the length of the tungsten wire and firmly pressed in place to ensure good contact between the barrel and electrodes. 3-4cm of the lengths of wire protruded beyond the edge of the barrel, which was fixed onto the arm of the etching unit (University of Nottingham, Medical Faculty Workshop). The etching bath contained a solution of 90g potassium nitrate (KNO_2, Sigma-Aldrich) in 80mL distilled water, filled to a level such that when the arm of the etching unit was lowered, 10 of the lengths of wire were in contact with the solution. A carbon electrode connected to the etching unit was placed in the solution to complete the circuit and allow current flow. Current was initially set to flow at 250mA while the barrel rotated, and etching was complete once the current dropped to 200mA, approximately 20-25 minutes later.

Glass coating

Glass capillaries (Harvard Apparatus) were stoppered at one end with plasticine and held in place in the jaws of an electrode puller (University of Nottingham, Medical Faculty Workshop). A length of etched tungsten wire was gently cut from the etching barrel and passed through a flame to remove residual adhesive, and dropped blunt end first into the stoppered capillary tube. The lower, weighted jaw of the electrode puller was lifted and clamped around the lower portion of the capillary tube. Current flowing through a coil around the capillary tube between the two jaws melted the glass, and the weighted jaw containing the etched wire dropped, coating the wire with glass.

Removal of glass tip from the coated electrode

The glass-coated electrode was secured on the microscope platform by placement of the glass butt in a v-clamp. The electrode was moved in the plane of view until both the electrode tip and a borax bead on a cauterising wire on the microscope platform were in sharp focus. Current was passed through the cauterising wire to melt the borax bead and the electrode was advanced towards it until the meniscus of the bead

was broken, at which point the current was switched off. The borax bead contracted, removing the glass tip from the electrode and exposing 1-2mm of tungsten wire. Only electrodes that had a smooth transition from the glass coating to the exposed wire (i.e. no protruding glass that could potentially damage the spinal cord), and electrodes with a straight, well-etched tip were used.

2.1.6. Choice of anaesthetic

General anaesthetics have been shown to have inhibitory effects of dorsal horn neuronal excitability (Collins *et al.*, 1995). It is therefore important to carefully consider the anaesthetic used when conducting electrophysiological studies, to minimise impact on neuronal responses. Many studies have avoided this issue with the use of decerebrate rats in order to investigate pain processing (Kawamata *et al.*, 2005; Silva *et al.*, 1997), however this has the disadvantage of also removing descending pain pathways which modulate spinal dorsal horn neuronal responses, and thus is not ideal.

Inhalation anaesthetics are a popular choice as they allow full control over the level of anaesthesia, an effect more difficult to achieve with injectable anaesthetics. Their mechanism of action is not entirely clear but they are thought to act on ion channels and involve augmentation of $GABA_A$ mediated transmission, decreasing spinal dorsal horn neuronal excitability (Wakai *et al.*, 2005). Until a few years ago, halothane and isoflurane were the most popular choices, however, the use of halothane is associated with hepatotoxicity and its use has all but ceased. Isoflurane, while slower in onset of anaesthetic action than halothane, has lower depressant effect on dorsal horn neuronal responses compared to halothane (Antognini & Carstens, 1999). Isoflurane is therefore used throughout the studies presented here, as it allows rapid and controllable anaesthesia, with minimal impact on neuronal responses. In addition, its popularity makes results obtained in these studies more readily comparable to more widespread literature.

2.2. The MIA model of OA pain

2.2.1. Anaesthetic system and surgical procedures

The anaesthetic system used was similar to that used for electrophysiological studies, except that a non-rebreathing tube was used, through which 3% isoflurane was delivered in 33% oxygen and 67% nitrous oxide, and expelled gases removed to a Cardiff aldasorber. The rat was held at the nosepiece and gently swayed until loss of consciousness (approximately 30 seconds). It was then placed on a heated blanket on its front, until deep anaesthesia (loss of withdrawal reflex to hindpaw toe pinch) had been achieved. The rat was then placed on its back and the knees were shaved with a hair clipper, and the knees and paws wiped clean with chlorhexidine. The ipsilateral paw was then extended, the base of the patella located and 1mg MIA (Sigma-Aldrich, Steinheim, Germany) in 50µL saline or saline alone was injected into the intra-articular space using a 29gauge insulin Kendall Monoject insulin syringe (Tyco Healthcare Group LP, Mansfield, MA, USA). MIA was made up fresh on the day of use by dissolution in saline, with vortexing. The experimenter was blinded to all treatments. Once the solution had been injected, the rat was placed on a veterinary bed until consciousness had been regained, before it was placed back in its cage and checked for signs of discomfort. Rats were checked again in the middle of the day and at the end of the working day. Vet beds were placed inside the cages, which were kept half on heated blankets in a quiet room with low level lighting for the remainder of the working day, before being returned to the holding rooms (vet bed removed). Rats were given *ad libitum* access to food mash (normal food pellets softened with water) in addition to their normal food and water for three days following injection. Weight and general health condition was checked daily for the following three days and then twice weekly (before behavioural testing, see below) until use in electrophysiological studies. Weight gain and general health condition did not vary between the treatment groups.

2.2.2. Behavioural testing

Behavioural tests were conducted 1 day before, on the day of injection, and 2, 3, 7, 9, 14, 16, 21, 23 and 28 days after injection of MIA or saline. Behavioural tests on the day before were primarily to allow acclimatisation of the rats to the behavioural equipment and methods. Behavioural tests were always conducted before midday, and usually between 7-9am, to minimise alterations in responses caused by time differences (and noise levels) between study days. Rats were tested for alterations in weight-bearing between ipsilateral and contralateral hindlimbs, and differences in paw withdrawal thresholds between ipsilateral and contralateral hindpaws.

Changes in weight-bearing between hindpaws is an indicator of joint discomfort and associated pain in an injured knee (Bove *et al.*, 2003; Clayton *et al.*, 1997; Kobayashi *et al.*, 2003). Naïve or saline-treated rats evenly distribute their weight between the hindpaws. The effects of joint damage caused by intra-articular injection of MIA were assessed using an incapacitance tester (Linton Instrumentation, Norfolk, U.K.). Rats were placed in a Perspex chamber with the two hindpaws on separate sensors. Once the rat was settled and positioned correctly with a forward facing posture, the mean force (in grams) exerted by each hindlimb over a 3-second period was calculated (Clayton *et al.*, 1997). Three readings were taken per rat and the average of these readings was calculated. Weight borne on the ipsilateral hindlimb is expressed as a percentage of the weight borne on the contralateral hindlimb, i.e.:

$$\text{Weight-bearing} = \frac{\text{weight (g) borne through ipsilateral hindlimb}}{\text{weight (g) borne through contralateral hindlimb}} \times 100$$

For testing of mechanical allodynia, rats were placed in individual Perspex boxes with wire mesh floors (Medical Engineering Unit, University of Nottingham). Once rats were settled (sitting, approximately 10 minutes), mechanical sensitivity of both ipsilateral and contralateral paws were determined with the use of a range of von Frey monofilaments exerting forces of 0.4g, 0.6g, 1g, 1.4g, 2g, 4g, 6g, 8g, 10g, and 15g. Each von Frey monofilament was applied in ascending order to the plantar surface of

the hindpaw for 3 seconds, 3 times, with a minimum of 3 seconds rest between each stimulus. If 4g did not elicit a response, the next highest weight was tested, up to a maximum of 15g (considered noxious in awake, freely moving adult rats - Chaplan *et al.*, 1994). Upon a withdrawal response, the paw was tested again with the next descending von Frey monofilament until no response occurred. The lowest weight of monofilament tested which elicited a withdrawal reflex was noted as the paw withdrawal threshold. In the case of no response following a 15g stimulus, a value of 16g was assigned as the paw withdrawal threshold for the purpose of analysis. Data were expressed as the difference in grams in withdrawal responses between ipsilateral and contralateral hindpaws, i.e. withdrawal threshold contralateral hindpaw (g) minus withdrawal threshold ipsilateral hindpaw (g).

2.3. Tissue analysis – EC and cytokine levels

Levels of ECs and ECLs, as well as cytokines were analysed in spinal cord, hindpaw skin and synovial fluid of naïve and MIA- or saline-treated rats. For materials and methods employed for the analysis of cytokine levels in these tissues, see Chapter 4, section 4.2.4.

2.3.1. Tissue collection

In the case of analysis of endocannabinoid levels where rats had been prepared surgically and a laminectomy performed for spinal drug administration as described above, rats were killed by anaesthetic overdose (5% isoflurane in a mix of 33% oxygen, 67% nitrous oxide until cessation of breathing) and decapitation. In the case of analysis of cytokines where no laminectomy had been performed, rats were killed by stunning and decapitation. The laminectomy was extended (in the former case) or performed and extended (in the latter case) to allow an approximately 3cm section of spinal cord, spanning from the lumbar enlargement upwards, to be removed. The spinal cord was then separated into ipsilateral and contralateral segments and snap-frozen on dry ice and stored at -80°C until analysis. An approximately 0.5-1cm^2

section of paw skin was also removed, snap-frozen on dry ice and stored at -80°C until analysis. Synovial fluid was removed using a method described by Barton *et al.*, (2007), with some alterations as described below. A needle perfusion system was constructed with a 25- and a 23-gague needle tips (BD Biosciences, Drogheda, Ireland) pulled apart from their plastic syringe-connectors. Firstly, the upper end of the 25-gague needle tip was bent approximately 135°, towards the bevel. The needles were bound with their tips 1-1.5mm apart, bevels facing away from each other, using epoxy resin (see Figure 2.1). The connector-portion of the 23-gague needle was attached to a length of flexible grade Portex nylon tubing, inner diameter 0.58mm, outer diameter 1.02mm, connected to a 1mL syringe filled with physiological saline, connected to a syringe pump (Harvard Apparatus, Kent, UK). The rat was placed on its back with the patella facing directly upwards for insertion of the perfusion needles into the joint space through the patella tendon. Physiological saline was infused at a constant rate of 400µL/minute until outflow of fluid through the 25-gague needle was achieved, which was collected into a 2mL glass vial (SLS, Nottingham, UK) for a period of 30 seconds. Fluid was immediately snap frozen on dry ice and stored at -80°C until analysis.

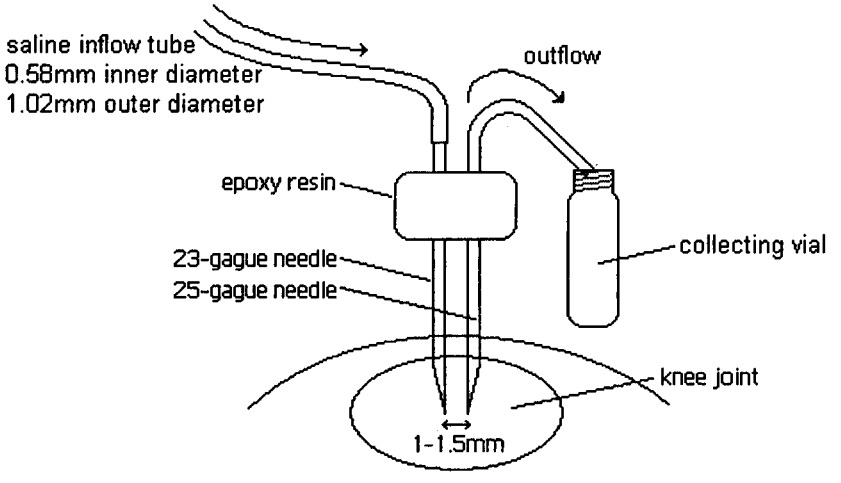


Figure 2.1 Perfusion system for synovial fluid removal from a rat knee. Saline was infuled at 400µL/minute into the joint space, forcing outflow of synovial fluid and saline through the 25-gague needle, collected in a 2mL glass vial for 30 seconds. Adapted from Barton et al., 2007.

2.3.2. Liquid chromatography – tandem mass spectrometry

Analysis of tissue samples for levels of AEA, OEA PEA and 2-AG was with a validated liquid chromatography tandem mass spectrometry (LC/MS-MS) analytical method of extraction and quantification (Richardson *et al.*, 2007). The assay method is in routine use and has been fully validated, demonstrating intra- and inter-day precision and accuracy of ≤15% RSD (relative standard deviation).

Extraction

All solvents and chemicals used were of HPLC grade or higher. First, all glassware was silanised using trichlorotrimethyl silane (Sigma-Aldrich, Steinheim, Germany), washed with toluene and methanol (both Fisher Scientific, Loughborough, UK). All tissues were kept on ice throughout. Tissues were weighed and homogenised in a hand-held glass homogeniser with 5mL 9:1 ethyl acetate: hexane (both Fisher Scientific, Loughborough, UK) mixture, 1mL high-performance liquid chromatography (HPLC)-grade water (Elga, High Wycombe, UK), and the internal standards deuterated anandamide (AEA-d8, Cayman Europe, Tallinn, Estonia, 15µL of 28µM = 0.42nmol prepared in ethanol - Fisher Scientific, Loughborough, UK) and deuterated 2-AG (2-AG-d8, Cayman Europe, Tallinn, Estonia, 100µL of 10µM = 1nmol prepared in acetonitrile - Fisher Scientific, Loughborough, UK), added to allow and correct for differences in recovery. Paw tissue was allowed to sit in this mixture for 1hour prior to homogenisation to soften the tissue and maximise recovery of the endocannabinoids. Synovial fluid was mixed rather than homogenised and the full volume recovered was noted and used. The homogenised mixture was transferred to a centrifugation tube, samples centrifuged (7000G, 15 minutes at 4°C) and supernatants collected in clean tubes. To optimise EC and ECL compound recovery, homogenisation and centrifugation was repeated two more times, using 2.5mL 9:1 ethyl acetate: hexane, and the supernatants pooled. Pooled supernatants were evaporated under nitrogen at 35°C. The supernatants were reconstituted in 1mL anhydrous chloroform (Fisher Scientific, Loughborough, UK) and vortexed for 1 minute, before undergoing solid phase extraction (SPE) for purification. Reconstituted samples were loaded into 100mg silica solid phase extraction cartridges (Phenomenex, Macclesfield, UK) placed in a Cerex SPE nitrogen positive-pressure manifold (Varian, UK). Cartridges were

washed with a further 3 additions of 1mL anhydrous chloroform, before elution into clean silanised glass tubes using 2x1mL 2% methanol in anhydrous chloroform, 4x1mL 2% methanol with 0.2% triethylamine (TEA, Fisher Scientific, Loughborough, UK), in anhydrous chloroform, and 4x1mL 2% methanol with 0.2% trifluoroacetic acid (TFA, Fisher Scientific, Loughborough, UK). Eluates were evaporated under nitrogen at 35$^{\circ C}$ and stored at -80$^{\circ C}$ until analysis, whereupon samples were reconstituted in 200µL acetonitrile, vortexed for 1 minute and transferred into 2mL glass vials (SLS, Nottingham, UK).

For calculation of EC and ECL concentrations in the extracted samples, EC and ECL compound extracted standards were prepared alongside tissue samples in the same manner as described above, in addition to non-extracted standards which bypassed extraction and SPE procedures, undergoing only evaporation under nitrogen at 35$^{\circ C}$ and reconstitution in 200µL acetonitrile prior to use in LC/MS-MS. Standards consisted of the following ECs and ECLs: AEA, 2-AG, noladin ether, Virodhamine (all from Tocris Cookson, Bristol, UK), OEA, PEA, 2LG, N-arachidonoyl dopamine (NADA), Arachidonyl Glycine, (all from Cayman Chemicals/Alexis, Nottingham, UK). 2-AG and 2LG were diluted in acetonitrile while all other compounds were diluted in ethanol, and made up to a 1mM stock standard stored at -80$^{\circ C}$ until use and dilution to include at least 8 concentrations covering the range of concentrations expected from the samples based on previous work, including 0.1nM, 0.5nM, 1nM, 5nM, 10nM, 50nM, 0.1µM, 0.5µM. In addition to extracted and non-extracted standards, quality control standards of human plasma were made up alongside extracted standards to allow intra-run comparison of LC/MS-MS system functionality and reproducibility.

Quantification

Mobile phases at pH 3.6 used throughout LC/MS-MS were made up as follows:
Mobile phase A = HPLC grade water + 1g/L ammonium acetate + 0.1% formic acid (both Fisher Scientific, Loughborough, UK).
Mobile phase B = acetonitrile + 1g/L ammonium acetate + 0.1% formic acid, and a little (~3mL /L) HPLC grade water to help dissolution of ammonium acetate).

Both mobile phases were filtered using 0.47µm nylon filters (Whatman, Maidstone, UK) before use.

A triple quadropole Quattro Ultima mass spectrometer (Waters Micromass, Manchester, UK) was used in conjunction with an Agilent 1100 LC system (Agilent Technologies, Waldbron, Germany). Source and desolvation temperatures of 125$^{\circ C}$ and 350$^{\circ C}$, respectively, were used. A tuning solution consisting 10µM AEA and 2AG in mobile phases A and B in a 1:1 ratio was infused at 250µL/minute into the mass spectrometer and source parameters (cone and desolvation gas flow rates) were optimised (typically around 200 and 800L/hour respectively), and the probe adjustment flange was adjusted to give the most intense signals for AEA and 2-AG. These settings were then maintained throughout analysis.

Samples were drawn and injected in a 5µL volume at 200µL/minute, from a cooled autosampler kept at 4$^{\circ C}$ through a liquid chromatography column (Thermo Hypersil-Keystone HyPurity Advance Column) with pre-column (100x2.1mm internal diameter, 3µm particle size), kept at 40$^{\circ C}$ and 250bar pressure with mobile phases A and B flowing at 300µL/minute used for a gradient elution. Gradient elution occurred over a 15 minute time-frame as follows: 55% A, 45% B initially, increasing linearly to 55% B at 2 minutes and 65% B at 6.5minutes, maintained until 9 minutes, followed by re-equilibration with 45% B for a further 6 minutes. Analyte quantification was undertaken using tandem electrospray mass spectrometry in positive mode (ES^+). The dominant product ion for each compound was selected for monitoring in multiple reaction monitoring (MRM) mode, and were subjected to dissociation by argon gas-induced dissociation in a collision reaction, to reveal product (daughter) ions. Compounds in the tissue samples were identified by comparison of on-column retention times and precursor (parent) and product ion mass: charge (*m/z*) values to that of compounds in the extracted and non-extracted standards prepared alongside tissue samples (see section 3.2.4.2). Typical precursor and product ion *m/z* values and on-column retention times are listed in Table 2.1. Entry and exit energies of 0 and 35eV respectively were used, with collision energies as listed in Table 2.1.

The injector was set to wash 3 times with 1:1 methanol to water between each sample, and two blank samples of 1:1 mobile phase A to mobile phase B were placed between each group of samples (i.e. ipsilateral spinal cord and contralateral spinal cord, or between treatment groups) to minimise carry-over between samples. Quantitative analysis was performed using the Quanlynx PLC system version 4 (Waters, Kilford, MA, USA). EC and ECL compound levels were calculated by comparing recovery of compounds in samples to those in the extracted and non-extracted standards, using the area under the curve for each analyte, and producing a concentration-response chart from which EC and ECL compound concentrations in samples could be extrapolated, taking into account the initial amount of tissue analysed. The area under the curve was also calculated for the internal (deuterated) standards, and corrections to EC and ECL compound levels were made accordingly.

Table 2. 1 Typical parameters of selected EC and ECL compounds identified using LC-MS/MS. Typical retention times, precursor and product ion mass:charge (m/z) values, cone voltages and collision voltages used in identification and quantification of EC and ECL compounds in spinal cord, paw tissue and synovial fluid.

Analyte	Retention time (min)	Precursor Ion (m/z)	Product Ion (m/z)	Cone Voltage (eV)	Collision Energy (eV)
AEA	5.43	348.33	62.00	35	11
AEA-d_8	5.43	356.33	62.00	48	21
2-AG	6.26	379.24	287.02	55	15
2-AG-d_8	6.26	387.35	96.17	43	34
Palmitoyl ethanolamide	6.13	300.26	62.00	35	15
Oleoyl ethanolamide	6.51	326.39	62.00	60	20
Virodhamine	1.79	348.33	62.00	35	11
2-Linoleoyl glycerol	6.32	355.52	263.18	55	15
Arachidonyl glycine	7.85	362.24	287.11	50	15
Noladin ether	6.51	365.20	273.17	63	14
Heptadecanoyl ethanolamide	7.15	314.47	62.00	60	20

2.4. Statistical analyses

Data were analysed using GraphPad Prism software, version 4.0. Details of statistical analyses used will be described in each results chapter (Chapters 3-6).

Chapter 3 Effects of spinal COX-2 inhibition on mechanically evoked responses of wide dynamic range neurones in naïve rats *in vivo*

3.1. Introduction

3.1.1. The role of COX-2 in endocannabinoid metabolism

The antinociceptive effects of the endocannabinoids (ECs) anandamide (AEA) and 2-arachidonoylglycerol (2-AG) have been well described in animal models of pain (see Chapter 1). Their therapeutic use is limited somewhat by a short-lived time of action, due to rapid metabolism. As a result, much study has centred on antinociceptive properties of inhibitors of the major NAE metabolic enzyme, FAAH (see Chapter 1). In addition to FAAH, NAEs are also substrates for catabolism by N-acylethanolamine-hydrolysing acid amidase (NAAA – Tsuboi *et al.*, 2007, Sun *et al*, 2005), and oxygenation by cyclooxygenase-2 (COX-2) (Kozak *et al.*, 2004; Yu *et al.*, 1997), lipoxygenases (LOX) (Edgemond *et al.*, 1998; Hampson *et al.*, 1995; Ueda *et al.*, 1995a) and cytochrome P450 (cP450 - Bornheim *et al.*, 1993; Snider *et al.*, 2008). The role of COX-2 in the metabolism of endocannabinoids is of particular relevance to pain processing as this enzyme is constitutively expressed in the spinal cord (Ghilardi *et al.*, 2004) and induced in chronic pain states. COX inhibitors are commonly used in the treatment of chronic pain states, but the role of endocannabinoids in mediating these effects is unclear. COX-2 has also been shown to contribute to the metabolism of 2-AG, which it metabolises as effectively as it does arachidonic acid, to biologically active metabolites such as prostaglandin H(2) glycerol ester (PGH(2)-G), which can further be processed further to form new prostaglandins (Kozak *et al.*, 2000, Prusakiewicz *et al.*, 2009).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to raise levels of endocannabinoids. Previous studies have demonstrated that ibuprofen can inhibit AEA hydrolysis in rat brain membrane preparations with a potency of the same order of magnitude as required for inhibition of COX-2, and at concentrations comparable to peak plasma concentrations following therapeutic dosing (Fowler *et al.*, 1999; Fowler *et al.*, 1997). The inhibitory effects of NSAIDS are blocked by CB_1 receptor antagonists, implicating a role of the endocannabinoids in mediating these effects (Guhring *et al.*, 2002; Telleria-Diaz *et al.*, 2009). NSAID-mediated increases in

endocannabinoid levels have been suggested to occur through a number of mechanisms; radioligand binding studies have shown that ibuprofen, ketorolac and flurbiprofen can block AEA metabolism, most likely by inhibiting FAAH activity, (Fowler *et al.*, 1997, 1999), while *in vivo* work with indomethacin has shown that NSAIDs reduce reuptake of endocannabinoids by decreasing levels of spinal nitric oxide, and shifting metabolism of the endocannabinoid precursor arachidonic acid towards endocannabinoid synthesis (Guhring *et al.*, 2002). As COX-2 is also responsible for EC metabolism, NSAIDs may raise EC levels independently of their actions on FAAH. Specific inhibition of the COX isoform COX-2 *in vivo* with rofecoxib, raised local levels of AEA, as well as that of the endocannabinoid-like compounds (ECLs) PEA and OEA in the formalin model of inflammation, an effect concurrent with antinociceptive effects (Guindon *et al.*, 2006b). Rofecoxib does, however, also have weak inhibitory effects on FAAH and so the contribution of COX-2 to endocannabinoid-mediated analgesia induced by NSAIDs remains unclear.

It has recently been shown that the COX-2 inhibitor nimesulide does not inhibit FAAH (Fowler *et al.*, 2003; Kim *et al.*, 2004) and so investigations with this drug would allow elucidation of the role of COX-2 in endocannabinoid metabolism, as well as the effect of this pathway in NSAID-mediated analgesia.

3.1.2. Nimesulide – effects on endocannabinoids and endocannabinoid-like molecules

The effects of nimesulide on ECs and ECLs have recently been studied both *in vitro* and *in vivo*. In rat hippocampal slices, nimesulide prolonged depolarisation-induced suppression of inhibition (DSI), an effect blocked by AM251 pre-treatment but not URB597, showing that the actions of nimesulide are mediated by the CB_1 receptor and are not mediated by FAAH (Kim *et al.*, 2004). In the carageenan model of acute inflammation, an intraplantar injection of 50μg nimesulide administered in a 50μL volume of saline significantly raised levels of PEA. AEA levels were also elevated, albeit non-significantly (Jhaveri *et al.*, 2008). These effects were blocked by the PPARα antagonist GW6471, suggesting that PEA acts at the PPARα receptor. Unlike

many COX-2 inhibitors and acidic NSAIDs which inhibit FAAH activity in the rat brain in a pH dependent manner, nimesulide does not inhibit FAAH at either pH6 or 8, consistent with the conclusion that non-ionised forms of the acidic NSAIDs are responsible for FAAH inhibition (Fowler *et al.*, 2003). This lack of action on FAAH makes nimesulide a good choice for the study of the effects of COX-2 inhibition on endocannabinoids.

3.1.3. Nimesulide - structure and selectivity

Nimesulide (4-nitro-2-phenoxymethanesulphonanilide – Figure 3.1) is a weak acid (pKa 6.5) which was first introduced in 1985 and belongs to the sulfonanilide group (see Rao, 2005). It is this group that confers its acidic nature, rather than by the presence of a carboxylic acid group which confers acidity in many of the NSAIDs (for review see Singla, 2000). It is a yellow, crystalline powder, with poor solubility in aqueous solutions - 0.01mg/mL (Piel *et al.*, 1997) which may cause bioavailability problems *in vivo*. However, this may be overcome by the formation of inclusion complexes with β-cyclodextrin. Nimesulide is readily soluble in acetone, chloroform and ethyl acetate, and slightly soluble in ethanol (Singla, 2000).

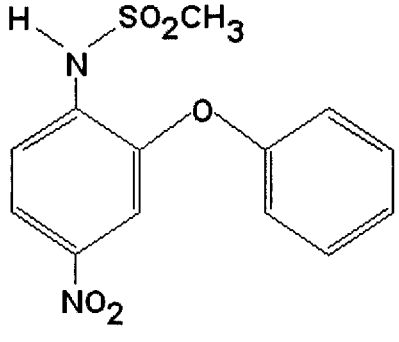


Figure 3.1 Nimesulide; 4-nitro-2-phenoxymethanesulphonanilide

Nimesulide was first shown to have higher selectivity for COX-2 over COX-1 in human leukocytes and in isolated gastric mucosal tissue (Tavares 1995). Since then, both *in vivo* and *in vitro* studies have shown that nimesulide is a relatively selective COX-2, versus COX-1 inhibitor at therapeutic doses (for review, Famaey, 1997; Kerola *et al.*,

2009; Shah *et al.*, 2001). Analogues include; NS-398, flosulide (CGP-28238), T-614, Fk-3311, L-745337 and MK-966 (rofecoxib), which was in use in the clinic for the treatment of symptoms of arthritis, including pain, inflammation, swelling and stiffness, but was withdrawn in 2004 due to safety concerns pertaining to toxic cardiac effects.

Nimesulide's selectivity for COX-2 is conferred via a good binding affinity, due to the isoleucine at the 253 position in COX-2, which in COX-1 is replaced by valine. The presence of isoleucine at this position results in a larger binding site which nimesulide can take advantage of (Garcia-Nieto *et al.*, 1999). In humans, selectivity has been shown in an acute dosing study (Kerola *et al.*, 2009), in which 100mg nimesulide was administered to 15 healthy, non-smoking volunteers (8 male) between the ages of 21 and 30 with a BMI <30, with blood analysis occurring before and after administration at 1, 3, 6, 24 and 48 hours. COX activity was measured indirectly, COX-1 via the measure of thromboxane-2 production during blood clotting, and COX-2 via endotoxin-induced PGE2 synthesis in blood leukocytes. Nimesulide resulted in almost complete COX-2 suppression and only a partial attenuation of COX-1 activity. Nimesulide has also been shown to have little effect on haemostatic activity, as well as lower levels of gastrointestinal damage and kidney toxicity, when compared to other NSAIDs such as naproxen (Bennett & Villa, 2000). Other evidence of the COX-2 selectivity of nimesulide has been demonstrated in a number of paradigms as reviewed by Famaey (1997):

Prostaglandin synthesis is more effectively inhibited by nimesulide in inflammatory areas than in gastric mucosa

Nimesulide inhibits COX-2 in preference to COX-1 with a ratio of 0.76 to 0.0004, conferring a 1.3-2,512 times higher selectivity for COX-2. This large variation in selectivity is largely due to variation in experimental conditions, including tissue type and preparation

In humans, following a dose of 100mg twice daily for two weeks (Bourgeois *et al.*, 1994), blood assays showed significantly lower COX-2-mediated PGE_2 production without effect on COX-1-mediated thromboxane TXB_2 production

3.1.4. Aims

Since many previous studies on the effects of NSAIDs on the EC system have used NSAIDs that inhibit both COX-2 and FAAH, the role of COX-2 in regulating levels of endocannabinoids and the contribution of endocannabinoids to the analgesic effects of NSAIDs *in vivo* is still unclear. The aim of this study was to determine whether the inhibition of COX-2 by nimesulide, which does not alter FAAH activity (Fowler *et al.*, 2003), attenuates innocuous and / or noxious-evoked responses of spinal neurones, and the contribution of CB_1 receptors in mediating these effects. Levels of endocannabinoids and related compounds in the spinal cord following treatment with nimesulide were also determined, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques.

3.2. Methods

3.2.1 *In vivo* electrophysiology and data acquisition

For detailed methodology of anaesthesia, surgery and identification of WDR neurones see Chapter 2. For most of the experiments in this chapter, one WDR neurone from the dorsal horn of the spinal cord was studied per rat. Where more than one neurone was studied, this has been stated.

3.2.2 Spinally administered nimesulide and mechanically evoked responses of WDR neurones in naïve rats

The effects of spinal nimesulide, prepared as described in Chapter 2, section 2.1.4, on mechanically evoked responses of WDR neurones were measured as a percentage change in firing rates compared to pre-drug control values. Previous studies with nimesulide include intraperitoneal, oral and intrathecal administration. Doses for the intraperitoneal route of administration were 2.5 and 3mg/kg (Tassorelli *et al.*, 2003, Gineste *et al.*, 2003), whereas the oral route used 5mg/kg (Bianchi *et al.*, 2007), which equates to a 1000µg dose for a 200g rat. Intrathecally the doses are much smaller, as is the volume of asministration – typically between 3 and 5µL fluid per rat or mouse, with the ED_{50} found to be around 9µg nimesulide in 5µL (Bujalska *et al.*, 2003, Miranda *et al.*, 2009, Pinardi *et al.*, 2005). Because nimesulide has not previously been applied to the spinal cord under similar conditions, a pilot study was conducted with a number of different doses to determine the appropriate range. Data in this chapter are presented from a range of doses; 1, 2, 3, 10, 25, 50 and 100µg/50µL (65 µM-6.5 mM) and all rats per dosing group (n=6) received the same combination of 2 or 3 doses. Although the actual doses of drug which bathes the surface of the spinal cord are high, the amount of drug which reaches the intracellular targets within neurones in the cord and the enzymes therein is likely to be far lower, especially given the distance of wide dynamic range neurones from the surface of the cord.

Each dose of nimesulide (or vehicle, n=6) was administered directly onto the exposed spinal cord using a 50µL Hamilton syringe (Hamilton-Bonaduz, Bonaduz, Switzerland) and was studied for 60 minutes before removal with absorbent paper and application of the next (higher) dose. Up to three doses were studied per rat, with 6 rats per dose expressed (total = 18 neurones from 18 rats). In a separate group of rats, the effects of spinal CB_1 receptor blockade on nimesulide-mediated effects were determined. The CB_1 antagonist AM251 (1µg/50µL (36µM), spinal, n=6) was administered directly onto the exposed spinal cord using a 50µL Hamilton syringe, 30 minutes before nimesulide (25µg/50µL). The dose and timepoint of administration of AM251 used was based on previous studies (Jhaveri *et al.*, 2006; Johanek & Simone, 2004).

3.2.3 Peripherally administered nimesulide and mechanically evoked responses of WDR neurones in naïve rats

Nimesulide (50 and 100µg/50µL) was prepared as above. Following identification, characterisation and the recording of stable control responses of WDR dorsal horn neurones as before, nimesulide (50µg n=8 neurones in 7 rats, 100µg n=12 neurones in 10 rats) or vehicle (50µL, 13 neurones in 13 rats) was injected slowly under the skin of the plantar surface of the paw using a 25 gauge needle (BD Microlance, Drogheda, Ireland). Care was taken to ensure the infused volume remained in the paw after withdrawal of the needle. Effects of nimesulide on mechanically evoked responses of neurones were studied for 60 minutes.

3.2.4 Spinally administered nimesulide and levels of endocannabinoid and endocannabinoid-like molecules in spinal cord and paw in the naïve rat

Rats were anaesthetised and surgically prepared as described above. On two separate occasions, nimesulide (25µg/50µL, n=8, 100µg/50µL, n=6), or vehicle (n=14), was applied to the spinal cord as described above. The maximal effect of nimesulide on mechanically evoked neuronal responses occurred 30 minutes post-

administration (see section 3.3.2) and so tissue was collected at this time point. Rats were killed by anaesthetic overdose with 5% isoflurane in 33.3% O_2 and 66.6% N_20. The spinal cord and, in the case of the 25µg dose, skin from the plantar surface of the hindpaw were rapidly removed. The spinal cord was separated into ipsilateral and contralateral segments, and both spinal cord and hindpaw skin were snap-frozen on dry ice and stored at -80°C until analysis. For analysis of the effects of 25µg/50µL nimesulide versus vehicle, (50µL, n=8 in each group), a validated liquid chromatography tandem mass spectrometry (LC/MS-MS) analytical method (Richardson *et al.*, 2007) was used to extract and measure levels of AEA, OEA PEA and 2-AG, as described in detail in Chapter 2. The assay method is in routine use and has been fully validated, demonstrating intra- and inter-day precision and accuracy of ≤15% relative standard deviation. For the analysis of the effects of 100µg/50µL nimesulide versus vehicle (50µL, n=6 in each group), tissue was analysed by Leonie Norris, School of Biomedical Sciences, with some alterations to the method as described below.

Briefly, tissue was weighed, finely minced and homogenised in 5mL acetonitrile (rather than 9:1 ethyl acetate: hexane) with 15µL of 28µM AEA-d8 and 100µL of 10µM 2-AG-d8 internal standards as before. The homogenised mixture was centrifuged, the supernatant collected, and the remaining pellet re-homogenised in 2.5mL acetonitrile before further centrifugation and collection. The solvent was evaporated and the remaining material reconstituted in 200µL acetonitrile before analysis by liquid chromatography-tandem mass spectrometry, by-passing the SPE stage, thus maximising recovery.

3.2.5 Statistical analysis

Data from electrophysiological studies are expressed as a percentage of the pre-drug control ± SEM. Statistical analyses comparing effects of different doses of spinal or peripheral nimesulide to vehicle were performed with a one-way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test. Statistical analysis comparing effects of 25µg spinal nimesulide to that of 25µg spinal nimesulide with CB_1 antagonist pre-treatment were

performed using a non-parametric Mann-Whitney test. Comparisons of the effects of treatment versus pre-drug controls were performed on raw data, using repeated measures ANOVA with post-hoc Dunnet's test. Data from analysis of the effects of nimesulide on levels of endocannabinoids and related compounds are expressed as nmol or pmol / g tissue. Statistical analyses comparing the effects of nimesulide versus vehicle on EC and ECL levels were performed using non-parametric Mann-Whitney test. In all cases, differences between data sets where $P<0.05$ were deemed to be statistically different. Data deviating from the mean by more than 2 standard deviations were excluded.

3.3. Results

3.3.1 Mechanically evoked responses of WDR neurones in the dorsal horn of naïve rats

In the studies for this chapter, a total of 101 neurones were studied electrophysiologically (including pilot studies) following spinal and peripheral nimesulide / vehicle administration. Mechanically evoked responses of WDR neurones to increasing intensities of mechanical stimuli were graded (n=101, Figure 3.2). A typical response of WDR neurones to von Frey stimulation of the receptive field can be seen in the example trace in Figure 3.3. The mean depth of WDR neurones studied was 960.2 ± 20.52μm from the dorsal surface, corresponding to laminae V-VI (n=94).

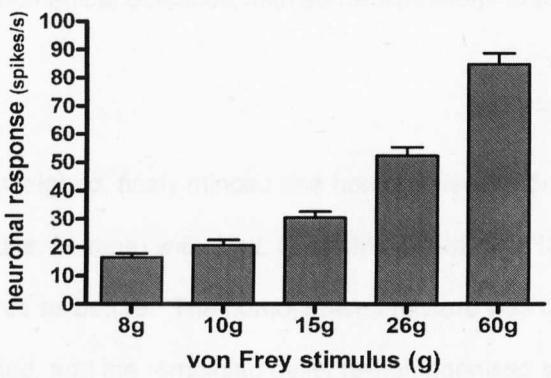


Figure 3.2 Graded responses of WDR neurones in naïve rats (n=101) following mechanical stimulation of the hindpaw receptive field. Data are expressed as the mean firing rate of the neurone per second (spikes/s) over a 10s stimulation, ± SEM.

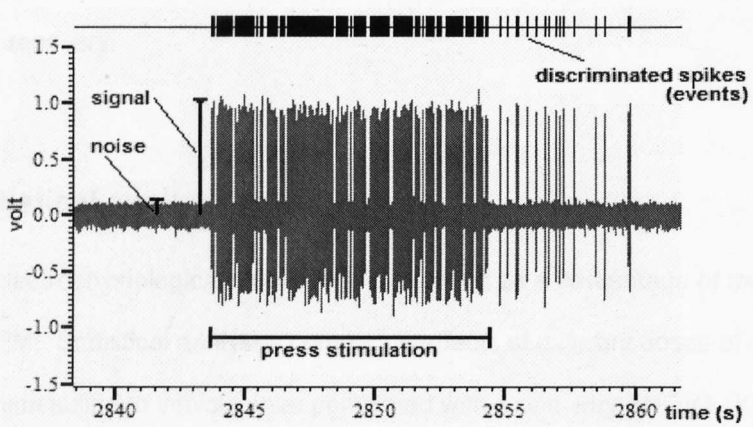


Figure 3.3 A spike train response of a WDR neurone following mechanical stimulation of the hindpaw receptive field with a von Frey monofilament (26g, pre-amp gain x1000).

3.3.2 Effects of spinally administered nimesulide on mechanically evoked responses of WDR neurones in naïve rats

Spinal nimesulide (1-100µg/50µL) dose-dependently attenuated mechanically evoked responses of WDR dorsal horn neurones compared to vehicle, both in the non-noxious (8 and 10g) and noxious (15, 26 and 60g) range (Figure 3.4). The maximal attenuation of mechanically evoked responses was produced by 25µg of nimesulide, maximal effects were observed at 34.6 ± 5.0 minutes post-drug administration. A typical single-unit response to nimesulide can be seen in Figure 3.5.

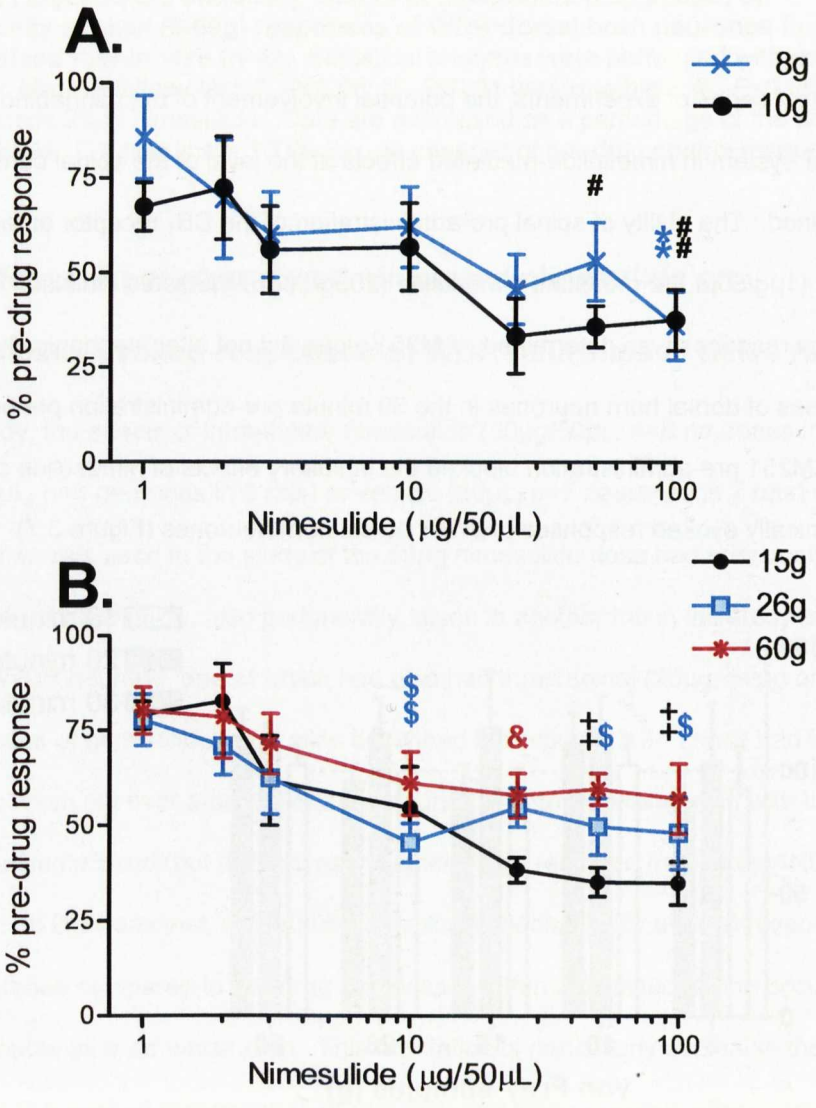


Figure 3.4 Mean maximal effects of spinal nimesulide on mechanically evoked responses of WDR dorsal horn neurones in naive anaesthetised rats. Responses to both non-noxious (A; 8g, 10g) and noxious (B; 15g, 26g, 60g) von Frey stimuli were tested. Nimesulide reduced mechanically evoked responses in a dose-dependent manner. Statistical analyses were performed with one-way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test; single symbol (#,$,&), $P<0.05$; double symbol (**, ##, ++, $$), $P<0.01$ versus vehicle (not shown, no significant difference to pre-drug controls) Data are expressed as a percentage of the pre-drug control ± SEM. (n=6 rats per dose, total n=18 rats)

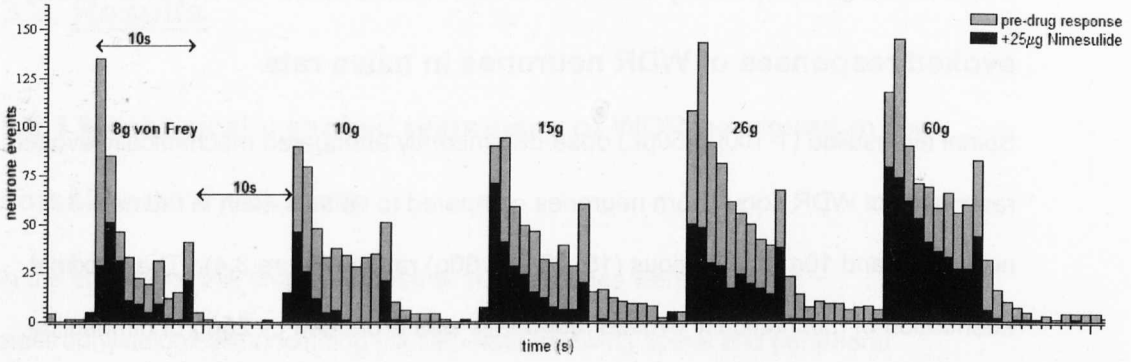


Figure 3.5 Example trace of mechanically evoked responses of a single WDR dorsal horn neurone in a naïve anaesthetised rat before (pre-drug response) and after spinal administration of nimesulide (25μg/50μL).

In the next series of experiments, the potential involvement of the cannabinoid receptor system in nimesulide-mediated effects at the level of the spinal cord was determined. The ability of spinal pre-administration of the CB_1 receptor antagonist AM251 (1μg/50μL) to modulate nimesulide (25μg/50μL)-mediated inhibition of neuronal response was determined. AM251 alone did not alter mechanically evoked responses of dorsal horn neurones in the 30 minute pre-administration period (Figure 3.6). AM251 pre-administration blocked the inhibitory effects of nimesulide on mechanically evoked responses of WDR dorsal horn neurones (Figure 3.7).

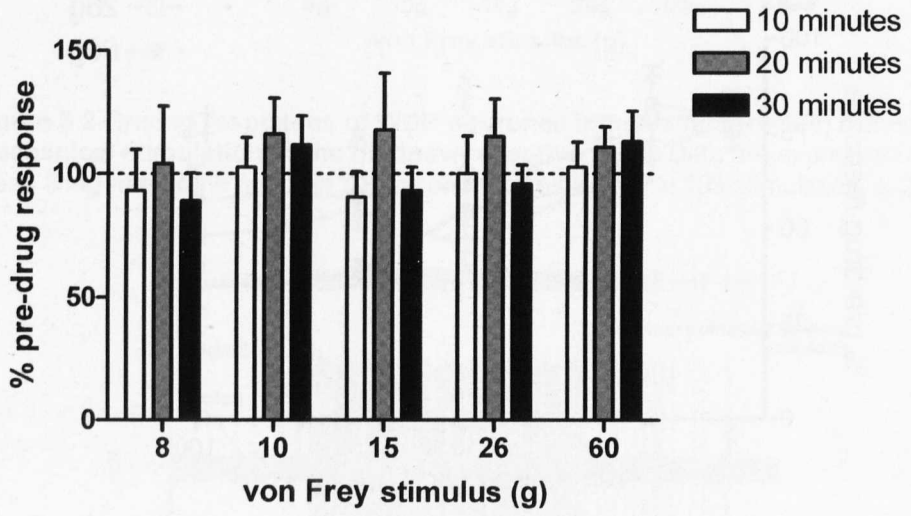


Figure 3.6 The CB_1 antagonist AM251 (1μg/50μL) alone had no effect on mechanically evoked (8-60g) responses of dorsal horn wide dynamic range neurones in naïve anaesthetised rats compared to pre-drug controls (n=6). Data are expressed as a percentage of the pre-drug control ± SEM. Statistical analyses were performed with a repeated-measures ANOVA with post-hoc Dunnet's test (all $P>0.05$). Dashed line = 100% (i.e. no change) of pre-drug control responses.

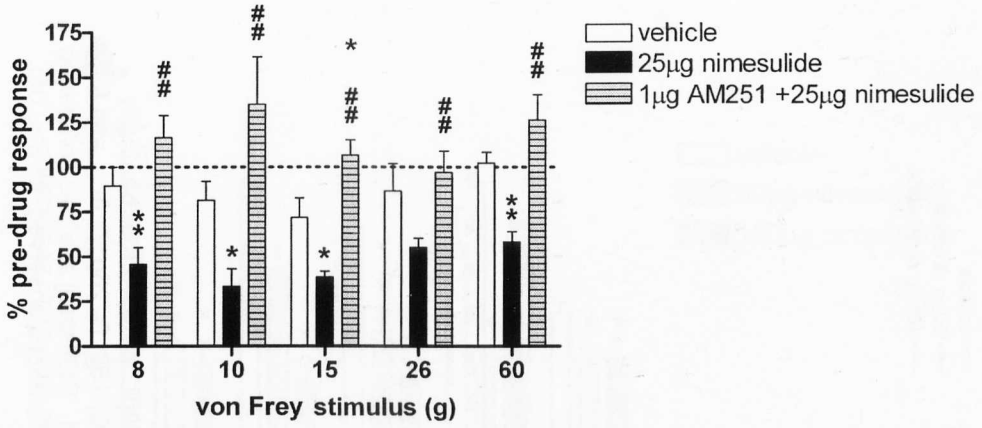


Figure 3.7 Spinal pre-treatment with the CB_1 receptor antagonist AM251 (1μg/50μL) blocked the inhibitory effects of nimesulide (25μg/50μL) on mechanically evoked (8-60g) responses of WDR dorsal horn neurones in naïve anaesthetised rats *in vivo* (n=6). Statistical analyses were performed with non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$ versus vehicle; # , $P<0.05$; ##, $P<0.01$ versus 25μg nimesulide. Data are expressed as a percentage of the pre-drug control ± SEM. Dashed line = 100% (i.e. no change) of pre-drug control responses.

3.3.3 Effects of peripherally administered nimesulide on mechanically evoked responses of WDR neurones in naïve rats

In this study, the effects of intraplantar nimesulide (50μg/50μL, n=8 neurones in 7 rats; 100μg/50μL, n=6 neurones in 5 rats) or vehicle (50μL, n=7 neurones in 7 rats) were studied. Two rats used in the study of the 50μg nimesulide dose had previously had a 100μg dose of nimesulide, also peripherally, given in another toe in the study of a separate WDR neurone, one of which had also had three spinal (25μg, 50μg and 100μg) doses of nimesulide for studies described in section 3.3.2. These had been allowed to wash out over a period of 2 hours prior to further investigation with this rat. Vehicle caused a trend (but did not reach significance, repeated measures ANOVA with post-hoc Dunnet's test, all $P>0.05$) to facilitate mechanically evoked responses of WDR neurones compared to pre-drug responses, with maximal facilitation occurring at 37 ± 3 minutes after administration. This facilitation is particularly evident in the time-course for 15g-evoked responses (Figure 3.8C), and when maximal effects are compared to time-matched nimesulide doses, is statistically significant (one way ANOVA (Kruskal-Wallis) with post hoc Dunn's test; $P<0.05$ and $P<0.01$) (Figure 3.9A). The difference is abolished when comparison between the effects of vehicle and nimesulide involve the maximal facilitation seen in both cases (Figure 3.9B).

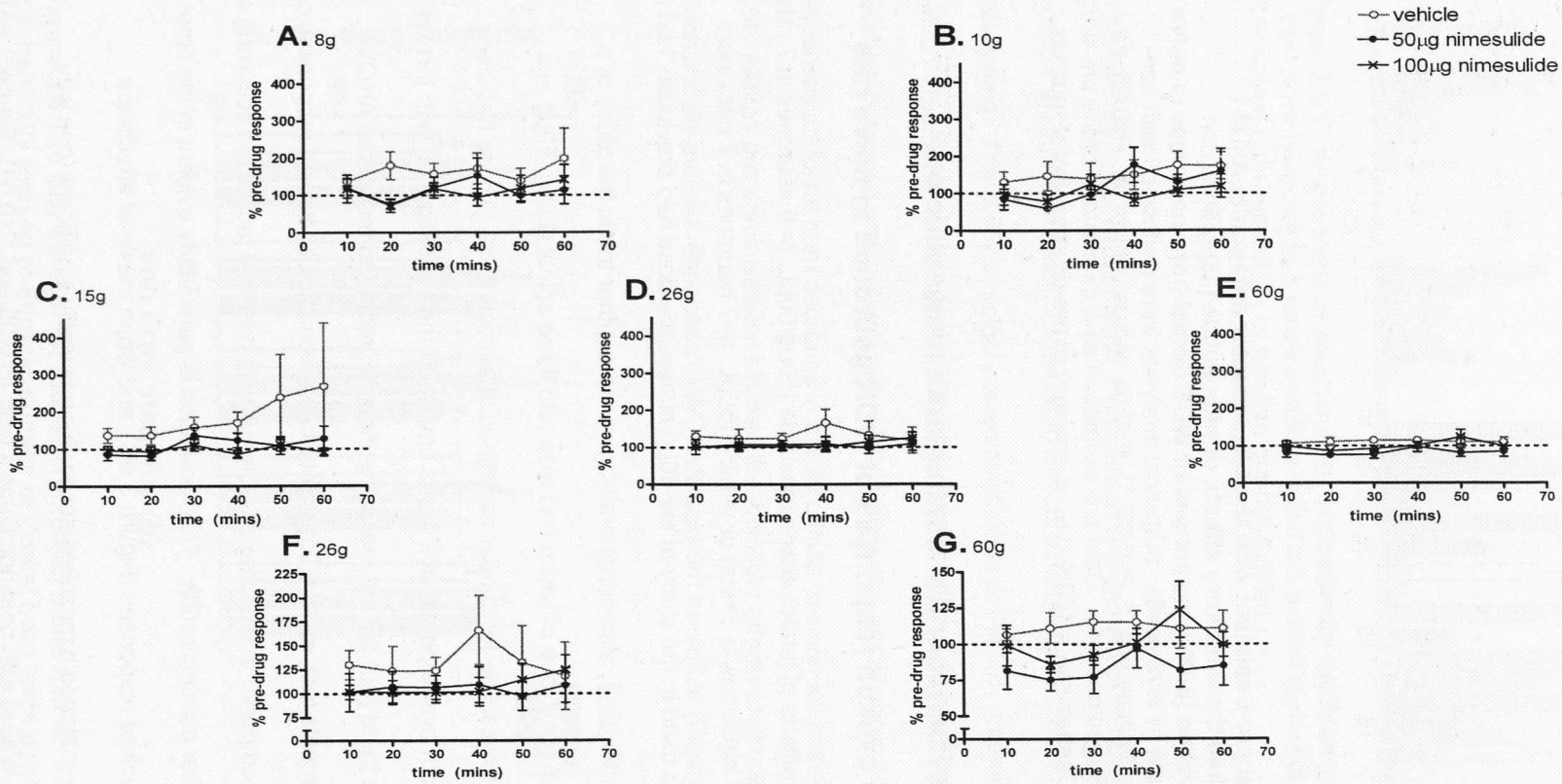


Figure 3.8 Time-course of the effects of peripherally administered vehicle (n=7) and nimesulide (50µg/50µL, n=8, 100µg/50µL, n=6) on mechanically evoked responses of WDR dorsal horn neurones in naïve rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g, and for a closer view; F, 26g; G, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analysis comparing effects of nimesulide to vehicle were performed with one-way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test (no statistical differences observed, all $P>0.05$). Dashed line = 100% (i.e. no change) of pre-drug control responses.

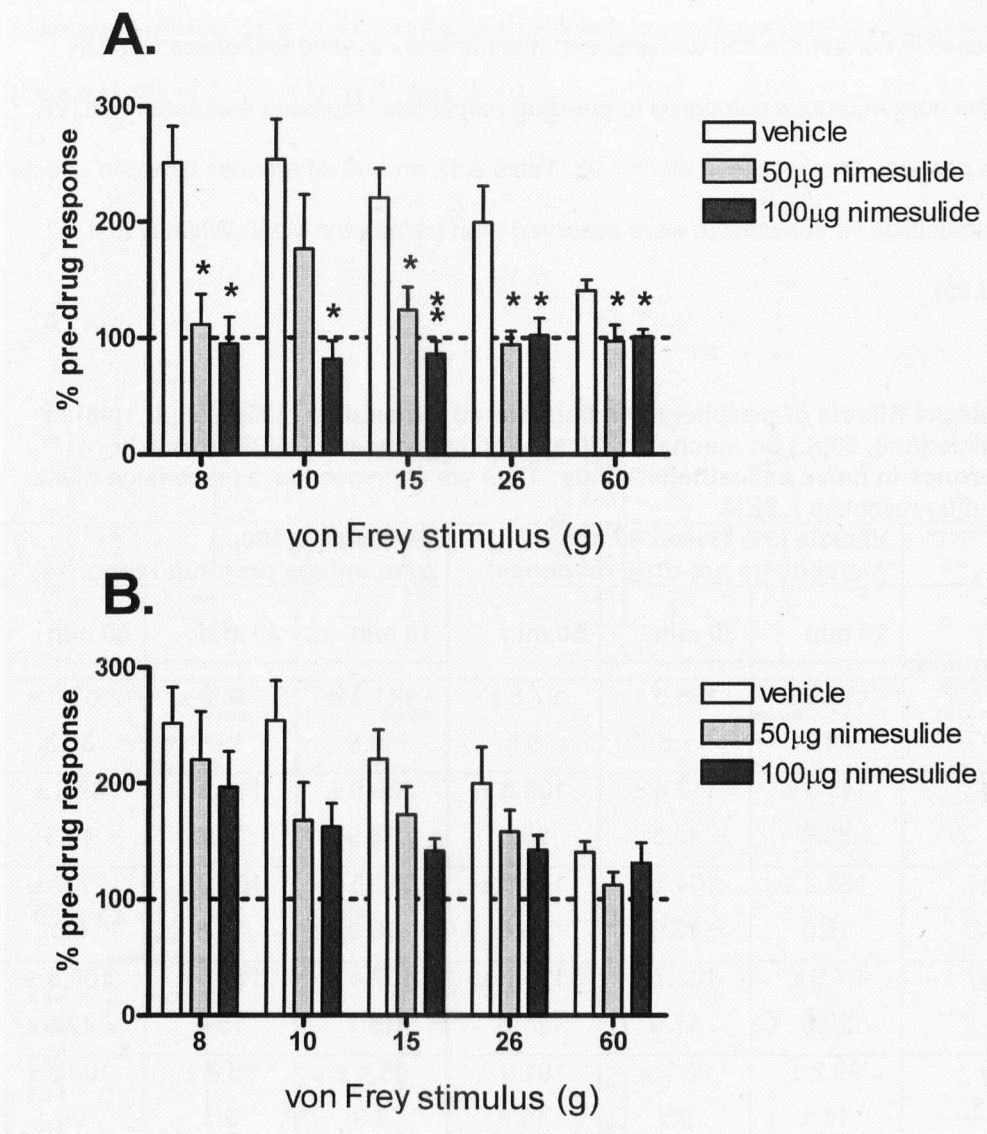


Figure 3.9 Mean maximal effects of intraplantar vehicle (n=8 neurones in 7 rats) and nimesulide (50μg/50μL (n=8), 100μg/50μL (n=6)) on mechanically evoked responses of WDR dorsal horn neurones in naive rats compared to; A, time-matched effects of nimesulide; **B**, maximal effects of nimesulide. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses comparing effects of nimesulide versus vehicle were performed with one-way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test; *, $P<0.05$; **, $P<0.01$. Dashed line = 100% (i.e. no change) of pre-drug control responses. Dashed line = 100% (i.e. no change) of pre-drug control responses.

To clarify these results, the experiment was repeated with an intraplantar injection of 100μg/50μL nimesulide (n=6 neurones in 5 rats) or 50μL vehicle (n=6 neurones in 6 rats), over a year after the initial study. In one rat, a 100μg dose nimesulide was administered peripherally twice, in separate toes studying a separate WDR neurone, and after a period of rest following the end of the first experiment. Data from this rat

were consistent with those obtained from other rats in this experiment. Neither nimesulide nor vehicle had any effect on mechanically evoked responses of WDR dorsal horn neurones compared to pre-drug responses (repeated measures ANOVA with post-hoc Dunnet's test, all $P>0.05$, Table 3.1), and no differences between effects of nimesulide versus vehicle were observed (non-parametric Mann-Whitney test, all $P>0.05$).

Table 3.1 Effects of peripherally administered nimesulide (100µg/50µL, n=6) or vehicle (n=6, 50µL) on mechanically evoked responses of WDR dorsal horn neurones in naïve anaesthetised rats. Data are expressed as a percentage of the pre-drug response ± SEM.

	Vehicle (3% Tween 80 in Saline) (percentage pre-drug response)			Nimesulide 100µg (percentage pre-drug response)		
von Frey stimulus	**10 min**	**30 min**	**50 min**	**10 min**	**30 min**	**50 min**
8g	112.9 ± 41.7	103.5 ± 7.8	97.8 ± 8.8	141.3 ± 53.9	138.0 ± 19.1	163.6 ± 35.2
10g	143.5 ± 38.8	112.4 ± 15.3	106.3 ± 20.5	168.8 ± 18.9	118.2 ± 16.9	87.8 ± 15.3
15g	128.2 ± 26.6	104.1 ± 13.0	138.8 ± 39.6	117.5 ± 19.9	104.9 ± 16.8	77.4 ± 9.8
26g	97.9 ± 20.6	102.7 ± 17.9	123.2 ± 21.6	107.4 ± 16.7	111.8 ± 15.4	103.8 ± 12.8
60g	99.2 ± 17.1	95.7 ± 9.5	101.9 ± 15.3	95.4 ± 8.9	95.4 ± 9.2	100.5 ± 9.7

3.3.4 Effects of spinally administered nimesulide on central and peripheral levels of endocannabinoid and endocannabinoid-like molecules in the naïve rat

To determine the potential contribution of changes in levels of endocannabinoids and endocannabinoid-like (ECL) molecules to the CB_1 receptor mediated effects of nimesulide, effects of spinal nimesulide (25µg/50µL, n=8; 100µg/50µL, n=8) versus vehicle (50µL, n=16) on spinal levels of endocannabinoids and related molecules *in vivo* were determined.

Spinal nimesulide (25μg) caused a decrease in spinal levels of AEA and PEA ipsilaterally ($P<0.005$), and a decrease in PEA levels contralaterally ($P<0.01$), without altering levels of 2-AG or OEA (Figure 3.10).

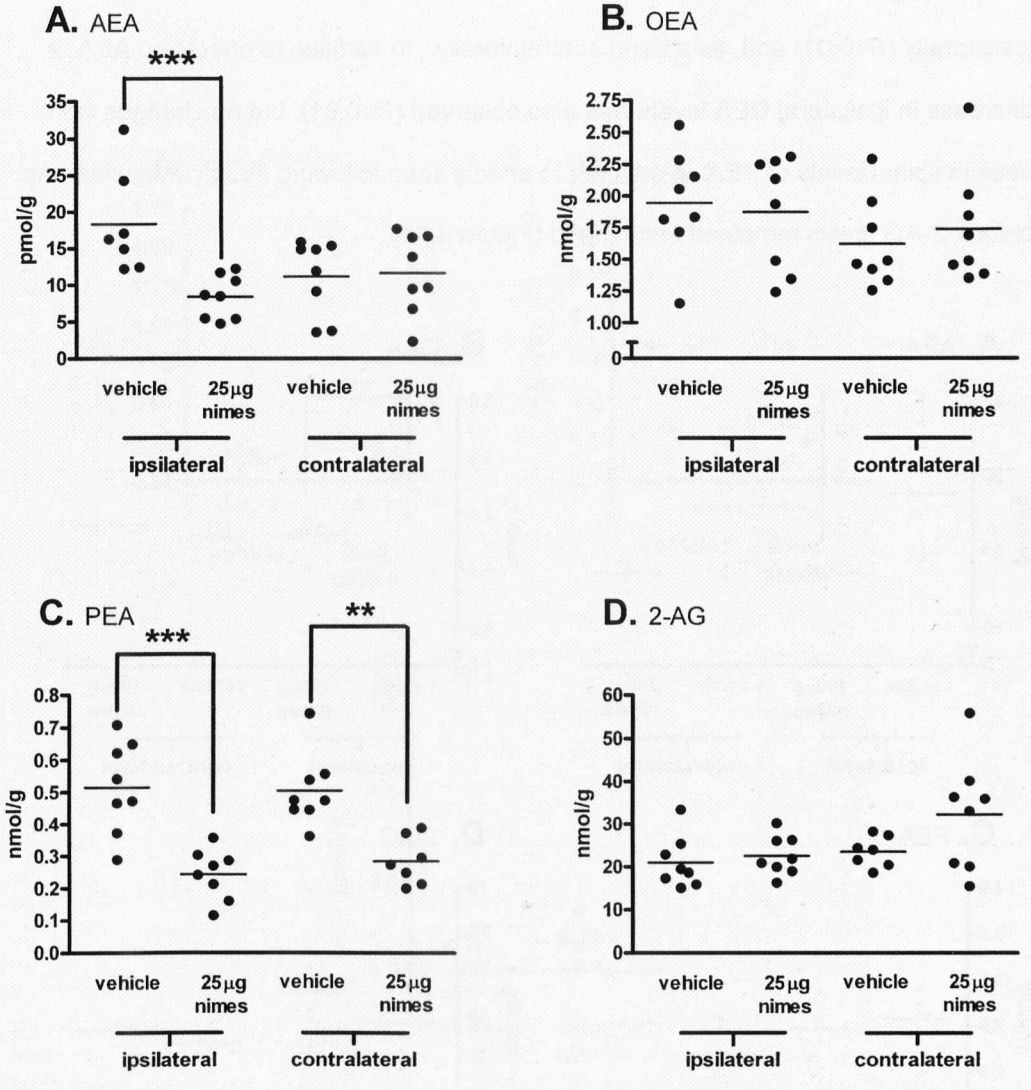


Figure 3.10 Effects of spinal nimesulide (25μg/50μL, n=8) on levels of; A, anandamide (AEA); B, N-oleoylethanolamine (OEA); C, N-palmitoylethanolamine (PEA; and D, 2-arachidonoylglycerol (2-AG) in spinal cord of naïve anaesthetised rats. Statistical analyses were performed with non-parametric Mann-Whitney test; **, $P<0.01$, ***, $P<0.005$ nimesulide versus vehicle (n=8). Data are expressed as individual values and median value is depicted by the line.

Quantification of spinal EC and ECL levels following a supramaximal dose of nimesulide (100µg/50µL, n=8) versus vehicle (50µL, n=8) used a slightly different extraction method (see section 3.2.4). Data varying from the mean by more than 2 standard deviations were excluded. In total this occurred in 3 out of 24 instances for AEA, 2 out of 24 for OEA, and 2 out of 24 instances for PEA. As seen with the 25µg dose, 100µg nimesulide decreased levels of AEA in the spinal cord, seen both ipsilaterally ($P<0.01$) and, as a trend contralaterally. In addition to effects on AEA, a decrease in ipsilateral OEA levels was also observed ($P<0.01$), but no changes were seen in spinal levels of PEA, in contrast to effects seen following 25µg nimesulide. As before, 2-AG levels remained unchanged (Figure 3.11).

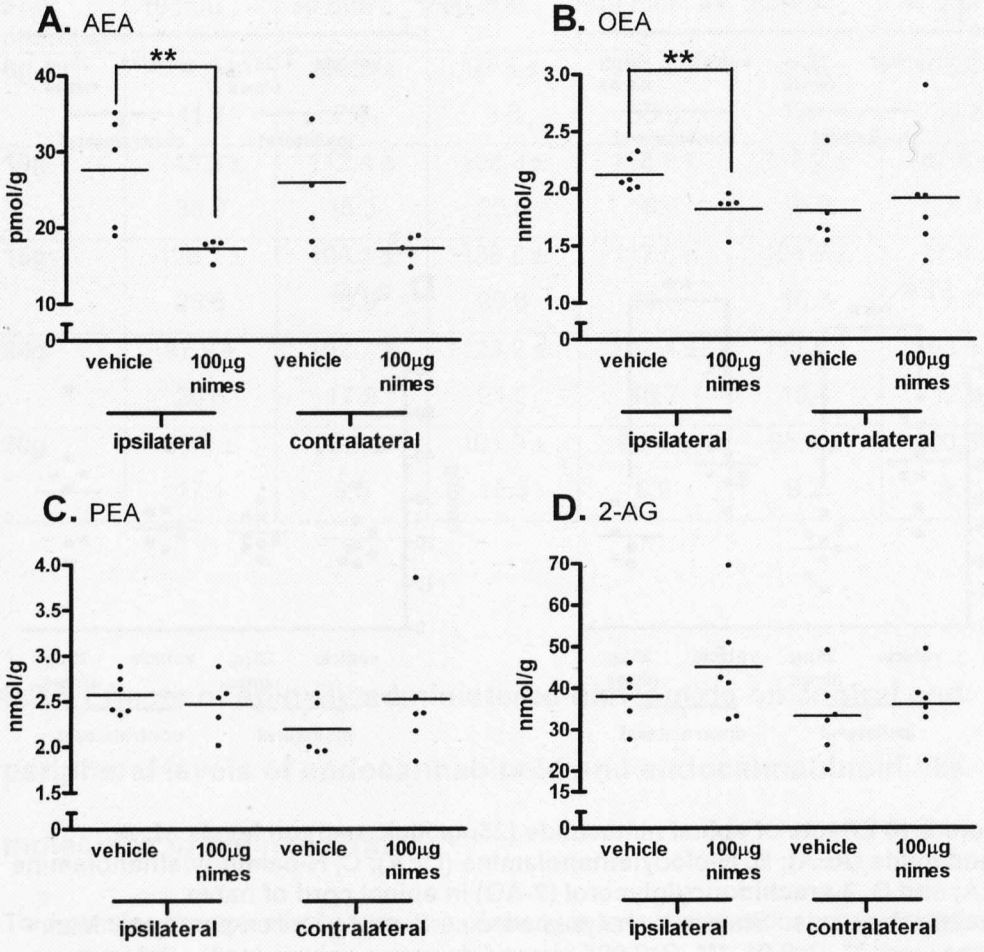


Figure 3.11 Effects of spinal nimesulide (100µg/50µL, n=6) on levels of; A, anandamide (AEA); B, N-oleoylethanolamine (OEA); C, N-palmitoylethanolamine (PEA; and D, 2-arachidonoylglycerol (2-AG) in spinal cord of naïve anaesthetised rats. Statistical analyses were performed with non-parametric Mann-Whitney test; **, $P<0.01$ nimesulide versus vehicle (n=6). Data are expressed as individual values and median value is depicted by the line.

To determine if spinal COX-2 inhibition altered peripheral levels of ECs and ECLs, effects of spinal nimesulide (25µg/50µL, n=8) versus vehicle (50µL, n=8) on levels of ECs and ECLs in the ipsilateral hindpaw *in vivo* were determined. Levels of AEA fell below limits of quantification and could not be determined. For 2-AG, OEA and PEA, at least 5 samples per group fell within the limits of quantification. Spinal nimesulide did not alter levels of OEA, PEA or 2-AG in the ipsilateral paw (Figure 3.12).

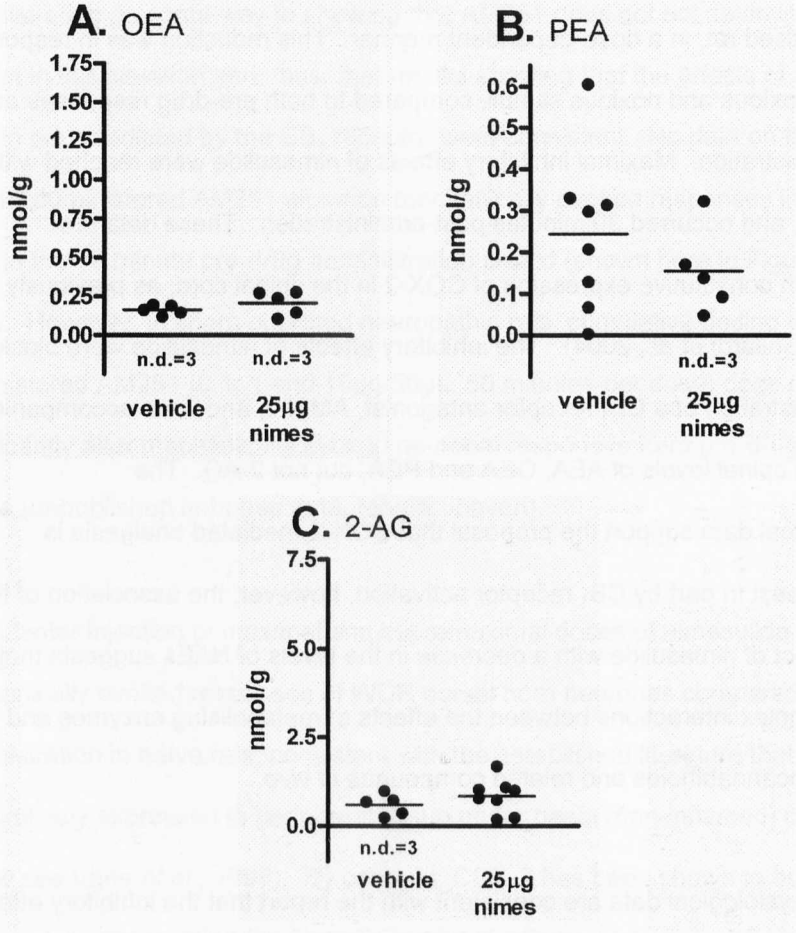


Figure 3.12 Effects of spinal nimesulide (25µg/50µL, n=8) on levels of; A, N-oleoylethanolamine (OEA); B, N-palmitoylethanolamine (PEA); and C, 2-arachidonoylglycerol (2-AG) in the ipsilateral paw of naïve anaesthetised rats. Statistical analyses comparing the effects of nimesulide to vehicle were performed with non-parametric Mann-Whitney test, all $P>0.05$. Data are expressed as individual values with median value depicted by the line. n.d. = number of samples falling below detection limits.

3.4. Discussion

3.4.1. CB_1-receptor-mediated attenuation of mechanically evoked responses of wide dynamic range dorsal horn neurones following spinal COX-2 inhibition

In the present study, spinal, but not peripheral, administration of the COX-2 inhibitor nimesulide reduced mechanically evoked responses of WDR dorsal horn neurones in the anaesthetised rat, in a dose-dependent manner. This reduction was in response to both non-noxious and noxious stimuli, compared to both pre-drug responses and vehicle administration. Maximal inhibitory effects of nimesulide were reached with a dose of 25µg, and occurred 30 minutes post-administration. These data are consistent with constitutive expression of COX-2 in the spinal cord, as previously established (Ghilardi *et al.*, 2004). The inhibitory effects of nimesulide were blocked by pre-administration of a CB_1 receptor antagonist, AM251, and were accompanied by a decrease in spinal levels of AEA, OEA and PEA, but not 2-AG. The pharmacological data support the proposal that COX-2-mediated analgesia is mediated at least in part by CB_1 receptor activation, however, the association of the inhibitory effect of nimesulide with a *decrease* in the levels of NAEs suggests that there are complex interactions between the effects of metabolising enzymes and levels of endocannabinoids and related compounds *in vivo*.

The electrophysiological data are consistent with the report that the inhibitory effects of spinal administration of indomethacin are also blocked by AM251 (Guhring *et al.*, 2002). It is important to note, however, that unlike indomethacin (Fowler *et al.*, 2003; Holt *et al.*, 2007), nimesulide does not inhibit FAAH (Fowler *et al.*, 2003) and, therefore, the data presented here implicate a direct role of COX-2 in the regulation of endocannabinoid function *in vivo*. Recently, the effects of two COX-2 inhibitors (NS398 and L-745,337) on evoked neuronal responses in naïve rats and in a model of joint inflammation have been reported (Telleria-Diaz *et al.*, 2009). In this study, spinal administration of the COX-2 inhibitor L-745,337 did not alter evoked neuronal responses in naïve rats, but attenuated responses following inflammation-induced

spinal hyperexcitability. Although this previous study only investigated the effects of the COX-2 inhibitor NS398 on the spinal release of 2-AG, it is of interest to note that spinal NS398 did not alter spinal release of 2-AG compared to the pre-drug application period (Telleria-Diaz *et al.*, 2009). These data are consistent with the observation that spinal nimesulide, at doses which attenuate evoked neuronal responses in a CB_1 receptor-dependent manner, does not alter levels of 2-AG in the spinal cord.

Data showing the effect of AM251 administration alone over the 30-minute pre-administration go some way to showing that AM251 does not act as an inverse agonist in this situation and, thus, that results showing that the effects of nimesulide were in part mediated by the CB_1 receptor were consistent. No data on the effects of spinally administered AM251 alone on mechanically evoked responses in naive rats beyond the 30 minute pre-drug administration period (shown here in Figure 3.6) exists. However, in sham operated neuropathic rats, cumulative dosing with spinally administered AM251 (0.1, 1 and 10µg/50µL, 50 minutes per dose) does not significantly alter mechanically evoked neuronal responses from pre-drug control values (unpublished in-house data, Maulik Jhaveri).

Intra-plantar injection of maximal and supramaximal doses of nimesulide did not alter mechanically evoked responses of WDR dorsal horn neurones compared with vehicle administration in naïve rats, consistent with the established literature that COX-2 is not constitutively expressed in peripheral tissue under basal (non-inflamed) conditions (for review see Vane *et al.*, 1998). By contrast, COX-2 has been shown to be active in inflammatory states using the formalin model of inflammatory pain, where hindpaw injection of the COX-2 inhibitor rofecoxib enhanced inhibitory effects of AEA on pain behaviour, and elevated hindpaw levels of AEA, OEA and PEA (Guindon & Beaulieu, 2006).

The first time the effects of intraplantar nimesulide on mechanically evoked neuronal responses were studied appeared to reveal a facilitatory effect of vehicle which was not seen in the nimesulide-treated rats. It was supposed that the nimesulide was counteracting an inflammatory response brought about by an injection of liquid into the

toe and further application of the von Frey monofilaments. In addition to this, control mechanically evoked WDR responses in these rats were not very stable, an effect magnified in the lower intensity stimuli tested, and cells were typically found late in the day due to inexperience both in searching for WDR neurones and the surgical techniques involved, resulting in an unstable setup. Together, these factors may have contributed to the unexpected results seen with peripheral injection of nimesulide and vehicle, and so the experiment was repeated over a year later, once experience in the technique had been gained, using only the higher dose (100µg/50µL), to ensure that maximal inhibition of COX-2 (if any was to be found peripherally) could be achieved. In this repeated experiment, responses to lower intensity stimuli again showed a trend towards being facilitated when compared to pre-drug responses, but there was no difference between treatment groups (nimesulide versus vehicle), showing that COX-2 is not constitutively active in the periphery. The facilitatory trend could be a result of irritation and sensitisation in the periphery following the injection and further stimulation, coupled with the pressure exuded on surrounding tissue by the volume of liquid injected. The greater magnitude of this effect in response to lower-intensity stimuli suggests a possible increase in Aβ-fibre responsiveness rather than C-fibres.

3.4.2. A putative role for CB_1-receptor-active metabolites of endocannabinoids in the modulation of nociceptive transmission

COX-2 promotes the production of the hyperalgesic prostanoids PGE_2 and PGI_2 from arachidonic acid (Taiwo and Levine, 1990), and it is widely accepted that COX-2 inhibitors exert their anti-inflammatory and anti-nociceptive actions via this pathway. However, NSAIDs have been shown to alter EC levels at clinically relevant doses (Guindon & LoVerme, 2006), although this effect is thought to be mediated largely by the inhibitory actions of NSAIDs on FAAH, and the contribution of COX-2 is not so clear. It is established from *in vitro* studies that COX-2 can metabolize AEA and 2-AG (for review see Kozak *et al.*, 2004; Yu *et al.*, 1997; Hu *et al.*, 2008). Furthermore, the physiological relevance of COX-2 regulation of endocannabinoids has been demonstrated in work using the hippocampal slice preparation, although levels of endocannabinoids were not measured (Slanina & Schweitzer, 2005). Evidence for a

role of COX-2 metabolism of endocannabinoids is further supported by the detection of COX-2 metabolites of AEA in FAAH knockout mice dosed with AEA (Weber *et al.*, 2004) and the presence of COX-2 metabolites of 2-AG in the rat (Hu *et al.*, 2008). Clearly, the data presented here, demonstrating a mismatch between the pharmacological effects of nimesulide mediated by the CB_1 receptor and levels of endocannabinoids suggest that, at least at the level of the spinal cord, COX-2 regulation of endocannabinoids is complex.

The mechanism by which inhibition of COX-2 by nimesulide can produce CB_1 receptor-dependent effects in the absence of overt increases in levels of AEA or 2-AG is unclear, but may involve catabolism via other pathways. COX-2 metabolises ECs via oxygenation (Yu *et al.,* 1997, Kozak *et al.*, 2004), a mechanism it shares with cytochrome P450 enzymes (Awumey *et al.*, 2008; Snider *et al.*, 2007; Snider *et al.*, 2009; Snider *et al.*, 2008; Stark *et al.*, 2008) and lipoxygenases (Hampson *et al.*, 1995; Ueda *et al.*, 1995b). Recently, a number of cP450- and LOX-metabolites of the endocannabinoids and their precursor, arachidonic acid, have been shown to have activity at the CB_1 receptor (Figure 3.13), and so one possible explanation for the data reported here is that COX-2 inhibition diverts metabolism of ECs and ECLs down cP450-and LOX-pathways, producing CB_1-active metabolites.

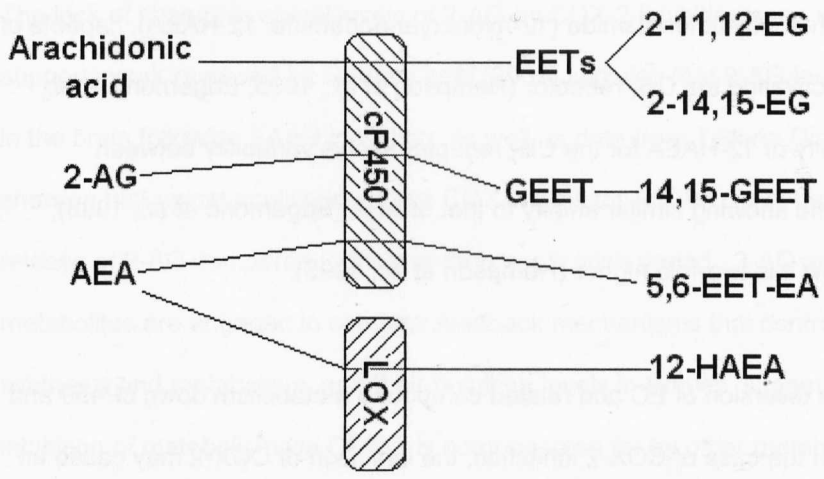


Figure 3.13 Production of CB_1 receptor ligands by metabolism of the endocannabinoids anandamide (AEA) and 2-arachidonoylglycerol (2-AG), and their precursor arachidonic acid, by the oxidative enzymes cytochrome P450 (cP450) and lipoxygenase (LOX). EET, epoxyeicosatrienoic acid; EG, epoxyeicosatrienol glycerol; EET-EA, epoxyeicosatrienol ethanolamide; GEET, glycerated epoxyeicosatrienoic acid; HAEA, hydroxyarachidonoylethanolamide.

Cytochrome P450 metabolism of AEA and its precursor arachidonic acid, results in the formation of eicosatrienoic acids (EETs), in particular the 2-epoxyeicosatrienol glycerols (2-EG) 2-(11,12-epoxyeicosatrienol)glycerol (2-11,12-EG) and 2-(14,15-epoxyeicosatrienol)glycerol (2-14,15-EG) from arachidonic acid (Chen *et al.*, 2008), and 2-(5,6-epoxyeicosatrienol)ethanolamide (5,6-EET-EA) from AEA (Snider *et al.*, 2009; Stark *et al.*, 2008), which are agonists at CB_1 receptors with comparable or greater binding affinity than 2-AG (Chen *et al.*, 2008). Although no involvement of cP450 in the metabolism of OEA has been reported, the structure of OEA contains one double bond (see figure 1.8, section 1.3.2, chapter 1) and may, therefore, also undergo oxidation by cytochrome p450. Metabolism of 2-AG by cP450 also produces EETs, of which 14,15-glycetated epoxyeicosatrienoic acid (14,15-GEET) shows agonist activity at the CB_1 receptor (Awumey *et al.*, 2008). The activity at CB receptors of many of these cP450 metabolites of ECs, ECLs and their precursor arachidonic acid, is comparable to, or greater than that of the endocannabinoids. The 2-EGs have comparable binding affinities to 2-AG for the CB_1 and CB_2 receptors, and *in vivo* studies show 2-14,15-EG to have greater activity at both CB_1 and CB_2 receptors than 2-AG (Chen *et al.*, 2008). 5,6-EET-EA is 300-fold more selective for CB_2 than CB_1 receptors, with a CB_2 receptor affinity 1000-times higher than that of AEA (Snider *et al.*, 2009). Its activity, as well as that of 14,15-GEET, relative to AEA at the CB_1 or CB_2 receptors is unknown. LOX-12 can metabolise AEA to produce 12-(S)-hydroxyarachidonoylethanolamide (12-hydroxyanandamide, 12-HAEA), capable of binding to and activating the CB_1 receptor (Hampson *et al.*, 1995; Edgemond *et al.*, 1998). The affinity of 12-HAEA for the CB_1 receptor shows variability between studies, with some showing similar affinity to that of AEA (Edgemond *et al.*, 1998), while others show it is two-fold higher (Hampson *et al.*, 1995).

In addition to the diversion of EC and related compound metabolism down cP450 and LOX-pathways in the case of COX-2 inhibition, the inhibition of COX-2 may cause an upregulation in cP450 and LOX activity. There appears to be a strong link between COX-2 inhibition by NSAIDs and the specific COX-2 inhibitor SC-236, and upregulation of 15-LOX activity (Shureiqi *et al.*, 2000, Wu *et al.*, 2003). It is unknown whether nimesulide can produce this effect, or whether 12-LOX may also be

upregulated in this way, which would result in increased AEA metabolism, and an increase in the production of the CB_1-active AEA metabolite, 12-HAEA.

In light of the still recent discovery of biological activity of cP450 and LOX-mediated EC metabolites at CB receptors, it is also important to consider the possibility that some of these metabolites, as well as those via COX-mediated metabolism, may interfere with, or enhance, EC and ECL synthesis. This is of importance particularly in light of the changes seen in PEA levels in experiments presented here, as the structure of PEA does not contain a double bond and so it cannot be a substrate for oxidation by cP450 or LOX (Chapman, 2004), and yet PEA levels in the spinal cord decreased following spinal application of 25µg, but not 100µg nimesulide. If COX-2 metabolites were to play a role in PEA synthesis then inhibition with nimesulide would cause a decrease in synthesis, and decreased PEA levels as seen following 25µg nimesulide. However, if this was the case, one might expect that increasing the amount of nimesulide administered would also cause a decrease in spinal PEA levels, which did not occur. Another possibility is that cP450/LOX-mediated metabolites of EC and ECLs inhibit PEA synthesis, and so if COX-2 inhibition pushes EC and ECL metabolism down cP450/LOX pathways as proposed above, PEA synthesis would be inhibited, possibly in a dose-dependent manner.

The lack of change in spinal levels of 2-AG by COX-2 inhibition seen in these studies supports work reviewed by Gaetani *et al* (2009) showing that 2-AG levels did not alter in the brain following FAAH inhibition, as well as data from Telleria-Diaz *et al.*, (2009) showing that spinal application of the COX-2 inhibitor NS398 did not alter spinal release of 2-AG compared to the pre-drug application period. 2-AG and its metabolites are engaged in complex feedback mechanisms that control 2-AG synthesis and metabolism, and their resulting levels in tissues, suggesting that inhibition of metabolism via COX-2 is compensated for by other metabolic pathways.

I therefore propose that metabolism of endocannabinoids via cP450 and LOX-pathways increases when COX-2 is inhibited by nimesulide. Increased catabolism of the NAEs AEA and OEA, as well as 2-AG via these additional metabolic pathways

may then result in the generation of alternative CB_1 receptor ligands which underpin the CB_1 mediated inhibition of neuronal responses, and occurs alongside the decreased levels of AEA and OEA observed in the spinal cord following treatment with nimesulide. At the present time it is not possible to confirm this hypothesis as deuterated standards for these proposed CB ligands are not currently available. The generation of these standards is crucial for further elucidation via LC/MS-MS techniques, of the role of these pathways in endocannabinoid regulation *in vivo*.

3.4.3. Conclusions

The data presented here establish a role for an endocannabinoid component in NSAID-mediated analgesia, mediated by the CB_1 receptor. It is proposed that COX-2 inhibition by NSAIDs shunts metabolism of endocannabinoids and their precursor arachidonic acid, down LOX and cytochrome p450 oxidative pathways, generating high affinity/efficacy CB_1 receptor ligands.

Chapter 4 Characterisation of the MIA model of OA pain with respect to WDR dorsal horn neuronal responses and cytokine levels in various tissues

4.1. Introduction

4.1.1. The MIA model of osteoarthritis

As described in Chapter 1, animal models currently in use for the study of osteoarthritis include those brought about spontaneously, or by means of surgical or chemical alteration of the joint. These models have their merits and uses, but, as described previously, the mechanisms underlying the pain associated with these models have not been widely studied. Interest in monosodium iodo-acetate (MIA) as a model of osteoarthritic symptoms in animals dates back to 1977 (Kalbhen and Blum), when it was injected into the knee of a chicken. The effects of MIA have also been studied in mice (Van der Kraan *et al.*, 1989) and horses (Penraat *et al.*, 2000) however, in horses it was deemed unsuitable as an experimental model of OA due to high rates of complication and the lack of bony fusion.

MIA is a metabolic inhibitor of chondrocyte glyceraldehyde-3-phosphate dehydrogenase, disrupting glycolysis and resulting in cell death (Kalbhen, 1987, Van der Kraan *et al.*, 1989). The MIA model of osteoarthritis has been extensively studied in the rat (Table 4.1) with doses ranging from 0.01 - 3mg. Doses of 0.1mg and above cause chondrocyte loss, which, together with decreases in proteoglycan concentration, produces many histopathological features of the clinical condition quickly (within 28 days) and in a dose-dependent manner (Guingamp *et al.*, 1997). Importantly, this model is associated with cartilage fibrillation and thinning (Janusz *et al.*, 2001) and subchondral bone changes, thought to be an important factor contributing to OA pain (see Chapter 1). Changes in subchondral bone include exposure, swelling and thickening, sclerosis, decrease in mineral content and density – indicative of chronic joint degeneration (Pomonis *et al.*, 2005), and the presence of osteophytes and cysts (Table 4.1). Changes in levels of osteoclasts and osteoblasts were evident from 7 days post-MIA injection, while degenerative changes such as collapse of bony traberculae appeared from 28 days onwards (Guzman *et al.*, 2003). These degenerative changes progressed with time, eventually showing large areas of bone remodelling, and loss of marrow hematopoietic cells at day 56 post-MIA injection

(Guzman *et al.*, 2003), indicating the MIA model to be one of progressive degeneration.

Functional effects following intra-articular injection of MIA are also evident, with decreases in motility (Guingamp *et al.*, 1997), weight-bearing (Bove *et al.*, 2003; Pominis *et al.*, 2005), and the presence of thermal hyperalgesia and tactile allodynia (Fernihough *et al.*, 2004). These behaviours have been shown to be pain-related, as morphine was able to attenuate mechanical hyperalgesia at days 3, 14 and 28 post-MIA injection, and reverse tactile allodynia at days 14 and 28 (Fernihough *et al.*, 2004). Changes in weight-bearing on the hindlimb following MIA injection occurs in two phases. The first phase peaks at day 3 or 4, and reverted to normal by day 7, while the second phase occurred from day 14 onwards (Pomonis *et al.*, 2005; Bove *et al.*, 2003). The biphasic nature and timing of changes in weight-bearing have been suggested to arise from different mechanisms. Nociceptive responses in the first phase are thought to result from an inflammatory response, while the second phase response is expected to be due to joint damage, based on their susceptibility to attenuation by analgesics (Fernihough *et al.*, 2004; Bove *et al.*, 2003). NSAID treatment (diclofenac and paracetamol) attenuated hyperalgesia and allodynia only in the earlier timepoints (3 days), coinciding with swelling on the knee, and not at day 14 or 28 post-MIA injection (Fernihough *et al.*, 2004). Radiographic evidence confirmed that MIA-treated joints had sustained damage, although no radiographic evidence exists to confirm that the first phase of altered weight-bearing is due to inflammation alone. Nevertheless, changes in levels of macrophage, neutrophil, plasma cell and lymphocyte infiltration, which cause synovial membrane expansion is present at earlier time-points and is largely resolved by day 7 (Bove *et al.*, 2003).

Table 4.1 A summary of studies of the MIA model of OA in rats. Where more than one dose is studied, those in bold were found to gave significant effect. Time points in **bold** denote days post injection at which maximal changes in behaviour were observed. It is for these doses that the behavioural / histological findings were observed, unless otherwise stated.

Author	Doses studied (mg), rat weight/strain	Time points studied	Behavioural findings	Histological findings
Guingamp *et al.*, 1997	0.01, 0.03, 0.1, **0.3, 3**mg 150g / Wistar	Not stated	0.1mg gave a functional impairment measured by functional telemetry, in a biphasic fashion. This finding was more robust at a 0.3mg dose.	↓ proteoglycan concentration Cartilage swelling followed by thinning and disappearance Osteophyte formation and clusters Subchondral bone exposure Human OA-like lesions
Janusz *et al.*, 2001	0.1, 0.25, 1 220-230g / Sprague-Dawley	Not stated	n/a	↓ proteoglycan concentration Chondrocyte necrosis Cartilage thinning, fibrillation calcification and ↓ cell density Subchondral bone resorption and remodelling
Bove *et al.*, 2003	0.1, 0.3, **1, 3** 175-200g / Wistar	1, 3, **7**, 14 days	1mg dose gave maximal change in weight distribution in a biphasic fashion	↓ proteoglycan concentration Loss of chondrocytes Cartilage fibrillation & loss of cellular detail
Guzman *et al.*, 2003	1 175-200g / Wistar	1, 3, 5, 7,14, 28, 56 days	n/a	Chondrocyte degeneration and necrosis ↑ osteoclasts and osteoblasts in bone Focal fragmentation and fibrosis Bone resorption, cysts and sclerosis

Author	Doses studied (mg), rat weight/strain	Time points studied	Behavioural findings	Histological findings
Kobayashi *et al.*, 2003	0.3, 3 7 week old female Wistar	15 days	n/a	↓ proteoglycan concentration Chondrocyte disorganisation Cartilage erosion and fibrillation Subchondral bone exposure
Dumond *et al.*, 2004	0, 0.3 150-175g / Wistar	2, 5, 10, 15, 20 days	n/a	COX-2 upregulation in the joint ↑ gelatinase activity ↓ proteoglycan concentration Cartilage lesions, fibrillation and matrix depletion Chondrocyte rarefaction and loss Subchondral bone sclerosis in some rats
Fernihough *et al.*, 2004	2 200 ± 60g / Wistar	Not stated	Mechanical hyperalgesia and tactile allodynia responsive to morphine	Cartilage loss (decrease in joint space) Cyst formation Bony outgrowths (osteophytes) Thickening of subchondral bone Joint misalignment
Pomonis *et al.*, 2005	0.3, 1, 3 126-150g / Sprague-Dawley	2, 4, 11, **21**, 28 days	Biphasic change in weight-bearing. First phase resolving fully by day 7, second phase spanning from day 7 to day 28.	Dose dependent joint degradation involving - bone swelling and lysis - decreased bone mineral content and density

The MIA model in the rat is now often used for OA studies involving changes in cytokine levels (Pelletier *et al.*, 1995; Caron *et al.*, 1996), and for testing pharmacological agents for cartilage preservation properties (Gencosmanoglu *et al.*, 2001; Janusz *et al.*, 2001). However, while the MIA model is excellent in terms of the clinically relevant histopathology, reproducibility, fast onset time, controllable severity and sensitivity to clinically used analgesics such as morphine, a dosing regime and onset period that produced both clinically relevant histology and behavioural changes consistently had not yet been fully established. This lab sought to establish such a dosing regime, in a study employing a range of doses from 0.3-3mg MIA, studying behavioural, electrophysiological, and histolopathological changes for up to 31 days after injection, in rats of different weights. An intra-articular dose of 1mg MIA in 50µL saline in 160-190g male Sprague-Dawley rats followed for 28 days was finally chosen. This dosing regime was found to produce time-dependent changes in pain behaviours (weight-bearing and allodynia), which will be discussed in more detail throughout this chapter (and see appendix).

4.1.2. The role of inflammation in cartilage pathology of OA

The role of inflammation in the progression of OA has been widely studied, and several clinical studies suggest an important association between inflammation of the synovial structures (synovitis), OA-associated inflammation, and the progression of structural changes. Several markers of synovial inflammation in OA have been studied, including cartilage oligomeric protein (COMP, Clark *et al* 1999, Sharif *et al.*, 1995), and changes in serum levels of C reactive protein (CRP – Spector, 1997) and hyaluronic acid (HA – Goldberg, 1991), however, at present the interest in such biomarkers is far outstripped by interest in the role of inflammatory mediators.

Inflammatory mediators associated with OA pathophysiology appear initially to be produced by the synovial membrane, from where they diffuse into the cartilage through the synovial fluid, activating the cartilage cells (chondrocytes) which then produce, and are the main source responsible for, these mediators (see Pelletier *et al.*, 2001). The location of chondrocytes in lacunae of articular cartilage, an avascular

and aneural tissue means that such production of inflammatory mediators is not associated with classic signs of inflammation (see Pelletier *et al.*, 2001). Inflammatory mediators produced by chondrocytes include matrix metalloproteases (MMPs) which are involved in the digestion of cartilage collagen (see Smith, 2006), and other members of serine- and cysteine-dependent protease families such as plasminogen-activating factor (PAF) and cathepsin P (but their involvement is primarily as activators of MMPs). Excessive amounts of cytokines and growth factors are also produced (see below), and play an important role in OA pathophysiology, acting within cartilage in an autocrine and paracrine manner to promote cartilage damage (Attur *et al.*, 1998). They are also associated with functional alterations in the synovium and subchondral bone. Chondrocytes from OA patients produce large amounts of nitric oxide (Amin *et al.*, 1995, Amin *et al.*, 1998, Pelletier *et al.*, 1996), prostaglandins (Pelletier *et al.*, 1998), and a vast array of pro-inflammatory cytokines including IL-1β, TNFα, IL-17 and IL-6 (Caron *et al.*, 1996; van de Loo *et al.*, 1995; Van bezooijen *et al.*, 1999; Goldring *et al.*, 1999).

Nitric oxide and osteoarthritis

Nitric oxide (NO) is very likely to play a role in cartilage catabolism seen in OA. NO is present at high levels in OA cartilage and synovium (Amin *et al* 1995, Pelletier *et al*, 1996), with serum and synovial fluid collected from arthritic patients revealing high levels of nitrites and nitrates (Farrell *et al.*, 1992). *Ex vivo,* OA cartilage produces NO without stimulation by typical inflammatory mediators such as IL-1. NO acts to inhibit proteoglycan synthesis, and causes chondrocyte cell death by apoptosis and necrosis, as well as increasing MMP and cytokine synthesis, while decreasing synthesis of IL-1Ra, a competitive antagonist of the cytokine IL-1 receptor (for review, see Amin *et al.*, 1998) (Figure 4.1).

Prostaglandins and osteoarthritis

Prostaglandins are produced by COX-2, which is upregulated in cartilage of OA patients (Amin *et al.*, 1997); OA cartilage produces 50x the levels of PGE_2 than normal

cartilage *ex vivo*. COX-2 upregulation and related increases in PGE_2 have also been observed in a canine model of OA (Pelletier *et al.*, 1998). Prostaglandins are not only involved in inflammation and pain but may also bring about structural changes seen in OA (see below, and Figure 4.1).

Prostaglandin PGE_2 is the best characterised in terms of OA, is primarily produced by osteoblasts (Pilbeam *et al.*, 2002) and has long been recognised as being important in joint pathology. Its production is stimulated by a rise in intracellular calcium which increases COX-2 expression via PKA pathways (Choudhary *et al.*, 2004). Its synthesis is enhanced by many cytokines such as IL-1 and IL-6, growth factors and hormones (Kwan *et al.*, 2004). PGE_2 also autoamplifies, stimulating its own production by cAMP-dependent activation of PKA which induces COX-2 mRNA transcription (Sakuma *et al.*, 2004), and in human OA has been shown to regulate the production of IL-1 and IL-6, adding further to its autoamplification (Amano *et al.*, 1996; Park *et al.*, 2004; Inoue *et al.*, 2002). In osteoblasts, PGE_2 acts on EP receptors, of which there are 4 subtypes, EP1-4 (Narumiya *et al.*, 1999). In humans, only the presence of EP3 and 4 have been shown immunohistochemically (Fortier *et al.*, 2004), while EPs 1, 2 and 4 have been shown in mice (Suda *et al.*, 1996). EP receptors have differential actions, involving cAMP and intracellular calcium pathways, mediating its many actions (see below, and for review, see Hikiji *et al.*, 2008).

PGE_2 has been shown to mediate bone resorption via cAMP signalling (Miyaura *et al.*, 2000) and stimulation of osteoclast formation (see Hikiji *et al.*, 2007). Osteoclast formation is stimulated by PGE_2 via induction of receptor activator of nuclear factor kappa B ligand (RANKL), a ligand for nuclear factor kappa B (NF-κB) (Krieger *et al.*, 2000; Yasuda *et al.*, 1998; Fuller *et al.*, 1998), the activation of which mediates osteoblast differentiation (Wu *et al.*, 2007; Suda *et al.*, 1996; Wani *et al.*, 1999). The role of PGE_2 in osteoclastic formation in humans is controversial, and seems to be dependent on the EP receptor subtype expressed by the cells (see Hikiji *et al.*, 2008). PGE_2 has also been implicated in OA-related loss of cartilage, by sensitising human OA chondrocytes to iNOS-mediated cell death *in vitro* (Notoya *et al.*, 2000).

PGE_2 not only has important roles in bone degradation, but has also been shown to be important in bone formation both *in vitro* and *in vivo*, by stimulating fibronectin synthesis, important in early bone formation (Tang *et al.*, 2005; Li *et al.*, 2003). PGE_2 has also been implicated in fracture healing and suppression of bone loss (see Hikiji *et al.*, 2008). PGE_2 also stimulates the formation of mineralised bone nodules, an *in vitro* indicator of *in vivo* calcification (Flanagan & Chambers, 1992; Nagata *et al.*, 1994; Wu *et al.*, 2007).

A large number of other prostaglandins have been implicated in bone metabolism and disease, including PGI_2, $PGF_{2\alpha}$, PGD_2, PGE_1 and thromboxane A2 (TxA_2). However, much less is known about their possible role in OA (for review, see Hikiji *et al.*, 2008).

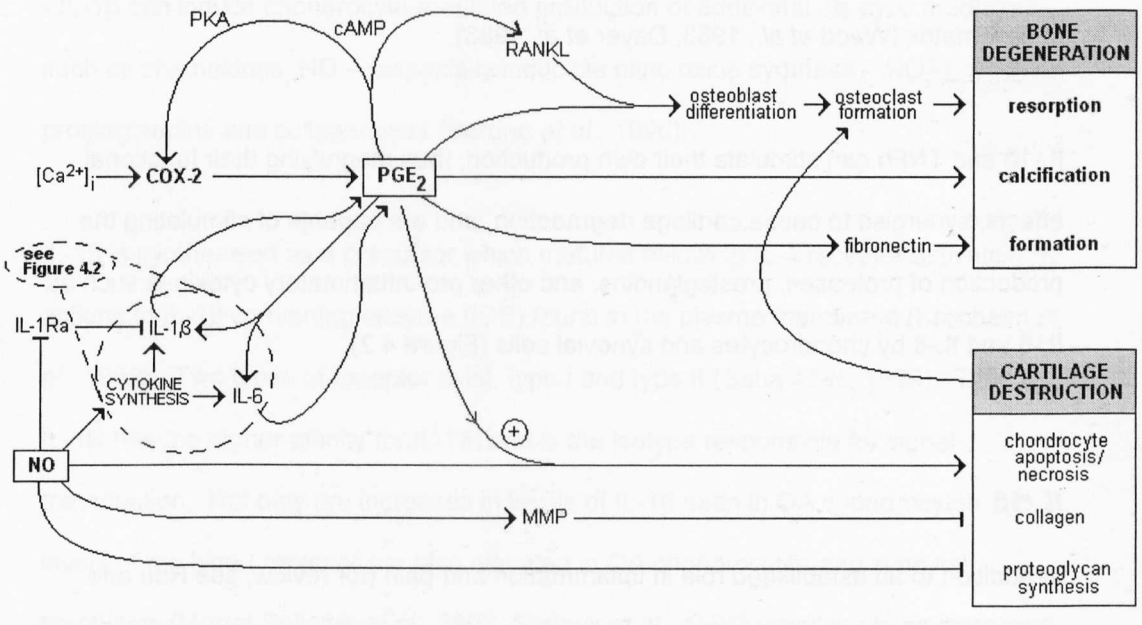


Figure 4.1 A summary schematic of the roles of NO and PGE_2 in bone and cartilage degradation in osteoarthritis. [Ca2+]$_i$, intracellular calcium; cAMP, cyclic adenosine monophosphate; COX-2, cyclooxygenase 2; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; MMP, matrix metalloprotease; NO, nitric oxide; PKA, protein kinase A; RANKL, receptor activator of nuclear factor kappa B ligand.

Pro-inflammatory cytokines and osteoarthritis

Cytokines are non-immunoglobulin soluble glycoproteins which non-enzymatically regulate host-cell function (Nathan and Sporn, 1991). Several cytokines have been implicated in the maintenance of cartilage loss. These include, in particular, IL-1β and TNFα, which in OA are mainly produced from synovial fibroblasts, in contrast to

cytokine production in RA, which originates from infiltrating monocytes and macrophages (Alaaeddine *et al.*, 1999). These cytokines are pivotal in the development of OA through their destructive effects on cartilage, with TNFα having a crucial role in the early stages and IL-1β throughout the disease (Caron *et al.*, 1996, van de Loo *et al.*, 1995). Sequestration of TNFα in early stage (but not established) collagen-induced arthritis in mice decreased joint pathology, determined by infiltration of inflammatory cells, cartilage damage and histological analysis (Joosten *et al.*, 1996). This early stage effect was independent of IL-1 receptor block, while anti-IL-1α/IL-1β was capable of suppressing damage in early phase and established arthritis, confirming the importance of their role throughout the disease (Joosten *et al.*, 1996). IL-1β acts to stimulate proteolytic pathways of extracellular matrix degradation, for example by stimulating collagenase and PGE_2 production, and inhibiting the formation of new matrix (Wood *et al.*, 1983, Dayer *et al*, 1986).

IL-1β and TNFα can stimulate their own production, thus magnifying their functional effects, synergise to cause cartilage degradation, and are capable of stimulating the production of proteases, prostaglandins, and other pro-inflammatory cytokines such as IL-6 and IL-8 by chondrocytes and synovial cells (Figure 4.2).

IL-1β

In addition to an established role in inflammation and pain (for review, see Ren and Torres, 2009), IL-1β has an established role in cartilage and bone destruction, largely due to the dys-regulation of the metabolic processes of chondrocytes, causing an increase in MMP-1, and MMP-13 expression which degrade components of cartilage including type I collagen (Holliday *et al.*, 1997). These events result in the initiation of osteoclast activation and the consequent bone resorption. The effects on MMPs were first noted as a correlation between the presence of IL-1 and MMP activity in OA synovium (Martel-Pelletier *et al.*, 1986, Wood *et al.*, 1983). Later, it was shown that OA chondrocytes have a higher sensitivity to IL-1β mediated production of MMP than non-OA chondrocytes, which is due to an increase in IL-1 receptor numbers (Pelletier *et al.*, 1993). In addition to effects on MMPs, IL-1β has other effects important in OA:

- stimulating the release of collagenase and plasminogen activator from cartilage and synovial cells (Dayer *et al.*, 1986, McCroskery 1985)
- suppressing cartilage-specific expression of type II and IX collagen (Goldring *et al.*, 1988), and increasing type I (the main component of bone) and III collagens in human articular cartilage cell cultures (Goldring *et al.*, 1988), causing changes in bone structure and bone remodelling
- mediating erosive processes in chronic arthritis, as shown by PGE_2- and leukotrieneB4-independent induction of cartilage proteoglycan degradation, and polymorphonuclear and mononuclear leukocyte infiltration in the synovial joint following intra-articular IL-1 injection into the rabbit knee (Pettipher *et al.*, 1986). Supportive *in vitro* studies have shown that IL-1-like factor stimulates bone resorption (Gowen *et al.*, 1983)
- IL-1β can induce chondrocyte-mediated production of additional catalytic mediators such as chemokines, NO – (especially inducible nitric oxide synthase - iNOS), prostaglandins and collagenases (Borden *et al.*, 1996)

IL-1β is synthesised as a precursor which matures following IL-1 receptor activation by actions of IL-1β converting enzyme (ICE) found in the plasma membrane (Kronheim *et al.*, 1992). Two types of receptor exist, type I and type II (Saha *et al.*, 1999). Type I IL-1R has the higher affinity for IL-1β, and is the isotype responsible for signal transduction. Not only are increases in levels of IL-1β seen in OA chondrocytes, but levels of the type I receptor are also elevated in OA chondrocytes and synovial fibroblasts (Martel-Pelletier *et al.*, 1992, Sadouk *et al.*, 1995), resulting in an increased sensitivity of the cell to stimulation by already-elevated levels of IL-1β (Martel-Pelletier *et al.*, 1992).

TNFα

TNFα induces osteoclastic bone resorption *in vitro* (Kronheim *et al* 1992) and as such, may be involved in remodelling of OA subchondral bone. This cytokine is less well studied than IL-1, but has been shown to have many effects that overlap with IL-1, namely the stimulation of collagenase and PGE_2 production by synovial fibroblasts

(Alaaeddine *et al.*, 1999), and can stimulate IL-1 and IL-6 production (for review, see Brouckaert *et al.*, 1993).

TNFα activates TNFR55 and TNFR75 receptors on the cell membrane (Loetscher *et al.*, 1990, Schall *et al.*, 1990). TNFR55 is the dominant form responsible for mediating TNFα activity in chondrocytes and synovial cells in OA, where it is upregulated (Alaaeddine *et al.*, 1997, Westacott *et al.*, 1994). TNFRs can be cleaved to form soluble TNFRs (sTNFR) which although themselves confer no effect, their presence has an effect which varies dose-dependently; at low concentrations sTNFR is postulated to stabilise the trimeric structure of TNFα thus prolonging its half-life; whereas at high concentrations sTNFR can decrease the half-life of TNFα by competing with membrane-bound receptors for TNFα binding (Loetscher *et al.*, 1990, Schall *et al.*, 1990). Release of sTNFR75 from OA synovial fibroblasts and chondrocytes are elevated (Alaaeddine *et al.*, 1997, Brennan *et al.*, 1995), but are still considered to be at "low" concentrations, and thus the effect is to prolong the half-life of TNFα in OA, potentiating its effects. TNFα effects are also prolonged in OA by upregulation of TNFα converting enzyme (TACE – Amin 1999), a member of the adamalysin family of metalloproteinases responsible for activating TNFα by proteolytic cleavage from its proform at the cell surface (Black *et al.*, 1997).

IL-17

IL-17 is produced almost solely by T-lymphocytes, unlike other cytokines which are commonly produced by monocytes and other innate cells, suggesting a role in adaptive or memory immune responses (Fossiez *et al.*, 1996). It has opposing roles in bone turnover, inferring protective roles by stimulation of various mediators involved in bone protection, and synergising by unknown mechanisms with IL-1β and TNFα to drive expression of inflammatory mediators including IL-6, PGE_2, NO, and IFNγ, promoting bone loss (Jovanovic *et al.*, 1998a; and for review, see Gaffen, 2004). Studies suggest that IL-17 may act through induction of membrane-bound and soluble Receptor Activator for Nuclear Factor κ B Ligand (RANKL - Nakashima *et al.,* 2000), an activator of osteoclasts which results in bone resorption. Levels of IL-17 are

elevated in a number of joint disease conditions including osteoarthritis (Van bezooijen *et al.,* 1999), rheumatoid arthritis, and periodontitis, where it has been implicated in contributing to bone destruction (Johnson 2004).

IL-6

IL-6 is known to be of importance in OA, with regulatory functions (Goldring *et al.*, 1999), and is a downstream effector of IL-17 (for review, see Gaffen, 2004). It is upregulated by IL-1 and PGE_2, and is synthesised and secreted by chondrocytes (Nietfeld *et al.*, 1990, Bender *et al.*, 1990). It confers both anti- and pro-inflammatory properties, and while it has no direct effects on the synthesis of prostaglandins, proteases, or matrix proteins, it has been shown to stimulate synthesis of **t**issue **i**nhibitors of **m**etallo**p**roteases (TIMP, Lotz and Guerne, 1991, Shingu *et al.*, 1993, Shingu *et al.*, 1995) and thus result in a reduction of MMPs. Other actions of IL-6 include:

stimulation of bone resorption (Ishimi *et al.*, 1990, Palmqvist *et al.*, 2002), an effect involving PGE_2 and effects on IL-1 and IL-17-mediated osteoclast formation (Devlin *et al.*, 1998)

co-administration (but not administration of IL-6 alone) with its soluble receptor sIL-6R (Taga *et al.*, 1989) also triggers osteoclast formation in mouse primary osteoblasts, which themselves confer low levels of membrane bound IL-6R mRNA (Udagawa *et al.*, 1995)

inhibiting proteoglycan synthesis in human cartilage (Nietfield *et al.*, 1990) and enhancing proteoglycan metabolism by enhancing catabolic effects of IL-1β and TNFα (Jikko *et al.*, 1998; Flannery *et al.*, 2000)

enhancing chondrocyte proliferation (Namba *et al* 2007), thus increasing synthesis of inflammatory mediators and degradative enzymes.

IL-6 is increased in early stage OA tissue (Scanzello *et al.*, 2009), particularly in synovial fluid, and is also found to be elevated throughout OA in synovial membranes and chondrocytes in equine OA (Ley *et al.*, 2009). There is evidence for a synergistic role for IL-6 with other pro-inflammatory cytokines in OA, in synovial fluid from early stage OA, IL-6 elevation is concomitant with an elevation in IL-15 levels (Scanzello *et*

al., 2009), and in blood serum from patients with active OA it is elevated alongside TNFα (Toncheva *et al.*, 2009). In an *in vitro* model of compression using primary calvaria osteoblasts from newborn mice, IL-6 mRNA was elevated alongside that of COX-2 (Sanchez *et al.*, 2009), which is known to be upregulated in human OA cartilage (Amin *et al.*, 1997), and in a canine model of OA (Pelletier *et al.*, 1998).

Anti-inflammatory cytokines and osteoarthritis

The anti-inflammatory cytokines IL-4, IL-10 and IL-13 act to decrease production of IL-1β, TNFα and the MMPs, and together with IL-6, increase synthesis of tissue inhibitor of MMP-1 (TIMP-1) and the IL-1 receptor antagonist, IL-1Ra (Hart *et al.*, 1989, Essner *et al* 1989, Vannier *et al.*, 1992, Hart *et al.*, 1995, Jovanovic *et al*, 1998b). These effects have been demonstrated both *in vitro* in cell lines, and *ex vivo* in cells cultured from OA patients. The effect of these mediators is to block a number of catalytic pathways associated with OA such as PGE_2 synthesis, and chondrocyte mediated production of collagenase and nitric oxide (Figure 4.2). The anti-inflammatory cytokines are spontaneously produced in OA synovial membrane and cartilage, and increased levels have been shown in synovial fluid (but not in the synovial membrane - Farahat *et al.*, 1993, Smith *et al.*, 1997, Pelletier *et al.*, 1995) of patients with OA (Schlaak *et al.*, 1996, Martel-Pelletier *et al* 1999), possibly as a response to the increased presence of the inflammatory mediators. However, the increased levels of anti-inflammatory cytokines are not concomitant with decreases in levels of the inflammatory cytokines (Schlaak *et al*, 1996), and while a high ratio of IL-1Ra: IL-1 is found in synovial fluid of patients with OA (Richette *et al.*, 2008), it is considered to be insufficient to negate the effects of the increased levels of IL-1β.

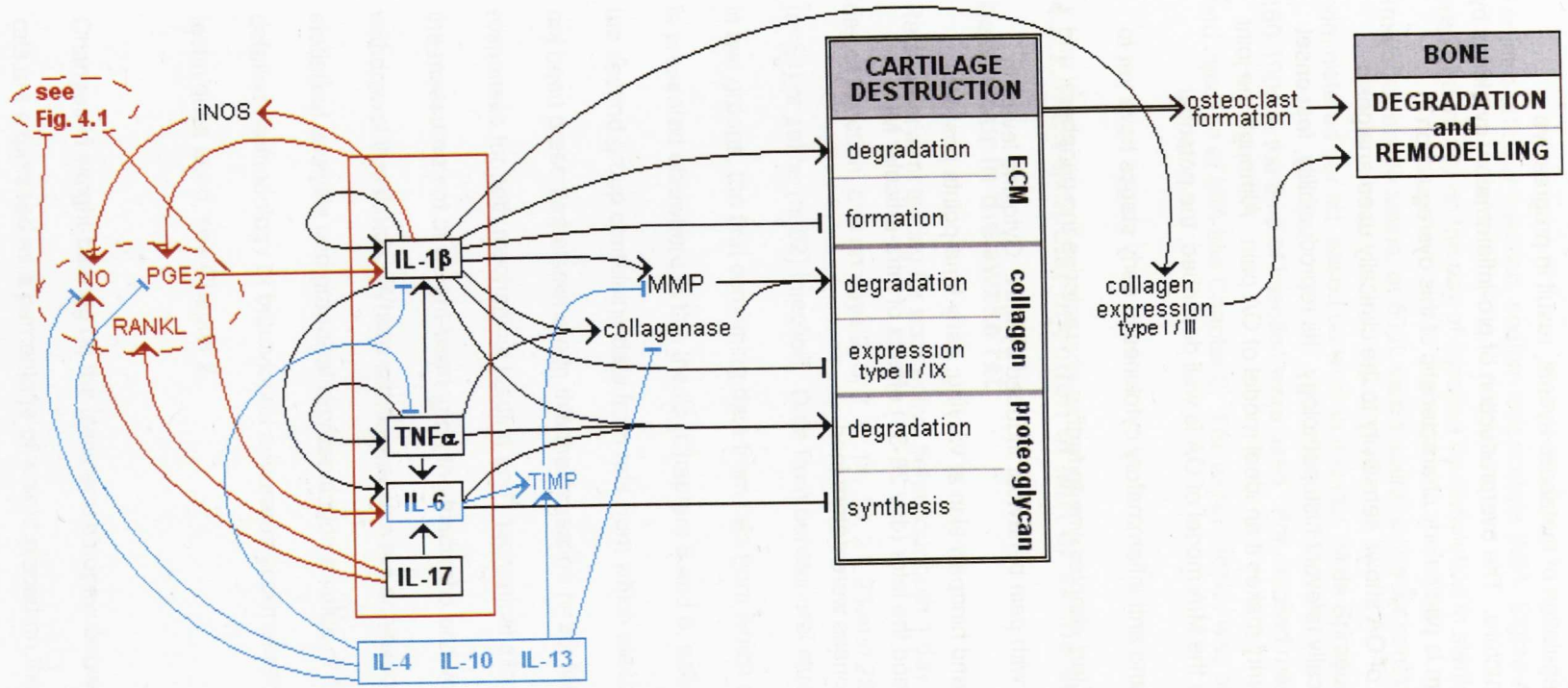


Figure 4.2 A summary schematic of the roles of cytokines in bone and cartilage integrity in osteoarthritis. Black denotes destructive effects of the pro-inflammatory cytokines; **blue** denotes protective effects of anti-inflammatory cytokines (IL-6 is both pro- and anti-inflammatory); **red** denotes effects on prostaglandins, nitric oxide (NO) and receptor activator of nuclear factor kappa B ligand (RANKL) as previously shown in Figure 4.1. The pro-inflammatory cytokines autoamplify and mediate cartilage destruction through effects on extracellular matrix (ECM), collagen and proteoglycan. Cartilage destruction triggers osteoclast formation, causing bone degradation and remodelling. IL-1β also directly causes bone changes and remodelling through activation of collagen types I and III. The pro-inflammatory cytokines also activate prostaglandin synthesis, of which PGE2 feeds back to amplify synthesis of IL-1β and IL-6. IL-1β is also involved in the generation of NO through inducible nitric oxide synthase (iNOS) activation, as is IL-17, which is also involved in activation of RANKL. The anti-inflammatory cytokines inhibit NO and PGE2 production, synthesis of collagenase and the pro-inflammatory cytokines IL-1β and TNFα and, together with IL-6, increase synthesis of tissue inhibitor of matrix metalloprotease (TIMP).

4.1.3. Aims

In osteoarthritis, the imbalance between anabolic and catabolic events in chondrocytes and a dysregulation of metabolic events, result in progressive destruction of the joint structure. The overproduction of pro-inflammatory cytokines by cartilage and the synovium is particularly characteristic of this dysregulation in patients. The MIA model of OA shows sensitivity to the clinically used analgesic morphine, as well as clinically relevant histopathology. Its reproducibility, fast onset time and controllable severity makes it an ideal model of OA pain. Although the joint pathology associated with the MIA model of OA is well described, the potential changes in levels of pro- and anti-inflammatory cytokines in early stages have yet to be described. The aim of this chapter was to further characterise the peripheral and central events associated with pain behaviour induced by MIA. Cytokine levels in synovial fluid, spinal cord and hindpaw skin at varying, early time-points post-MIA injection were measured, and the later (day 28-31) effects of intra-articular MIA injection on neuronal responses were determined.

4.2. Methods

4.2.1. Induction of the MIA model of osteoarthritis pain

For intra-articular injection, sodium iodoacetate (MIA, Sigma-Aldrich, Dorset, UK), was prepared freshly on the day of injection by dissolution in sterile saline. A dose of 1mg/50µL sterile saline, or 50µL sterile saline alone for control groups, was administered per rat, based on work in this lab. Male Sprague-Dawley rats weighing 160-190g at the time of injection were used. For detailed methodology of anaesthesia and injection of MIA see Chapter 2. The experimenter was blinded to all treatments.

4.2.2. Intra-articular injection of MIA or saline and nociceptive behaviour in the awake rat

Rats underwent testing for nociceptive behaviour both 1 day before injection, on the day of injection, and on days 2, 3, 7, 9, 14, 16, 21, 23 and 28 following injection of MIA (n=63) or saline (n=62) injection. Data from behavioural studies herein are expressed in two groups, the first containing data from rats from which electrophysiological data is presented elsewhere in this thesis (Chapters 5 and 6, saline n=36, MIA n=36), and the second group containing data from rats from which electrophysiological data has *not* been presented elsewhere in this thesis (saline n=26, MIA n=27). Nociceptive responses for both mechanical loading and mechanical allodynia were tested, through the measurement of weight-bearing on the hindlimbs and von Frey monofilament withdrawal thresholds. Where withdrawal was not achieved with a stimulus of 15g, in statistical analysis and graphical representation a value of 16g was assigned. For a detailed methodology of behavioural apparatus acclimatisation and behavioural testing techniques used, see Chapter 2.

Changes in weight-bearing on the ipsilateral hindpaw between MIA and saline treated rats are expressed as a percentage of weight placed on the contralateral paw in the same rat. Responses to application of von Frey monofilaments to the plantar surface of the hind paw are expressed as the difference between withdrawal thresholds of

ipsilateral paw, compared to the contralateral paw, in grams. Statistical analysis comparing the effects of intra-articular injection of MIA and saline in both weight-bearing and withdrawal threshold measurements were performed using a one-way ANOVA Kruskal-Wallis, with post-hoc Dunn's test.

4.2.3. Intra-articular injection of MIA or saline and electrically and mechanically evoked responses of WDR neurones in the anaesthetised rat

For detailed methodology of anaesthesia, surgery, and identification and recording of WDR neuronal responses, see Chapter 2. In many of the experiments in this chapter, data from more than one WDR neurone from the dorsal horn of the spinal cord was studied per rat, thus maximising the obtainable data from the animals used. The responses of neurones to electrical and mechanical stimulation of the hindpaw were characterised. For electrical characterisation, the threshold (mA) and latency (ms) of electrical stimulation of C fibres (as described in Chapter 2) were compared between rats receiving an intra-articular injection of MIA (n=70 neurones in 58 rats) and those receiving saline alone (n=66 neurones in 47 rats). The number of action potentials of Aβ-, Aδ-, and C-fibres, as well as the total action post-discharge (as defined in Chapter 2) was also compared between the groups, following a train of 16 stimulations at 3x the C-fibre threshold, delivered at a rate of 0.5Hz, as described in Chapter 2 (saline n=70 neurones in 47 rats, MIA n=80 neurones in 58 rats). In all of the electrical characterisation of neurones, statistical comparisons were performed using a non-parametric Mann-Whitney test.

The effects of intra-articular injection of MIA or saline on mechanically evoked responses were studied as described in Chapter 2. In addition, post-stimulus neuronal responses were studied following mechanical stimulation with each of the von Frey stimuli presented, and the effects of intra-articular injection of MIA or saline on post-stimulus responses were compared. Four measures of post-stimulus response were studied: the duration of post-stimulus neuronal response (s), the post-

stimulus neuronal response count, the average rate of post-stimulus neuronal response (spikes per second) for the first 10s after stimulus removal (regardless of whether or not the response had ceased before that time had passed), as well as the rate of post-stimulus neuronal response (spikes per second) over the total duration of post-stimulus response (Figure 4.3, located at end of section 4.2). Statistical comparisons of the effects of MIA- and saline-injection on during- and post-stimulus responses were performed using non-parametric Mann-Whitney test.

4.2.4. Intra-articular injection of MIA or saline and levels of cytokines in the synovial fluid, hindpaw skin and spinal cord

In a separate group of rats to those used for behavioural and electrophysiological characterisation of the MIA model of OA pain, the effects of intra-articular injection of MIA or saline on levels of cytokines in the synovial fluid, hindpaw skin and spinal cord of the rat were analysed, on two separate occasions.

On the first occasion, the effects were studied at 6 and 24 hours post injection. A total of 32 Male Sprague-Dawley rats weighing 130-150g were used, 16 rats received 1mg MIA in 50µL sterile saline, prepared and injected as described above, and 16 rats received 50µL sterile saline alone, acting as controls. Six or 24 hours after injection (n=8 rats MIA and 8 rats saline per time-point), rats were killed by stunning and decapitation, and the synovial fluid, hindpaw skin and spinal cord (ipsilateral and contralateral) were collected as described in Chapter 2, and stored at -80°C until further analysis. Not all tissue was analysed, due to difficulty in removal of tissue (particularly synovial fluid), as well as limitations of the analytical technique used (96 well plate with space for 88 samples). The analysis groups consisted of the following: MIA treated, 6 hours post-injection, n=7 rats; MIA treated, 24 hours post-injection, n=7 rats; saline treated, 6 hours post-injection, n=6 rats; saline treated, 24 hours post-injection, n=6 rats. From each rat, ipsilateral and contralateral synovial fluid, hindpaw skin and spinal cord samples were analysed by electrochemiluminescence, using the MSD® 96-well MULTI-SPOT® Standard MS6000 "Rat Demonstration 7-PlexUltra-Sensitive Kit" (Meso Scale Discovery, Maryland, US), a cytokine assay kit allowing

simultaneous analysis of the cytokines IL-1β, TNFα, IL-13, IL-4, IL-5, IFNγ and GRO/KC, of which the pro-inflammatory IL-1β and TNFα, and the anti-inflammatory IL-13 were of particular interest.

Samples were prepared for analysis as follows:

Spinal cord samples were weighed, thawed and agitated using a pipette in 100µL High Performance ELISA buffer (Europa Bioproducts Ltd., Ely, Cambridge, UK) with Complete EDTA protease inhibitor (Roche Diagnostics GmbH, Mannheim, Germany), sonicated on ice and centrifuged for 14,000xg for 15 minutes at 4°C, and the supernatant collected for analysis.

Paw tissue was prepared using cryogenic pulverisation: samples were frozen further in liquid nitrogen and pulverised using the CryoPrep™ apparatus (Covaris, Massachusetts, US). Samples were weighed, before pipette-agitation, sonication and centrifugation, in 100µL ELISA buffer, as described above, and the supernatant collected for analysis.

Synovial fluid was used neat, without processing.

Samples were analysed using the "tissue culture protocol" recommended with the kit, using kit reagents, as follows:

25µL of each sample (synovial fluid) or supernatant (spinal cord and paw tissue) was pipetted into a well on the 96 well plate along with standard concentration curve samples spanning cytokine concentrations of 0-10,000pg/mL over 8 wells. The plate was then sealed with an adhesive plate seal and incubated on a shaker at 800 r.p.m. for 1.5hours at room temperature. 25µL of the secondary antibody (detection antibody solution) was then added to each well, the plate re-sealed and incubated as above for a further 1.5 hours. Each well was then washed three times with 100µL 0.05% Tween 20 in PBS (NaCl 137mM, KCl 2.7mM, Na_2HPO_4, 8.1mM, KH_2PO4 1.5mM; pH7.3) and 150µL of the 2x Read Buffer provided in the kit was added to each well. After careful removal of any bubbles with a sterile needle tip, luminescence was analysed in the SECTOR® Imager 6000 (Meso Scale Discovery, Maryland, US).

Results are expressed as raw concentrations of cytokine (ng or pg) per mL (synovial fluid) or gram (hindpaw skin, spinal cord) of tissue. The lower limits of detection of the

cytokines analysed were as follows: rIL-1β, 25pg/mL; rTNFα, 10pg/mL; rIL-13, 6pg/mL. Statistical analyses were performed using a non-parametric Mann-Whitney test, or one way ANOVA Kruskal-Wallis test with post-hoc Dunn's test, where appropriate.

Following the results of this analysis (section 4.3.4) the study was repeated with some alterations. The range of time-points post-injection was increased to include tissue collection 3 hours post-injection, in recognition of early inflammatory events in OA. Analysis switched to a Bio-plex™ cytokine assay system (Bio-Rad Laboratories Inc., California, US), with higher sensitivity and flexibility over which cytokines could be analysed. In light of this, the focus was on the cytokines IL-1β and TNFα as before, with the addition of IL-6, which could not be analysed using the MSD multi-spot assay system. Samples analysed were also scaled back to include only synovial fluid samples, which in human OA conditions has frequently been found to contain elevated levels of cytokines (see section 4.1.2) and spinal cord samples, as injection of the Feline Immunodeficiency Virus (FIV) into mouse temporomandibular joint, or cisterna magna, induced IL-1β expression in the dorsal horn and spinal horn respectively, coinciding with arthritic behaviour (Fiorentino *et al.*, 2008), lending credence to the hypothesis that central changes in cytokines may contribute to the progression of OA.

A total of 91 male Sprague-Dawley rats weighing 130-150g were used, 42 received 1mg MIA in 50μL sterile saline, prepared and injected as described above, and 49 rats received 50μL sterile saline alone, acting as controls. 3, 6 or 24 hours after injection, rats were killed as above, and the ipsilateral and contralateral spinal cord and synovial fluid were collected and stored at -80°C until further analysis, as above. For analysis, at least 6 samples per group (treatment group and per time-point) were studied.

Samples were analysed using immunosorbence assay, by Ian Spendlove, City Hospital, Nottingham UK following the protocol supplied for the Bio-Plex Cytokine Assay kit (Bio-Rad Laboratories Inc., California, US). Spinal cord samples were weighed, thawed and agitated using a pipette in 1mL 1% Bovine Serum Albumin (BSA – Sigma-Aldrich, UK) in PBS, sonicated and centrifuged at 14,000xg for 15 minutes at

4°C and the supernatant collected. Synovial fluid samples were used neat, without processing.

Samples were analysed using the "tissue culture protocol" recommended with the kit, using kit reagents, as follows:

Vacuum pressure under a flat-bottomed 96-well plate was set to 2 inches Hg, and the filter plate pre-wet with 100µL Bio-Plex assay buffer as supplied in the kit. Anti-cytokine (IL-1β, TNFα and IL-6) beads prepared in Bio-Plex Assay Buffer A were added to each well in a 50µL volume, followed by 1x vacuum filtration and 2x filter wash with 100µL Bio-Plex wash buffer. 50µL of each sample (synovial fluid) or supernatant (spinal cord) was pipetted into a well, along with standard concentration curve samples spanning cytokine concentrations of 0-20,000pg/mL over 9 wells. The plate was then sealed with an adhesive plate seal, covered with foil and incubated on a shaker at 1100 r.p.m for 30 seconds decreasing to 300 r.p.m. for a total of 30 minutes. Next, the plates were filter washed 3x 100µL Bio-Plex wash buffer, followed by addition of 25µL of the secondary, detection antibody, prepared in Bio-Plex Detection Antibody Diluent, from the antibody stock solution, into each well. Plates were re-sealed and incubated as above for a further 30 minutes before filter washing 3x with 100µL Bio-Plex wash buffer, and addition of 50µL Streptavadin-PE, prepared in Bio-Plex Assay buffer, into each well. Plates were re-sealed and incubated as above for a further 30 minutes, then washed as before and the beads resuspended in 125µL Bio-Plex Assay Buffer, re-sealed and shaken at room temperature for 30 seconds at 1100 r.p.m, before luminescence was measured in the Bio-Plex reader. Results are expressed as raw concentrations of cytokine (ng or pg) per gram spinal cord or mL synovial fluid. Statistical analyses were performed using a non-parametric Mann-Whitney test.

Throughout the studies presented herein, data deviating from the mean by more than two standard deviations were excluded. For statistical comparisons, statistical significance was accepted at $P<0.05$.

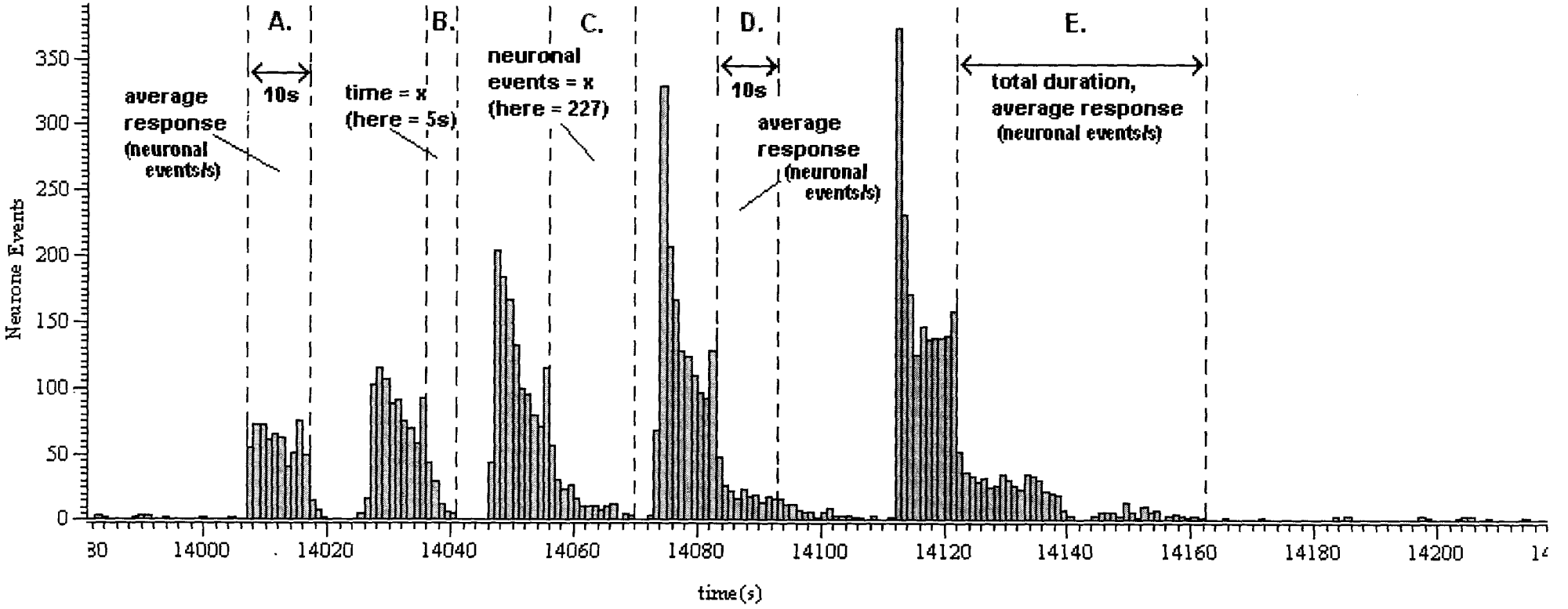


Figure 4.3 Study of stimulus-evoked and post-stimulus responses of WDR dorsal horn neurones. **A**, stimulus-evoked response over the 10s stimulus period; **B**, duration of post-stimulus neuronal response; **C**, post-stimulus neuronal response count; **D**, average rate of post-stimulus neuronal response over the first 10s period following stimulus removal; **E**, average rate of post-stimulus neuronal response over the total duration of post-stimulus neuronal response. (bin size = 1s).

4.3. Results

4.3.1. Effects of intra-articular injection of MIA or saline on nociceptive responses of awake rats

The effects of intra-articular injection of 1mg MIA in 50µL saline, or saline alone, on weight-bearing through the ipsilateral hindlimb, and withdrawal threshold of the ipsilateral hindpaw in response to punctuate mechanical stimuli on the plantar surface of the foot, were studied over a 28 day period. MIA caused a significant drop in weight borne on the ipsilateral hindlimb (Figure 4.4; saline, n=62; MIA, n=63, one-way ANOVA Kruskal-Wallis with post-hoc Dunn's test). Diminished weight-bearing on the ipailateral hindlimb was biphasic, with the first, more pronounced phase reducing by day 9. Weight-bearing on the ipailateral hindlimb remained reduced throughout the duration of the 28-day study period post-injection. MIA also caused a significant decrease in the withdrawal threshold of the ipsilateral hindpaw (Figure 4.5; saline, n=62; MIA, n=63, one-way ANOVA Kruskal-Wallis with post-hoc Dunn's test) from day 2 after injection. The withdrawal threshold remained significantly lower than in saline-treated rats for the duration of the study.

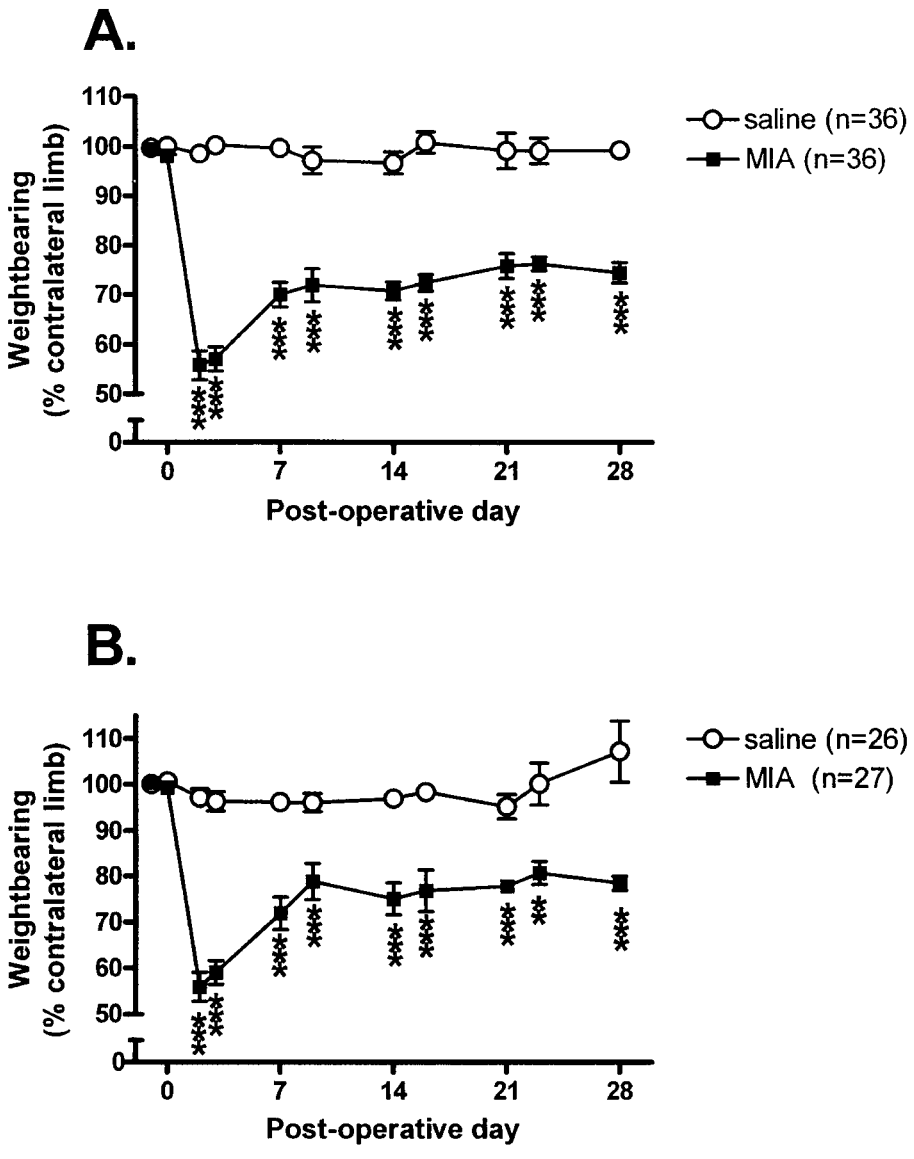


Figure 4.4 Reduced weight-bearing on the ipsilateral hindlimb of rats receiving intra-articular injection of 1mg MIA in 50µL saline (black squares, n=63) versus 50µL saline alone (open circles, n=62), over a 28-day period post-injection. A, Data from rats used in electrophysiological studies described in Chapters 5 and 6; **B,** Data from rats not used in electrophysiological studies elsewhere in this thesis. Statistical analysis comparing the effects of saline to MIA on weight-bearing through the ipsilateral hindlimb was performed using a one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test, $^{**}P<0.01$; ***, $P<0.005$. No statistically significant differences in weight-bearing deficiencies were seen between data presented in panels A and B (one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test). Weight-bearing through the ipsilateral hindlimb is expressed as a mean percentage of the weight borne on the contralateral hindlimb, averaged from 3 readings per rat, per time-point, ± SEM.

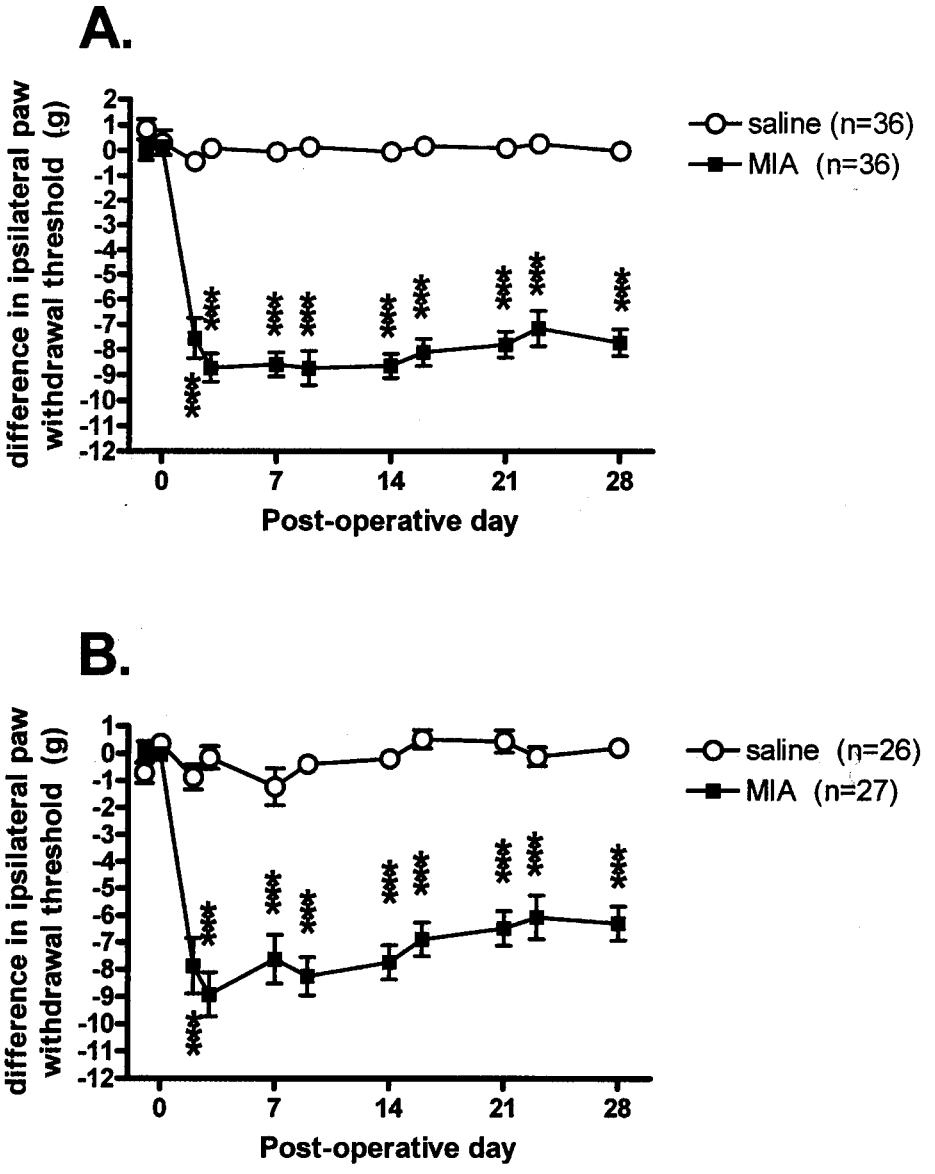


Figure 4.5 Reduced withdrawal thresholds to mechanical stimuli of the ipsilateral hindpaw of rats receiving intra-articular injection of 1mg MIA in 50µL saline (black squares, n=63) versus 50µL saline alone (open circles, n=62), over a 28-day period post-injection. A, Data from rats used in electrophysiological studies described in Chapters 5 and 6; **B**, Data from rats not used in electrophysiological studies elsewhere in this thesis. Statistical analysis comparing the effects of saline to MIA on withdrawal threshold differences between ipsilateral and contralateral hindpaws was performed using a one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test; ***, $P<0.005$. No statistically significant differences in withdrawal thresholds were seen between data presented in panels A and B (one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test). Data are expressed as the mean difference in grams between withdrawal thresholds of the ipsilateral and contralateral hindpaws ± SEM.

4.3.2. Effects of intra-articular injection of MIA or saline on electrically evoked responses of WDR dorsal horn neurones 28-31 days post-injection

The effects of intra-articular injection of 1mg MIA in 50μL saline, or saline alone, on the electrical threshold (mA) and latency of C fibre-evoked responses of WDR dorsal horn neurones were studied. There were no differences in the electrical threshold for C fibre activation of WDR neurones in saline- versus MIA-treated rats (saline, 1.17 ± 0.03mA, n=66 neurones in 47 rats; MIA, 1.25 ± 0.04mA, n=70 neurones in 58 rats, non-parametric Mann-Whitney, $P>0.05$). There was, however, a decrease in the latency of C fibre-evoked responses of WDR neurones in MIA-treated rats: saline, 204.7 ± 7.19ms, n= 70 neurones in 47 rats; MIA, 186.76 ± 7.49, n=79 neurones in 58 rats, $*P<0.05$, non-parametric Mann-Whitney test.

Aβ, Aδ, C fibre and post-discharge responses of WDR neurones during a train of 16 electrical stimulations, given at 3 times the C fibre threshold at a rate of 0.5Hz into the toes of the hindpaw, were also compared between the two treatment groups. No differences were observed (Table 4.2.). The mean depths of recording of WDR neurones in saline- and MIA-treated rats are indicated in Table 4.2.

Table 4.2 Mean depth of recording and number of action potentials evoked by supramaximal electrical stimulation of C fibres in the Aβ, Aδ, C fibre and post-discharge latency bands. Statistical comparisons between the treatment groups were performed using a non-parametric Mann-Whitney test, no significant differences were observed (all $P>0.05$). Data are expressed as mean evoked spike count ± SEM.

	Firing (count)	
	Saline-treated (n=70 neurones, 47 rats) Mean depth = 839.0 ± 23.0 μm	MIA-treated (n=80 neurones, 58 rats) Mean depth = 834.8 ± 24.7μm
Aβ (0-20ms)	187.5 ± 6.3	190.5 ± 5.6
Aδ (20-90ms)	157.6 ± 9.2	148.6 ± 8.59
C fibre (90-300ms)	346.2 ± 29.2	326.5 ± 19.1
post-discharge (>300ms)	347.0 ± 37.2	313.87 ± 27.35

4.3.3. Effects of intra-articular injection of MIA or saline on mechanically evoked responses of WDR dorsal horn neurones

The effects of intra-articular injection of 1mg MIA in 50µL saline, or saline alone, on hindpaw mechanically evoked responses of WDR dorsal horn neurones, were studied using data from a total of 42 neurones in 41 saline-treated rats 48 neurones in 47 MIA-treated rats. Data deviating from the mean by more than two standard deviations were excluded, in total 11 out of 210 neurones were excluded from saline-treated rats, and 10 out of 240 neurones were excluded from MIA-treated rats. Mechanically evoked responses of neurones were graded to increasing stimuli applied to the hindpaw, and there was a trend for responses in MIA-treated rats to be higher than in saline-treated rats. Significant differences between the treatment groups were observed for the noxious 15g and 26g-evoked responses (Figure 4.6). An example of a typical trace obtained from saline- and MIA-treated rats is shown in Figure 4.7, with an arrow indicating the visible increase in post-stimulus response of WDR neurones in MIA-treated rats.

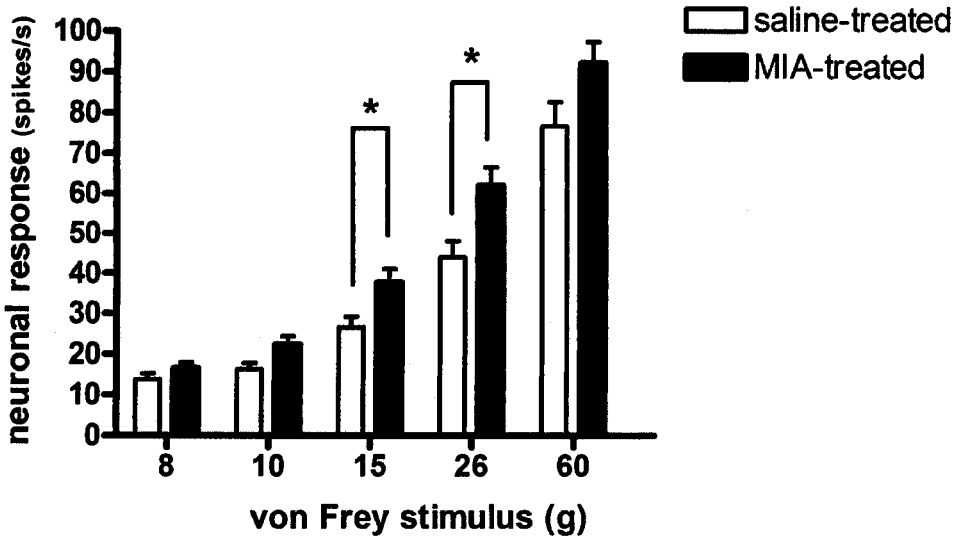


Figure 4.6 Graded responses of WDR neurones in saline (white bars, n=42) and MIA- (black bars, n=48) treated rats, following mechanical stimulation of the hindpaw receptive field. Statistical analysis comparing responses in saline- versus MIA-treated rats were performed using a non-parametric Mann-Whitney test; *, $P<0.05$. Data are expressed as the mean firing rate of the neurone per second (spikes/s) over a 10 second stimulation period, ± SEM.

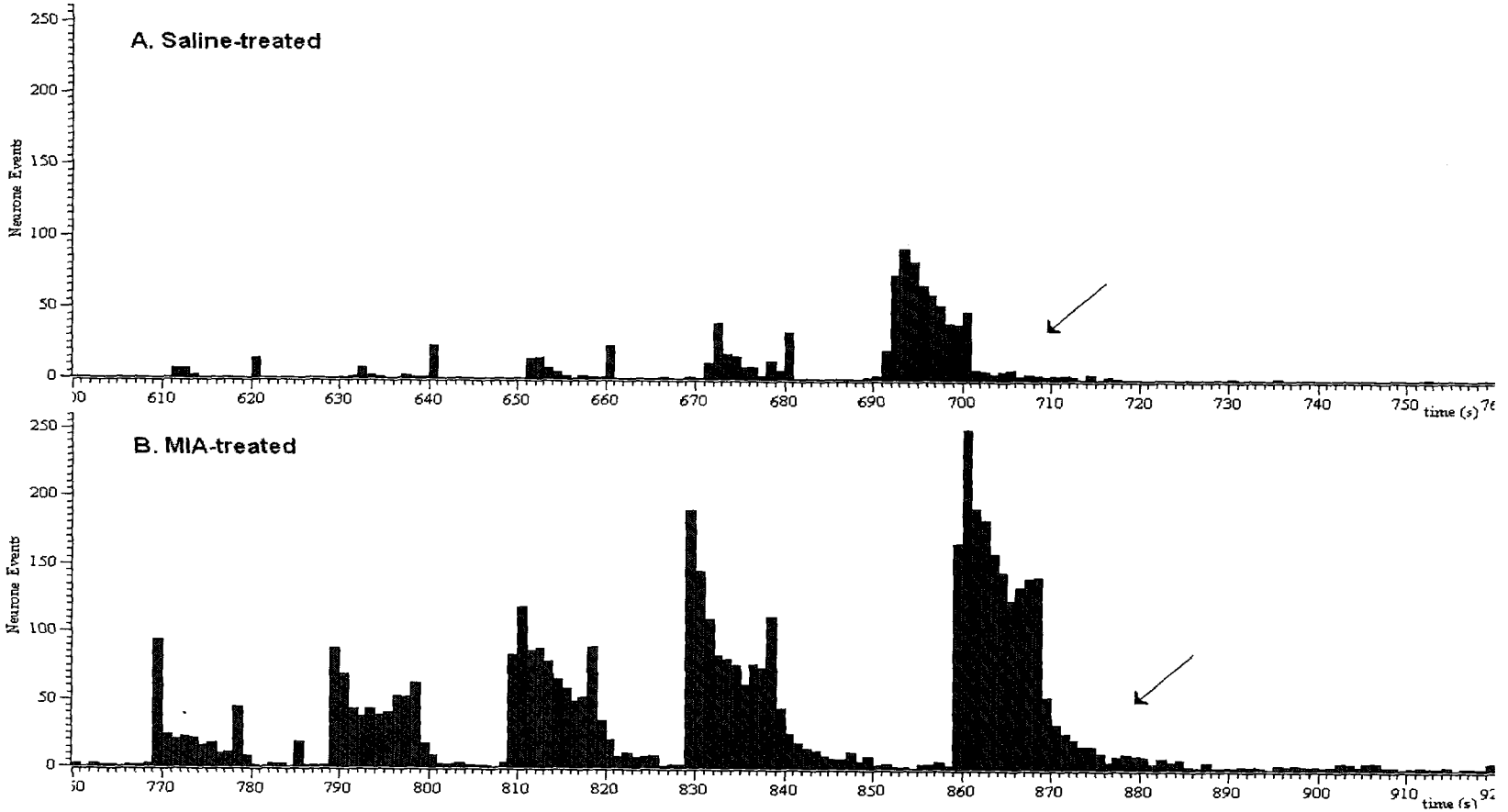


Figure 4.7 Example traces of the effects of intra-articular saline- and MIA- injection on mechanically evoked and post-stimulus responses of WDR dorsal horn neurones. Neuronal responses to mechanical stimuli are clearly elevated following MIA-injection (B) versus saline-injection (A). Post-stimulus responses in MIA-treated rats are also clearly larger and longer lasting than in saline-treated rats (indicated by the arrows). Bin width = 1s.

The post-stimulus responses of neurones were compared in MIA- and saline-treated rats. Data are from 36 MIA- and 36 saline-treated rats, used in studies outlined in Chapters 5 and 6. Data points more than 2 standard deviations from the mean of the group were excluded; in total for the four paradigms this meant exclusion of 47 out of 360 data points for the duration of post-stimulus neuronal firing; 53 out of 360 data points for the total post-stimulus neuronal firing count; 56 out of 360 data points for the average rate of neuronal firing over the total duration of firing; and 48 out of 360 points for the average rate of neuronal firing over the first 10s period following stimulus removal. MIA injection was associated with an increase in the duration of post-stimulus neuronal response in the MIA-treated rats compared to saline-treated rats (Figure 4.8, **A**), which reached significance for all von Frey weights, with the exception of the 8g stimulus. MIA injection also caused an increase in the number of post-stimulus neuronal events (Figure 4.8 **B**) following innocuous and noxious stimulation, although this did not reach significance for 8g and 60g stimuli. The rate of post-stimulus action potential firing was also calculated, both for the duration of post-stimulus response (Figure 4.8 **C**) and for the first 10 seconds once stimulation had ceased, regardless of whether or not the neuronal response continued up to, and beyond that duration, as a measure of post-stimulus neuronal response intensity (Figure 4.8 **D**). MIA injection caused an increase in the rate of post-stimulus neuronal response to all stimuli intensities, with significant effects observed for all weights apart from the 8g evoked response over the first 10 seconds, and the 60g evoked response for the duration of response.

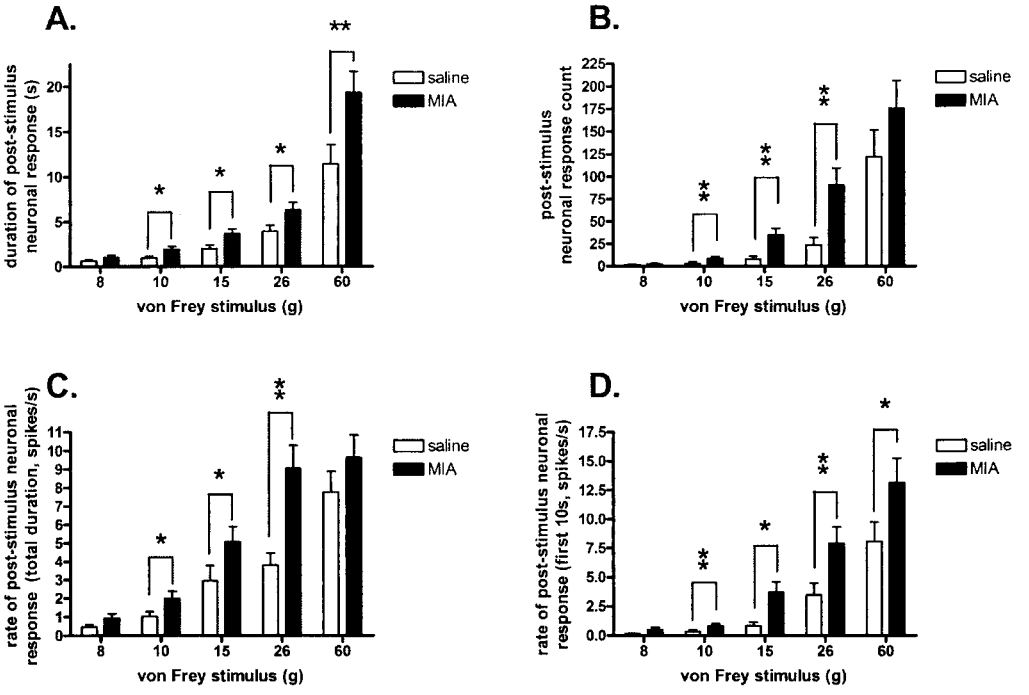


Figure 4.8 Post-stimulus responses of WDR neurones in saline (white bars, n=36) and MIA- (black bars, n=36) treated rats, following removal of mechanical stimuli from the hindpaw receptive field. A, duration of post-stimulus neuronal response; **B**, post-stimulus neuronal response count; **C**, rate of post-stimulus neuronal response over total duration of post-stimulus response; **D**, rate of post-stimulus neuronal response in the first 10-second post-stimulus period. Statistical analysis comparing the effects of saline to MIA were performed using a non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$. Data are expressed mean ± SEM.

4.3.4. Effects of intra-articular injection of MIA or saline on levels of cytokines in the synovial fluid, hindpaw skin and spinal cord

The effects of intra-articular injection of 1mg MIA in 50µL saline, or saline alone, on levels of cytokines in synovial fluid, spinal cord and paw tissue within the first 24 hours after injection, were studied in two separate studies.

In the first study, MIA caused a decrease in levels of the anti-inflammatory cytokine IL-13 in synovial fluid 6 hours post-injection, compared to saline-treated controls (Saline 6hr n=6, MIA 6hr n=6) (Figure 4.9, left panel). By contrast, levels of IL-13 in the synovial fluid were comparable at 24 hours post-injection (Saline 24hr n=6, MIA 24hr n=7). Levels of the pro-inflammatory cytokines IL-1β or TNFα in synovial fluid were comparable in MIA- and saline-treated rats at 6 and 24 hours post-injection. An

important observation was that levels of cytokines in the ipsilateral joint were differentially influenced at 6 hours and 24 hours after intra-articular injection of saline (Figure 4.9, left panel), this was significant for IL-13 (Figure 4.9**A**). Thus comparisons between MIA-treated groups at 6 and 24 hours post-injection are not readily interpretable. Nevertheless, it is worth noting that there were significant differences between levels of IL-1β and TNFα at 6 and 24 hours post-MIA injection (Figure 4.9**B**, **C**). In synovial fluid from the contralateral joint, cytokine levels did not differ between the two treatment groups, or between the two time-points studied (Figure 4.9, right panel). Comparison of ipsilateral and contralateral synovial fluid indicates that injection *per se* causes rapid, transient changes in levels of the pro-inflammatory cytokines: in both MIA- and saline-treated rats, IL-1β levels were higher in the ipsilateral joint than in the contralateral joint 6 hours after injection (Saline, $P<0.05$; MIA, $P<0.01$), and TNFα levels were higher ipsilaterally in MIA-treated rats ($P<0.01$), with a trend towards higher ipsilateral TNFα levels in saline-treated rats. After 24 hours, no differences in cytokine levels were observed ipsilaterally versus contralaterally for either treatment group. There were no differences between ipsilateral and contralateral levels of IL-13 in either treatment group, at either time-point (Figure 4.9).

MIA treatment did not alter levels of IL-1β in the hindpaw, compared to saline controls, ipsilaterally or contralaterally, at either time-point. Levels of IL-1β however, significantly higher at 24 hours compared to 6 hours following MIA injection for both the ipsilateral and contralateral hindpaw. Note however, that in saline-treated rats, levels of IL-1β were also higher in the contralateral hindpaw at 24 hours following injection, compared to 6 hours (Figure 4.10 **A**). 6 hours after MIA injection, TNFα and IL-13 levels were below the limits of detection in the hindpaw of MIA treated rats, but was detectable at 24 hours, both ipsilaterally and contralaterally (Figure 4.10). There were no significant differences between ipsilateral and contralateral levels of cytokines at any time-point studied.

Levels of all cytokines in the spinal cord were below detection limits for both MIA- and saline-treated groups at both time-points studied (data not shown).

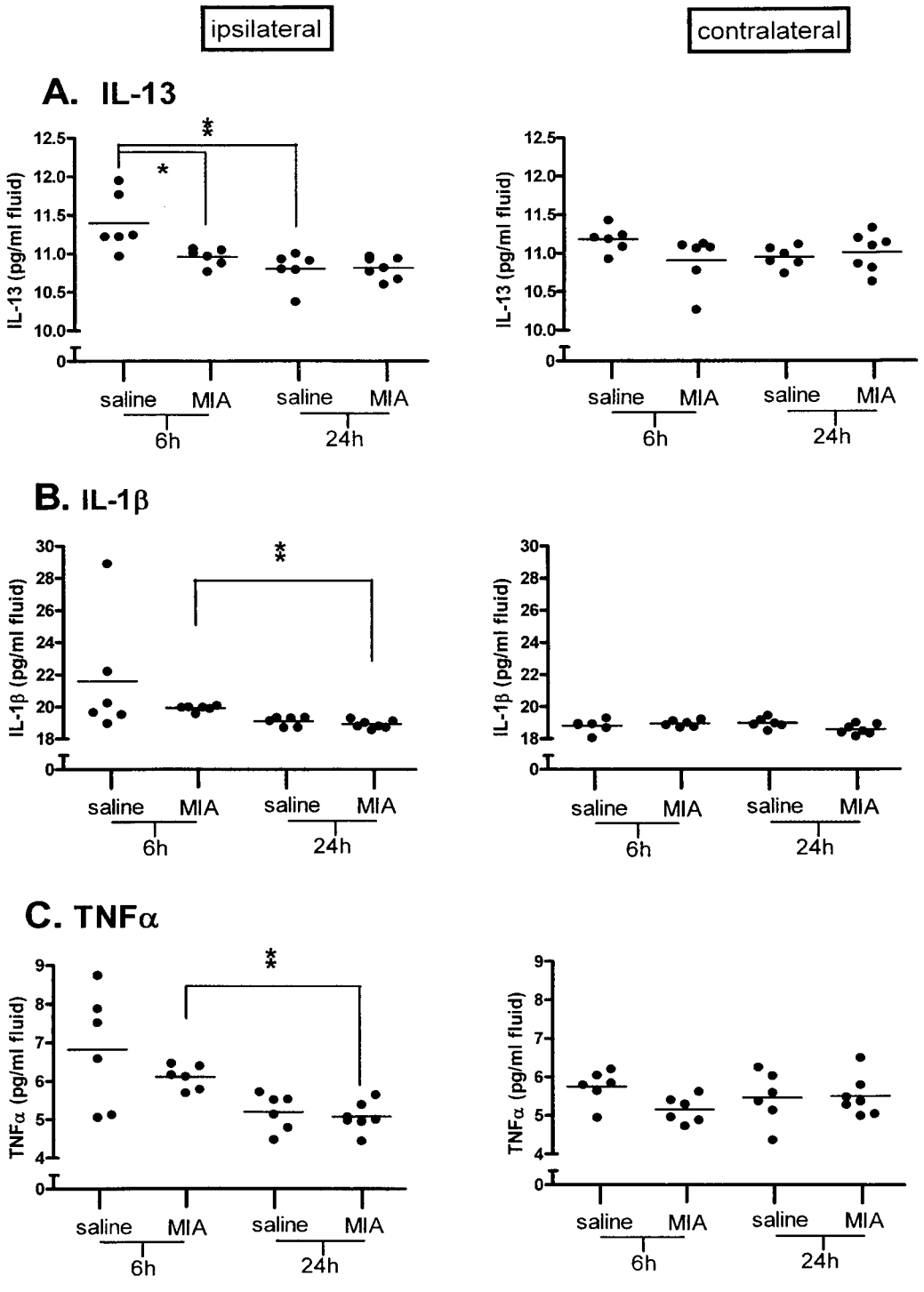


Figure 4.9 Cytokine levels in ipsilateral (left panel) and contralateral (right panel) synovial fluid 6- and 24-hours after injection with MIA (n=13 rats) or saline (n=12 rats). A, IL-13 **B**, IL-1β; **C**, TNFα. Statistical analyses comparing effects of MIA to those of saline at each time-point (*, $P<0.05$), and comparing the effects at 6h versus 24h intra-treatment; (**, $P<0.01$) were performed using a non-parametric Mann-Whitney test. Data are expressed as individual values and the line indicates the mean value.

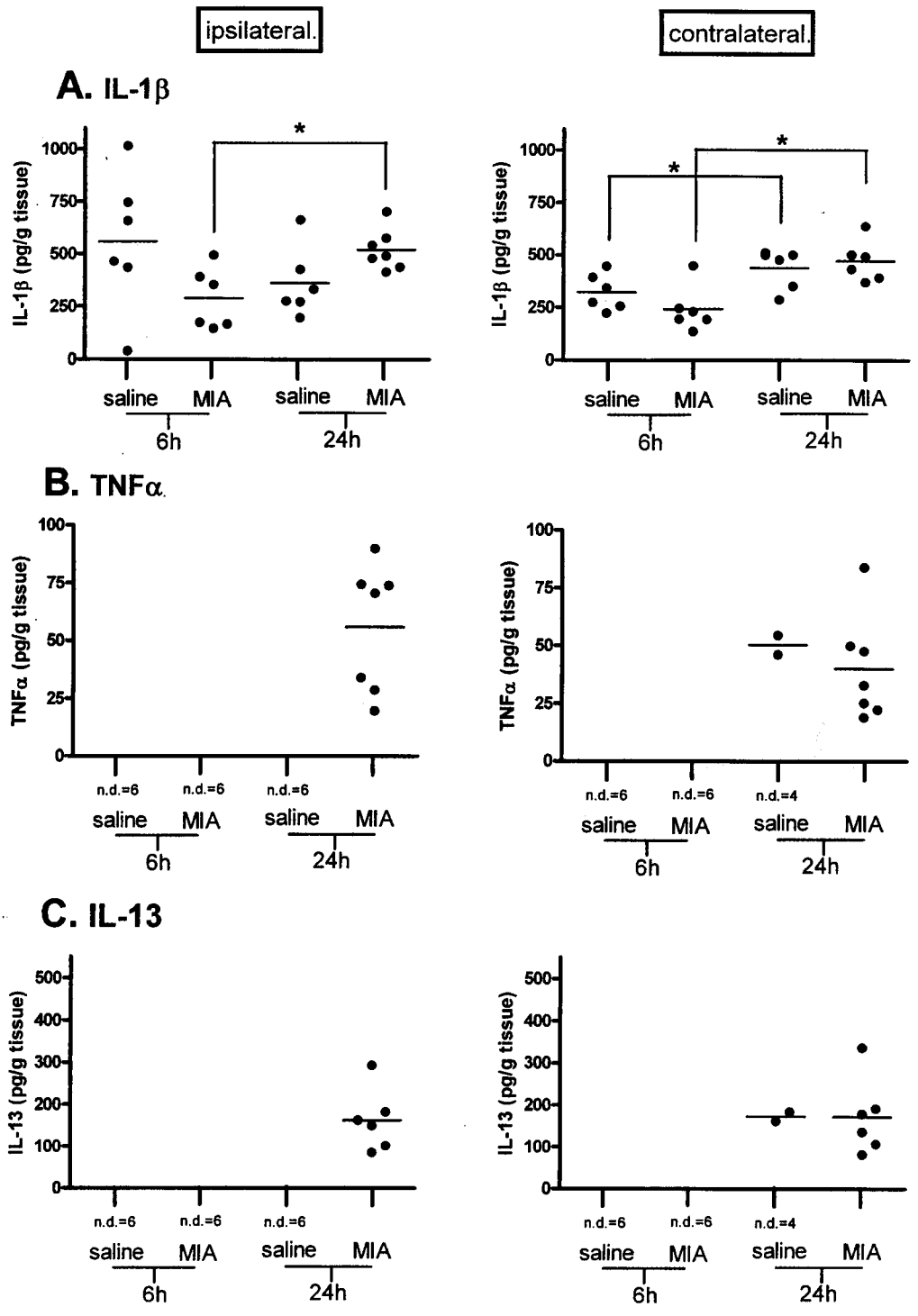


Figure 4.10 Cytokine levels in ipsilateral (left panel) and contralateral (right panel) hindpaw skin 6- and 24-hours after injection with MIA (n=13 rats) or saline (n=12 rats). A, IL-1β; **B**; TNFα; **C**, IL-13. Statistical analyses were performed comparing effects of MIA to those of saline at each time-point, and comparing the effects at 6h versus 24h intra-treatment, were performed using a non-parametric Mann-Whitney test; *, $P<0.05$. There were no differences in cytokine levels between the ipsilateral and contralateral joints within time-points and treatment groups studied. Data are expressed as individual values and the line indicates the mean value. n.d.= number of samples with levels of cytokines out of range of detection.

In the second study, focussing on synovial fluid, effects of MIA injection on IL-1β, TNFα, and also IL-6 levels, at more timepoints (3 hours n=14; 6 hours n=14; 24 hours n=14; total n=42 rats) were studied. Due to analystical constrictions (96-well plate), not all samples were analysed. The numbers of samples analysed per treatment group and per time-point are indicated below.

Treatment group	Time post-injection (hours)	Synovial fluid (no. of samples)		Spinal cord (no. of samples)	
		ipsilateral	contralateral	ipsilateral	contralateral
MIA	3	7	7	6	6
	6	6	6	6	6
	24	7	7	7	7
saline	3	7	7	7	7
	6	6	7	7	7
	24	6	7	7	7

There were no differences in levels of IL-1β in the synovial fluid between MIA- and saline-treated rats at any timepoint, except after 6 hours in the contralateral joint. However, there was a trend towards a decrease in synovial fluid levels of IL-1β in both treatment groups over time (Figure 4.11 panel **A**). Levels of IL-1β exhibited a trend towards being lower in ipsilateral synovial fluid than in the contralateral side, significantly so in saline-treated rats 6h, and MIA-treated rats 24h after injection. Effects of MIA or saline on levels of TNFα in the synovial fluid could not be ascertained using this method, as only 15 out of 80 samples reached detectable levels (Figure 4.11, panel **B**).

A trend towards an increase in IL-6 levels in synovial fluid of MIA- versus saline-treated rats was observed ipsilaterally at 3- and 6-hours post-injection (Figure 4.11, panel **C**), although this did not reach significance, and only 3 or 4 samples in each group were detectable (saline: 3h = 5 of 7 samples detectable, 1 value excluded; 6h = 5 of 6 detectable, 1 excluded; 24h = 2 of 6 detectable. MIA: 3h = 5 of 7; 6h = 3 of 6; 24h = 1 of 6). There was a trend towards decrease (non-significant) in IL-6 levels in ipsilateral synovial fluid with time in both treatment groups, most notably with fewer samples reaching detection limits at later time-points, and very few samples (saline, n=2; MIA, n=1) reaching detectable limits 24 hours after injection. Ipsilateral synovial fluid levels of IL-6 tended to be higher than contralateral levels, although this did not reach significance.

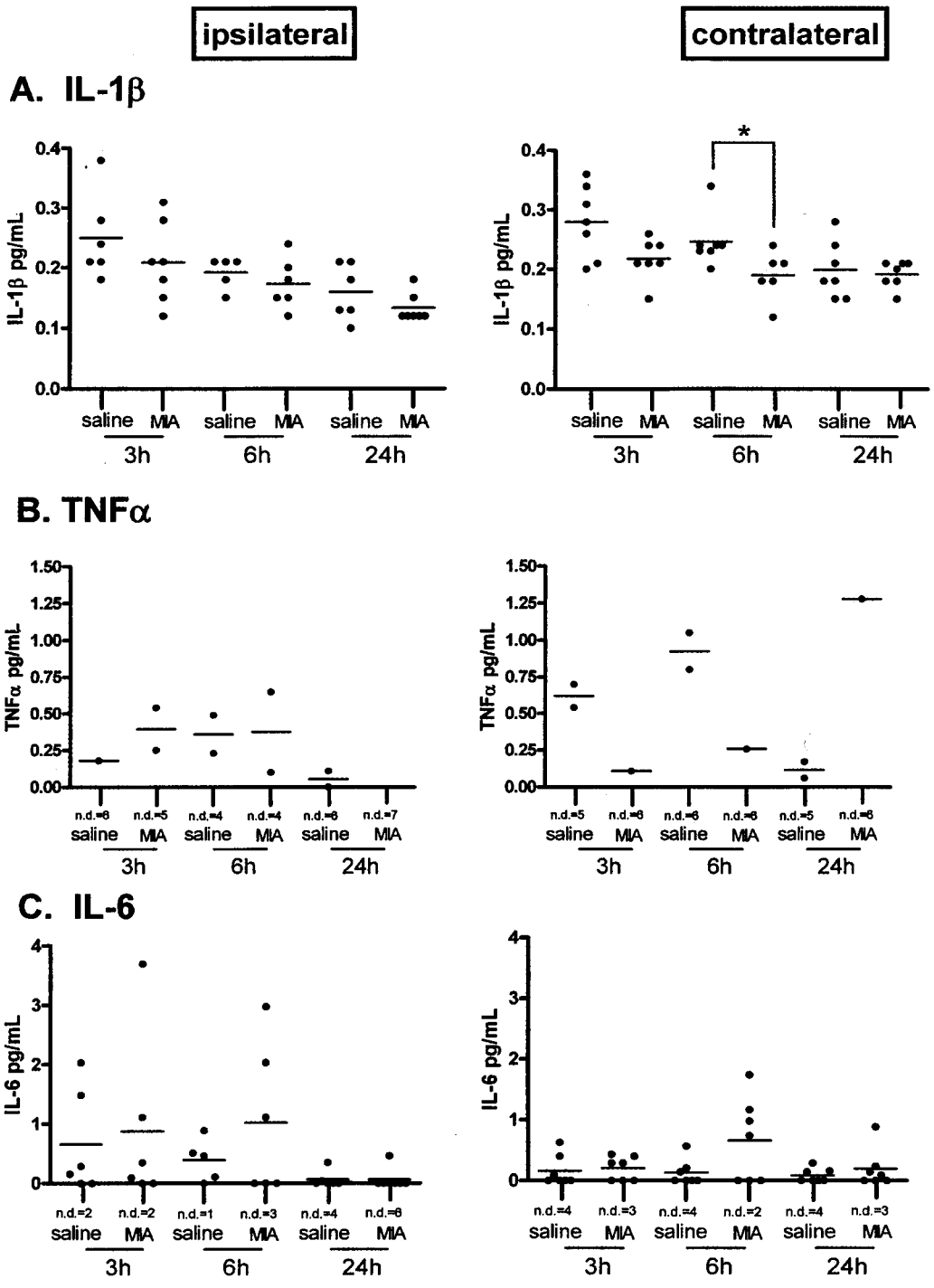


Figure 4.11 Cytokine levels in ipsilateral (left panel) and contralateral (right panel) synovial fluid 3-, 6- and 24-hours after injection with MIA (n=20) or saline (n=21). A, IL-1β; **B,** TNFα; **C**, IL-6. Statistical analyses comparing effects of MIA to those of saline at each time-point (*, $P<0.05$), and comparing the effects ipsilaterally versus contralaterally; (#, $P<0.05$; ##, $P<0.01$), were performed using a non-parametric Mann-Whitney test. Statistical analysis comparing cytokine levels between different time-points (3h versus 6h versus 24h) for MIA- and saline-treated rats were performed using non-parametric Kruskal-Wallis with post-hoc Dunn's test – no statistical differences were observed. Data are expressed as individual values and the line indicates the mean value. n.d. = number of samples with levels of cytokines out of range of detection.

In the spinal cord, IL-1β could not be detected with this method. For TNFα and IL-6, all samples in all groups fell within detectable limits. No effect of MIA was observed on spinal cord IL-6 or TNFα levels at any time-point.

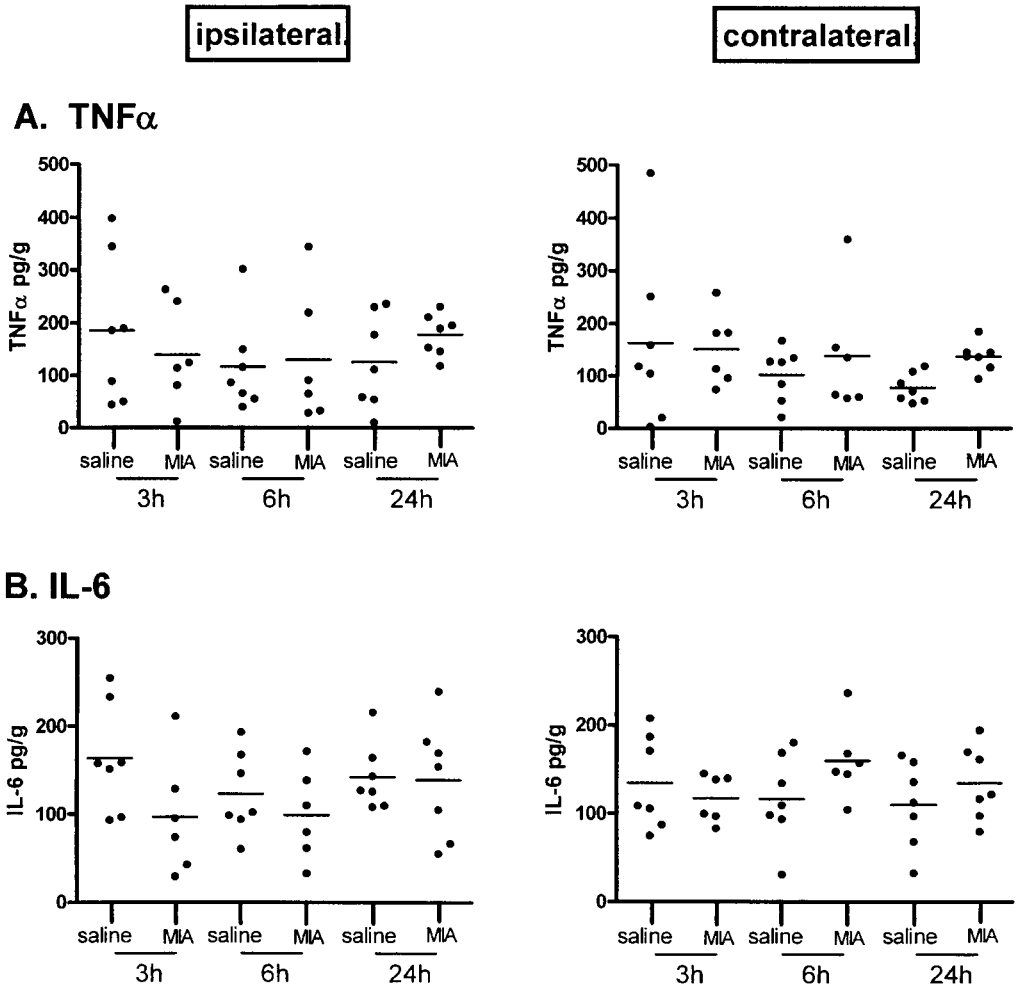


Figure 4.12 Cytokine levels in ipsilateral (left panel) and contralateral (right panel) spinal cord 3-, 6- and 24-hours after injection with MIA (n=19) or saline (n=21). A, TNFα; **B,** IL-6. Statistical analyses were performed comparing effects of MIA to those of saline at each time-point, and comparing the effects ipsilaterally versus contralaterally were performed using a non-parametric Mann-Whitney test (no significant differences). Statistical analysis comparing cytokine levels between different time-points (3h versus 6h versus 24h) for MIA- and saline-treated rats were performed using a non-parametric Kruskal-Wallis with post-hoc Dunn's test (no statistical differences). Data are expressed as individual values and the line indicates the mean value.

4.4. Discussion

The present study aimed to further characterise the MIA model of osteoarthritis pain with respect to behavioural and neurological responses, and the early changes in levels of cytokines of notable importance in the human condition (for review, see Pelletier *et al.*, 1991). Intra-articular injection of 1mg MIA brought about a robust, reproducible change in nociceptive responses, in two paradigms of nociception in the awake rat. Changes in withdrawal thresholds to punctuate mechanical stimuli were observable by the second day following injection of MIA, and were maintained, without sign of recovery, throughout the 28-day study period. Similarly, changes in hindlimb weight-bearing were observed. Changes in weight-bearing were biphasic, suggesting an initial inflammatory response following injection of the metabolic inhibitor, which is succeeded by structural changes within the knee joint itself (Guzman *et al.*, 2003; Ivanavicius *et al.*, 2007; Clements *et al.*, 2009) to produce a disease state in the knee similar to that seen in human OA. MIA injection was associated with neuronal hyperactivity in several paradigms of electrophysiological study. These data suggest changes in central mechanisms of nociception may be partly responsible for the nociceptive behaviour observed in the period preceding the electrophysiological studies. Increased responsiveness of WDR dorsal horn neurones in response to mechanical stimuli were observed, both during and after the period of stimulation. However, there were no alterations in responses to supra-maximal electrical stimulation of the receptive field, which may be due to a number of factors (see later). Injection into the knee was followed by time-dependent alterations in levels of some cytokines in the synovial fluid and putatively in the hindpaw skin and spinal cord, although these events appear to be due to the trauma of injection rather than an effect of MIA, as similar changes were evident in saline-treated controls. The work outlined here, together with previous studies studies using MIA (Guingamp *et al.*, 1997; Bove *et al.*, 2003; Fernihough *et al.*, 2004; Pomonis *et al.*, 2005), supports the use of MIA as a pre-clinical model of osteoarthritis pain, and suggests that central mechanisms may be partly responsible for underlying chronic pain responses.

4.4.1. MIA injection caused behavioural deficits translatable to human OA pain

MIA caused a decrease in weight borne on the ipsilateral hindlimb, as well as a decrease in the withdrawal threshold in response to punctuate mechanical stimuli. These changes in behavioural responses were rapidly induced, present from day 2 after injection. In weight-bearing studies, the weight borne on the ipsilateral hindlimb is initially reduced to approximately 55% of that borne on the contralateral hindlimb on the second day following injection, recovering to between 70 and 80% over the next few days, and remaining there from 9 days post-injection onwards. The short-lasting nature of this initial drop in weight-bearing suggests it may, at least in part, be mediated by an inflammatory response (Bove *et al.*, 2003). This biphasic nociceptive response was not observed following punctuate stimulation of the plantar surface of the hindpaw, suggesting the inflammation may be localised to the knee joint itself. These behavioural responses are comparable to other studies assessing behavioural deficits in the MIA model. Similar to the results presented here, 1mg MIA produced a biphasic change in weight-bearing through the hindlimbs which was maximal within 3 days of injection and somewhat recovered by day 7 (Bove *et al.*, 2003; Pomonis *et al.*, 2005). Biphasic functional impairment was also seen in a functional telemetry test (Guingamp *et al.*, 1997), with loss of mobility peaking at days 1-2, recovering slightly over the next 7 days and then maintaining at a steady, impaired state. Similar to results presented herein, withdrawal thresholds to punctate stimulation of the plantar surface of the hindpaw, following a 2mg dose of MIA, decreased in a monophasic fashion, with impairment seen on the first day of testing (Day 3) after injection, dropping further by day 5 and maintaining that level of impairment over a 32 day study period (Fernihough *et al.*, 2004). In human OA, changes in weight-bearing are frequently reported (see Jüni *et al.*, 2006) and patients often also report referred pain (pain in non-injured areas) and touch hypersensitivity. The deficits in behavioural responses presented herein are therefore clinically relevant in the study of OA-related pain syndromes, and may provide a useful tool in research into pharmacological management of OA-pain syndromes, and studies could be improved by the inclusion of more spontaneous pain measures. Locomotor behaviour is frequently measured in

the study of spontaneous pain, and gait analysis, stride frequency, and travel distance in over-ground and in running-wheel conditions have been used to study impaired mobility in a mouse strain susceptible to OA (Costello *et al.*, 2010). The inclusion of such locomotor tests in future work could help to further characterise behavioural changes in the MIA model of OA pain.

4.4.2. Are changes in cytokine levels in synovial fluid and paw tissue contributing to MIA-related pain behaviour?

In the present study, the only changes in cytokine levels following MIA treatment were a decrease in anti-inflammatory IL-13 in ipsilateral synovial fluid at 6 hours following MIA injection compared to saline-treated controls. In addition, I observed a decrease in levels of IL-1β in the contralateral synovial fluid at 6 hours post-MIA injection versus saline-treated controls. However, while levels of IL-1β were analysed in both studies, this result was only obtained in the second study, and not seen at all in the first. This discrepancy makes understanding what effect if any, injection of MIA has on cytokine levels in synovial fluid, based on the studies herein, impossible, and may be explained by a number of factors, which will be discussed in more detail in section 4.4.5.

While no changes were observed in cytokine levels between MIA- and saline-treated rats in synovial fluid, hindpaw skin or spinal cord, differences in cytokine levels measured at different time-points was frequently evident. In the first study, synovial fluid levels of IL-1β, TNFα and IL-13 were lower at 24 hours compared to 6 hours post-MIA injection. This effect was most evident in MIA treated-groups where statistical significance was reached, except for in the case of IL-13, where levels in synovial fluid of rats treated with MIA was already lower at 6 hours than in saline-treated rats. However, a trend was visible in saline-treated rats. The presence of these time-dependent changes in cytokine levels in both MIA-treated rats and the saline-treated controls, and the lack of such overtly visible trends in synovial fluid removed from the contralateral knee, suggests injection *per se* has marked effects on the levels of cytokines, which then rapidly resolve. It is important to note that saline injection does not produce significant changes in weight-bearing or allodynia even at

day 2 and, therefore, the changes in cytokines observed in saline-treated rats appear to have negligible effect on early stage pain behaviour. This effect extended to other tissues analysed, although rather than a decrease in levels, an increase in paw tissue levels of IL-1β was observed between 6 hours and 24 hours post-injection, in MIA-treated rats ipsilaterally, and both MIA- and saline-treated rats contralaterally. The rise in cytokine levels so soon in tissues away from the site of trauma suggests an inflammatory response that does not remain localised. These increases in paw tissue cytokine levels may be extended to TNFα and IL-13 as well. Although levels 6 hours post-injection are below detection limits, a few paw tissue samples from saline-treated rats. Most, if not all samples from MIA-treated rats taken 24 hours post-injection, have levels of these cytokines within the detectable range. This suggests the possibility that injection causes either an immediate decrease in levels of paw tissue cytokines which recover with time, occurring sooner in MIA-treated rats than in saline-treated rats, or that injection causes a slow increase in these cytokines in the paw, with MIA-treated rats displaying a more rapid response than saline-treated rats. Unfortunately, as no samples from naïve rats were obtained and thus pre-injection levels are unknown, it is not possible to ascertain which of these is the case. However, once again, due to the presence of this trend in both MIA- and saline-treated rats, and in both ipsilateral and contralateral hindpaw skin tissue, it appears that these changes in cytokine levels have negligible effects on early stage pain behaviour, but the effects on late stage behaviour, and neuronal responses, are unknown. The changes in cytokine levels in contralateral hindpaw skin, suggests that injection may cause peripheral systemic effects on cytokines. Analysis of cytokine levels in the fore-paws could help to ascertain whether this is the case.

The trend for time-dependent changes in cytokine levels does not appear to be present in the spinal cord, as no patterns in levels of TNFα or IL-6 over time emerged (IL-1β in the spinal cord were below limits of detection). This suggests the possibility that an inflammatory reaction beyond the site of injection may be restricted peripherally, and enhanced as a result of repetitive use, as, following recovery from injection anaesthesia, the rats are freely moving.

Cytokine levels have not previously been analysed in the MIA model of OA. In the aged guinea pig model, levels in blood serum have been analysed and are approximately 2-100 fold higher than levels seen in the synovial fluid in the present study (Huebner & Kraus, 2006; Huebner *et al.,* 2007). Cytokine levels in synovial fluid have, however, been measured in human OA (Scanzello *et al.*, 2009). Levels of cytokines observed in the synovial fluid in this study are comparable to those in human OA synovial fluid, although variations in levels between the two separate analyses in this study are large. In human OA, changes in levels of cytokines are observed in several joint structures such as synovial fluid, synovial fibroblasts and chondrocytes. Much of the research in this area has focused on end-stage OA, and as such, very little is known about cytokines in early OA. However, in the synovial fluid, IL-6 has been shown to be elevated in both early and end stage OA, with a trend to being higher in early stage OA (Scanzello *et al.*, 2009). Both TNFα and IL-1β have also been observed in both early and endstage-OA synovial fluid, with levels of TNFα similar at both timepoints while IL-1β is more predominantly seen in endstage OA (Scanzello *et al.,* 2009). However, IL-1β was observed in only one out of fifteen early stage OA synovial fluid samples, and four out of ten endstage-OA synovial fluid samples. In endstage OA, IL-1 is increased in synovial fibroblasts (Sadouk *et al.*, 1995), and in *ex vivo* OA chondrocyte explants, a two-fold increase in IL-1 receptor density is concomitant with a 3-4 fold decrease in the concentration of IL-1required for half-maximal stimulation of MMP (Martel-Pelletier *et al.*, 1992). TNFα and TNFα convertase (TACE - an activator of TNFα) are elevated in endstage-OA cartilage (Amin *et al.*, 1999), while in the synovial joint cell cultures from end-stage OA patients, TNFα elevations were present together with increased levels of soluble TNF receptors (sTNFR) - endogenous inhibitors of TNFα (Brennan *et al.*, 1995). Changes in cytokine levels have also been observed in a spontaneous murine model of OA, where IL-6 and IFNγ were upregulated in the cartilage of an OA-susceptible strain of mouse versus a closely related strain, not overly susceptible to development of OA (Takahashi *et al.*, 1997).

4.4.3. Elevated mechanically evoked responses of WDR dorsal horn neurones in MIA-treated rats are indicative of central sensitisation

Punctuate stimulation of the hindpaw by von Frey monofilaments in electrophysiological studies outlined here have shown mechanically evoked WDR neuronal responses in MIA-treated rats to be higher than in saline-treated rats, and while this did not reach significance in response to lower (8g and 10g) stimuli, a trend is clearly visible. Indeed, this lack of statistical significance may be explainable by the observation that treatments eliciting a facilitatory effect in neuronal responses outlined in this thesis tended to do so with greater variation in intensity than treatments eliciting an inhibitory effect, thus increasing the range and standard error of values obtained. While it is still unclear how nociceptive mechanisms relate to nociceptive behaviours (Costigan *et al.*, 2009), the correlation between neuronal responses and pain behaviours in a number of studies strongly suggests that elevated sensory neuronal responses translates to increases in pain-related behaviours (Guindon & Hohmann, 2008; Asiedu *et al.*, 2010; Radtke *et al.*, 2010). Increases in spinal and peripheral neuronal activity, both spontaneous and evoked, have been described in several models of OA and RA, including the MIA model itself (Kelly *et al.*, 2007; Kelly *et al.*, 2008; McDougall *et al.*, 2009; Schuelert & McDougall, 2009).

The presence of both punctuate allodynia and mechanical hyperalgesia in MIA-treated rats, together with increased mechanically evoked responses of WDR neurones in these rats suggests that central events may contribute to the changes in hindpaw withdrawal responses described in the MIA model. Structural alterations in the knee joint caused by intra-articular injection of MIA may produce changes in sensory input from mechanical stimulation of the hindpaw. Together with the presence of punctuate allodynia in the awake rat, the changes in neuronal responses are indicative of the phenomena of referred pain, frequently observed in human OA in the thigh, leg and foot following knee-OA, and a phenomenon of central sensitisation (Farrell *et al.*, 2000; Bajaj *et al.*, 2001). It is thought that referred pain may be a result of crosstalk

between afferents from the knee and hindpaw, the cell bodies of which co-localise in DRGs of L3-L5 (Salo & Theriault, 1997; Bajrovic & Sketelj, 1998).

Central sensitisation occurs due to a number of mechanisms involving both pre-synaptic and post-synaptic events (see Chapter 1). Pre-synaptically, the involvement of increased C-fibre activation and recruitment of Aβ-fibres has been implicated. While no changes in electrically evoked responses of these fibre types were observed between MIA- and saline-treated rats, this may be because facilitatory effects of MIA treatment may be difficult to discern following such a large stimulus, particularly with smaller sample sizes. It may also suggest the activation of descending inhibitory control of neuronal responses or that sensitisation at the level of peripheral nerve endings contribute to elevated responses. In the studies presented herein, supra-maximal electrical stimulation is primarily given to establish that the neurone of interest is WDR, and so the lack of observed difference between MIA- and saline-treated rats may be due to the lack of suitability of this type of stimulus for the study of electrically evoked responses. Electrically evoked responses of sensory neurones have been demonstrated in the aged (36 months) guinea pig model of OA (McDougall *et al.*, 2009), as well as in the MIA model itself, with data in the mouse showing increased electrically evoked responses of A- and C-fibre (Harvey & Dickenson, 2009), and from the rat demonstrating increased mechanical responses of C and Aδ-fibres 14 days after MIA treatment (Schuelert & McDougall, 2008; Kelly *et al.*, 2008).

Phenotypic changes in Aβ-fibres have been implicated in the development of central sensitisation, and after nerve injury these fibres release excitatory amino acids such as substance P (Noguchi *et al.*, 1995; Malcangio *et al.*, 2000), the expression of which is usually localised to C-fibres. Elevations in levels of excitatory amino acids (EAAs) and substance P has been observed in human OA joints, as have altered levels of receptor expression. Levels of substance P were found to be higher in synovial fluid of patients with endstage OA than non-OA control subjects (Marshall *et al.*, 1990). Substance P expression has also been found in subchondral bone sensory nerve fibres taken from endstage OA (Ogino *et al.*, 2009). Glutamate and aspartate levels are elevated in synovial fluid in endstage OA (McNearney *et al.*, 2000), and elevated

levels of these excitatory substances have been shown to be crucial in the development of hyperalgesia in models of neuropathic pain (Meller *et al.*, 1994).

The NMDA receptor is naturally present in human articular cartilage *in vivo* (Salter *et al.*, 2004), however, differences in the receptor subtypes present in normal and endstage human OA chondrocytes have been observed, resulting in altered receptor activity (Salter *et al.*, 2004; Ramage *et al.*, 2008). In OA chondrocytes, altered NMDA receptor activity results in downstream activation of tetrodotoxin-sensitive Na^+ and small conductance Ca^{2+}-activated potassium (SK) channels, which does not occur in normal chondrocytes (Ramage *et al.*, 2008). Increased activation of NMDA receptors through altered expression and elevated levels of excitatory amino acids (EAAs) and substance P, results in reductions in fibre thresholds, thus increasing neuronal hyperexcitability (peripheral sensitisation), and has been shown to be of importance in the development of central sensitisation (Woolf & Salter, 2000) and associated behavioural deficits (Gao *et al.*, 2005). Upregulation of other receptor types is also thought to contribute to central sensitisation, including expression of the ion channel TRPV1 on A- and C-fibres (Hudson *et al.*, 2001; Hong & Wiley, 2005). While the TRPV1 receptor is typically associated with heat and chemically induced hyperalgesia, after activation it has been shown to play a role in mechanical hyperalgesia, particularly in chronic pain states (Ro *et al.*, 2009). Backlabelling staining techniques have shown an increase in TRPV1-positive cells in L4 DRG in the MIA model (Fernihough *et al.*, 2005). TRPV1 expression has also been observed in synovial fibroblasts from patients with symptomatic (endstage) OA, and stimulation of these synovial fibroblasts *in vitro* with the TRPV1 receptor agonist capsaicin resulted in increased IL-6 mRNA expression and protein presence in cell culture supernatants (Engler *et al.*, 2007).

While it is unknown whether changes in EAAs and the NMDA receptor occur following MIA- injection, it has been shown to occur in other models of OA, including the anterior cruciate ligament transaction (ACLT) model in rabbits (Jean *et al.*, 2008). In this model, levels of glutamate and aspartate were elevated (compared to sham) approximately 2-fold in knee joint dialysates. Protein expression of the

glutamate/aspartate transporter GLAST, and the glutamate transporter GLT-1 were also elevated in chondrocytes from ACLT knees (Jean *et al.*, 2008).

4.4.4. Post-stimulus neuronal responses in the MIA model

Anecdotal evidence from human OA sufferers suggests that after painful stimulus, the sensation of pain lasts longer than in healthy subjects (Parks *et al.*, 2009). To test whether mechanical stimuli had a longer-lasting post-stimulus effect in the MIA model, baseline responses of dorsal horn WDR neurones to hindpaw stimulation of the receptive field were characterised in MIA- and saline-treated rats. MIA not only caused an increase in the total number and duration of post-stimulus events, but also increased the rate of events, both in the first 10 seconds after stimulation and for the duration of post-stimulus response, thus making the increase in the number of events a result not only of the increase in duration but also an increase in response rate. These increases were seen following stimulation across the whole range of stimulus intensities used, although significance was not reached for the lowest (8g) weight, and in some cases following stimulation by the highest, 60g weight. A possible reason for this is that following higher intensity noxious stimuli, descending inhibitory controls play a large role in modulating responses of WDR dorsal horn neurones, preventing further facilitation of response. Descending inhibition may be stimulated by increased neurotransmitter release following higher intensity noxious stimuli, and the activation of different receptor subtypes by these neurotransmitters at higher concentrations, which can result in anti-nociceptive effects, as outlined in Chapter 1, section 1.1.4.

The increase in post-stimulus responses seen here further implies that central sensitisation may be responsible in the development of nociceptive responses in this model. It is also possible that these increases in post-stimulus responses may be observable and characterised as behavioural changes in the awake rat. Certainly, during behavioural tests outlined here, it was noticed that rats with lower withdrawal thresholds to punctuate stimuli also tended to display nociceptive and nocifensive responses following stimulation, for greater periods of time than rats with withdrawal thresholds at 15g or even 10g. This was evident by increased shaking, licking and

general guarding of the paw, displayed by a curled paw position. These rats also appeared to be slower to replace the paw on the testing cage floor following withdrawal; unfortunately, this behaviour was not quantified during the study, and exists only as anecdotal observations. It could however be a viable addition to the behavioural tests employed in such studies, and has frequently been used in other pain models as a measure of nociception, including in the formalin model of inflammatory pain (Roussy *et al.*, 2009) and following intrathecal administration of excitatory amino acids (Osborne & Coderre, 2003).

4.4.5. Experimental limitations

Analysis of cytokine levels in synovial fluid, hindpaw skin and spinal cord of MIA- and saline-treated rats proved problematic, with varying levels of cytokines, particularly IL-1β recorded between assays. A number of factors may have contributed to these disparities in absolute levels, and may go some way to explaining the different results obtained from the analyses:

Processing and analysis conditions between assays varied. In the first, tissue samples travelled overnight, unprocessed, on dry ice prior to processing and analysis. In the second analysis, sample supernatants were prepared (in the case of spinal cord, while synovial fluid samples were used neat) on the morning of use and transported for a short period of time (<2hours) on dry ice before analysis. While it is unlikely that these minor differences and temperature fluctuations will be solely responsible for the variability of results obtained here, they may have been a contributing factor. Of particular note is that samples in the first analysis were split over two plates, and fluorescence readings from the control standard concentrations varied between these two plates. In addition, the levels of cytokines in many of the samples were below the lowest concentration used for preparation of the standard curve, so that the vast majority of samples had their cytokine concentrations deduced by extrapolation of the curve. Whilst the levels presented were within the limits of detection for the relevant analysis method, extrapolation is subject to the assumption that the standard curve does not vary below the lowest concentration used to create it

and that accuracy of fluorescence readings is equal between high and low concentrations. It is recommended that in future, a number of test runs be conducted to ascertain the likely cytokine concentrations in tissue samples, and the concentrations used for preparation of standard curves be adjusted accordingly.

A major source of variability with the synovial fluid samples comes from the method of synovial fluid withdrawal (see Chapter 2 for details, Barton *et al.*, 2007). Synovial fluid is a viscous substance, and the small joint space in the knee of a rat made it impossible to withdraw without an infusion to counteract the vacuum-like conditions. It was not possible to ascertain what proportion of the extracted liquid was synovial fluid versus saline, which was used as the infusion agent. It is also a particularly difficult technique with regards to reproducibility, and time between starting saline infusion and obtaining synovial fluid outflow can vary considerably, adding to the variation in synovial fluid: saline proportions in the final obtained samples. Together with the small sample sizes, this variability caused difficulties in comparisons between the effects of treatments, particularly as the changes, if any, appear to be very small. It is therefore recommended that some method development on the technique of synovial fluid removal should be undertaken to allow reproducible withdrawal of a quantifiable, undiluted volume of synovial fluid, or ensure complete removal of fluid in a known amount of diluent. It is also possible that removal and analysis of the entire knee may prove to be a beneficial in this case, as in the human condition, cytokine level changes are not only seen in the synovial fluid but also in other knee structures, such as the chondrocytes and synovial fibroblasts. This technique however, would incur the obvious disadvantage that analysis of a larger selection of tissue incurs; changes in analyte levels may occur in discrete regions, and at such low levels that distinguishing an alteration amongst possibly larger, naturally occurring background levels would not be possible. It is also recommended that any future analysis in the same vein make use of more sensitive analytical techniques, possibly with the amplification power provided by PCR, with larger sample sizes to counter the effects of variability between a minority of samples on the analytical comparison of particularly small changes.

4.4.6. Conclusions

Intra-articular MIA injection causes behavioural pain responses representative of the symptoms reported by OA patients (Hendiani *et al.*, 2003). MIA-treated rats displayed decreased nociceptive thresholds, changes in weight-bearing and increased responses of spinal neurones innervating sites distal from the site of injury. The behavioural data together with electrophysiological data demonstrating increased mechanically evoked and post-stimulus responses in MIA-treated rats suggests that central sensitisation contributes to the development of behavioural deficits. This is the first time that post-stimulus responses of WDR dorsal horn neurones have been studied in this, or any other model of OA, and its use may prove a useful pharmacological and experimental paradigm in future work.

I was unable to detect any changes in levels of cytokines that have been highlighted to be of importance in human OA, in the synovial fluid, hindpaw skin or spinal cord in the first 24 hours following MIA-injection. Analysis of cytokine levels throughout the development of the MIA model may provide more insight into any role of cytokines in pain behaviour. In addition, it is important to remember that cytokine effects can be altered by levels of soluble receptors, and so quantification of their presence would also be required to make a rounded judgement on the effects of cytokines in the MIA model of OA pain. Nevertheless, the data presented suggests that the rapid development of tactile allodynia and weight-bearing deficits is independent of effects on those cytokines studied.

Chapter 5 Spinal and systemic action of COX-2 inhibition on nociceptive processing of dorsal horn neurones in the MIA model of OA

5.1. Introduction

5.1.1. Effects of spinal COX-2 inhibition on pain processing

COX-2, typically thought of as the inducible isoform of COX, has long been known to be induced in tissues following inflammation or injury (Xie *et al.*, 1991; Kujubu *et al.*, 1991; Sirois & Richards, 1992). COX-2 is important in the development of associated pathologies, such as oedema and pain; peripheral inflammation following intraplantar injection of complete Freund's adjuvant (CFA) or carrageenan is accompanied by an increase in local COX-2 mRNA, COX products such as prostaglandins and local oedema which is COX-2 dependent (Seibert *et al*, 1994). COX-2 inhibition can abolish inflammatory pathologies such as oedema, and abolish acute nociceptive transmission. Thermal hyperalgesia following intraplantar carrageenan administration or spinal delivery of substance P or NMDA is suppressed by intrathecal or systemic inhibition of COX enzymes (Yaksh *et al.*, 2001).

5.1.2. Upregulation of spinal COX-2 and the development of central pain

Following peripheral inflammation, COX-2 mRNA has been shown to be upregulated in the spinal cord and dorsal root ganglion (DRG), in a number of animal models, including the MIA model (Seibert *et al.*, 1994; Hay *et al.*, 1997; Hay & de Belleroche, 1997; Ghilardi *et al.*, 2004; Beloeil *et al.*, 2009; Vanegas & Schaible, 2001; Prochazkova *et al.*, 2009). Spinal COX-2 upregulation can be rapid, occurring within the first few hours after inflammation (Vanegas & Schaible, 2001). COX-2 mRNA upregulation is also observed in DRGs following TNFα and IL-1β application *in vitro* (Fehrenbacher *et al.*, 2005). Spinal prostaglandin synthesis and activation of a number of neuropeptides including dynorphin, enkephalin (also preprodynorphin and preproenkephalin) and c-fos are also increased following peripheral inflammation (Iadarola *et al.*, 1988; Draisci & Iadarola, 1989; Ruda *et al.*, 1988). The increased levels of COX products, particularly PGE_2, is thought to contribute significantly to the development of spinal hyperexcitability (Ghilardi *et al.*, 2004; Samad *et al.*, 2001,

Svensson & Yaksh, 2002; Vanegas & Schaible, 2001). Increases in spinal COX-2 and prostaglandin synthesis have been shown to be responsible for the rapid (maximal at 6h) development of allodynia in the complete Freund's adjuvant (CFA) model of OA (Hay *et al.*, 1997), with COX-2 inhibition abolishing the development of allodynia in these models. Hyperalgesia following intraplantar carrageenan is also COX-2 dependent, and follows increased COX-2 mRNA levels and prostaglandin synthesis in the lumbar spinal cord within 4h (Hay & de Belleroche, 1997).

Direct application of PGE_2 to the spinal cord results in inflammatory-mediated generation of spinal hyperexcitability (Vasquez *et al.*, 2001). COX inhibitors (non-specific and both COX-1 or COX-2 specific) block the generation of spinal hyperexcitability when administered during the development of inflammation, suggesting that prostaglandins play a vital role in its development. The importance of COX-2 in this process is evident by the development of behavioural deficits associated with spinal hyperexcitability; pre-administration of the COX-2 selective inhibitor SC398 prevented the development of both mechanical allodynia and thermal hyperalgesia following contusion of the T13 spinal segment, over a 4-week course following injury (Hains *et al.*, 2001). In addition, in neuronal and glial COX-2 knockout mice, while the extent of peripheral inflammation was unchanged, inflammation-induced COX-2 expression was reduced and mechanical hypersensitivity was abolished (Vardeh *et al.*, 2009).

5.1.3. The importance of spinal COX-2 in the maintenance of central pain

Once neuronal hyperexcitability has been established, COX-2 is important in its maintenance, but these effects are not solely prostaglandin dependent. This has been demonstrated by a number of studies showing varying efficacy of COX-1 and COX-2 inhibitors on neuronal hyperexcitability following inflammation, irrespective of their ability to reduce prostaglandin production; in the CFA and kaolin/carrageenan models of joint inflammation, once inflammation had been established, only specific COX-2 inhibitors (and not non-specific COX inhibitors, or specific COX-1 inhibitors) were able

to reduce neuronal hyperexcitability, even though the reduction in spinal PGE_2 by various specificities of COX inhibitor was equal, and did not vary between pre- and post-development of inflammation (Telleria-Diaz *et al.*, 2010). The lack of effect of COX-1 inhibition or non-selective COX inhibition on spinal hyperexcitability in established inflammation was also observed *in vivo* following spinal PGE_2 application (Vasquez *et al.*, 2001) and in rat spinal cord preparations taken following intraplantar carrageenan-induced inflammation (Lopez-Garcia & Laird, 1998). It is also demonstrable in behavioural paradigms – in LPS-induced reactive arthritis, only a specific COX-2 inhibitor (etoricoxib, oral dose) was able to reduce nociceptive responses (Bressan & Tonussi, 2008).

In both the CFA and kaolin/carrageenan models of joint inflammation, the importance of rapid endocannabinoid breakdown by elevated COX-2 expression in increased neuronal responses has been demonstrated *in vivo* (Telleria-Diaz *et al.*, 2010). L-745,337 (COX-2 inhibitor)-mediated reduction in neuronal response was abolished with co-administration of the CB_1 antagonist AM251 (Telleria-Diaz *et al.*, 2010). Spinal AM251 alone in these models did not alter spinal neuronal responses, suggesting either the lack of endocannabinoid tone in this model, or its presence concomitant with an increased rate of endocannabinoid metabolism, possibly by the upregulation of COX-2 (Telleria-Diaz *et al.*, 2010; Jhaveri *et al.*, 2007).

Nimesulide, which preferentially inhibits COX-2 and importantly does not inhibit FAAH, would be useful in elucidating the importance of COX-2-mediated endocannabinoid metabolism in central sensitisation. Many of the studies conducted with nimesulide focus on its anti-inflammatory effects, however, some research focussing on antinociceptive properties have also been published, using the CFA model and formalin models of inflammatory pain (Gineste *et al.*, 2003, Bianchi & Broggini, 2002). Given intraperitoneally, 2.9mg/kg nimesulide completely inhibited thermal hind paw hyperalgesia following intravenous administration of formalin through the tail vein, and reduced mechanical hyperalgesia following intraplantar injection of CFA (Gineste *et al.*, 2003). Nimesulide also showed significant analgesic effects in tail-flick and

formalin tests under baseline conditions, and counteracted nitroglycerin-induced hyperalgesia in both tests in the rat (Tassorelli *et al.*, 2003).

5.1.4. Aim

The aim of this chapter was to compare the effects of spinal and systemic nimesulide on mechanically evoked responses of WDR neurones in a model of chronic pain, using the MIA model of osteoarthritis pain *in vivo*.

5.2. Methods

5.2.1. Induction of the MIA model of osteoarthritis pain

For intra-articular injection, sodium iodoacetate (MIA, Sigma-Aldrich, UK), was prepared freshly on the day of injection by dissolution in sterile saline. A dose of 1mg MIA in 50µL sterile saline or 50µL sterile saline alone for control groups was administered per rat, based on previous work done in this lab (see appendix). The experiments were carried out on male Sprague-Dawley rats weighing 130-150g at the beginning of the study. For detailed methodology of anaesthesia and injection of MIA see Chapter 2. The experimenter was blinded to all treatments.

5.2.2. *In vivo* electrophysiology and data acquisition

For detailed methodology of anaesthesia, surgery and identification and recording of WDR neurone responses, see Chapter 2. For most of the experiments in this chapter, one WDR neurone from the dorsal horn of the spinal cord was studied per rat. Where more than one neurone was studied, this has been clearly stated. Effects of nimesulide on both during- and after-stimulus neuronal responses were studied.

5.2.3. Spinally administered nimesulide or vehicle and mechanically evoked WDR neurone responses in MIA- and saline-treated rats

Nimesulide was freshly prepared in 3% Tween in physiological saline in doses of 3, 10, 25 and 100µg/50µL. Doses were chosen to allow comparison to the range of doses studied in the naïve preparation (Chapter 3), including a low, minimally-effective dose in the naïve rat (3µg), a maximal and supramaximal dose (25µg and 100µg), and an intermediary dose (10µg) sufficiently distant from the 3 and 25µg doses so to avoid interference between doses. Nimesulide or vehicle was administered directly to the spinal cord as described previously (Chapter 2). The effects of nimesulide on mechanically evoked responses of WDR neurones were examined every 10 minutes

for 40 minutes, based on findings of the mean time point of maximal effect of spinally administered nimesulide in naïve rats occurring 30 minutes post-administration (Chapter 3). The drug was then carefully removed from the cord using tissue paper and a higher dose of nimesulide (or sequential dose of vehicle) was added. Results from saline-treated rats with spinal vehicle (n=6) were also used for experiments detailed in Chapter 6 to reduce the total number of rats used and avoid replication of experiments inline with the 3R principle (reduce, reuse, refine). For these experiments, each spinal application of vehicle was followed for 60 minutes before removal and application of the next dose. Assurances were made that this did not affect experimental data (see results, section 5.3.4). In total, four groups of rats were studied; MIA-treated rats dosed with spinal nimesulide (n=6), or vehicle (n=6), saline-treated rats dosed with spinal nimesulide (n=6) or vehicle (n=6).

5.2.4. Subcutaneous nimesulide or vehicle and mechanically evoked responses of WDR neurones in MIA and saline-treated rats

For subcutaneous administration, nimesulide was prepared freshly on the day of study. Nimesulide was dissolved directly in 3% Tween in physiological saline, to create 1mg/kg and 10mg/kg doses, vortexed and ultrasonicated for 15 minutes, ensuring it had entered solution. Drugs were administered subcutaneously under the scruff of the neck in a 2.5mL volume, using a 25 gague needle (BD Microlance, Drogheda, Ireland). No studies with subcutaneous administration of nimesulide have been reported, however, the ED50 of oral nimesulide on hyperalgesia in the adjuvant arthritis model in rats is 2.4mg/kg. Recommendations for studies with systemic use of nimesulide in arthritic pain are for dosing between 1-10mg/kg/day (Prof K.D. Rainsford, Shefield Hallam University, personal communication). Since nimesulide has not previously been applied subcutaneously under similar experimental conditions, a pilot study was performed in MIA- and saline-treated rats (n=8, 5 of which had been used earlier in the day for study into the effects of spinally administered CB_1- or CB_2-receptor antagonist (Chapter 6) following a 1-2 hour washout period, and after stable mechanically evoked responses of WDR neurones had again been obtained, and 3 MIA-treated rats which had not previously been used

in any other study), 2 hours per dose. 1mg/kg and 10mg/kg doses were then chosen for further study. Nimesulide was injected subcutaneously as described before, and the effects on mechanically evoked responses of WDR neurones were examined every 10 minutes for 120 minutes, based on findings of the mean time point of maximal effect from the preliminary study. The higher dose of nimesulide (or sequential dose of vehicle) was then administered. Four groups of rats were studied; MIA-treated rats dosed with subcutaneous nimesulide (n=6), or vehicle (n=6), saline-treated rats dosed with subcutaneous nimesulide (n=6) or vehicle (n=6). The clearance of intravenously- and orally administered nimesulide is much slower than the time between doses in this study (Rainsford *et al.*, 2005), and is unlikely to be much faster following subcutaneous administration. To reflect this fact, doses of 1mg/kg and 11mg/kg are displayed on graphical representations of these data.

5.2.5. Spinal or subcutaneous nimesulide and post-stimulus WDR neuronal responses in MIA- and saline-treated rats

The effects of both spinal and subcutaneous nimesulide on post-stimulus responses was also studied, comparing effects to pre-drug post-stimulus duration of response, response count, and response rate, as described in Chapter 4. The total duration of post-stimulus response, rate of post-stimulus response over the entire response duration, rate of post-stimulus response over the first 10 seconds after stimulus removal, and total stimulus count were analysed.

5.2.6. Statistical analyses

Data are expressed as a mean percentage of the pre-drug response ± standard error of the mean (SEM). Statistical analyses were performed using a non-parametric Mann-Whitney test or Kruskal-Wallis test, where appropriate. Differences with P values <0.05 were considered statistically significant.

5.3. Results

Mean depths of recordings were as follows: spinal nimesulide studies = 843 ± 34μm for saline-treated rats (n=11) and 785 ± 46μm for MIA-treated rats (n=11); subcutaneous nimesulide studies = 876 ± 30μm for saline-treated rats (n=11) and 906 ± 35μm for MIA-treated rats (n=11). There were no significant differences between the depths of recording in any of the groups of neurones studied (non-parametric Mann-Whitney test, all P>0.05).

5.3.1. Effects of intra-articular injection of MIA or saline on nociceptive responses in awake rats

In order to ensure that intra-articular injections of MIA were correctly placed and that the MIA model of OA pain progressed as previously described (Chapter 4), the behavioural responses of each rat was compared to that of all MIA- and saline-treated rats used throughout this thesis (presented together in Chapter 4, section 4.3.1). Injection of 1mg MIA in 50μL saline significantly reduced weight borne on the ipsilateral hindlimb, compared to saline-treated rats (Figure 5.1A and B) in a biphasic manner, with the first phase clearing by day 9 post-injection. MIA also caused a significant decrease in withdrawal threshold to punctuate stimuli of the plantar surface of the ipsilateral hindpaw versus contralateral, whilst there was no difference in withdrawal thresholds of saline ipsilateral hindpaw versus contralateral (Figure 5.2A and B). There were no differences in behaviour for groups of rats which subsequently received spinal or subcutaneous nimesulide (one-way ANOVA (Kruskal Wallis) with post-hoc Dunn's test, all $P>0.05$). Results from both weight bearing and withdrawal threshold studies were comparable to those obtained from all MIA- and saline-treated rats presented in Chapter 4 (one way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test, all $P>0.05$).

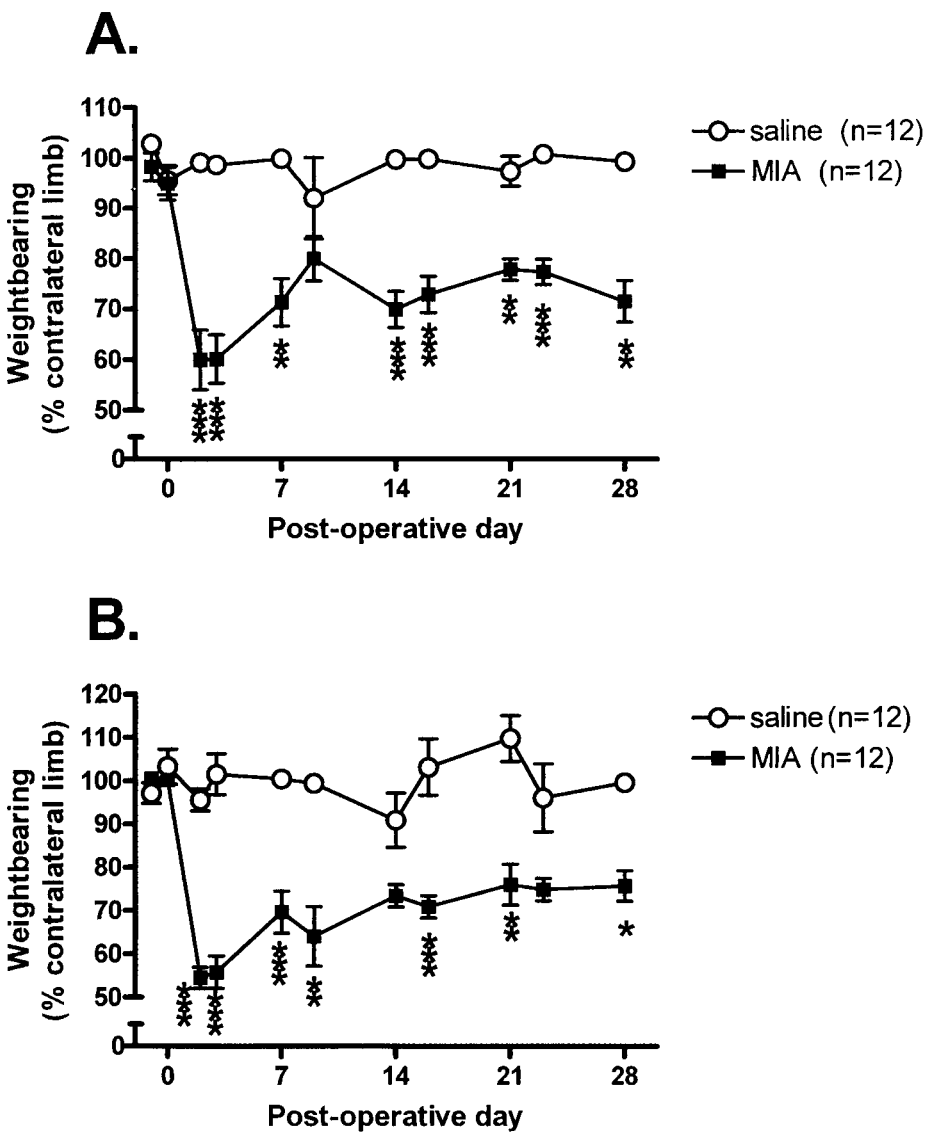


Figure 5.1 Reduced weight-bearing on ipsilateral hindlimb of rats receiving intra-articular injection of 1mg MIA in 50µL saline (black squares, n=12 per electrophysiological study group) versus 50µL saline alone (open circles, n=12 per electrophysiological study group), over a 28-day period post-injection. A, rats used in spinal nimesulide electrophysiological studies; **B**, rats used in subcutaneous nimesulide electrophysiological studies. Statistical analysis comparing the effects of saline to MIA on weight-bearing through the ipsilateral hindlimb was performed using a one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test, *, $P<0.05$; **, $P<0.01$; ***, $P<0.005$. Weight-bearing through the ipsilateral hindlimb is expressed as a mean percentage of the weight borne through the contralateral hindlimb, averaged from 3 readings per rat, per time-point, ± SEM. There were no significant differences in weight-bearing between rats used in spinal studies and those used in subcutaneous studies.

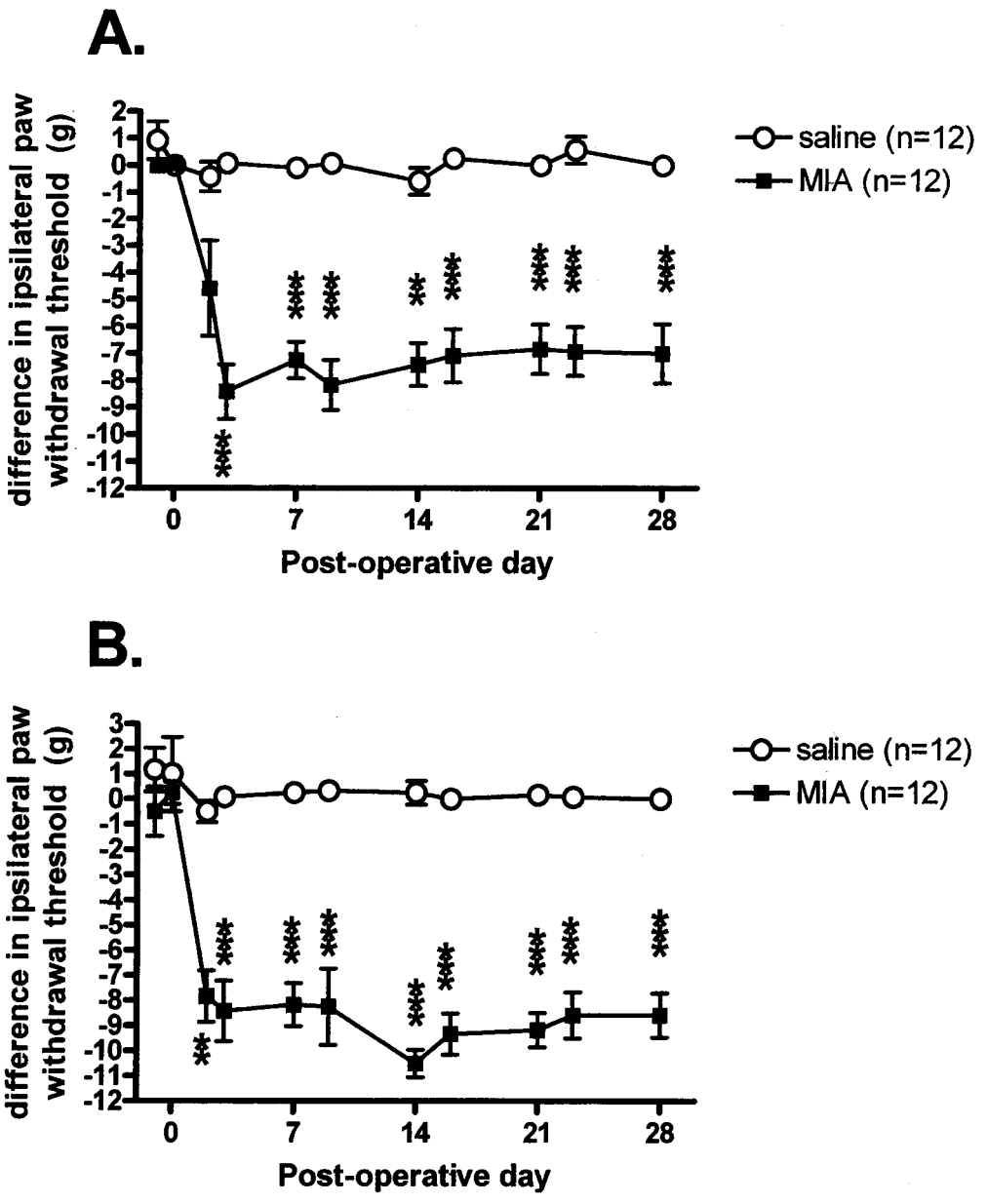


Figure 5.2 Reduced thresholds for withdrawal to mechanical stimuli of the ipsilateral hindpaw of rats receiving intra-articular injection of 1mg MIA in 50µL saline (black squares, n=12 per electrophysiological study group) versus 50µL saline alone (open circles, n=12 per electrophysiological study group), over a 28-day period post-injection. A, rats used in spinal nimesulide electrophysiological studies; **B**, rats used in subcutaneous nimesulide electrophysiological studies. Statistical analysis comparing the effects of saline to MIA on withdrawal thresholds of ipsilateral and contralateral hindpaw was performed using a one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test, **, $P<0.01$; ***, $P<0.005$. Data are expressed as the mean difference in grams between withdrawal thresholds of the ipsilateral and contralateral hindpaws ± SEM. There were no significant differences between ipsilateral thresholds of MIA-treated rats used in spinal studies and those used in subcutaneous studies.

5.3.2. Effects of intra-articular injection of MIA on mechanically evoked responses of WDR dorsal horn neurones in the spinal cord

To further ensure the integrity of induction of the MIA model of OA pain in the rats used in electrophysiological studies in this chapter, the effects of MIA and saline injection on baseline mechanically evoked responses of WDR dorsal horn neurones of each rat was compared to that of all MIA- and saline-treated rats used throughout this thesis (presented together in Chapter 4, section 4.3.1). MIA caused a trend to increase evoked responses of WDR neurones (Figure 5.3), particularly in response to higher stimuli, which reached significance following 26g stimulation in the group used for subcutaneous nimesulide electrophysiological studies (Figure 5.3B, non-parametric Mann-Whitney, $P<0.05$). Neuronal responses differing by more than 2 standard deviations from the mean were excluded from analysis. In total this happened in 4 instances of 120 values obtained from saline-treated rats, and 4 instances of 120 values obtained from MIA-treated rats. There were no significant differences between these data and those presented in Chapter 4 (non-parametric Mann-Whitney test, $P>0.05$).

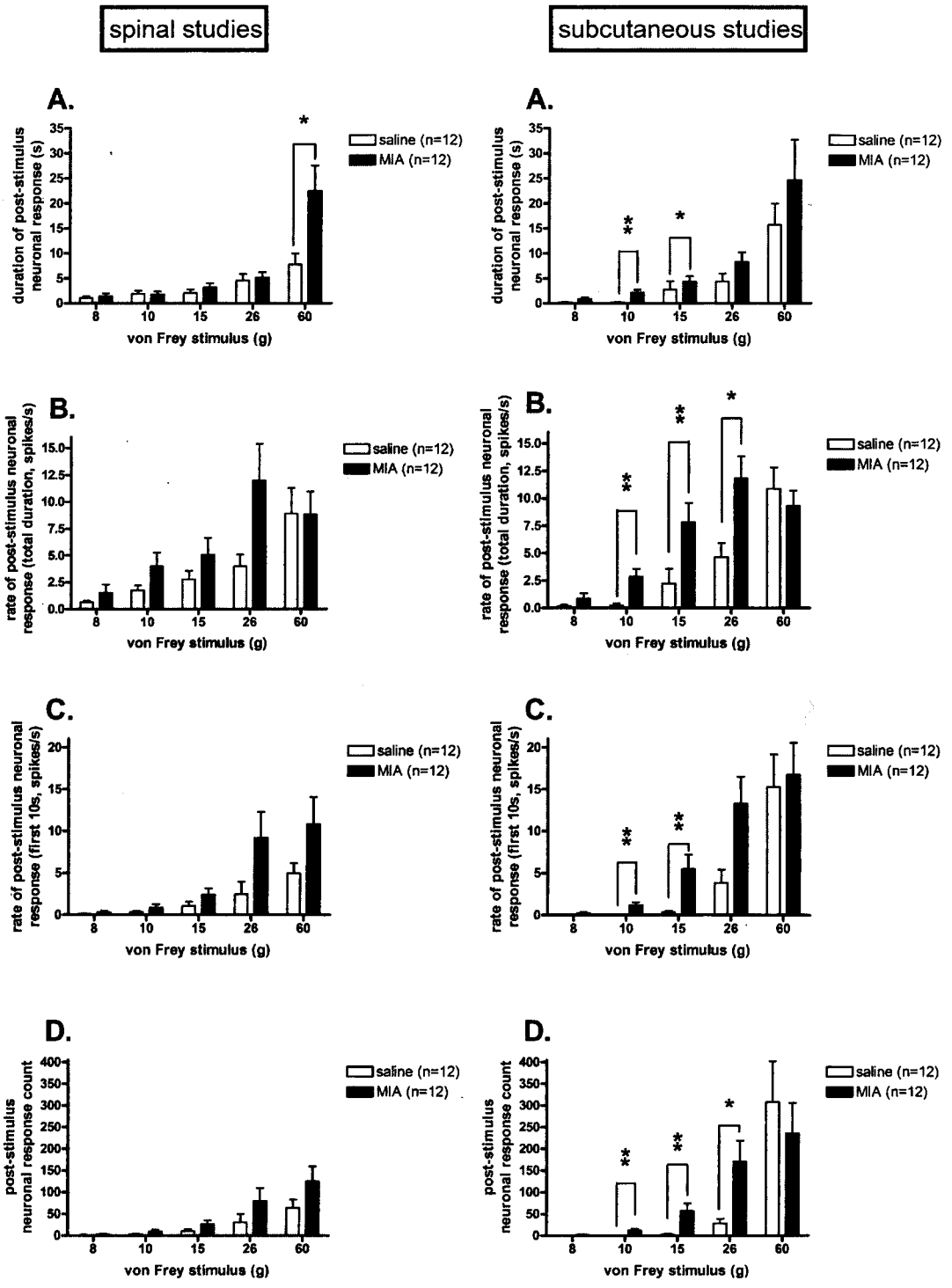


Figure 5.4 Post-stimulus responses of WDR dorsal horn neurones in saline- (n=12) and MIA- (n=12) treated rats, following the removal of mechanical stimuli from the hindpaw receptive field. A, duration of post-stimulus neuronal response (s); **B**, rate of post-stimulus neuronal response (spikes/s) for the total duration of response; **C**, rate of post-stimulus neuronal response (spikes/s) in the first 10 seconds following stimulus removal; **D**, total post-stimulus neuronal response count. Left column ("spinal studies') shows data from rats used in spinal nimesulide electrophysiological studies; right column ("subcutaneous studies") shows data from rats used in subcutaneous nimesulide electrophysiological studies. Data are expressed as mean ± SEM. Statistical comparisons were performed using a non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$.

5.3.4. Effects of spinal nimesulide or vehicle on mechanically evoked responses of WDR neurones in MIA- and saline-treated rats

In this study, 24 neurones from 27 rats were studied. Data from 3 experiments were excluded: Neurone 1, a blood clot on the cord could not successfully be removed, obscuring access of the drug to the spinal cord; neurone 2, behavioural data suggested that the MIA injection was misplaced; neurone 3, as the experiment progressed it became apparent that two cells were being detected.

Spinal nimesulide dose-dependently attenuated 8-26g-evoked responses of WDR dorsal horn neurones in MIA-treated rats compared to time-matched vehicle controls (Figure 5.5). Spinal nimesulide did not attenuate 60g-evoked responses of spinal neurones (Figure 5.5E), however, earlier in the course of the experiment (lower doses) nimesulide prevented the increase in response following repeated stimulation ($P<0.05$, 10µg nimesulide versus vehicle alone). Maximal effects of nimesulide in MIA-treated rats occurred at 31.3 ± 0.7 minutes post-drug administration.

In saline-treated rats, spinal nimesulide produced a trend to attenuate all mechanically evoked responses of WDR neurones compared to vehicle controls, significantly so following 15, 26 and 60g stimuli (Figure 5.6). Maximal effects of nimesulide occurred at 32.8 ± 0.7 minutes. Effects of spinal nimesulide on 8-26g-evoked responses were comparable in saline- and MIA-treated rats. However, 60g-evoked responses of neurones were attenuated by nimesulide in saline- but not MIA-treated rats (Figure 5.6 inset). The maximal effect of nimesulide was observed with 25µg in both groups.

Spinal administration of vehicle had no effect on mechanically evoked responses of WDR neurones in either saline- or MIA-treated rats. In MIA-treated rats, each vehicle dose was administered for 40 minutes while in saline-treated rats each dose was administered for 60 minutes. Nevertheless, no differences were observed in their time-courses (Figure 5.7).

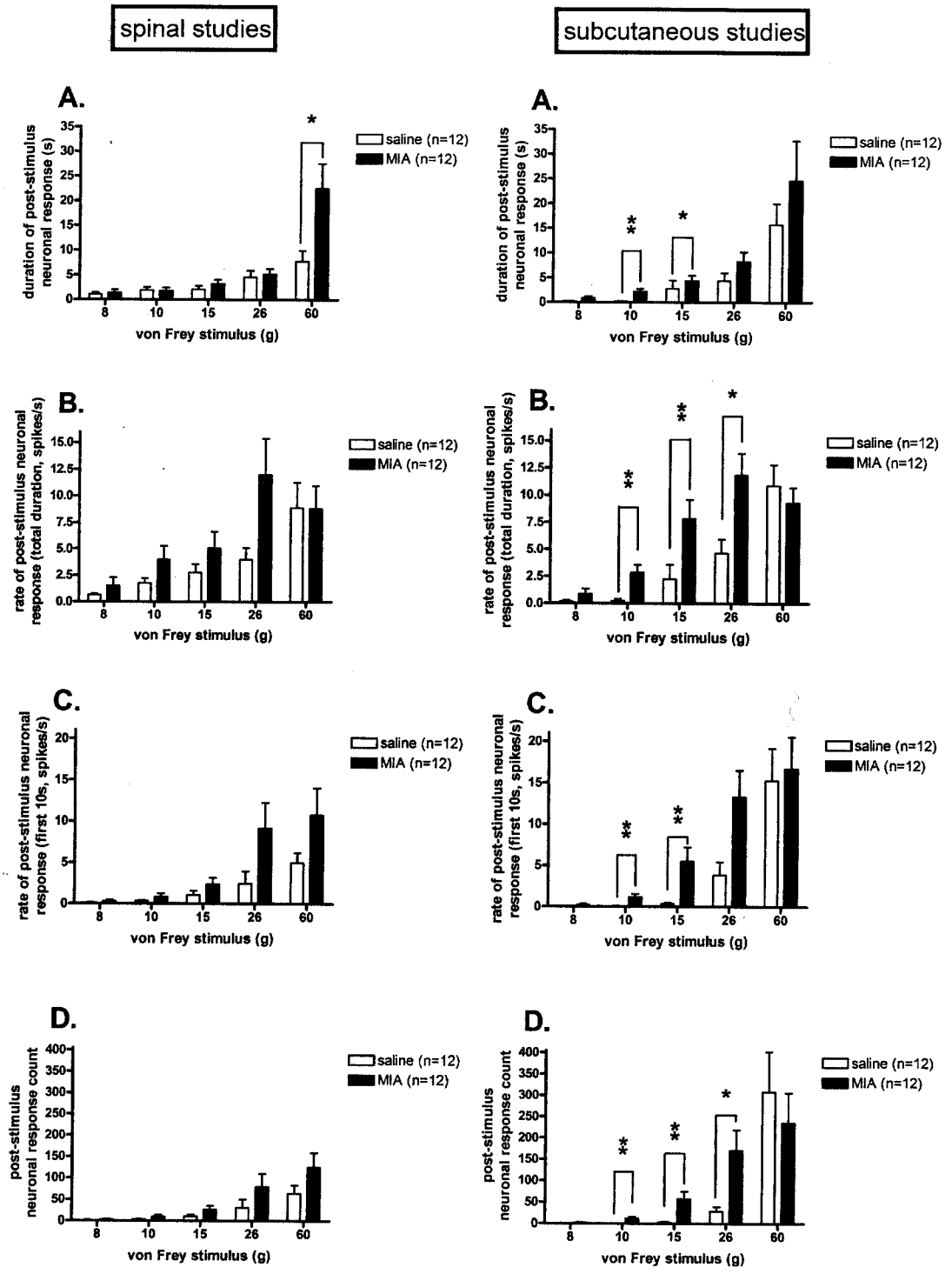


Figure 5.4 Post-stimulus responses of WDR dorsal horn neurones in saline- (n=12) and MIA- (n=12) treated rats, following the removal of mechanical stimuli from the hindpaw receptive field. A, duration of post-stimulus neuronal response (s); **B**, rate of post-stimulus neuronal response (spikes/s) for the total duration of response; **C**, rate of post-stimulus neuronal response (spikes/s) in the first 10 seconds following stimulus removal; **D**, total post-stimulus neuronal response count. Left column ("spinal studies') shows data from rats used in spinal nimesulide electrophysiological studies; right column ("subcutaneous studies") shows data from rats used in subcutaneous nimesulide electrophysiological studies. Data are expressed as mean ± SEM. Statistical comparisons were performed using a non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$.

5.3.4. Effects of spinal nimesulide or vehicle on mechanically evoked responses of WDR neurones in MIA- and saline-treated rats

In this study, 24 neurones from 27 rats were studied. Data from 3 experiments were excluded: Neurone 1, a blood clot on the cord could not successfully be removed, obscuring access of the drug to the spinal cord; neurone 2, behavioural data suggested that the MIA injection was misplaced; neurone 3, as the experiment progressed it became apparent that two cells were being detected.

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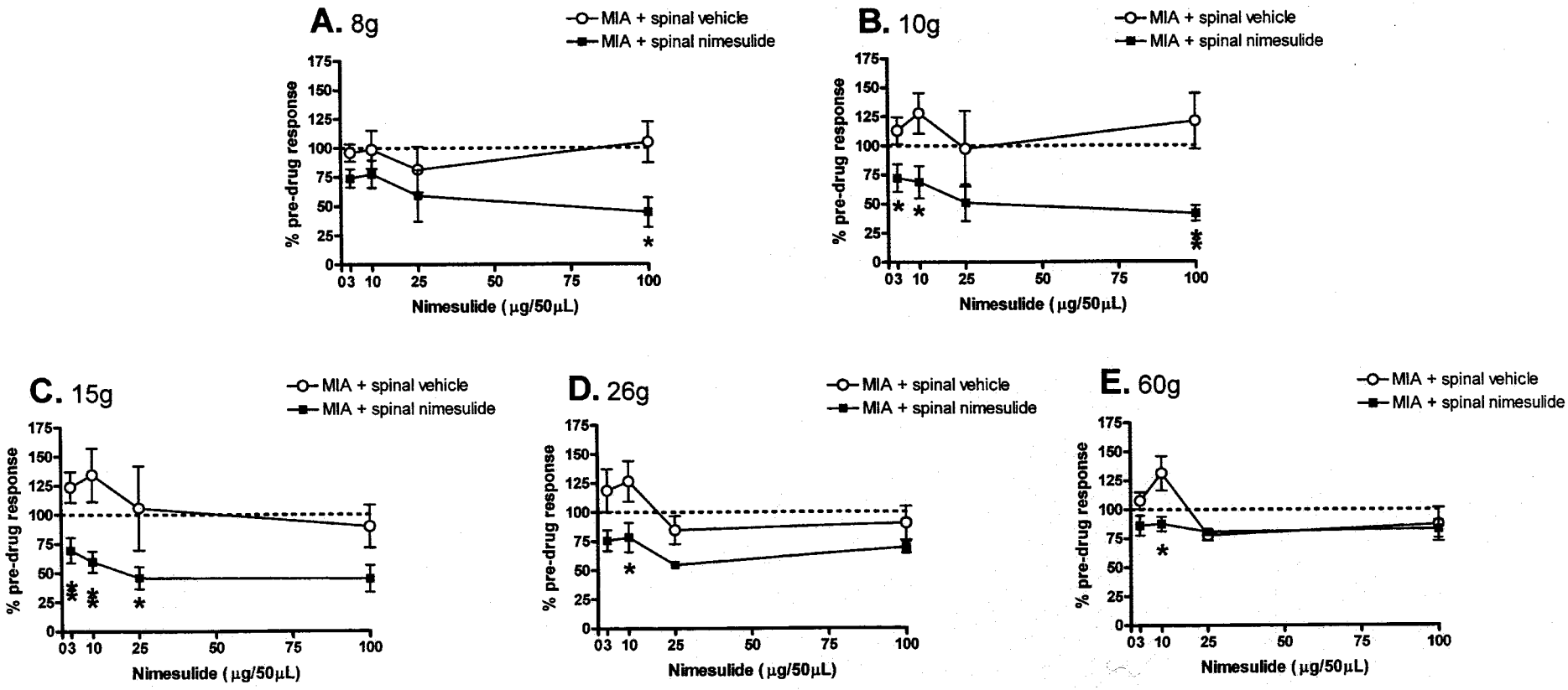

Figure 5.5 Spinally administered nimesulide attenuated 8-26g-evoked responses of WDR dorsal horn neurones in MIA-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses comparing the effects of nimesulide to time-matched vehicle treatment were performed using a non-parametric Mann-Whitney test; **P*<0.05; ***P*<0.01. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control responses.

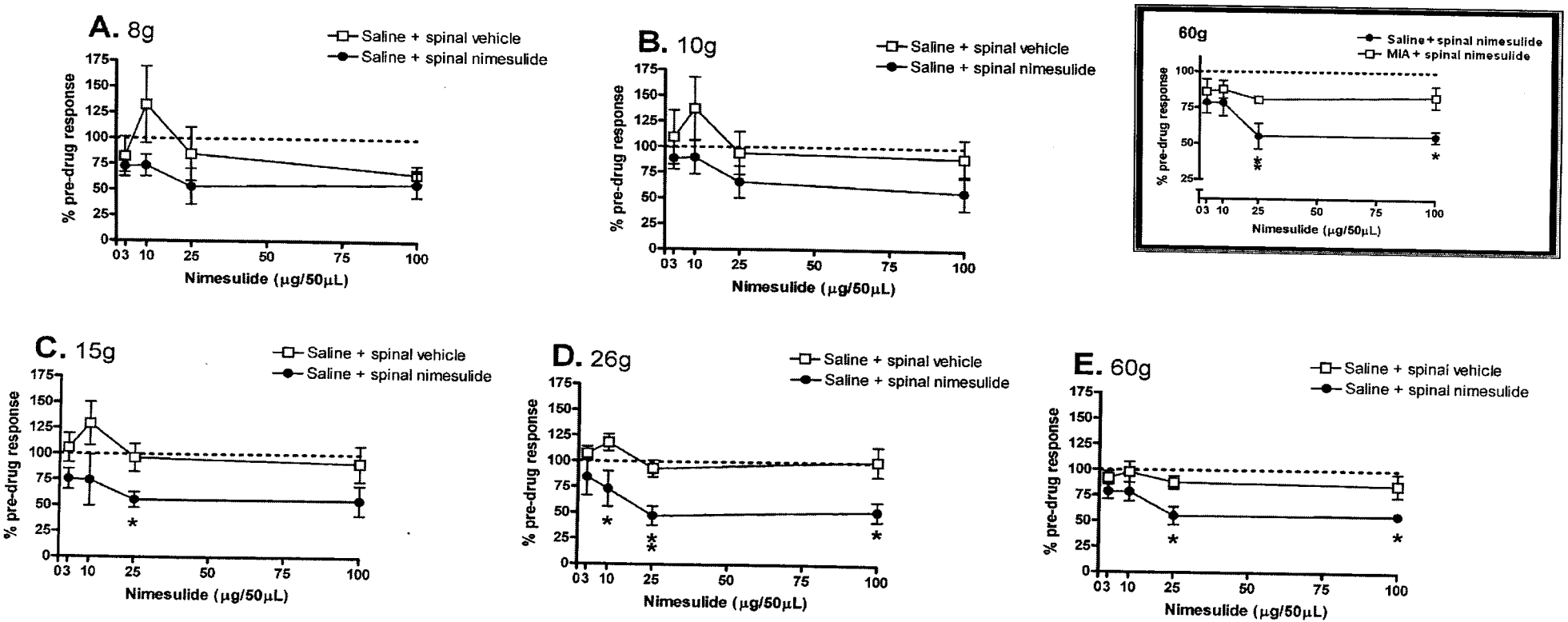

Figure 5.6 Mean maximal effects of spinal nimesulide versus time-matched vehicle on mechanically evoked WDR dorsal horn neurones in saline-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. INSET: Comparison of effect of nimesulide on 60g-evoked responses in saline- and MIA-treated rats. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses comparing the effects of nimesulide versus vehicle treatment in saline-treated rats (panels A-E), and the effects of nimesulide on 60g-evoked responses in saline- versus MIA-treated rats (inset) were performed using a non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control responses.

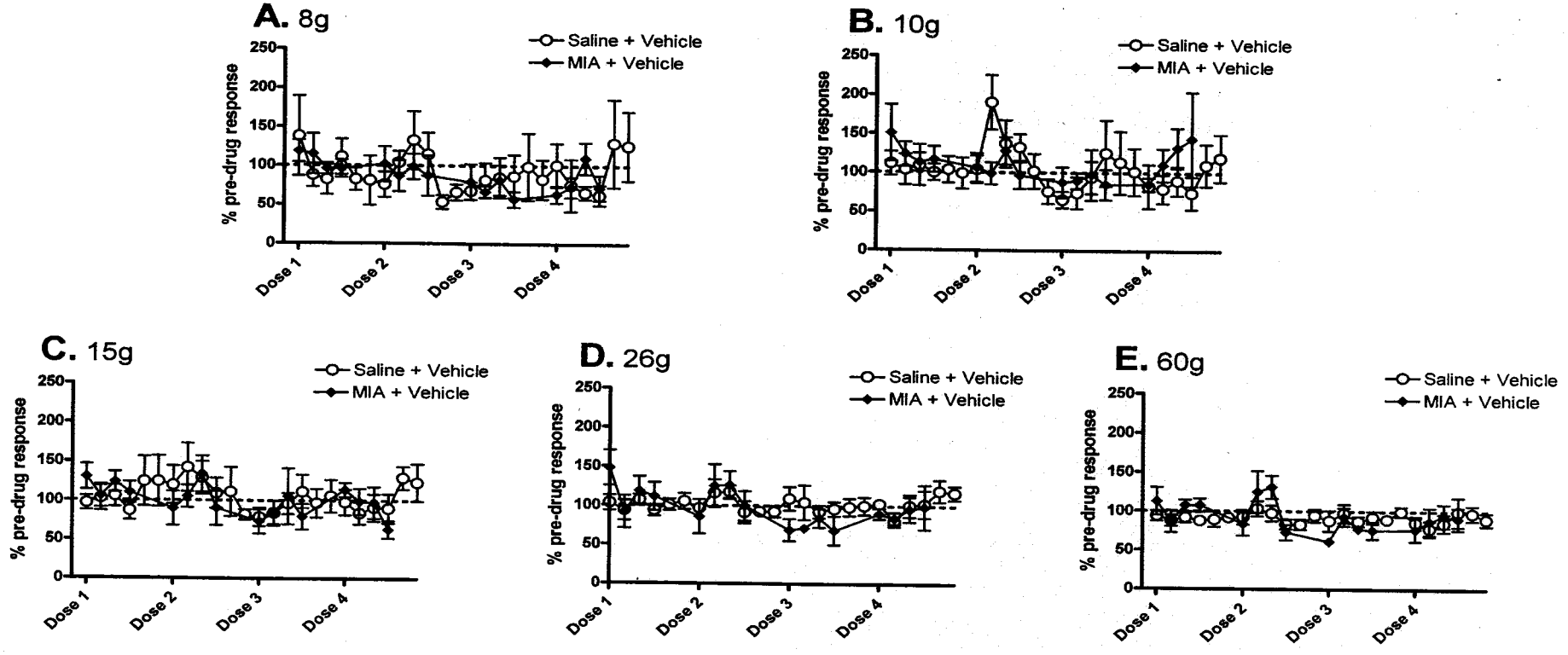

Figure 5.7 Spinal vehicle had no effect on mechanically evoked responses of WDR dorsal horn neurones in saline- or MIA-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses comparing the effects of vehicle in saline-treated rats to the effects of vehicle in MIA- treated rats, and comparing the effects of vehicle in both saline- and MIA-treated rats to their pre-drug controls, were performed using a one-way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test (no statistically significant differences). n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control responses.

5.3.5. Effects of spinal nimesulide on post-stimulus WDR neuronal responses in MIA-treated rats

The variability of pre-drug post-stimulus responses of MIA- and saline-treated rats used in this study highlighted problems with low sample sizes when studying post-stimulus responses (Figure 5.4). Overall, there were no differences in the effects of nimesulide on any of the post-stimulus response paradigms (duration of response, rate of response over total duration, rate of response in first 10s, and total response count) in MIA- or saline-treated rats. The effects of nimesulide versus vehicle on post-stimulus responses in MIA- and saline-treated rats are shown in Figure 5.8.

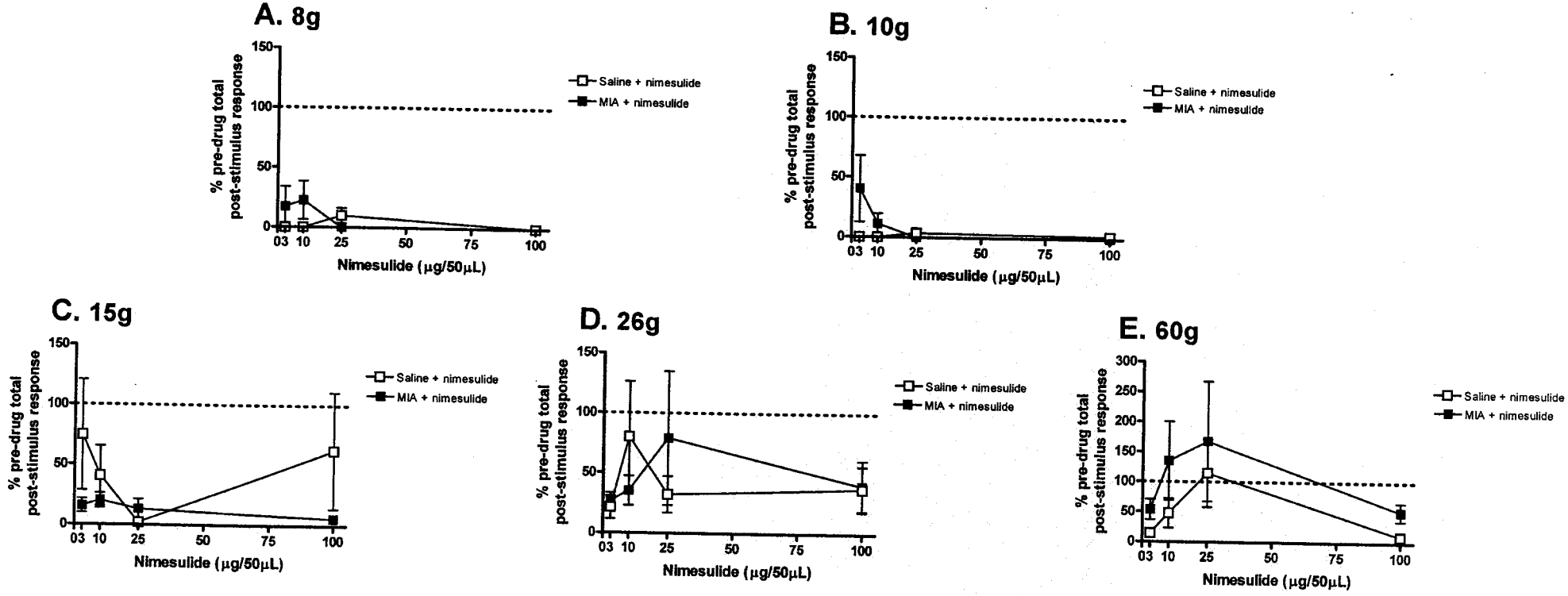

Figure 5.8 Mean maximal effects of spinal nimesulide on post-stimulus neuronal response count of WDR dorsal horn neurones in MIA- and saline-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g; note change of scale in panel E) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug response count ± SEM. Statistical analyses comparing the effects of nimesulide on post-stimulus neuronal count in MIA-treated rats to the effects in saline-treated rats were performed using non-parametric Mann-Whitney test. (no statistically significant differences). n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control responses.

5.3.6. Effects of subcutaneous administration of nimesulide or vehicle on mechanically evoked responses of WDR neurones in MIA- and saline-treated rats

In MIA-treated rats, subcutaneously administered nimesulide significantly attenuated mechanically evoked responses of WDR dorsal horn neurones compared to time-matched vehicle controls (Figure 5.9). Responses to all intensities of von Frey stimuli were robustly and dose-dependently attenuated. 1mg/kg nimesulide produced its maximal effect 84.83 ± 5.72 minutes post-drug administration, while the maximal effect of 10mg/kg nimesulide occurred 95.67 ± 4.98 minutes post-drug administration. The 80- and 100-minute time point of the first and second dose of vehicle respectively, were used for comparison.

As can be seen from a time-course comparing the effects of subcutaneous nimesulide to that of vehicle in MIA-treated rats, nimesulide appears to have a biphasic effect, seen particularly clearly in response to lower stimuli (8, 10 and 15g, Figure 5.10). Peak effects occurred 34.67 ± 2.24 minutes and 32.33 ± 1.96 minutes after administration in the first phase, and 97.50 ± 3.19 minutes and 103.83 ± 1.97 minutes after administration in the second phase, for the 1mg/kg and 10mg/kg doses respectively. The inhibitory effects of nimesulide were higher in the second phase than in the first, although this difference did not reach significance (non-parametric Mann-Whitney, all $P>0.05$).

In saline-treated rats, both subcutaneous nimesulide and vehicle treatments facilitated 8-15g-evoked WDR dorsal horn neurones from pre-drug responses (repeated measures ANOVA with post-hoc Dunnet's test, Figure 5.11). There were no significant differences between the effects of nimesulide versus vehicle (area under the curve, non-parametric Mann-Whitney test, all $P>0.05$) on evoked responses (Figure 5.12).

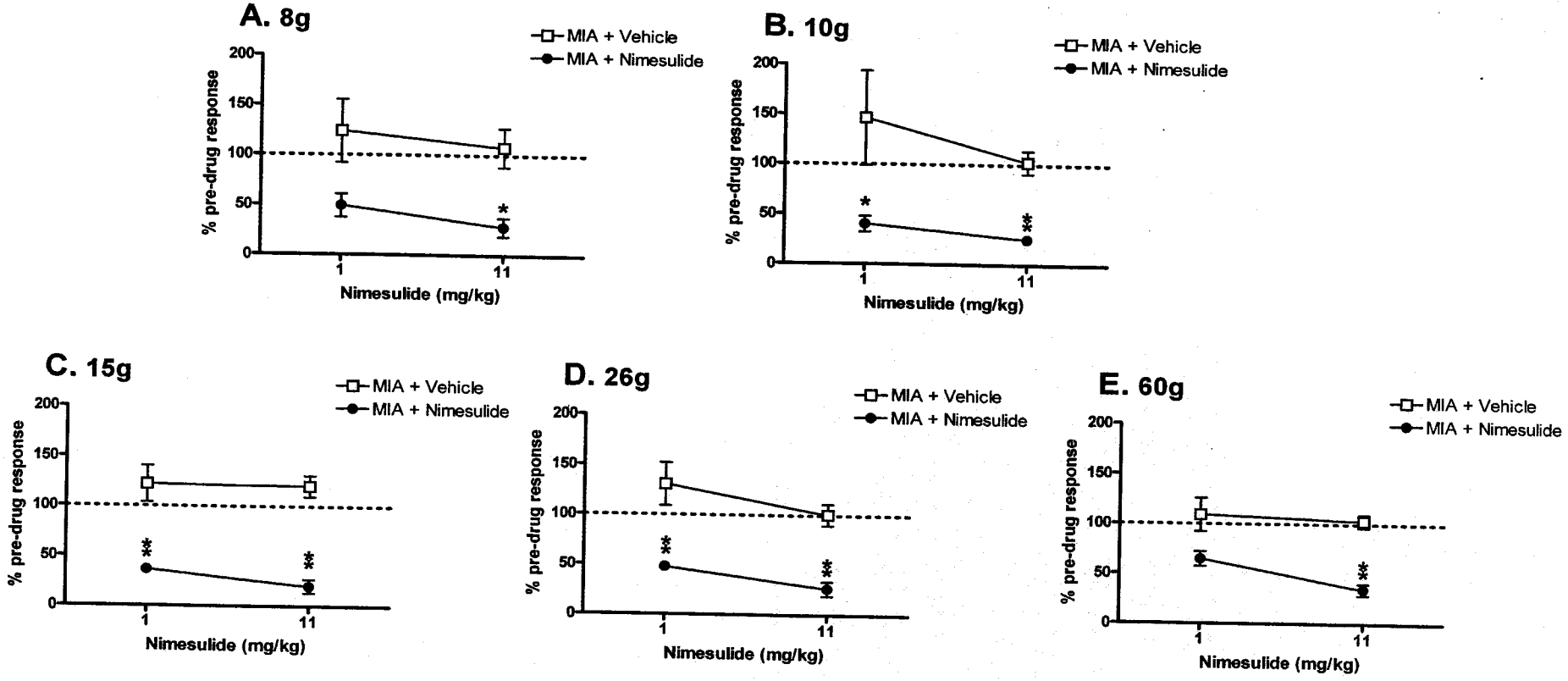

Figure 5.9 Subcutaneously administered nimesulide attenuated 8-60g-evoked responses of WDR dorsal horn neurones MIA-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses comparing the effects of nimesulide to vehicle treatment were performed using a non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0`.01$. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control responses.

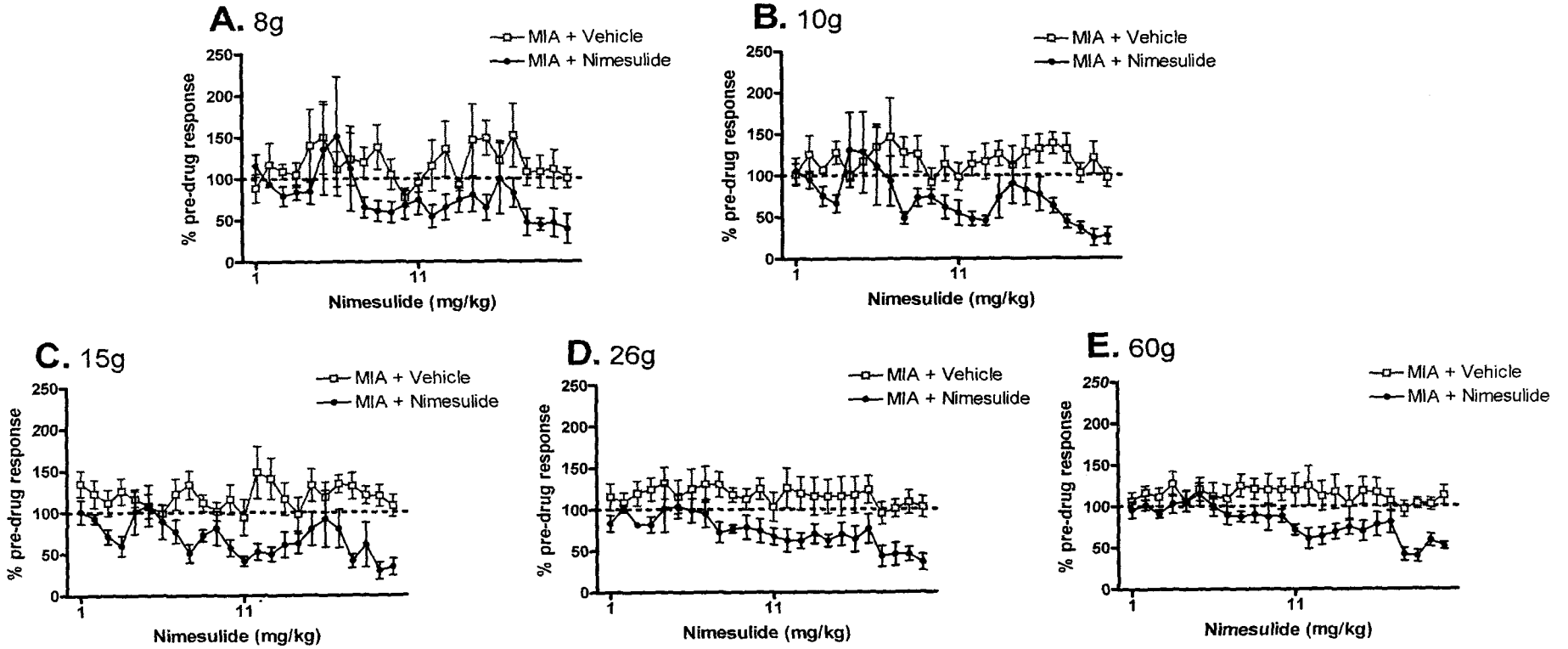

Figure 5.10 Time-course of the effects of subcutaneously administered vehicle and nimesulide on mechanically evoked responses of WDR dorsal horn neurones in MIA-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control responses.

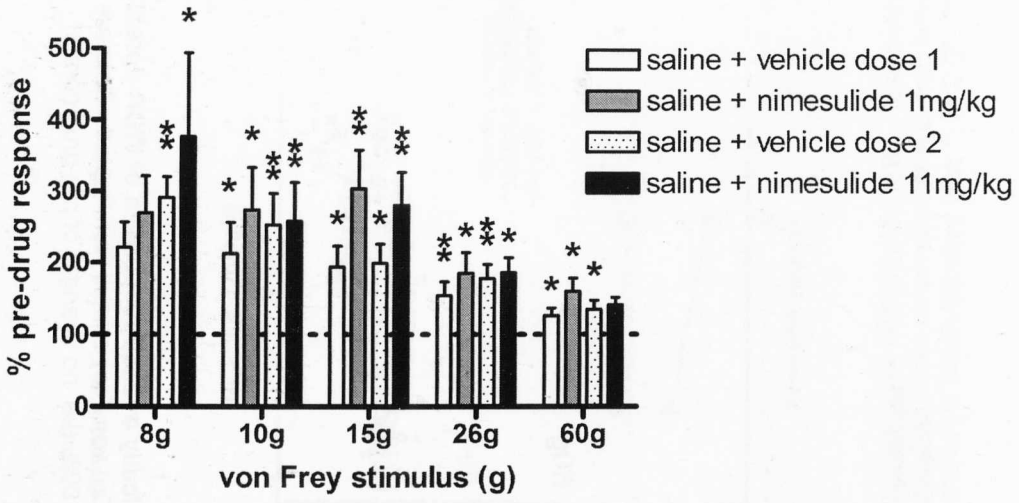

Figure 5.11 Mean maximal facilitatory effects of subcutaneously administered nimesulide or vehicle on mechanically evoked responses of WDR dorsal horn neurones in saline-treated compared to pre-drug responses (dashed line). Data are expressed as mean percentage of pre-drug rate of response ± SEM. Comparisons of the effects of nimesulide or vehicle to pre-drug responses were performed using repeated measures ANOVA with post-hoc Dunnet's test, *, $P<0.05$; **, $P<0.01$. n=6 neurones in 6 rats per group.

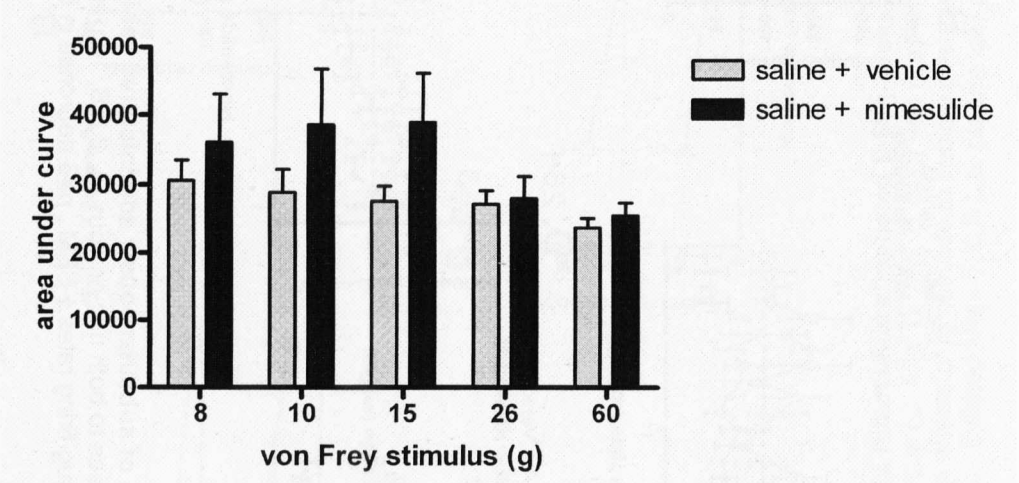

Figure 5.12 Area under curve of time-courses of subcutaneous nimesulide versus vehicle on mechanically evoked responses in saline-treated rats. Responses to both non-noxious and noxious von Frey stimuli were tested. Data are expressed as an area under the curve of the mean percentage of pre-drug firing rates ± SEM. Statistical analyses comparing the effects of nimesulide to vehicle treatment were performed using a non-parametric Mann-Whitney test (all $P>0.05$). n=6 neurones in 6 rats per group.

5.3.7. Effects of subcutaneous nimesulide or vehicle on post-stimulus responses of WDR neurones in MIA- and saline-treated rats

In MIA-treated rats, nimesulide attenuated post-stimulus responses in all four paradigms compared to pre-drug controls (repeated measures ANOVA with post-hoc Dunnet's test, Figure 5.13). The mean times of maximal effect of nimesulide in MIA-treated rats in all four paradigms of post-stimulus response are shown in Table 5.1. In many cases, post-stimulus responses following nimesulide administration fell to zero over the course of the study. Following subcutaneous administration of vehicle, post-8+10g-stimuli responses were facilitated, compared to pre-drug control values for all 4 measures, although these also did not reach significance (repeated measures ANOVA with post-hoc Dunnet's test). Higher weight evoked responses following 15-60g stimuli were not altered by vehicle, compared to pre-drug values. The effects of subcutaneous nimesulide and vehicle on post-stimulus responses in MIA-treated rats are shown in Figure 5.13. The results from all four measures of post-stimulus activity are presented in Table 5.2.

Table 5.1 Mean time of maximal effect of nimesulide on post-stimulus responses of WDR dorsal horn neurones in MIA-treated rats.

	nimesulide (mg/kg)	Time (minutes)
Duration of post-stimulus neuronal response	1	80.6 ± 6.1
	11	73.5 ± 4.5
Rate of post-stimulus neuronal response (total duration)	1	83.5 ± 4.7
	11	75.1 ± 4.9
Rate of post-stimulus neuronal response (first 10 seconds)	1	83.8 ± 4.8
	11	73.6 ± 4.6
Post-stimulus neuronal response count	1	81.0 ± 4.9
	11	72.5 ± 4.7

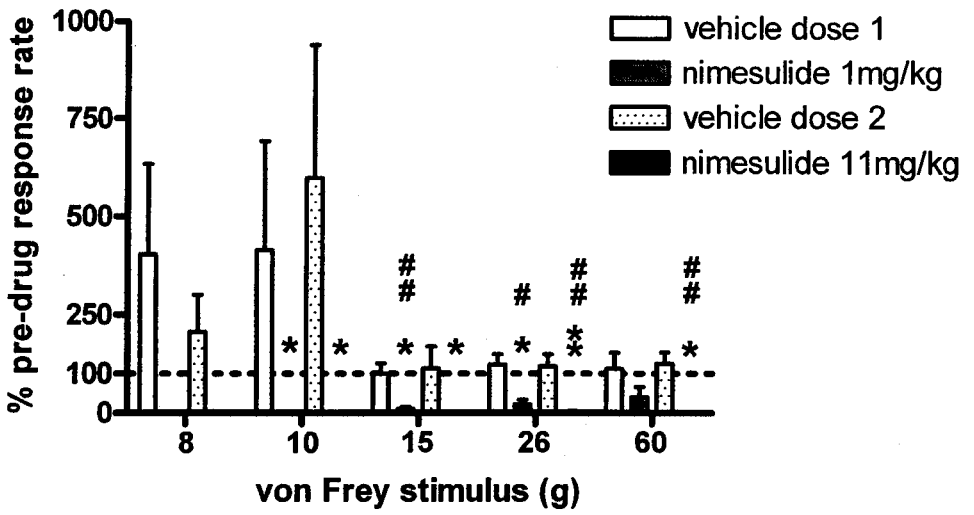

Figure 5.13 Rate of post-stimulus neuronal response (total duration) of WDR neurones in MIA-treated rats following subcutaneous administration of nimesulide or vehicle. After nimesulide administration, post-stimulus responses were attenuated compared to pre-drug responses (dashed line). After vehicle administration, post-8+10g-stimulus responses showed a trend towards facilitation compared to pre-drug responses. Data are expressed as mean percentage of pre-drug rate of response ± SEM. Comparisons of the effects of nimesulide or vehicle to pre-drug responses were performed using repeated measures ANOVA with post-hoc Dunnet's test, *, $P<0.05$; **, $P<0.01$. Comparisons of the effects of nimesulide versus vehicle were performed using a non-parametric Mann Whitney test, #, $P<0.05$; ##, $P<0.01$. n=6 neurones in 6 rats per group.

Table 5.2 Effects of subcutaneous nimesulide and time-matched vehicle controls on post-stimulus responses of WDR dorsal horn neurones in MIA-treated rats. Nimesulide attenuated post-stimulus responses following all intensity stimuli in all paradigms (**blue**). Following vehicle treatment, 8+10g-post-stimulus responses tended to be elevated (**red**). Data are expressed as mean percentage of pre-drug response ± SEM. Comparisons of the effects of nimesulide or vehicle to pre-drug responses were performed using repeated measures ANOVA with post-hoc Dunnet's test, *, $P<0.05$; **, $P<0.01$. Comparisons of the effects of nimesulide versus vehicle were performed using a non-parametric Mann-Whitney test, #, $P<0.05$; ##, $P<0.01$. n=6 neurones in 6 rats per group.

		% pre-drug control			
	stimulus	**vehicle (dose 1)**	**vehicle (dose 2)**	**nimesulide (1mg/kg)**	**nimesulide (11mg/kg)**
Duration of post-stimulus neuronal response	**8g**	**396.0 ± 172.8**	163.4 ± 98.8	-	-
	10g	167.8 ± 56.1	63.3 ± 31.9	- *	- *
	15g	124.4 ± 39.5	51.2 ± 18.7	**8.1 ± 5.6 * ##**	- **
	26g	113.1 ± 18.8	124.3 ± 41.8	**25.0 ± 13.2 #**	**12.0 ± 12.0 ***
	60g	75.9 ± 20.9	244.2 ± 114.5	**23.96 ± 10.38 ****	**8.14 ± 8.14 ****
Rate of post-stimulus neuronal response (total duration)	**8g**	**404.0 ± 229.4**	206.5 ± 93.98	-	-
	10g	**414.3 ± 277.9**	**598.3 ± 340.6**	- *	- *
	15g	99.7 ± 26.7	113.5 ± 33.4	**10.0 ± 6.2 * ##**	- *
	26g	122.5 ± 26.6	118.8 ± 30.8	**22.0 ± 12.4 * #**	**2.7 ± 2.6 ** ##**
	60g	113.2 ± 40.8	125.7 ± 29.2	**40.4 ± 26.7**	**1.9 ± 1.52 * ##**
Rate of post-stimulus neuronal response (first 10 secs)	**8g**	**816.3 ± 458.7**	**530.1 ± 357.2**	**1.4 ± 1.1**	-
	10g	**430.3 ± 282.2**	244.0 ± 180.5	-	-
	15g	127.3 ± 52.7	88.2 ± 44.0	**2.8 ± 1.7 * ##**	- *
	26g	144.3 ± 46.8	136.5 ± 60.7	**13.6 ± 8.9 ##**	**6.9 ± 6.9 ***
	60g	90.5 ± 31.0	212.8 ± 131.3	**10.0 ± 6.5 ****	**9.6 ± 9.6 ***
Post-stimulus neuronal response count	**8g**	**816.3 ± 458.7**	530.1 ± 357.2	-	-
	10g	**430.6 ± 282.1**	**248.2 ± 80.1**	-	-
	15g	137.6 ± 52.1	84.3 ± 41.9	**3.4 ± 2.1 * ##**	- *
	26g	143.2 ± 49.3	161.8 ± 57.6	**11.8 ± 7.8 #**	**6.4 ± 6.4 ***
	60g	102.7 ± 39.9	391.0 ± 287.2	**22.0 ± 15.47**	**0.6 ± 0.6**

In saline-treated rats, as with mechanically evoked responses, both subcutaneous nimesulide and vehicle facilitated all four measures of post-stimulus responses compared to pre-drug responses. This effect was greatest following 8-15g stimuli, corresponding with observations in stimulus-evoked responses. The effects of nimesulide on post-stimulus response in saline-treated rats are shown in Figure 5.14. There were no differences between the maximum facilitation of post-stimulus neuronal responses after subcutaneous nimesulide compared to the effects of time-matched vehicle controls (Kruskal-Wallis, all P>0.05). Effects of subcutaneously administered nimesulide and vehicle in MIA- and saline-treated rats on post-stimulus responses of WDR neurones were comparable to effects seen on stimulus-evoked response.

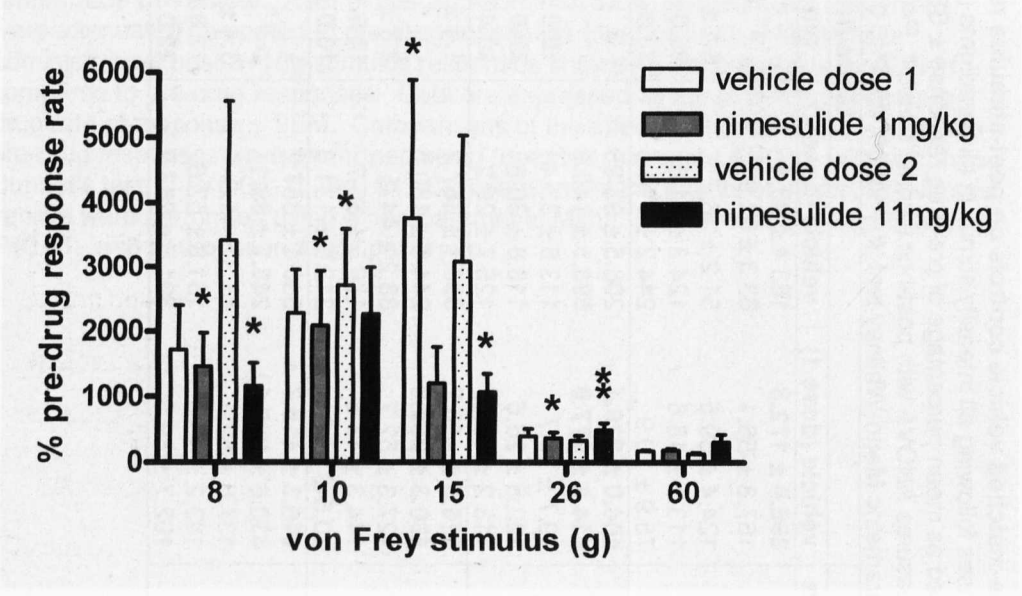

Figure 5.14 Mean maximal effects of subcutaneous nimesulide or time-matched vehicle on post-stimulus responses of WDR neurones in saline-treated rats. After both nimesulide or vehicle administration, post-stimulus responses were facilitated compared to pre-drug responses. Data are expressed as mean percentage of pre-drug rate of response ± SEM. Comparisons of the effects of nimesulide or vehicle to pre-drug responses were performed using repeated measures ANOVA with post-hoc Dunnet's test, *, $P<0.05$; **, $P<0.01$. Comparisons of the effects of nimesulide versus vehicle were performed using a non-parametric Mann-Whitney test, with no significant differences observed. n=6 neurones in 6 rats per group.

5.4. Discussion

The present study aimed to determine the effects of spinal and subcutaneous application of nimesulide on WDR dorsal horn neuronal responses in saline- and MIA-treated rats. Spinal nimesulide dose-dependently attenuated innocuous and noxious mechanically evoked responses of neurones in both saline- and MIA-treated rats. Subcutaneous nimesulide dose-dependently attenuated innocuous and noxious mechanically evoked responses in MIA-treated rats, and also produced a trend to reduced post-stimulus responses. However, in saline-treated rats, subcutaneous nimesulide did not attenuate mechanically evoked responses of DH neurones, in fact, both nimesulide and vehicle facilitated evoked- (significantly) and post-stimulus- (non-significantly) responses of DH neurones.

5.4.1. Validation of model induction

In order to ensure that MIA injections were correctly placed, and in order to ascertain that electrophysiological studies in MIA rats were a true representation of the model, behavioural and electrophysiological characteristics of these rats were compared to those from all MIA- or saline-treated rats used throughout this thesis (see Chapter 4). Behavioural data from hindpaw weight-bearing and von Frey withdrawal thresholds from each rat used were comparable to, and did not differ statistically from, the mean response seen within their group. Neuronal responses in saline- and MIA-treated rats were also comparable to those seen previously, both during stimulation and in the four paradigms of post-stimulus response, with MIA-treated rats displaying higher responses than saline-treated rats. The similarity between data obtained from saline- and MIA-treated rats used for study in this chapter to those used throughout the thesis show them to be representative of the MIA model of OA pain, and their controls.

5.4.2. Mechanisms of attenuation of neuronal responses following spinal COX-2 inhibition

Mechanically evoked responses of WDR dorsal horn neurones before spinal nimesulide or vehicle application were larger in MIA-treated rats, compared to saline-treated rats. In MIA-treated rats, spinal nimesulide dose-dependently attenuated 8-26g-evoked responses of WDR dorsal horn neurones compared to time-matched vehicle controls, while in saline-treated rats nimesulide attenuated 8-60g-evoked responses of neurones. The relative levels of inhibition of neuronal responses by spinal nimesulide following 8-26g stimuli were comparable in MIA- and saline- treated rats, as well as in naïve rats as seen in Chapter 3 (Table 5.3).

Table 5.3 Mean maximal effects of spinal nimesulide on 10+60g-evoked responses of WDR dorsal horn neurones in naïve, saline, and MIA-treated rats.

Stimulus	Treatment	% pre-drug control with spinal nimesulide 100µg/50µL
10g	naïve	37.5 ± 8.0
	saline	56.5 ± 16.5
	MIA	41.3 ± 6.7
60g	naïve	57.2 ± 9.2
	saline	55.5 ± 4.0
	MIA	82.8 ± 7.6 *

Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses comparing the effects of nimesulide in saline- and MIA-treated rats to the effects in naïve rats were performed using one-way ANOVA (Kruskal Wallis) with post-hoc Dunn's test; *, $P<0.05$. n=6 neurones in 6 rats per group.

The inhibitory effects of nimesulide support a role for COX-2 products in mediating responses to both innocuous and noxious stimuli. The lack of effect of nimesulide on 60-g evoked responses in MIA-treated rats suggests that COX-2 inhibition is unable to modulate the responses following frank noxious stimuli. The reason for this may be that higher doses of nimesulide are required to block the contribution of COX-2 in mediating this response. However, it may also suggest that COX-2 products increase neuronal responses to low-intensity stimuli but have little effect on already high neuronal responses following supramaximal-intensity stimuli. The ability of blockade of COX-2 to alleviate low weight evoked responses indicates a role of COX-2 products in the facilitation of 8-26g, but not 60g-evoked responses. This may be due to a sensitising effect of COX-2 on low threshold Aβ, Aδ and C-fibres.

Upregulation of low threshold mechanoreceptor activity is commonly seen in many models of chronic pain (see Chapter 1) and has been attributed to a number of factors including increased expression of sodium channels, voltage gated calcium channels - particularly the α2δ-1 subunit (Matsumoto *et al.*, 2006; Luo *et al.*, 2002), and vanilloid receptors, as well as the increased production and release of several neuropeptides including CGRP and substance P (Noguchi *et al.*, 1995; Miki *et al.*, 1998; Ma *et al.*, 1999). However, much conflicting evidence exists in this area (Allen *et al.*, 1999; Malcangio *et al.*, 2000; Hughes *et al.*, 2007) suggesting that the effects are specific to the type of nerve injury sustained (Malcangio *et al.*, 2000, Devor *et al.*, 2005). The data presented here suggests that nimesulide has an inhibitory effect on central sensitisation, reducing responses of post-synaptic neurones to elevated primary afferent fibre inputs. COX-2 inhibition has previously been shown to reverse behaviours associated with central sensitisation in several models, including chronic constriction injury and partial ligation models of neuropathic pain (Bingham *et al.*, 2005).

5.4.3. Peripheral COX-2 upregulation contributes to pain mechanisms in the MIA model of OA

In MIA-treated rats, subcutaneous nimesulide dose-dependently inhibited mechanically evoked responses of WDR dorsal horn neurones compared to time-matched vehicle controls. Subcutaneous nimesulide did not have an inhibitory effect in saline-treated rats. These novel effects of systemic nimesulide in MIA- versus saline-treated rats would suggest that effects are unlikely to be mediated by a spinal site of action, as spinal nimesulide attenuated neuronal responses equally in MIA- and saline-treated rats. A possible explanation is that subcutaneously administered nimesulide acts to inhibit COX-2 in the joint, where COX-2 is upregulated following MIA injection (Dumond *et al.*, 2004). COX-2 is also upregulated in human end-stage OA within the joint tissues, including cartilage, synovial tissue and subchondral bone (Brenner *et al.*, 2004; Amin *et al.*, 1997; Ogino *et al.*, 2009), and in subchondral bone in a naturally occurring canine model of OA (Lascelles *et al.*, 2009). The findings

presented in this thesis support a role for non-spinal COX-2 in contributing to established pain mechanisms in the MIA model.

It is also possible that following subcutaneous administration, some of the nimesulide reaches the spinal cord, where COX-2 is upregulated in MIA-treated rats, reaching a maximum at day 5 and maintaining thereafter (Prochazkova *et al.*, 2009). A spinal site of action for subcutaneously administered nimesulide together with spinal COX-2 upregulation in MIA-treated rats may explain the differential effects of nimesulide on neuronal responses seen in MIA- and saline-treated rats. Subcutaneously administered nimesulide may also act supraspinally to attenuate neuronal responses. Systemically administered nimesulide has been shown to reach the brain (see section 5.4.4), where COX-2 is expressed by excitatory neurones, and is found post-synaptically in several areas including the cerebral cortex, amygdala, hippocampus, brainstem, and dorsal raphe nucleus (Breder *et al* 1995; Kaufmann *et al.*, 1996).

COX-2 is a common target for OA-pain treatments, with the use of celecoxib and lumiracoxib commonplace in the clinic (Stengaard-Pedersen *et al.*, 2004; Tannenbaun *et al.*, 2004). COX-2 inhibition has also been shown to attenuate OA-related pain behaviour in the MIA model, with repeated dosing with the COX-2 inhibitor celecoxib and the non-selective COX-inhibitor indomethacin reducing alterations in weight-bearing on the ipsilateral side (Pomonis *et al.*, 2004). However, in this previous study, acute systemic administration did not alleviate weight-bearing deficiencies, suggesting that the effects of peripheral COX-2 upregulation on nociceptive transmission of primary afferent fibres do not contribute greatly to behavioural deficits. Chronic dosing may allow drug accumulation and COX inhibition at more central sites such as the brain or spinal cord. Here, upregulation of COX-2 contributes to central sensitisation mechanisms, with the time-course of COX-2 gene expression in the spinal cord following intra-articular MIA injection mirroring that of the development of pain behaviours (Prochazkova *et al.*, 2009)(see next section).

5.4.4. Possible secondary mechanisms of subcutaneous-nimesulide-mediated attenuation of evoked responses of WDR dorsal horn neurones in the MIA model of OA

Visual examination of the time-courses of subcutaneously administered nimesulide reveals a possible two-phase mode of action of nimesulide on mechanically evoked responses of WDR dorsal horn neurones in MIA-treated rats. While these observations were not statistically significant, the errors (particularly following 26-60g stimuli) at each timepoint following the second dose of nimesulide are quite small, suggesting this trend to be a real effect of nimesulide and not due to variability. It suggests that nimesulide may act in two distinct compartments and the time between phases of inhibitory action is a factor of nimesulide's latency of distribution. Based on what is known of the distribution of nimesulide throughout tissues following i.v. and p.o administration, it is expected that following subcutaneous administration, nimesulide distributes throughout peripheral tissues over a period of 1-4 hours (Rainsford, 1999, and personal communication). Data on its distribution within the spinal cord following systemic administration is unavailable, however as the volume of distribution of nimesulide is quite low (~10% body weight, Bernareggi & Rainsford, 2005), and following the observation in these studies that at these doses, subcutaneous nimesulide does not produce an effect at the spinal cord within 4 hours, it is expected that the primary effects of nimesulide under the current study conditions will occur in the periphery. After systemic administration very low concentrations of nimesulide can be found in the brain (Bernareggi & Rainsford, 2005), and nimesulide has been shown to inhibit neuronal activation in the brain after systemic (i.p.) administration, in nociceptive areas such as the PAG and locus coeruleus (Tassorelli *et al.*, 2003). The second phase of inhibitory effect of nimesulide could be due to the time taken for nimesulide to reach the brain after systemic administration, and suggests that nimesulide may modulate both peripheral and central mechanisms of nociceptive transmission in the MIA model. An alternative explanation (which is not mutually exclusive of a putative central site of action for nimesulide) is that nimesulide may act on two distinct molecular systems. One would certainly be the classical prostaglandin inhibition pathway (although the actions of nimesulide here are relatively weak – for

review see Bevilacqua & Magni, 1993) and therefore also effects on the synthesis of cytokines and other inflammatory mediators (see Chapter 4), and the other could possibly be via the endocannabinoid system, with an increased production of CB_1-receptor ligands through a shunting of endocannabinoid metabolism through LOX and cP450 metabolic pathways as outlined in Chapter 3. Nimesulide has also been shown to have anti-inflammatory actions by inhibiting oxidant release from activated neutrophils; reducing histamine release from mast cells; inhibiting production of platelet activating factor from basophils; and in cartilage, inhibiting stromelysin release and blocking MMP activity (Bevilacqua & Magni, 1993; Bennett & Villa, 2000). Interestingly, at low concentrations, nimesulide was shown to increase nitric oxide production *in vitro* in an inflammatory model (Boje *et al.*, 2003), and as a result it has been suggested that *in vivo*, inhibition of COX-2 alone may promote NO toxicity. The mechanisms for this action are unclear but COX-2 products may contribute to an inhibitory feedback loop for iNOS under inflammatory conditions, modulating NO synthesis. The importance of endocannabinoid modulation of several nociceptive mediators including NO is known (for review, see La Rana *et al.*, 2008), and it has been shown that prolonging endocannabinoid action reduces NO release (Ortega-Gutiérrez *et al.*, 2005) in a CB_1-receptor-dependent manner (Molina-Holgado *et al.*, 2002; Waksman *et al.*, 1999). It is possible in the studies outlined in this chapter, that after COX-2 inhibition by nimesulide, inhibition of prostaglandin synthesis accounts for the initial attenuation of WDR mechanically evoked responses observed within the first hour, while an increase in NO synthesis triggered by COX-2 inhibition results in the recovery of neuronal response from this first phase. The increase in CB_1-receptor activation following nimesulide administration (as described in Chapter 3, results section 3.3.2) could feedback on this increased NO synthesis and result in the second phase of attenuative effect of nimesulide on evoked responses of WDR dorsal horn neurones in the MIA-treated rat. This possibility is especially attractive in light of findings that the endocannabinoid system is upregulated in the MIA model (see Chapter 6),

5.4.5. Vehicle induced facilitation

In saline-treated (as opposed to MIA-treated) rats, not only did subcutaneous nimesulide fail to attenuate stimulus-evoked responses, but both subcutaneous nimesulide and vehicle increased mechanically evoked neuronal responses from pre-drug responses, and produced a trend towards increase post-stimulus responses. The effect was most pronounced in response to low-weight stimuli suggesting the involvement of Aβ fibres. The basis for vehicle mediated facilitation of neuronal responses is puzzling. The facilitatory effect on evoked responses of WDR dorsal horn neurones did not occur in saline-treated rats receiving spinal nimesulide or vehicle, indicating that this response is due to the model. If it were, we might expect that in saline-treated rats, this rise in evoked-responses throughout the course of the experiment would also be evident in rats receiving spinal vehicle, but this is not the case. The effect could be due to the solubilising agent for nimesulide in these experiments – Tween80, and specifically, its effects in peripheral tissues. Tween has previously been shown to be associated with several hypersensitivity reactions (Gelderblom *et al.*, 2001), including pain phenomena such as itch and burning sensations, and so may be a candidate for the phenomena seen here.

5.4.6. Conclusions

Data presented here suggest a role for both peripheral and spinal upregulation of COX-2 expression in the increase of nociceptive transmission in the MIA model of pain. Spinal COX-2 upregulation contributes to central sensitisation in this model, increasing spinal excitability of WDR dorsal horn neurones to normally non-noxious events, while peripheral COX-2 upregulation appears to be responsible for increasing both non-nociceptive and nociceptive input from the periphery to the dorsal horn. Spinal actions of nimesulide in the studies presented here, together with the findings of a CB_1 receptor-dependent mechanism of spinal nimesulide activity in naïve rats suggest a role for the endocannabinoid system in the modulation of aberrant nociceptive transmission in the MIA model. The role of the endocannabinoid system in the MIA model will be discussed further in Chapter 6.

Chapter 6 Functional effects of endocannabinoid tone on signalling mechanisms in a rat model of osteoarthritis

6.1. Introduction

As described in Chapter 1, the involvement of the EC system in pain has been well established both in human painful human conditions and in animal models. Studies into its involvement in neuropathic and chronic pain have been conducted in various models including nerve injury, disease-related models including diabetes, cancer- and HIV-treatment-related pain, demyelination disorders, multiple sclerosis and postherpetic neuralgia, with antinociceptive effects observed following prevention of EC metabolism, or receptor stimulation by exogenous compounds (for an in-depth review, see Rahn and Hohmann, 2009). Studies have involved the use of exogenous CB receptor ligands including endocannabinoid and fatty acid amide compounds, herbal cannabis constituents such as Δ^9-THC and synthetic analogues thereof, and synthetic agonists, both receptor subtype specific and mixed. Studies have also been undertaken with inhibitors of endocannabinoid transport and metabolism. Both exonegous application of CB receptor ligands and modulation of EC metabolism were able to suppress or reverse established mechanical hyperalgesia and allodynia, and thermal hyperalgesia and cold allodynia in a number of chronic pain models including the nerve injury models (for an in-depth review, see Rahn and Hohmann, 2009). For example, in the chronic constriction injury (CCI) model of neuropathic pain, nociceptive behaviours were blocked with mixed CB receptor agonists, CB_2 receptor-selective agonists, and FAAH/MAGL inhibitors. Suppression of nociception in these models was mediated not only by the CB receptors, but also (and in some cases, solely) by the TRPV1 receptor. In the CCI model, antihyperalgesic effects of cannabidiol, a non-psychoactive component of marijuana, were blocked by co-administration with the TRPV1 receptor antagonist capsazepine, but not by CB_1 or CB_2 receptor antagonists (Costa *et al.*, 2007; Comelli *et al.*, 2008). Antinociceptive effects of CB receptor activation accompanied neurophysiological changes, with the CB receptor agonist WIN 55,212-2 dose-dependently inhibiting neuronal wind-up (Strangman & Walker, 1999) and reversing elevated spontaneous activity of WDR dorsal horn neurones associated with the CCI model (Liu & Walker, 2006) in a CB_1-dependent manner.

6.1.1. Alterations in the cannabinoid system brought about by experimental models of chronic pain

Not only have the antinociceptive effects of endocannabinoid system manipulation in models of neuropathic pain been evaluated, but the effects of neuropathic pain models on the endocannabinoid system, particularly CB receptor expression and endocannabinoid levels, have also been studied. Endocannabinoid levels in some models were altered, for example in Theiler's murine encephalomyelitis viral infection, a model of MS, upregulation of CB_2 receptor mRNA was observed, as were elevated levels of 2-AG and PEA (Loria *et al.*, 2008). Increased CB_2 receptor expression in the spinal cord has also been shown in other models of neuropathic pain, including spinal nerve transection (Romero-Sandoval *et al.*, 2008), CCI and spinal nerve ligation (SNL) (Zhang *et al.*, 2003), in which activation of CB_2 receptors attenuated neuronal activity (McGaraughty *et al.*, 2009) and capsaicin-evoked calcium responses (Sagar *et al.*, 2005). Changes in spinal and supraspinal levels of endocannabinoids and fatty acid amides in the CCI model of neuropathic pain have also been observed, with 2-AG and AEA levels elevated in spinal cord and PAG three days postoperatively, and also in the rostro-ventral medulla (RVM) at day seven, when thermal hyperalgesic and mechanical allodynic responses are at a maximum, while levels of PEA were decreased in the spinal cord at post-operative day three, and also in the dorsal raphe and RVM at day seven (Petrosino *et al.*, 2007). The SNL model of neuropathic pain has also been linked with increases in AEA levels in dorsal root ganglion (DRG, Mitrirattanakul *et al.*, 2006), and the spinal cord (Guasti *et al.*, 2009).

Endocannabinoid tone has also been demonstrated in models of both acute (kaolin/carrageenan) and chronic (Freund's complete adjuvant) joint inflammation, with the use of CB_2 receptor agonists JWH015 and JWH133. In naïve rats, these agonists caused CB_2 and TRPV1 receptor-mediated increase in synovial blood flow, whereas in acute and chronic joint-inflamed rats, this reaction was attenuated, possibly due to receptor internalisation following prolonged exposure to elevated endocannabinoid levels (McDougall *et al.*, 2008).

6.1.2. The endocannabinoid system in the MIA model of osteoarthritic pain

Peripheral endocannabinoid tone has been demonstrated in the MIA model of OA, with CB_1 receptor antagonist application directly to the knee increasing neuronal responses to non-noxious knee rotation in MIA- but not saline-treated rats (Schuelert & McDougall, 2008). In addition, CB_1 receptor agonists reduced neuronal responses to noxious rotation with greater antinociceptive effects in MIA-treated than control rats (Schuelert & McDougall, 2008). Work from our laboratory has shown that the MIA model of osteoarthritic pain is associated with elevated levels of the endocannabinoids AEA and 2-AG, and the related molecules PEA and OEA, in the spinal cord and in the hindpaw (see appendix). Interestingly, while levels of the *N*-acylethanolamines are elevated both ipsilaterally and contralaterally in the spinal cord, levels of 2-AG contralaterally remain unaltered. This suggests that not only are afferent inputs are involved in central sensitisation, but that descending nociceptive pathways also play a role in the neuronal plasticity seen in this model.

6.1.3. Aims

Mounting evidence from our group strongly supports a role of endocannabinoids in the MIA model of osteoarthritic pain. The aim of work presented here was to determine whether the elevated levels of endocannabinoids and related compounds act tonically to modulate the responses of WDR dorsal horn neurones in the MIA model of osteoarthritic pain, via the activation of CB_1 or CB_2 receptors.

6.2. Methods

6.2.1. Induction of the MIA model of osteoarthritis pain

For intra-articular injection, monosodium iodoacetate (MIA, Sigma-Aldrich, UK), was prepared freshly on the day of injection by dissolution in sterile saline. A dose of 1mg MIA in 50µL sterile saline (n=18) or 50µL sterile saline alone for control groups (n=18) was administered per rat, based on work done in this laboratory (see Chapter 4). Male Sprague-Dawley rats weighing 160-190g at the time of injection were used. For detailed methodology of anaesthesia and injection of MIA, refer to Chapter 2. The experimenter was blind to all treatments.

6.2.2. Ensuring correct placement of injection and development of MIA-induced changes in nociceptive behaviour

To ensure that MIA- or saline- treated rats used in electrophysiological studies were true representations of their treatment groups, rats underwent testing for nociceptive behaviour encompassing changes in hindlimb weight-bearing and von Frey monofilament withdrawal threshold, both before injection and for 28 days thereafter. Data were gathered and analysed in an identical manner to that described in Chapter 4, and compared with data therein at the end of the experiment.

6.2.3. Measuring functional effects of EC tone in the spinal cord on neuronal responses following intra-articular MIA injection

For detailed methodology of anaesthesia, surgery, and identification and recording of WDR neuronal responses, refer to Chapter 2. One WDR neurone from laminae V and VI of the dorsal horn of the spinal cord was recorded per rat for all of the data presented in this chapter. The depth of neurones was recorded and checked at the end of the experiment. Effects of intra-articular injection of MIA (versus saline) on endocannabinoid tone of the spinal cord were evaluated with the CB_1 receptor antagonist AM251 (n=12 rats: 6 in MIA-treated rats; 6 in saline-treated rats), and the

CB_2 receptor antagonist SR144528 (n=12: 6 in MIA-treated rats; 6 in saline-treated rats), and compared to the effects of the vehicle alone (3% Tween 80 / physiological saline, n=12: 6 in MIA-treated rats; 6 in saline-treated rats) on mechanically evoked responses of WDR dorsal horn neurones in the spinal cord. Data are expressed as a percentage of the average pre-drug neuronal response over the 10 second stimulation period. The effects of the antagonists on post-stimulus responses were also studied, comparing effects to pre-drug post-stimulus duration of response, firing count, and firing rate, as described in Chapter 4. The same vehicle was used for studies of both AM251 and SR144528. In order to minimise the number of animals used, data gathered from vehicle treatments in MIA- and saline-treated rats used in this study were also used in studies outlined in Chapter 5. AM251 and SR144528 were prepared freshly on the day by dissolution in the vehicle to give final concentrations of 0.1, 1, and 10μg/50μL (3.6, 36 and 360μM) for AM251, and 0.001, 0.01 and 0.1μg/50μL (42nM, 420nM and 4.2μM) for SR144528. Doses were based on previous literature which administered these antagonists spinally in naïve rats and models of neuropathic pain in rats (AM251 - Jhaveri *et al.*, 2006, Johanek and Simone, 2004; SR144528 – Sagar *et al.*, 2005), and were administered directly onto the exposed spinal cord using a 50μL Hamilton syringe (Hamilton-Bonaduz, Switzerland). Drugs were applied for 60 minutes to the spinal cord, before removal and application of the next (higher) dose. In saline-treated rats, vehicle was also applied for 60 minutes per dose whereas in MIA-treated rats each dose of vehicle was applied for 40 minutes, as these data were gathered in earlier experiments outlined in Chapter 5. Assurances were made that this did not affect experimental data, see Chapter 5, section 5.3.4.

6.3. Results

Mean depths of recordings were as follows: AM251 = 793 ± 39μm in MIA-treated rats, 891 ± 54μm in saline-treated rats, n=6 neurones in 6 rats per group; SR144528 = 807 ± 82μm in MIA-treated rats, 898 ± 78μm in saline-treated rats, n=6 neurones in 6 rats per group; vehicle = 812 ± 70μm in MIA-treated rats, 790 ± 36μm in saline-treated rats, n=6 neurones in 6 rats per group.

6.3.1. Effects of intra-articular injection of MIA or saline on nociceptive responses of awake rats

In order to ensure that intra-articular injections of MIA were correctly placed and that the MIA model of OA pain progressed as previously described, the behavioural responses of each rat were compared to that of all MIA- and saline-treated rats used throughout this thesis (presented together in Chapter 4, section 4.3.1). Injection of 1mg MIA in 50μL saline significantly reduced weight borne on the ipsilateral hindlimb, compared to saline-treated rats (Figure 6.1; saline, n=18; MIA n=18, one way ANOVA Kruskal-Wallis with post-hoc Dunn's test) in a biphasic manner, as previously described. MIA also caused a significant decrease in withdrawal threshold to punctuate stimulation of the plantar surface of the ipsilateral hindpaw compared to the contralateral hindpaw, whilst there was no difference between ipsilateral and contralateral hindpaw withdrawal thresholds in saline-treated rats (Figure 6.2; saline, n=18; MIA, n=18). Results from both weight -bearing and withdrawal threshold studies were comparable to those obtained from all MIA- and saline-treated rats presented in Chapter 4, with no statistically significant differences existing between them (one way ANOVA (Kruskal-Wallis) with post-hoc Dunn's test, all $P>0.05$)

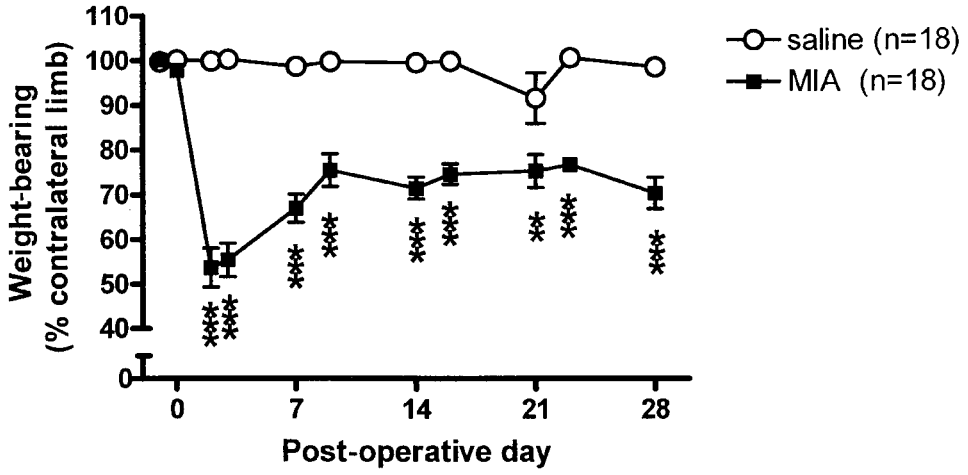

Figure 6.1 Reduced weight-bearing on the ipsilateral hindlimb of rats receiving intra-articular injection of 1mg MIA in 50µL saline (black squares, n=18) versus 50µL saline alone (open circles, n=18), over a 28-day period post-injection. Statistical analysis comparing the effects of saline to MIA on weight-bearing through the ipsilateral hindlimb was performed using a one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test, **, $P<0.01$; ***, $P<0.005$. Weight-bearing on the ipsilateral hindlimb is expressed as a mean percentage of the weight borne on the contralateral hindlimb, averaged from 3 readings per rat, per time-point, ± SEM.

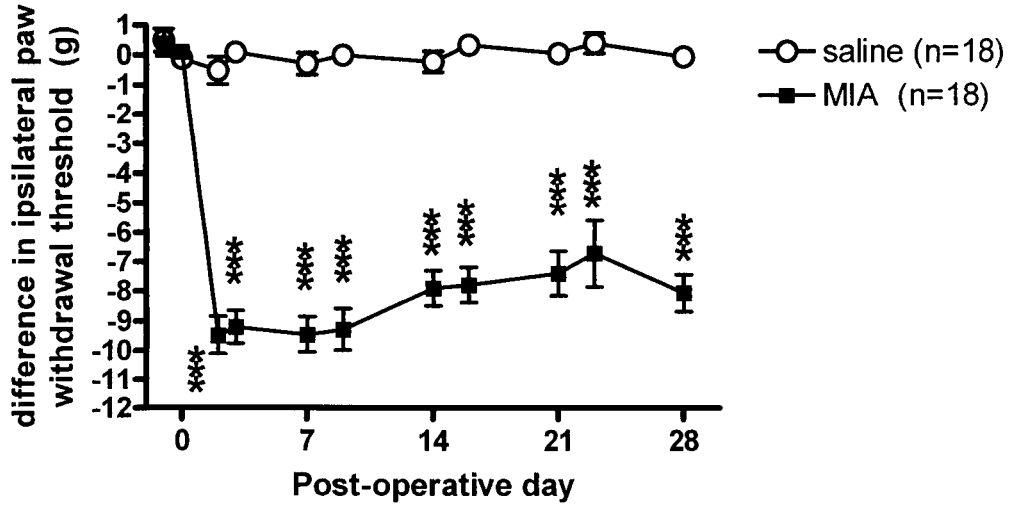

Figure 6.2 Reduced withdrawal thresholds to mechanical stimulation of the ipsilateral hindpaw of rats receiving intra-articular injection of 1mg MIA in 50µL saline (black squares, n=18) versus 50µL saline alone (open circles, n=18), over a 28-day period post-injection. Statistical analysis comparing the effects of saline and MIA on hindpaw withdrawal thresholds of the ipsilateral and contralateral hindpaws was performed using a one-way ANOVA Kruskal-Wallis, with post-hoc Dunns test, *, $P<0.05$; **, $P<0.01$; ***, $P<0.005$. Data are expressed as the mean difference in grams between withdrawal thresholds of the ipsilateral and contralateral hindpaws ± SEM.

6.3.2. Effects of intra-articular injection of MIA on mechanically evoked responses of WDR dorsal horn neurones in the spinal cord

To further ensure the integrity of induction of the MIA model of OA-like pain in the MIA-treated rats used in electrophysiological studies in this chapter, the effects of MIA and saline injection on baseline mechanically evoked responses of WDR dorsal horn neurones of each rat were compared to those of all MIA and saline-treated rats used throughout this thesis (presented together in Chapter 4, section 4.3.1). MIA caused a trend to increased evoked responses of WDR neurones, particularly in response to higher weight stimuli, although this did not reach statistical significance (non-parametric Mann-Whitney, $P>0.05$). Neuronal responses greater that 2 standard deviations from the group mean response to any stimulus, per group, were excluded from analysis. In total this happened in 6 instances of 90 values obtained from saline-treated rats, and none from MIA-treated rats. There were no significant differences in these data and those presented in Chapter 4 (non-parametric Mann-Whitney test, $P>0.05$).

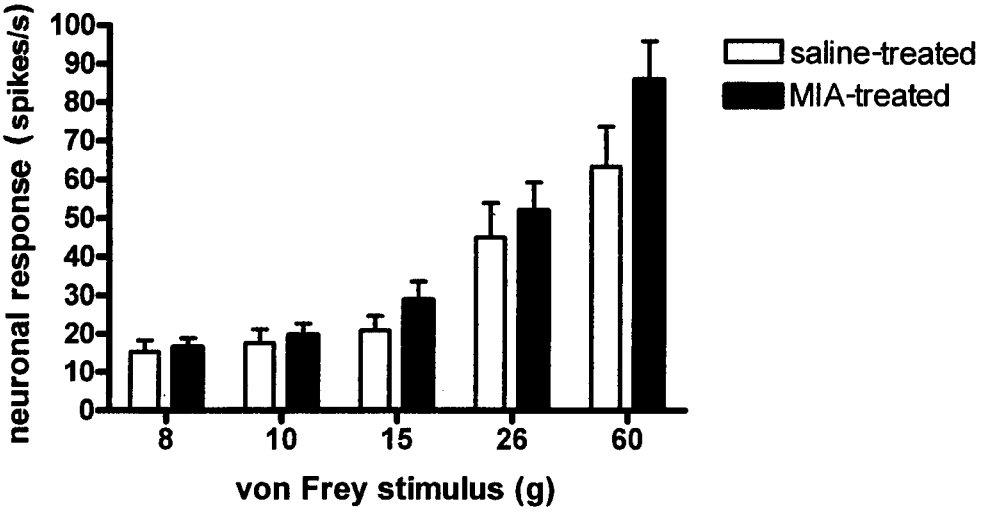

Figure 6.3 Graded responses of WDR neurones in saline (white bars, n=18) and MIA- (black bars, n=18) treated rats following mechanical stimulation of the hindpaw receptive field, between 28 and 31 days after injection. Statistical analyses comparing the effects of saline to MIA on evoked responses were performed using a non-parametric Mann-Whitney test (all $P>0.05$). Data are expressed as the mean firing rate of the neurone per second (spikes/s) over a 10 second stimulation period, ± SEM.

6.3.3. Effects of intra-articular injection of MIA on post-stimulus responses of WDR dorsal horn neurones in the spinal cord

The effects of MIA and saline injection on post-stimulus responses of WDR dorsal horn neurones from the current study were also compared to that of all MIA and saline-treated rats used throughout this thesis. In the present study, post-stimulus responses of WDR neurones in MIA-treated rats were not always elevated compared to saline-treated rats; however, significant elevation was observed following 60g stimuli in 3 of the 4 parameters (Figure 6.4, **Left panel A**, **C**, and **D**), while the remaining parameter (rate of response over the total duration) showed a trend towards greater responses in MIA-treated rats (Figure 6.4, **Left panel B)**. However, following 10, 15 and 26g stimuli, the post-stimulus response in MIA-treated rats was often lower than in saline-treated rats, and much lower than the average of all rats used throughout the thesis. There were significantly lower responses in MIA-treated rats used in this study versus all MIA-treated rats presented throughout this thesis, in 3 out of 4 parameters, following the 15g stimulation (Figure 6.4, **Right panel**). Statistical comparisons between responses in MIA- and saline-treated rats within this study, and between these data and those presented in Chapter 4 were performed using a non-parametric Mann-Whitney test; *, $P<0.05$, **, $P<0.01$.

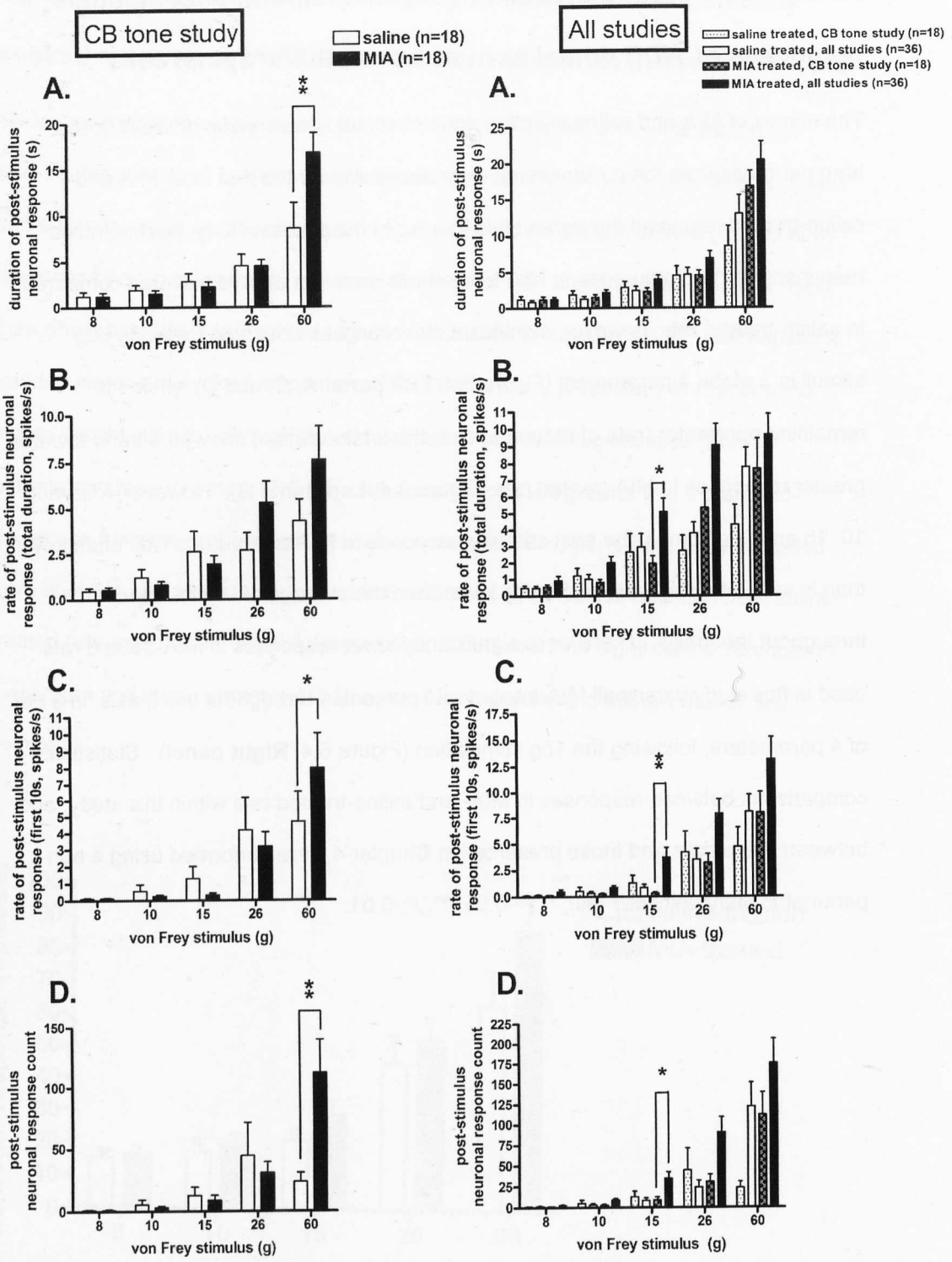

Figure 6.4 Post-stimulus responses of WDR dorsal horn neurones in saline- and MIA-treated rats following the removal of mechanical stimuli from the hindpaw receptive field. A, duration of post-stimulus neuronal response (s); B, rate of post-stimulus neuronal response for the total duration of response (spikes/s); C, rate of post-stimulus neuronal response in the first 10 seconds following stimulus removal (spikes/s); D, post-stimulus neuronal response count. Left panel ("CB tone study") shows data from rats used in the present study; right panel ("All studies") shows data from the present, CB tone study compared to data from all MIA- and saline-treated rats used throughout this thesis (including CB tone study data). N.B. difference in scales between left and right panels. Data are expressed as mean ± SEM. Statistical comparisons were performed using a non-parametric Mann-Whitney test; *, $P<0.05$, **, $P<0.01$.

Post-stimulus responses in MIA- and saline-treated rats showed differential effects to repeated hindpaw stimulation following spinal vehicle administration. In saline-treated rats, following spinal administration of vehicle, post-stimulus responses remained at around 100% of the pre-vehicle control values. However, in MIA-treated rats, post-stimulus responses often fell to zero following spinal administration of vehicle. Comparison of the area under the curves for the time-courses of effects of spinal vehicle in MIA- and saline-treated rats revealed a decrease in post-8g neuronal responses of MIA-treated rats following spinal vehicle administration in all four post-stimulus paradigms tested, compared to saline-treated rats, and there was a trend for decreased 15-60g post-stimulus neuronal responses in MIA-treated versus saline-treated rats following spinal vehicle in some paradigms (non-parametric Mann-Whitney test, Figure 6.5).

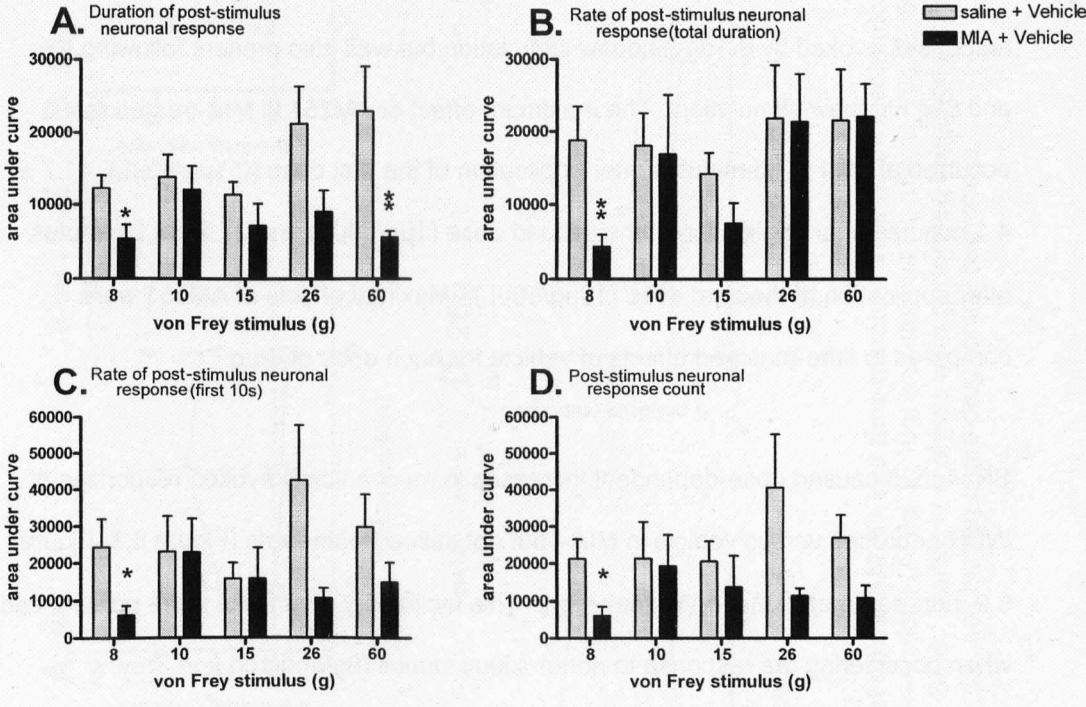

Figure 6.5 Post-stimulus responses of WDR dorsal horn neurones in saline- and MIA-treated rats following vehicle application. **A**, duration of post-stimulus neuronal response (s); **B**, rate of post-stimulus neuronal response for the total duration of response (spikes/s); C, rate of post-stimulus neuronal response in the first 10 seconds following stimulus removal (spikes/s); D, post-stimulus neuronal response count. Data are expressed as the area under the curve for the duration of the testing period ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test, *, $P<0.05$; **, $P<0.01$. n=6 neurones in 6 rats per group.

6.3.4. Effects of intra-articular injection of MIA on endocannabinoid tone in the spinal cord: mechanically evoked responses of WDR dorsal horn neurones

The effects of the CB_1 and CB_2 receptor antagonists AM251 and SR144528 on mechanically evoked responses of WDR dorsal horn neurones in MIA- and saline-treated rats were evaluated using 3 consecutive, increasing doses of the antagonist in 50µL vehicle (3% Tween in saline, n=6 neurones per antagonist in 6 saline-treated rats, n=6 neurones per antagonist in 6 MIA-treated rats), and compared to the effects of 50µL vehicle alone (n=6 neurones in 6 saline-treated rats, n=6 neurones in 6 MIA-treated rats). Spinal AM251 caused dose-dependent increases (peaking with the 1µg dose) in mechanically evoked responses of WDR neurones in MIA-, but not saline-treated rats (Figure 6.6, Figure 6.7, non-parametric Mann-Whitney test). The facilitation produced by spinal AM251 in MIA-treated rats was most pronounced for the responses evoked by 8-15g hindpaw stimulation but was also present following 26g and 60g hindpaw stimulation. The maximum effect of AM251 in MIA-treated rats occurred at 39.4 ± 4.5 minutes after application of the first dose (0.1µg/50µL), 41.7 ± 4.1 minutes after application of the second dose (1µg/50µL) and 27.2 ± 3.2 minutes after application of the third dose (10µg/50µL). Maximal effects of AM251 were compared to time-matched effects of vehicle for each dose of drug.

SR144528 caused dose-dependent increases in mechanically evoked responses of WDR neurones versus vehicle in MIA-, but not saline-treated rats (Figure 6.8, Figure 6.9, non-parametric Mann-Whitney test). This facilitatory effect was more pronounced when considering the response to non-noxious stimuli (8g and 10g von Frey monofilaments), but was also present in response to 15g and 26g stimuli. The maximum effect of SR144528 in MIA-treated rats occurred at 32.6 ± 3.1 minutes after application of the first dose (0.001µg/50µL), 37.8 ± 3.6 minutes after application of the second dose (0.01µg/50µL) and 35.6 ± 3.3 minutes after application of the third dose (0.1µg/50µL). Maximal effects of SR144528 were compared to time-matched effects of vehicle for each dose of drug.

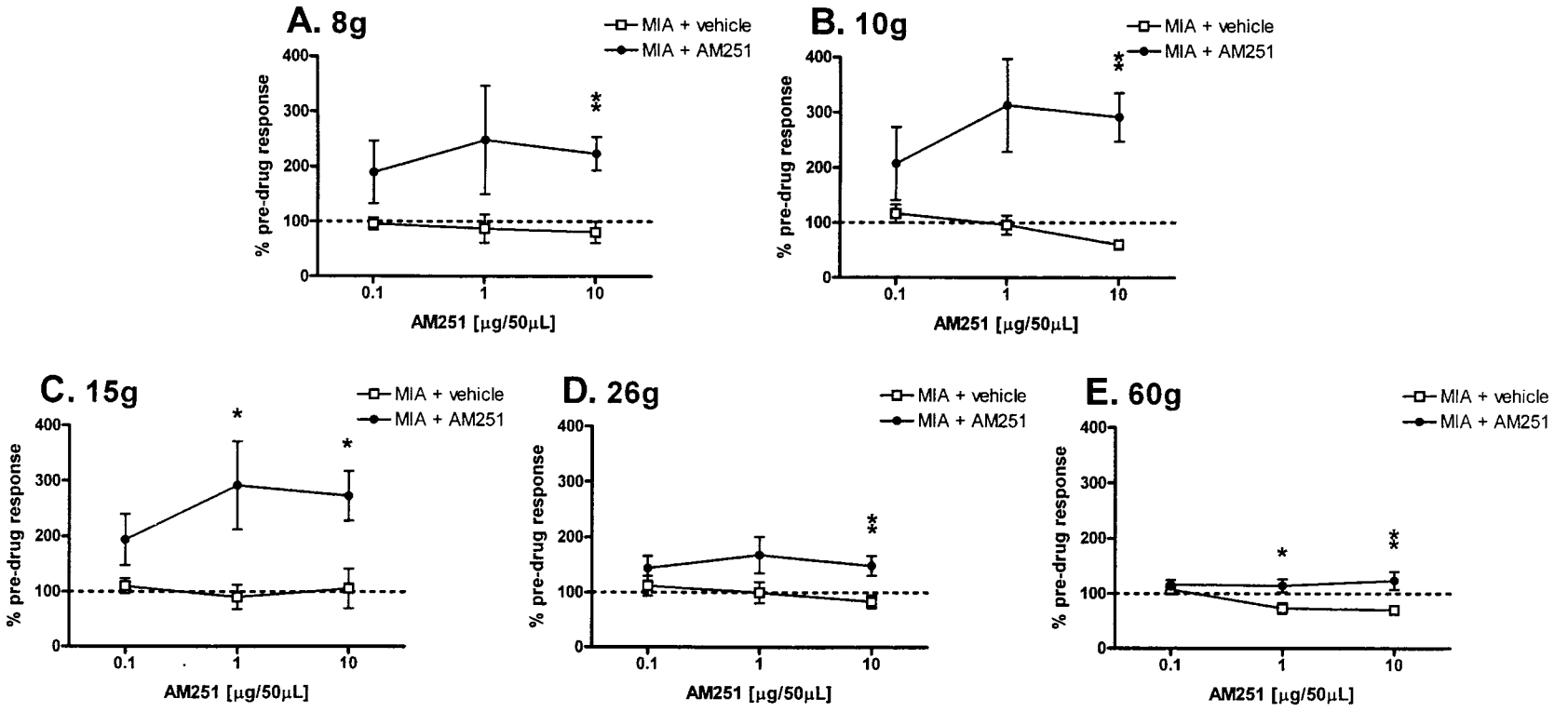

Figure 6.6 Mean maximal effects of spinal AM251 and time-matched vehicle on mechanically evoked responses of WDR dorsal horn neurones in MIA-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control responses.

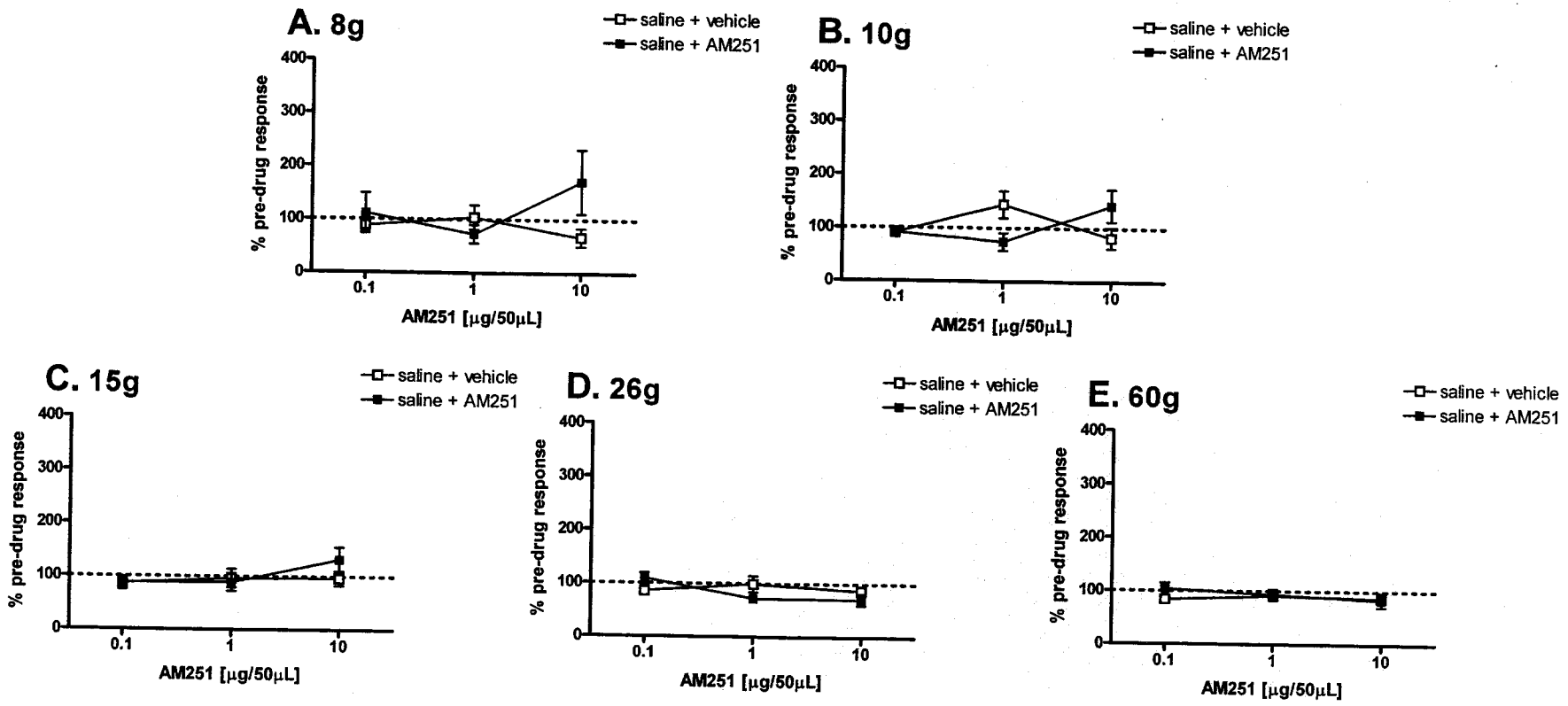

Figure 6.7 Mean maximal effects of spinal AM251 and time-matched vehicle on mechanically evoked responses of WDR dorsal horn neurones in saline-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test, no significant differences (all $P>0.05$). n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control values.

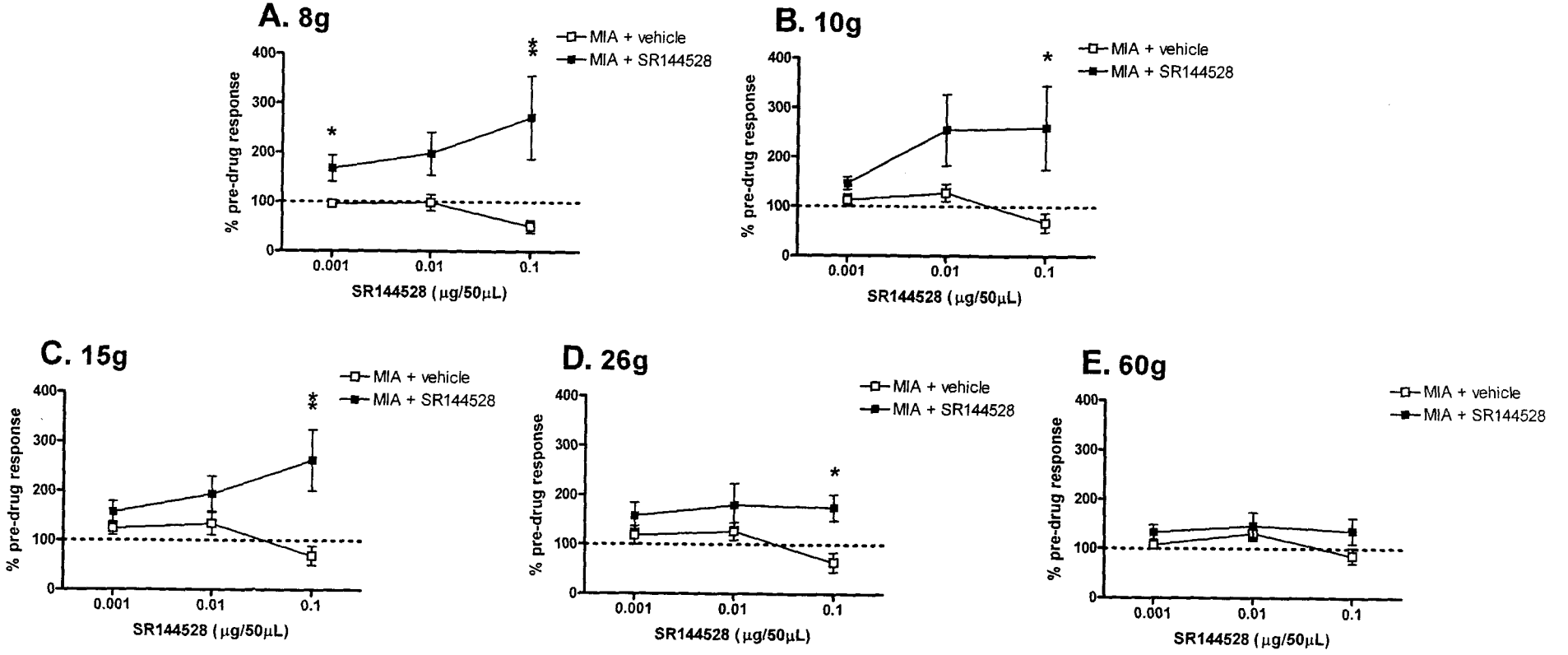

Figure 6.8 Mean maximal effects of spinal SR144528 and time-matched vehicle on mechanically evoked responses of WDR dorsal horn neurones in MIA-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control values.

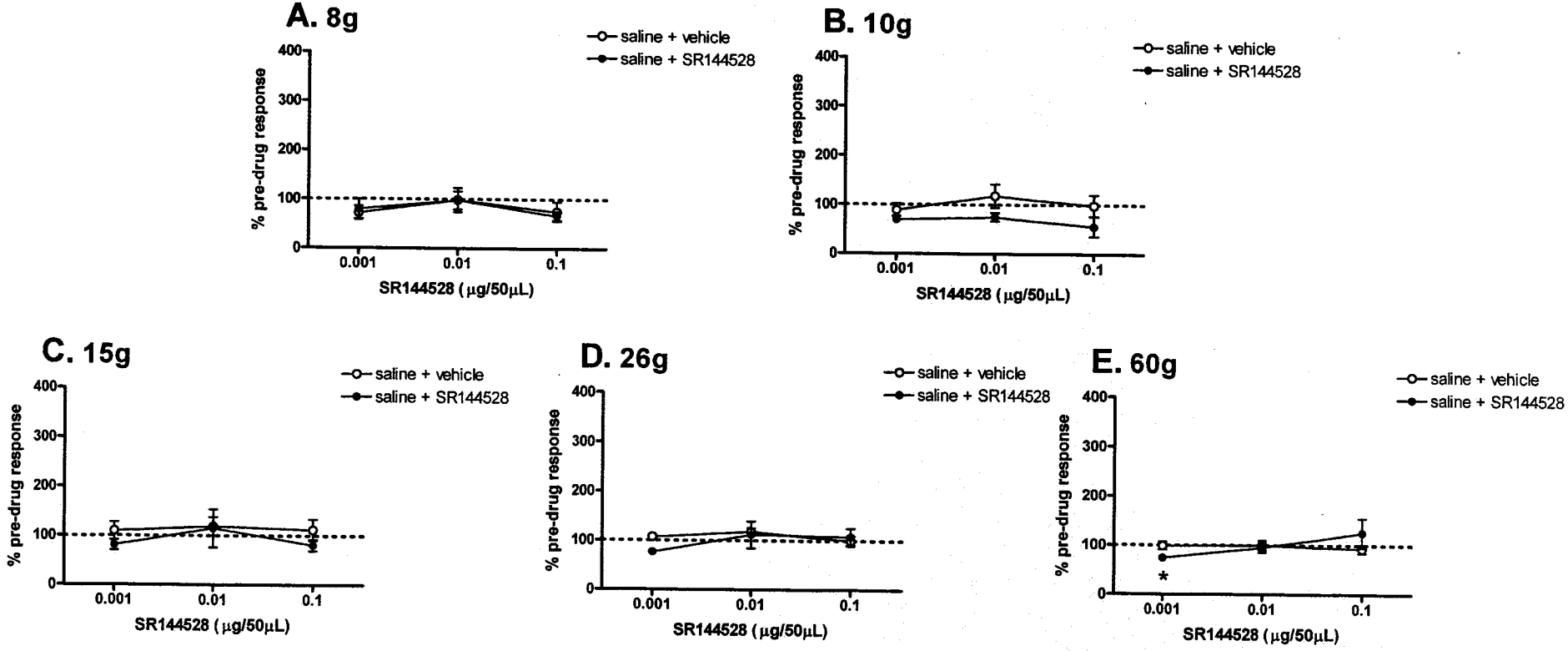

Figure 6.9 Mean maximal effects of spinal SR144528 and time-matched vehicle on mechanically evoked responses of WDR dorsal horn neurones in saline-treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug firing rates ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test; *, $P<0.05$. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control values.

6.3.5. Effects of intra-articular injection of MIA on endocannabinoid tone in the spinal cord: effects on post-stimulus responses of WDR dorsal horn neurones

The effects of AM251 and SR144528 on post-stimulus responses of WDR dorsal horn neurones in MIA- and saline-treated rats presented in section 6.3.4 were also evaluated. The effects on four parameters were studied as described in chapter 4. Data from each neurone are expressed as a percentage of the average pre-drug responses from three sets of control values.

In MIA- (but not saline-) treated rats, AM251 increased post-stimulus neuronal responses in all four measures evaluated. AM251 had greatest effect following lower-intensity (8-15g) stimuli. An example can be seen in Figure 6.10 and shows the effects of spinal AM251 compared to vehicle on the average rate of neuronal response over the total duration of post-stimulus response in MIA-treated rats. The time at which AM251 had a maximal effect on each post-stimulus response parameter is presented in Table 6.1. AM251 had no effect on post-stimulus neuronal responses in saline-treated rats compared to the effects of vehicle, on any of the parameters tested (data not shown). Statistical comparisons were performed with a non-parametric Mann-Whitney test.

Table 6.1 Average time (minutes) of maximal effect of spinal application of AM251 in MIA-treated rats (n=6) on four measures of post-stimulus response

	Time following 0.1µg AM251 (minutes)	Time following 1µg AM251 (minutes)	Time following 10µg AM251 (minutes)
Duration of post-stimulus neuronal response	36.2 ± 3.23	37.1 ± 3.51	33.0 ± 2.47
Rate of post-stimulus neuronal response (total duration)	35.6 ± 3.61	36.8 ± 3.08	33.0 ± 3.0
Rate of post-stimulus neuronal response (first 10 seconds)	34.2 ± 3.46	33.2 ± 3.2	34.6 ± 2.76
Post-stimulus neuronal response count	34.2 ± 3.36	32.8 ± 3.9	34.6 ± 2.3

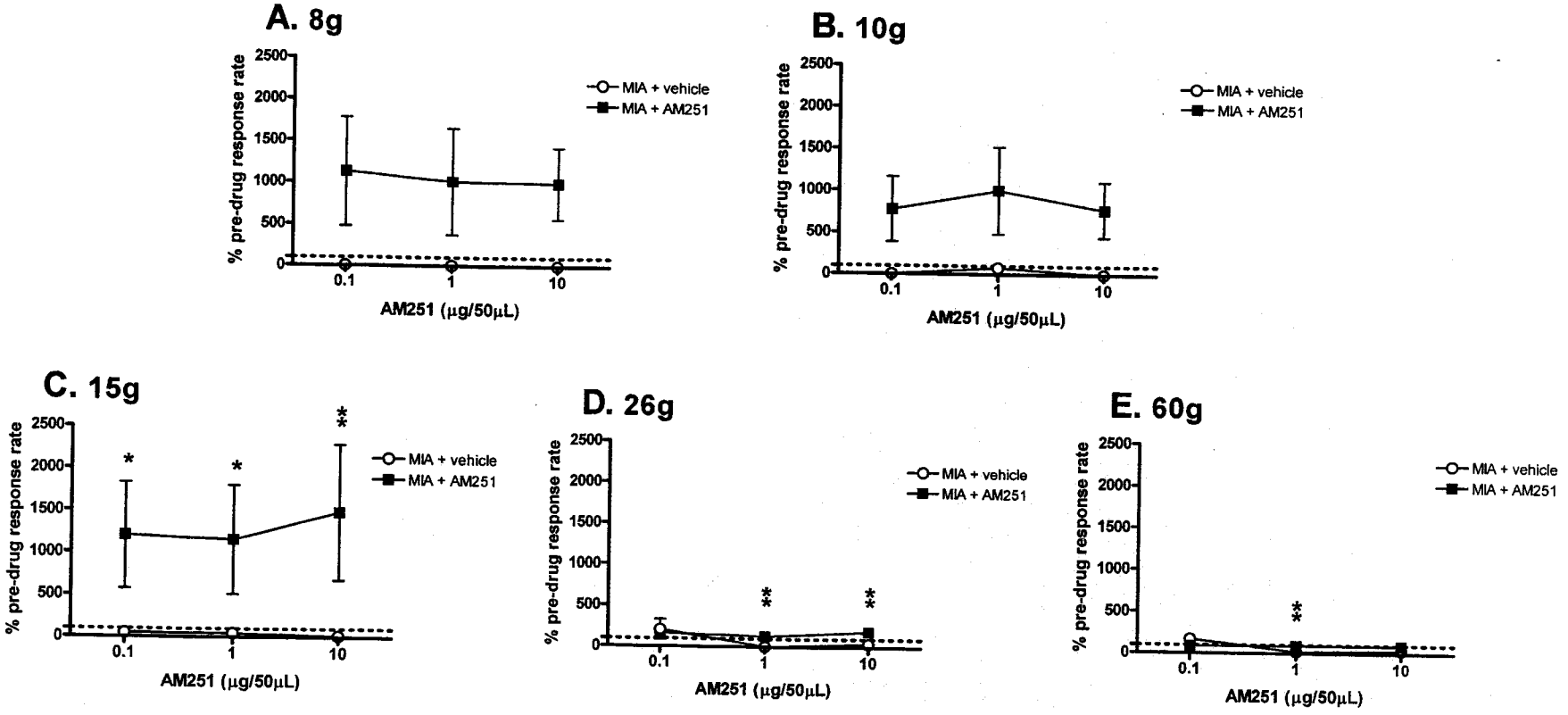

Figure 6.10 Mean maximal effects of AM251 (black squares, n=6) and time-matched vehicle controls (open circles, n=6) on rate of post-stimulus neuronal response (over the total duration of response) of WDR dorsal horn neurones in MIA treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug duration ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control values.

Post-stimulus neuronal responses following spinal SR144528 administration were similar to those observed following AM251. In MIA- (but not saline-) treated rats, SR144528 increased the post-stimulus responses in all four measures, particularly following low-intensity stimuli. An example can be seen in Figure 6.11 and shows the effects of spinal SR144528 compared to vehicle on the average rate of neuronal response over the total duration of post-stimulus response in MIA-treated rats. The time at which SR144528 had its maximal effect on each post-stimulus response parameter is presented in Table 6.2. SR144528 had no effect on post-stimulus neuronal responses in saline-treated rats compared to the effects of vehicle, on any of the parameters tested (data not shown). Statistical comparisons were performed with a non-parametric Mann-Whitney test.

Vehicle control data presented for comparison with effects of SR144528 are from the same neurones as the data presented previously for comparison with effects of AM251, but from different time-points (time-matched to the maximal effects of SR144528 on post-stimulus neuronal responses in each paradigm).

Table 6.2 Average time (minutes) of maximal effect of spinal application of SR144528 in MIA-treated rats (n=6) on four measures of post-stimulus response

	Time following 0.001µg SR144528 (minutes)	Time following 0.01µg SR144528 (minutes)	Time following 0.1µg SR144528 (minutes)
Duration of post-stimulus neuronal response	33.6 ± 2.9	41.4 ± 3.4	39.0 ± 3.0
Duration of post-stimulus neuronal response (total duration)	33.1 ± 2.9	38.4 ± 3.4	37.8 ± 3.1
Rate of post-stimulus neuronal response (first 10 seconds)	36.0 ± 2.6	44.4 ± 3.1	38.7 ± 3.3
Post-stimulus neuronal response count	34.4 ± 3.1	42.6 ± 2.7	37.1 ± 3.0

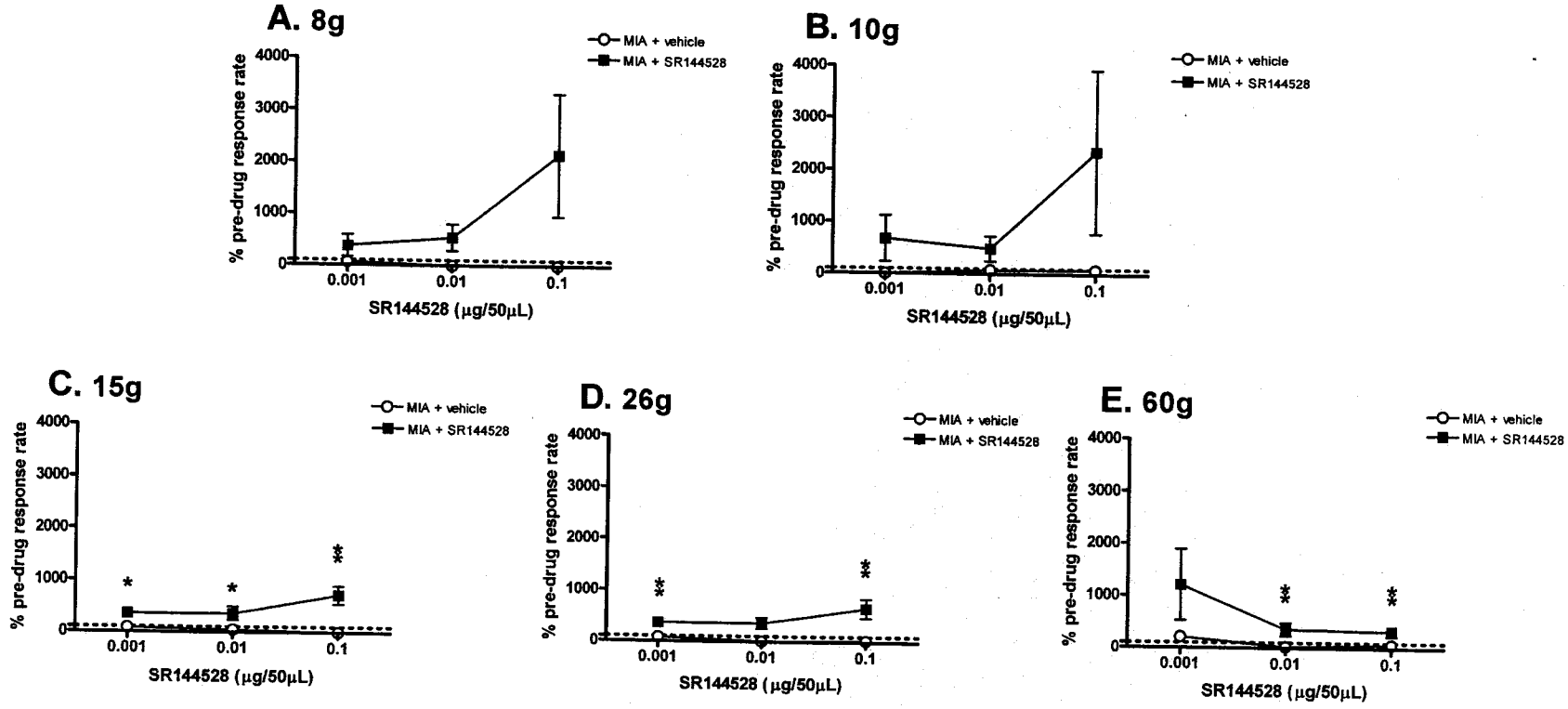

Figure 6.11 Mean maximal effects of SR144528 (black squares, n=6) and time-matched vehicle controls (open circles, n=6) on rate of post-stimulus neuronal response (over the total duration of response) of WDR dorsal horn neurones in MIA treated rats. Responses to both non-noxious (A, 8g; B, 10g) and noxious (C, 15g; D, 26g; E, 60g) von Frey stimuli were tested. Data are expressed as mean percentage of pre-drug duration ± SEM. Statistical analyses were performed using non-parametric Mann-Whitney test; *, $P<0.05$; **, $P<0.01$. n=6 neurones in 6 rats per group. Dashed line = 100% (i.e. no change) of pre-drug control values.

6.4. Discussion

The present study aimed to determine the effects of intra-articular MIA injection on endocannabinoid tone in the rat spinal cord, following reports of altered endocannabinoid levels in both animal models of neuropathic pain and in human OA.

6.4.1. Validation of model induction

In order to ensure that MIA injections were correctly placed and that data from the electrophysiological studies in MIA rats were a true representation of the model, behavioural and electrophysiological characteristics of these rats were compared to those from all MIA- or saline-treated rats used throughout this thesis. Behavioural data from hindpaw weight-bearing and von Frey withdrawal threshold tests from each rat were comparable to, and did not differ statistically from, the mean response seen within their group. This was also true for pre-drug mechanically evoked responses of neurones: however, differences between the MIA- and saline-treated rats used in the studies outlined in this chapter did not reach statistical significance as previously observed. A tendency for higher responses in the MIA-treated rats was still observed, and the smaller sample size (18 per group in this study versus 42 saline and 48 MIA previously used) may account for the greater variation in response, and thus the lack of statistically significant differences between evoked responses in the MIA versus saline-treated rats. Post-stimulus responses were, however, not always comparable to those seen in other rats used throughout this thesis: in MIA-treated rats, response rates and total response count were lower in this study than the average for all rats used throughout the thesis, significantly so for 15g-evoked responses. Post-stimulus responses in MIA-treated rats also showed a trend to being lower than post-stimulus responses in saline-treated rats, particularly following 8-15g stimuli. The evoked responses of neurones in saline-treated rats used in this study were comparable to previous studies. These differences in the magnitudes and duration of the post-stimulus response in this sub-group of MIA-treated rats may arise as a result of the smaller sample size of neurones studied and the inherent variation between neuronal responses between different cells in an animal and between individual animals.

Other parameters of neuronal response (other than spikes per second) may better highlight the difference in stimulus-evoked responses of MIA- versus saline-treated rats, particularly in cases of smaller sample size. Possible parameters of analysis include interspike interval, and the peak rate of response (spikes per second) over a 1s bin-period from a total 10s stimulation period.

6.4.2. Possible differences in neuronal desensitisation between MIA- and saline-treated rats

In MIA-treated rats, following spinal administration of vehicle alone, post-stimulus responses decreased, while in saline-treated rats, no change compared to pre-vehicle administration was observed. This suggests there may be a difference in mechanisms of desensitisation of spinal neurones in MIA-treated versus saline-treated rats. A possible explanation is that elevated stimulus-evoked responses in MIA-treated rats due to sensitisation of spinal neurones cause rapid and transient desensitisation of post-synaptic ion channels, thus reducing the generation of post-stimulus post-synaptic action potentials. This may also explain why post-stimulus responses in the MIA-treated rats used in the studies presented here had lower post-stimulus responses than saline-treated rats even before spinal administration of vehicle. Another possibility is that upregulation of cannabinoid receptors in MIA-treated rats acts to attenuate post-stimulus responses following repeated stimulation. The CB_2 receptor has been shown to be upregulated in the spinal cord in models of neuropathic pain (see section 6.4.3) with the functional effect of attenuating neuronal responses in these animals (McGaraughty *et al.*, 2009).

6.4.3. Endocannabinoid tone in the MIA model of OA

Previous work from this group has shown that endocannabinoid levels are elevated in the spinal cord of MIA-treated rats (see appendix). In order to establish whether the increased endocannabinoid tone in the spinal cord functionally impacts upon neuronal responses in MIA-treated rats, effects of blockade of spinal cannabinoid receptors with the CB_1 and CB_2 receptor antagonists AM251 and SR144528, on neuronal responses

both during and immediately after hindpaw stimulation were studied. Both AM251 and SR144528 facilitated stimulus-evoked responses, and post-stimulus responses of WDR dorsal horn neurones, to varying degrees in MIA- and saline-treated rats.

Stimulus-evoked responses

Spinal administration of the CB_1 receptor antagonist AM251 (0.1-10µg/50µL) caused a trend to facilitate non-noxious (8g and 10g) mechanically evoked responses, and significantly facilitated noxious (15g and 26g, but not 60g) mechanically evoked responses of WDR dorsal horn neurones in the spinal cord of MIA-treated treated rats, compared to spinal vehicle administration. In saline-treated rats, the highest dose of AM251 also had a facilitatory effect on mechanically evoked responses to 8, 10 and 15g stimuli, however this did not reach significance when compared to vehicle. Spinal administration of the CB_2 receptor antagonist SR144528 (0.001-1 µg/50µL) also significantly facilitated evoked responses of WDR neurones in MIA- but not saline-treated rats, following both non-noxious and noxious stimuli. The facilitatory effects of both AM251 and SR144528 were most pronounced following stimulation by the lower weight von Frey monofilaments (8, 10, 15g) than by the higher weights (26, 60g).

Post-stimulus responses

In MIA- (but not saline-) treated rats, spinal administration of the CB_1 and CB_2 receptor antagonists greatly facilitated post-stimulus responses of WDR dorsal horn neurones in all paradigms of post-stimulus response. The effect was most clearly seen following lower-intensity stimuli (8-15g). Responses were elevated to a lesser extent following stimulation by 26 and 60g filaments, but they were still significantly higher than following vehicle administration. Neither AM251 nor SR144528 had any effect on post-stimulus responses in saline-treated rats, for any of the parameters measured, mirroring the lack of effect on evoked-responses.

The level of facilitation observed (typically between 200% and 2000%) is perhaps surprising, given that stimulus-evoked responses were typically "only" around 150-300% larger than pre-drug responses. It is not possible to compare these data to those of other groups as no studies investigating post-stimulus neuronal responses

following mechanical stimulation have been published. However, taking into account the finding that post-stimulus responses before antagonist administration were usually far lower than responses evoked during stimulus application, it is possible to see how an elevation of post-stimulus response would have a much greater proportional effect.

6.4.4. Endocannabinoid modulation of neuronal excitability

The work presented here demonstrates a functional role of endocannabinoids in limiting hyperexcitability of spinal neurones in the MIA model, via CB_1 and CB_2 receptors. The effects are particularly apparent following low-weight evoked responses, supporting a functional role for the upregulated endocannabinoid system in modulating allodynic behavioural responses seen in the MIA model of OA pain. The anti-allodynic effects of CB receptor activation have long been recognised (Herzberg *et al.*, 1997; Bridges *et al.*, 2001; Guindon & Beaulieu, 2006), although the mechanisms by which this occurs are still not fully understood. A possible mechanism is via the effects of endocannabinoids on inhibitory GABA circuits, changes to which are known to underpin allodynia in neuropathic pain states. Inhibitory GABAergic interneurones are lost from the dorsal horn following nerve injury (Scholz *et al.*, 2005), and altered synthesis, storage and release of GABA has been shown in neuropathic spinal dorsal horn neurones, particularly in lamina II (Castro-Lopes *et al.*, 1993, 1995; Eaton *et al.*, 1998; Moore *et al.*, 2002). $GABA_B$ receptors co-localise with CB_1 receptors in the brain (Katona *et al.*, 1999, 2001; Hajos *et al.*, 2000; Nyiri *et al.*, 2005) where it is established that cross-talk between these two systems occurs (Cinar *et al.*, 2008). Colocalisation between CB_1 and $GABA_B$ receptors has also been demonstrated in the dorsal horn of the spinal cord (Salio *et al.*, 2002), where the antinociceptive effects of intrathecal administration of the $GABA_B$ agonist baclofen have been shown to be mediated, in part, by the CB_1 receptor (Naderi *et al.*, 2005). It is possible that in the present study, intrathecal MIA-injection may cause disinhibition of spinal neurones by decreased GABA synthesis or release, contributing to increased neuronal transmission following low-weight stimulation of the hindpaw. The facilitatory effect of spinal CB antagonists on WDR neuronal responses following low-weight stimulation may indicate a role for endocannabinoid tone in the MIA model in

stimulating $GABA_B$ receptors, activating inhibitory interneurones and counteracting a putative loss in inhibitory GABAergic transmission.

Upregulation of CB_1 receptor expression in the ipsilateral spinal cord has been demonstrated following central sensitisation, in neuropathic pain models such as CCI (Lim *et al.*, 2003, Wang *et al.*, 2007). While the CB_2 receptor has been found to be expressed in the brain under normal conditions (see Chapter 1), there are no reports to suggest that it is constitutively expressed in the spinal cord. Whilst further work is needed for confirmation, the elevation in neuronal response following spinal CB_2 receptor blockade seen in the studies presented herein demonstrates, for the first time, a functional role of the CB_2 receptor in the spinal cord of MIA-treated rats. Elevation of cannabinoid receptor expression has been reported in models of neuropathic pain, and spinal expression of the CB_2 receptor has been shown following SNL (Romero-Sandoval *et al.*, 2008; Wotherspoon *et al.*, 2005; Zhang *et al.*, 2003), and CCI (Zhang *et al.*, 2003). In these models, CB_2 receptor activation is restricted to non-neuronal cells such as activated microglia (Zhang *et al.*, 2003) but it has been shown to attenuate neuronal activity (McGaraughty *et al.*, 2009). Activation of inflammatory cells following intra-articular injection of MIA has been demonstrated in the rat spinal cord (Hsieh *et al.*, 2010) and so it is possible that the CB_2 receptor expression in the spinal cord of MIA-treated rats observed here, as in models of neuropathic pain, is microglial.

Together with upregulation of CB receptor expression, levels of endocannabinoids and related molecules have been shown to be elevated in neuropathic pain models. These include 2-AG and AEA in the CCI model (Petrosino *et al.*, 2007), AEA in the SNL model (Guasti *et al.*, 2009), and 2-AG and PEA in a model of MS (Loria *et al.*, 2008). In osteoarthritis, levels of endocannabinoids and related compounds are found to be elevated in the synovial fluid of patients with osteoarthritis compared to healthy volunteers (Richardson *et al.*, 2008). Levels of endocannabinoids and related compounds in the spinal cord of MIA- and saline-treated rats were investigated by our group (see appendix), and were found to be elevated following injection of MIA, from 14 days post-injection onwards, 2-AG ipsilaterally and AEA, PEA and OEA bilaterally.

It is likely that CB-receptor mediated suppression of neuronal hyperexcitability seen in the MIA model is a combination of elevated spinal CB receptor expression and elevated levels of spinal endocannabinoids and related compounds.

EC synthesis has long been known to be stimulated by neuronal activation, first shown in brain neurones (Giuffrida *et al.*, 1999; Stella & Piomelli, 2001; Stella *et al.*, 1997), and activity-dependent EC synthesis may be partly responsible for the elevated EC levels seen in the MIA model of OA pain. Endocannabinoid synthesis can be stimulated by several mechanisms, including post-synaptic depolarisation (i.e. Ca^{2+} dependent EC synthesis), activation of post-synaptic G_q-coupled receptors and synergism between these two mechanisms (for review, see Hashimotodani *et al.*, 2007). EC synthetic enzymes have been located in the rat spinal cord, with DAGL mRNA identified post-synaptically by *in situ* hybridization in nociceptive primary afferents in the superficial dorsal horn (Nyilas *et al.*, 2009) and upregulated microglia after spinal cord injury (Garcia-Ovejero *et al.*, 2009). NAPE-PLD expression has recently been demonstrated in a subset of capsaicin-sensitive dorsal root ganglion neurones, indicating that AEA production may occur in sensory neurones of the spinal cord, although its expression here is yet to be described (Nagy *et al.*, 2009). CB receptor mRNA has also been located in the spinal cord, with the CB_1 receptor present in abundance in the superficial laminae of the spinal cord, known to be of importance in nociceptive processing, being the locus of termination of primary nociceptive Aδ – and C-fibre afferents (Lever & Malcangio, 2002).

The increased levels of endocannabinoids and their increased functional role in the MIA model as shown by the experiments presented here support the role of ECs as a useful target for the treatment of pain. Given their rapid metabolism, a possible target would be EC metabolic enzymes. Much research has been conducted into the antinociceptive effects of FAAH inhibitors in models of neuropathic pain, proving its inhibition to be a viable, effective target in pain (see Chapter 1).

Finally, in relation to the work presented here, the EC system has been shown to have important roles in regulation of bone mass, bone loss and osteoclast activity (Idris *et*

al., 2005), and so the upregulation of the EC system in the MIA model of OA might have functions in protection of structural integrity of the joint as well as suppression of neuronal responses. Both receptor genes (Cnr1 and Cnr2) are associated with human osteoporosis, with a large number of incidences of single polymorphisms and haplocytes on the Cnr2 gene seen in postmenopausal osteoporosis (Karsak *et al.*, 2005). Deletion of either of these in the mouse results in alteration of bone mass (Karsak *et al.*, 2005). Osteoclast activity and bone mineral density may be regulated by agonists of CB_1 and CB_2 receptors (Karsak *et al.*, 2005) and may prevent ovariectory-induced bone loss in mice by promoting osteoclast apoptosis and by inhibiting the production of several osteoclast survival factors (Idris *et al.*, 2005).

6.4.5. Conclusions

In summary, data from these studies have demonstrated a functional role of endocannabinoid receptors in the modulation of neuronal activity following intra-articular injection of MIA. This is supported by work in this lab demonstrating changes in levels of endocannabinoids in MIA-treated rats. The functional effects of EC tone to decrease hyperexcitability of spinal neurones, both during and after stimulation, particularly following low-intensity stimuli, suggests that endocannabinoid tone in this model may also be important in modulation of allodynic behaviours in the MIA-treated rat. Taken together with observations of altered endocannabinoid levels and receptor expression in human OA patients, the possible implications of these findings are that the endocannabinoid system may modulate painful experience in the human OA condition, supporting the validity of the endocannabinoid system as a clinical target.

Chapter 7 General Discussion

The work presented in this thesis has demonstrated an important modulatory role of the endocannabinoid system in the MIA model of OA. The contribution of oxidative metabolism of endocannabinoids by COX-2 has also been demonstrated.

The sensitivity of neuronal responses to COX-2 inhibition by nimesulide in naïve, saline- and MIA-treated rats suggests that the products of COX-2 influence responses of spinal neurones. Traditionally, COX-2 is thought to enhance neuronal responses due to the production of pro-nociceptive substances such as prostaglandins. Work in this thesis demonstrated that COX-2 effects on neuronal responses are also mediated by the catabolism of inhibitory compounds such as endocannabinoids. Analgesic effects of COX-2 inhibition by nimesulide were blocked by AM251, showing them to be mediated by CB_1 receptor activation, and suggesting that COX-2 inhibition caused an increase in CB_1 ligands. Levels of ECs and ECLs in the spinal cord, however, decreased following spinal administration of nimesulide. This work suggested that following COX-2 inhibition, EC and ECL oxidation is shunted down LOX and cP450 metabolic pathways, with the production of CB_1-active metabolites.

A major finding of this thesis is that the endocannabinoid system is dynamically regulated in a model of chronic pain and that the alteration in levels of endocannabinoids has functional consequences on neuronal responses and by extension may influence pain behaviour. The work in this thesis has expanded our understanding of the role of spinal endocannabinoids and provides evidence for their complex regulation by COX-2, as well as the more traditional routes of catabolism.

7.1. Clinical relevance of the MIA model of OA pain

The MIA model is a well-described model of OA pain, allowing the study of analgesic potential of pharmacological interventions. There is also the potential for its use in the study of causes of OA-induced pain. The poor relationship between structural damage and pain in clinical OA make clinical rationale of any model difficult. Nevertheless, the MIA model produces both clinically relevant structural changes as well as clinically relevant and reproducible changes in behavioural paradigms indicative of OA-related pain. Other models of OA have been well described in terms of structural changes (see Chapter 1), however, of those, only the meniscectomy (MNX) model have also been described in terms of OA-related pain. The MNX model is a mechanical joint alteration model employing surgical techniques to accelerate the course of spontaneous OA (Bendele *et al.*, 1987). It reproduces all of the structural features of spontaneous OA pathology in the guinea pig, but at a much faster rate. A comparison of the MIA, MNX and spontaneous degeneration models may be seen in Table 7.1 (page 204).

Intra-articular injection of 1mg MIA produces robust, reproducible behavioural changes in hindpaw weight-bearing and paw withdrawal thresholds ipsilaterally versus contralaterally. These behaviours are indicative of pain associated with knee joint damage which in clinical OA manifests itself as pain on standing, walking or other weight-bearing exercises, as well as secondary allodynia – allodynia at a site distal to the site of initial damage, indicative of the phenomena of central sensitisation. While both the MIA and MNX model have previously demonstrated rapid and sustainable asymmetry in hindlimb weight-bearing (peaking at day 3 and maintaining throughout the study period and sensitive to morphine treatment), the MIA model holds advantage over the MNX model with regards to the reproducibility of these behaviours. In the studies presented here, all MIA-treated rats developed weight-bearing deficiencies, while in the MNX model, results are conflicting, with one study showing changes from as early as 7 days post-operatively onwards, another from 21 days PO onwards, while another failed to see any alteration in weight-bearing at any time-point studied (Bove *et al.*, 2006; Mapp *et al.*, 2010; Fernihough *et al.*, 2004). Intra-articular MIA also

produced rapid and sustainable mechanical hyperalgesia and tactile allodynia, while in the MNX model no mechanical hyperalgesia was observed (Fernihough *et al.*, 2004), and only a subset of animals developed mechanical allodynia - 50% of animals displaying changes at day 3, 70% at day 21 (Bove *et al.*, 2006). The "subset" nature of development of secondary allodynia is similar to the clinical condition, where only a subset of patients report the presence of pain distal to the original site of OA (Kosek & Ordeberg, 2000; Bajaj *et al.*, 2001). However, to minimise the numbers of animals used in research and maximise obtainable results from studies, it is important that the relevant pathologies in which we are interested are robust and reproducible, making the MIA model a better choice. In addition to tactile allodynia, thermal hyperalgesia is present in the MNX model, but lacking in the MIA model, although cooling hypersensitivity has been observed (Bove *et al.*, 2006; Vermeirsch *et al.*, 2007; Harvey & Dickenson, 2009; Vonsy *et al.*, 2009). The presence, or lack thereof, of thermal hyperalgesia in human OA patients has not been quantified and so while this lack of thermal hyperalgesia in the MIA model may indicate a difference in hyperalgesic mechanisms to that seen in the MNX model, it does not diminish the clinical relevance of the MIA model.

The spontaneous model of degeneration in the guinea pig resembles the human OA condition very closely with respect to structural alterations and time-course, developing over the life-span of the animal (McDougall, 2009). While nociceptive behaviours have not been studied in this model, nociception has been studied electrophysiologically with peripheral nerve recordings. Like the MIA model, where responses of spinal neurones are recorded, neuronal responses to both innocuous and noxious mechanical stimuli were higher in OA than non-OA animals. The similarity of results obtained from the MIA- and spontaneous degeneration models support the validity of the MIA model as a clinically relevant model of OA pain.

The ease of model induction is of important consideration. The MNX model involves exposure and transection of the medial collateral ligament (MCL) and the meniscus. By contrast, the MIA model requires one intra-articular injection which is much simpler to perform, quicker (typically taking between 20-30 seconds per rat once

anaesthetised), and causes much less damage to the joint capsule. The added complication of induction of the MNX model not only adds to experimental considerations of time and expertise required to set up a study, but adds to inter-study variability between different laboratories, highlighted by the difference in observations regarding weight-bearing asymmetry described above. One study attributed weight-bearing asymmetry 7 days post-operatively to the effects of the surgical technique itself with respect to capsular damage and ensuing synovitis (Mapp *et al.*, 2010) highlighting the extent of damage caused by MNX model induction, and suggests that inflammatory mediators could play a large role in the development of related pathologies. This is in contrast to the MIA model, in which all inflammatory effects of the effects of injection are cleared within 7 days (Bove *et al.*, 2003; Pomonis *et al.*, 2005). The spontaneous degeneration in the guinea pig model requires no intervention and therefore is not associated with inter-experimental differences in damage caused upon induction, and is the easiest to setup. However, owing to the time required for generation of disease pathology, it is more costly and labour-intensive in terms of animal care, than either the MIA or MNX models. In addition, the spontaneous nature of disease progression means that not all animals will develop structural abnormalities, and the sheer length of time required for maturation of the model means that some animals may die before data is obtained.

Models that bring about OA-like structural changes by enzymatic means may be criticised for being aetiologically irrelevant to the clinical condition. The disease progression of human OA is also much longer than the time-course of MIA-model induction. It is important to weigh up the benefits of a model versus its failings, and in the case of the MIA model, while the starting point may not be clinically relevant, and the disease progression much faster than in the clinical condition, the end-point is clearly sympathetic to clinical OA with respect to both structural and behavioural deficits. In this case, one must ask if the starting-point and speed of onset should be considered an important factor, given the late-stage close-resemblance of this model to the clinical condition.

Taking into account the reproducibility and speed of model induction, as well as the presence of structural, behavioural and electrophysiological alterations, I conclude that the MIA model of OA is a robust, reproducible, quick, relatively simple, and above all, clinically relevant model of OA. Its use could be of great value not only in the elucidation of effects of pharmacological intervention on OA pain, but also in the understanding of the causes of pain in this disease state.

Table 7.1 Comparison of the MIA, MNX and spontaneous degeneration models of OA with respect to model induction, alterations in joint structure, behavioural responses and electrophysiological observations.

MODEL	**MIA**	**MNX**	**Spontaneous degeneration**
INTERVENTION	Injection	*Surgery*	No intervention
TIME *required for model maturation*	1 month	1-3 months	Typically 18 months
STRUCTURAL ALTERATION			
Cartilage degradation	✓ In all animals Full thickness cartilage loss over total cartilage area, References: see Chapter 4	✓ In most animals Full thickness cartilage loss focal to central weight-bearing region only Bendele *et al.*, 2001 Fernihough *et al.*, 2004	✓ In most animals Ding *et al.*, 2005 Muraoka *et al.*, 2007 McDougall *et al.*, 2009
Bone degeneration	✓ In all animals *References: see Chapter 4*	✓ *In some animals at 1 month* ✓ In all animals at 3 months Pastoureau *et al.*, 1999 Bolbos *et al.*, 2007	✓ In most animals Ding *et al.*, 2005 Muraoka *et al.*, 2007 McDougall *et al.*, 2009
NOCICEPTIVE BEHAVIOUR			
Weight Bearing asymmetry	✓ Peaking at day 3 References: see Chapter 4	**?** Variable results Fernihough *et al.*, 2004 Bove *et al.*, 2006 Mapp *et al.*, 2010	**?** No pain behaviour tested

	MIA	MNX	Spontaneous degeneration
NOCICEPTIVE BEHAVIOUR contd. Mechanical hyperalgesia	√ In all animals Bove *et al.*, 2003	**X** No mechanical hyperalgesia present Fernihough *et al.*, 2004	**?** No pain behaviour tested
Tactile allodynia	√ In all animals Bove *et al.*, 2003 Fernihough *et al.*, 2004	√ In some animals – 70% at day 21 Bove *et al.*, 2006 Fernihough *et al.*, 2004	**?** No pain behaviour tested
Thermal hyperalgesia	**X** No thermal hyperalgesia present Vermeirsch *et al.*, 2007 Harvey & Dickenson, 2009 √ Cooling hypersensitivity present, Vonsy *et al.*, 2009	√ Bove *et al.*, 2006	**?** No pain behaviour tested
Sensitivity to morphine	√ Fernihough *et al.*, 2004, Vonsy *et al.*, 2009	√ Bove *et al.*, 2006, Fernihough *et al.*, 2004	n/a
ELECTROPHYSIOLICAL EFFECTS Increased responses to innocuous and noxious stimuli	√ In all animals Spinal recordings	**?** Electrophysiological data unavailable	√ In some animals Peripheral recordings McDougall 2008; 2009

7.2. Mechanisms of nociception in the MIA model of OA pain

7.2.1. Intra-articular injection of MIA causes central sensitisation

The work carried out in this thesis demonstrated the presence of central sensitisation in the MIA model of OA pain. The evidence for this central sensitisation was the presence of allodynia and increased neuronal responses following stimulation of the ipsilateral hindpaw. As described earlier, central sensitisation has been shown in OA patients and, therefore, these novel data further support the clinical validity of this model. There are several potential mechanisms for the central sensitisation observed in these studies. The NMDA receptor is known to be involved in central sensitisation and alterations in receptor subtype expression is known to occur both in human OA chondrocytes (Salter *et al.*, 2004; Ramage *et al.*, 2008) as well as in experimental models of OA (Jean *et al.*, 2008). Other likely candidates for alteration in MIA-induced central sensitisation include the TRPV1, PPAR, and GABA receptor systems. The TRPV1 receptor system (pro-nociceptive) is known to be elevated pre-synaptically in both the MIA model of OA pain and in human OA (see Chapter 1). The PPAR receptor system (anti-nociceptive) is peripherally downregulated in human OA cartilage, and PPAR activation in the guinea pig partial meniscectomy model has been shown to reduce the progression of experimental OA (Afif *et al.*, 2007; Kobayashi *et al.*, 2005), however, it is unknown if PPARs are altered in the MIA model of OA, and characteristics of this receptor system in the spinal cord, particularly in chronic pain states, remain to be elucidated. Intra-articular injection of MIA may also alter GABA synthesis/ release, causing disinhibition of WDR neuronal responses. GABA receptor activation attenuates neuronal responses and nociceptive behaviours in MIA-treated rats (Rahman *et al.*, 2009; Vonsy *et al.*, 2009), possibly by inhibition of increased descending serotonergic facilitation (Chapter 1).

7.2.2. The importance of microglia in central sensitisation

It is known that following increased neuronal activity, microglia and astrocytes are activated (McMahon *et al.*, 2005; Hansson, 2006). Activated microglia release pro-nociceptive substances such as nitric oxide, ATP and pro-inflammatory cytokines, which modify neuronal signalling. Activated astrocytes express inflammatory markers such as nitric oxide synthase and COX-2, as well as proteases, protease inhibitors, growth factors and cytokines, which contribute to neuronal sensitivity (Anneser *et al.*, 2001; Sasaki *et al.*, 2001; Maihöfner *et al.*, 2003; Dong & Benveniste, 2001; Ridet *et al.*, 1997). Whilst it is unknown whether microglial and astrocyte activation occurs in the MIA model of OA, microglial activation has been proposed in the CFA model of joint pain (Sun *et al.*, 2007). It is also known that microglial and astrocyte activation is crucial to the initiation and maintenance of central sensitisation and related behaviours (Marchand *et al.*, 2005; Meller *et al.*, 1994), and so may play a role in the elevated neuronal responses seen in MIA-treated rats in this thesis. Of important consideration to the studies herein, is that inhibitory effects of nimesulide on microglial activation have been described (Scali *et al.*, 2000). Inhibitory effects of nimesulide on evoked responses of spinal WDR neurones may, therefore, be partly mediated by inhibitory effects on microglia themselves, resulting in decreased release of neuronal excitatory substances, as well as direct inhibition of astrocyte and microglial COX-2.

7.2.3. Interactions between microglia and the EC system

The importance of the endocannabinoid system in the function of activated microglia has been established, and work from this laboratory has demonstrated EC upregulation in the MIA model (see Appendix). The expression of CB_2 receptor mRNA by activated spinal cord microglia in neuropathic pain models has been shown (Zhang *et al.*, 2003), and may be up- or down-regulated by the actions of certain pathogens and cytokines (for review, see Stella, 2009). The expression of CB_1 receptors and orphan (cannabinoid) receptors on microglia has also been suggested (see Stella, 2009). The relationship between ECs and microglia are complicated, and endocannabinoid action on microglia has both pro- and anti-inflammatory effects.

Activation of microglial CB_2 receptors by 2-AG results in microglial migration and proliferation (Carrier *et al.*, 2004), while inhibiting release of detrimental factors such as TNFα and free radicals (Eljaschewitch *et al.*, 2006; Ramirez *et al.*, 2005). Thus it has been hypothesised that stimulation of the CB_2 receptor on microglia results in an accumulation of the anti-inflammatory (M_2) microglial phenotype at the site of lesion (Stella, 2009). 2-AG has also been shown to suppress COX-2 elevation *in vitro* following excitatory or cytotoxic stimulation by glutamate or IL-1β in microglia, an effect mediated through CB_1 and MAPK/NF-κB signalling pathways (Zhang & Chen, 2008). In this way, 2-AG limits COX-2-elevation-enhanced glutamatergic transmission. Differential actions of other endocannabinoids on microglia have also been described, with NADA inhibiting and AEA enhancing microglial production of PGE_2 and PGD_2 (Navarette *et al.*, 2009) while inhibiting NF-κB activation (Correa *et al.*, 2010). AEA also enhances microglial IL-10 production, which retrogradely inhibits microglial activation (Correa *et al.*, 2010). In addition, activated microglia play a role in endocannabinoid turnover, and *in vitro* are involved in both EC synthesis and catabolism (Carrier *et al.*, 2004; Muccioli *et al.*, 2007). In neuropathic rats, following inhibition of microglial activation using minocycline, spinal EC and ECL levels were altered (Guasti *et al.*, 2009). Levels of 2-AG were decreased while PEA was increased, and AEA remained unchanged. Given the putative entourage effects of PEA (see Chapter 1), increases in PEA levels here may serve to enhance AEA activity at microglia, thus further regulating their activation.

7.2.4. Endocannabinoid tone modulates neuronal responses in the MIA model of OA pain

A functional role for endocannabinoid tone in the MIA model to modulate neuronal responses has been demonstrated by the studies described in this thesis. The comparable effects of spinal nimesulide in MIA-treated, saline-treated and naïve rats (Chapters 3 and 5) indicates that there are no additional spinal mechanisms of nimesulide-mediated attenuation of neuronal response in MIA-treated rats over and above that which is already present in controls. In light of the evidence of a CB_1-dependent effect of spinal nimesulide in naïve rats (Chapter 3), the data presented in

this thesis support a role for the endocannabinoid system in spinal nimesulide-mediated inhibition of WDR dorsal horn neurones in this model. In MIA-treated rats, mechanically evoked and post-stimulus WDR neuronal responses were elevated following spinal AM251 and SR144528 administration (particularly following lower weight stimuli, Chapter 6), and by extension, these data may indicate a role of endocannabinoid tone for modulation of mechanical allodynia behaviour in this model. Given also that spinal nimesulide attenuated lower weight evoked responses but not 60g evoked responses (Chapter 5), the data presented in this thesis could indicate that post-synaptic increases in CB_1 receptor ligands following nimesulide administration act in a retrograde fashion to reduce excitatory transmission from primary Aβ-fibre (as well as C fibre) inputs to the spinal cord in the MIA-treated rat. AEA and 2-AG levels have been shown to be elevated in the spinal cord (and some areas of the brain, including the PAG) in models of neuropathic pain (Petrosino *et al.*, 2007). In-house data from studies manipulating the endocannabinoid system in experimental models of neuropathic pain using FAAH inhibitors or CB receptor agonists frequently show inhibitory effects only on responses to lower intensity stimuli (data not shown), suggesting the EC system is upregulated in central sensitisation to counteract abnormal input from low-threshold mechanoreceptors, and while it can alleviate some C fibre responses, it cannot alleviate very high stimulus C-fibre responses. A recent study suggests that spinal endocannabinoids *potentiate* C-fibre-induced nociception following nociceptive stimuli, by activating CB_1 receptors on dorsal horn neurones, reducing GABAergic and glycinergic transmission (Pernía-Andrade *et al.*, 2009). An upregulation of spinal endocannabinoids in the MIA model therefore may well serve to decrease nociceptive input from traditionally non-nociceptive fibres and *increase* input from nociceptive sources via C-fibre activation, resulting in decreased low- but not high-intensity-evoked responses, as seen here.

The endocannabinoid system is known to modulate pre-synaptic neurotransmitter release. It is also involved in pre- and post-synaptic activation of inward rectifying K^+ channels and inhibition of Ca^{2+} channels, thus further inhibiting pre-synaptic neurotransmitter release, and reducing post-synaptic neuronal responsiveness. High concentrations of AEA are also known to activate TRPV1 and so the anti-nociceptive

effects of increased endocannabinoid tone in the MIA model, while robust, may have limits, supported by the observation of increased baseline WDR neuronal responses in MIA- versus saline-treated rats. However, this baseline elevation of WDR neuronal responses may have been greater without the putative upregulation of the endocannabinoid system in this model, supported by the data herein, describing elevated responses following spinal CB receptor blockade. Interactions between the endocannabinoid and GABA receptor systems have prevously been demonstrated (see Chapter 1). Elevated endocannabinoid tone may act to stimulate GABAergic neurones, counteracting the putative loss in GABAergic function and increase in descending serotonergic facilitation.

7.2.5. Proposed central mechanisms of nociception in the MIA model of OA pain

I propose that following intra-articular administration of MIA, joint damage causes increased input from afferent fibres in the joint and paw to the spinal cord, supported by work in the spontaneous degeneration model of OA (McDougall *et al.*, 2008; McDougall *et al.*, 2009). The increased spinal activity and central sensitisation is associated with activation of microglia and astrocytes, and may involve changes in receptor systems such as NMDA, TRPV1, PPAR, GABA and 5-HT. Increased expression of enzymes such as COX-2 may also occur, resulting in the release of pro-nociceptive mediators which positively feed back into increased neuronal activity. The endocannabinoid system is also upregulated, the functional effects of which are to modulate the increase in neuronal responses. However, due to rapid metabolism by the proposed increased activity of COX-2, the modulatory effects of elevated spinal endocannabinoids on neuronal responses may have limits, and so neuronal responses in MIA-treated rats are higher than in saline-treated rats. Spinal administration of nimesulide reduces neuronal responses, due to inhibition of COX-2 mediated synthesis of nociceptive substances such as prostaglandins, the putative shunting of endocannabinoid oxidation through LOX and cP450 pathways to produce CB_1-active metabolites, as well as putative off-target effects such as inhibiting the activation of microglia (Figure 7.1).

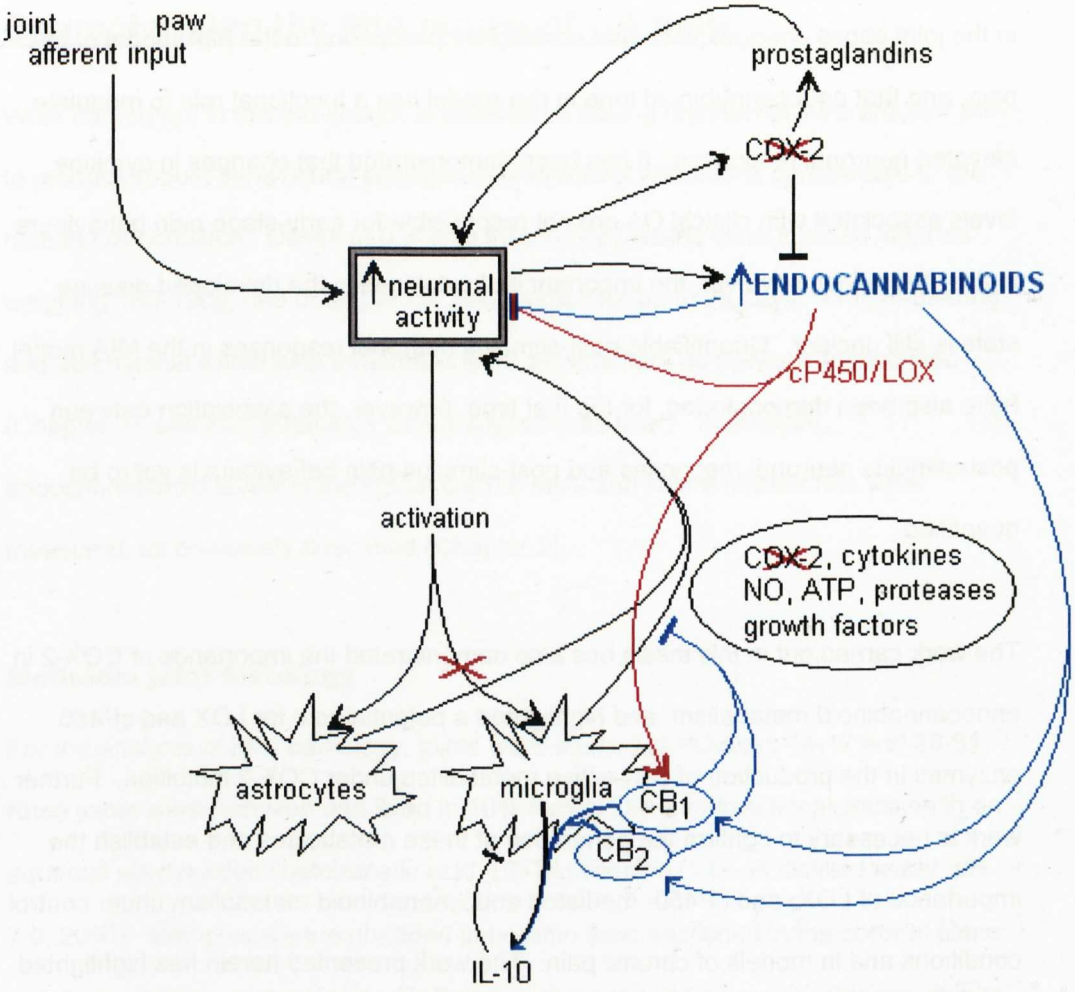

Figure 7.1 Proposed effects of intra-articular MIA injection on neuronal activity and the involvement of the endocannabinoid system. MIA injection causes joint damage, resulting in an increase in afferent input from the joint and paw and increasing neuronal activity. Increased neuronal activity putatively activated microglia and astrocytes, which express or release pro-nociceptive mediators such as COX-2, cytokines, nitric oxide (NO), ATP, proteases and growth factors. Upregulation of enzymes involved in pro-nociception such as COX-2 also occurs as a direct result of increased neuronal activity, causing further release of pro-nociceptive mediators such as prostaglandins. The endocannabinoid system (blue) is also upregulated, and has a functional role to limit baseline neuronal activity. Pink = effects of the COX-2 inhibitor nimesulide. Inhibition of COX-2 by nimesulide reduces prostaglandin production as well as inhibiting COX-2-mediated metabolism of endocannabinoids. This decreased COX-2 oxidation of endocannabinoids putatively shunts oxidation through LOX and cP450 pathways, resulting in CB_1-receptor mediated attenuation of neuronal responses. Nimesulide may also have off-target effects, e.g. direct inhibition of microglial activation.

7.3. Conclusions

The work presented in this thesis has demonstrated that peripheral structural changes in the joint cause changes in central nociceptive processing in the MIA model of OA pain, and that endocannabinoid tone in this model has a functional role to modulate elevated neuronal responses. It has been demonstrated that changes in cytokine levels associated with clinical OA are not responsible for early-stage pain behaviours in the MIA model, however, the importance of cytokines in the developed disease state is still unclear. Quantifiable post-stimulus neuronal responses in the MIA model have also been demonstrated, for the first time, however, the association between post-stimulus neuronal responses and post-stimulus pain behaviours is yet to be quantified.

The work carried out in this thesis has also demonstrated the importance of COX-2 in endocannabinoid metabolism, and highlighted a potential role for LOX and cP450 enzymes in the production of CB_1-active metabolites under COX-2 inhibition. Further work is necessary to confirm the generation of these metabolites and establish the importance of LOX- and cP450- mediated endocannabinoid metabolism under control conditions and in models of chronic pain. The work presented herein has highlighted the pharmacological potential of the EC system as a target in OA pain.

Appendix

Characterising the MIA model of OA pain

Work carried out in this lab sought to establish a dosing regimen of intra-articular MIA to produce robust behavioural changes and structural alterations comparable to the human OA condition. Doses of 0.3-3mg MIA in 50µL saline were injected into rats weighing 160-190g, one dose per rat, and studied for up to 28 days. Weight-bearing and mechanical withdrawal thresholds were ascertained, as previously described (Chapter 2), and joint pathology was analysed (see later). In addition, endocannabinoid levels in the spinal cord of MIA- and saline-treated rats were measured, as previously described (Chapter 2).

Methods: joint histology

For the analysis of joint pathology, joints were studied at PO days 14-17 and 28-31. Knee joints were removed and fixed in 10% formal saline before decalcification in an aqueous ethylenediaminetetracetic acid (EDTA) solution (14% in distilled water; pH 7.0, 20°C). Samples were embedded in paraffin wax, sectioned in the coronal plane in 5-8µm sections, and stained with Safranin-O (red) and fast green. Scoring of medial and lateral knee compartment tibial plateaux cartilage, tibial subchondral bone and joint synovium were undertaken, ensuring a comprehensive histopathological analysis of MIA-induced joint pathology. The Osteoarthritis Research Society International (OARSI) Cartilage Histopathology Assessment System (OOCHAS) (Mainil-Varlet *et al.*, 2003) was used, and consists of a grade (0-6) which defines the depth progression of OA histopathology features into the cartilage (0, normal; 1, surface intact; 2, surface discontinuity; 3, vertical fissures; 4, erosion; 5, denudation; 6, deformation) and a stage (0-4) which defines the surface extent of joint involvement; 0, no activity; 1, <10%; 2, 10-25%; 3, 25-50%; 4, >50%. Since this system does not assess the histopathological features of subchondral bone or synovium, these were scored separately. Subchondral bone lesions were assessed using an established method (Janusz *et al.*, 2001) with a scale of 0–4; 0, no subchondral lesions with

cellular infiltration; 1, minimal subchondral lesions (1–2): <5% of the tibial plateau involved; 2, mild subchondral lesions (2–3): <15% of the tibial plateau involved; 3, moderate subchondral lesions (4–5): <25% of the tibial plateau involved; 4, severe subchondral lesions (5 or more): >25% of the tibial plateau involved. The synovium scoring assessed the level of hyperplasia in the synovium, hypercellularity was indicative of synovitis. The synovium scoring system produced a mean score for the medial and lateral knee joint compartments and consisted of: 0, lining cell layer 1-2 cells thick; 1, lining cell layer 3-5 cells thick; 2, lining cell layer 6-8 cells thick and / or mild increase in cellularity; 3, lining cell layer >9 cells thick and/or severe increase in cellularity as previously described (Mapp *et al.*, 2008).

Results

MIA caused time-related asymmetry between ipsilateral and contralateral weight-bearing, and decreases in mechanical withdrawal thresholds (Figure 8.1 A, B).
A 1mg dose significantly increased noxious von Frey evoked responses of single WDR dorsal horn neurones at 28 days (but not 14 days – data not shown) post-injection (Figure 8.2), and was associated with increased levels of endocannabinoids and related compounds in the hindpaw and spinal cord, compared to saline-treated rats (Figure 8.3).

Joint pathology was dose-dependent. 1mg MIA produced pathology comparable to joint degeneration seen in OA patients; articular cartilage exhibited hypocellular focal lesions with reduced glycosaminoglycan content, identified by reduced safranin O staining. These lesions were often associated with a horizontal subchondral cleft. In addition, fibrous tissue interspersed the subchondral bone trabeculae (Figure 8.4).
MIA caused dose-dependent changes in cartilage and subchondral bone at days 14 and 28, and was accompanied by mild synovitis at day 14, which was cleared by day 28 following a 1mg, but not 3mg, dose of MIA (Table 8.1).

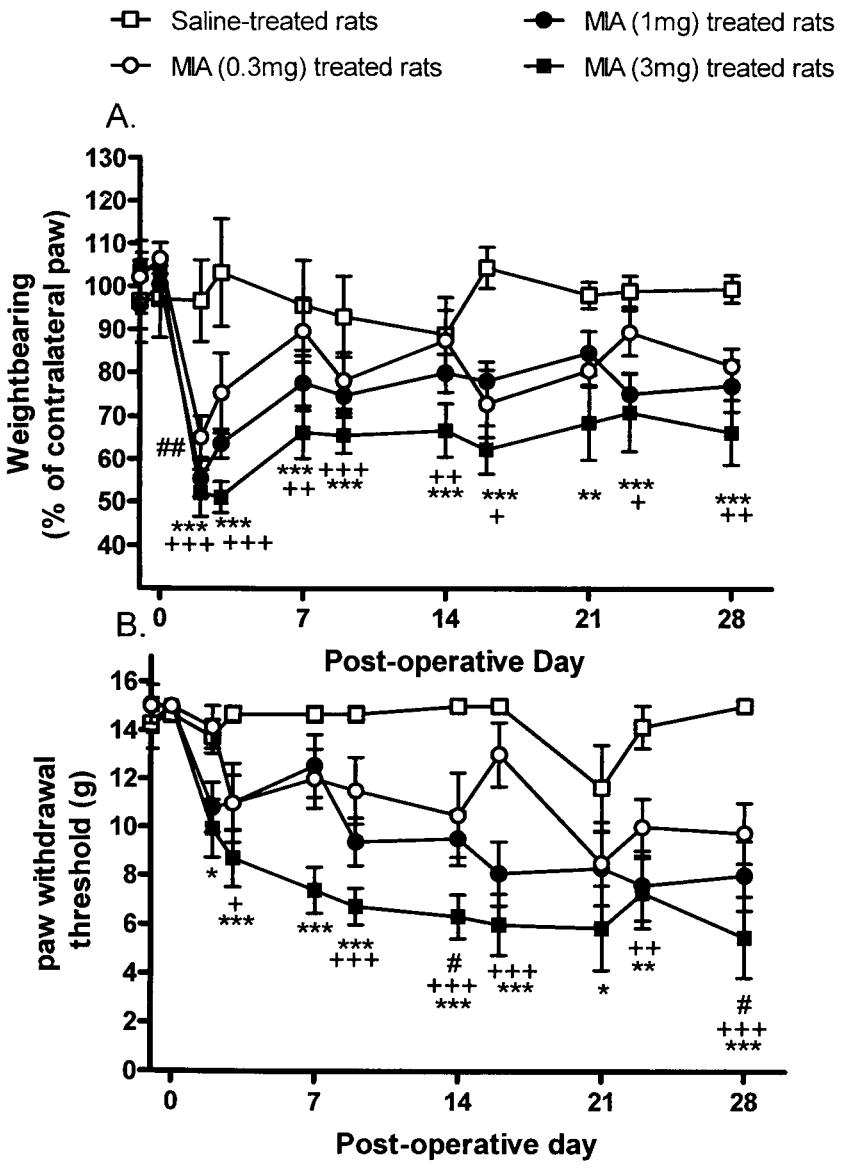

Figure 8.1 Time course of the changes in (A) weight distribution on the ipsilateral hindlimb and (B) hindpaw withdrawal thresholds of the ipsilateral hindpaw following intra-articular injection of MIA (0.3-3mg/50µL) in rats. Statistical analyses comparing MIA-treated rats to saline-treated rats were performed using a 2-way ANOVA with a Bonferroni post hoc test: #, $P<0.05$; ##, $P<0.01$ for 0.3mg MIA; +, $P<0.05$; ++, $P<0.01$ and +++, $P<0.001$ for 1mg MIA; *, $P<0.05$; **; $P<0.01$ and ***, $P<0.001$ for 3mg MIA- versus saline-treated rats.

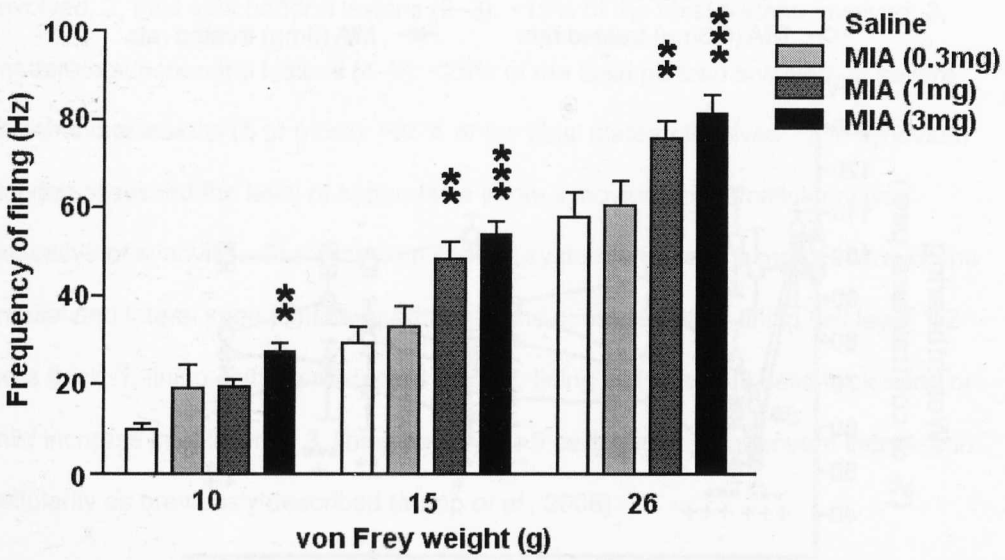

Figure 8.2 Mechanically evoked responses of WDR neurones in saline- and MIA (0.3-3mg) -treated rats following punctate stimulation (10-26g) of the hindpaw 28-31 days after intra-articular injection. Statistical analyses comparing neuronal responses in MIA-treated rats and saline-treated rats were performed using a 2-way ANOVA with a Bonferroni post hoc test: *, $P<0.05$, **; $P<0.01$; ***, $P<0.001$.

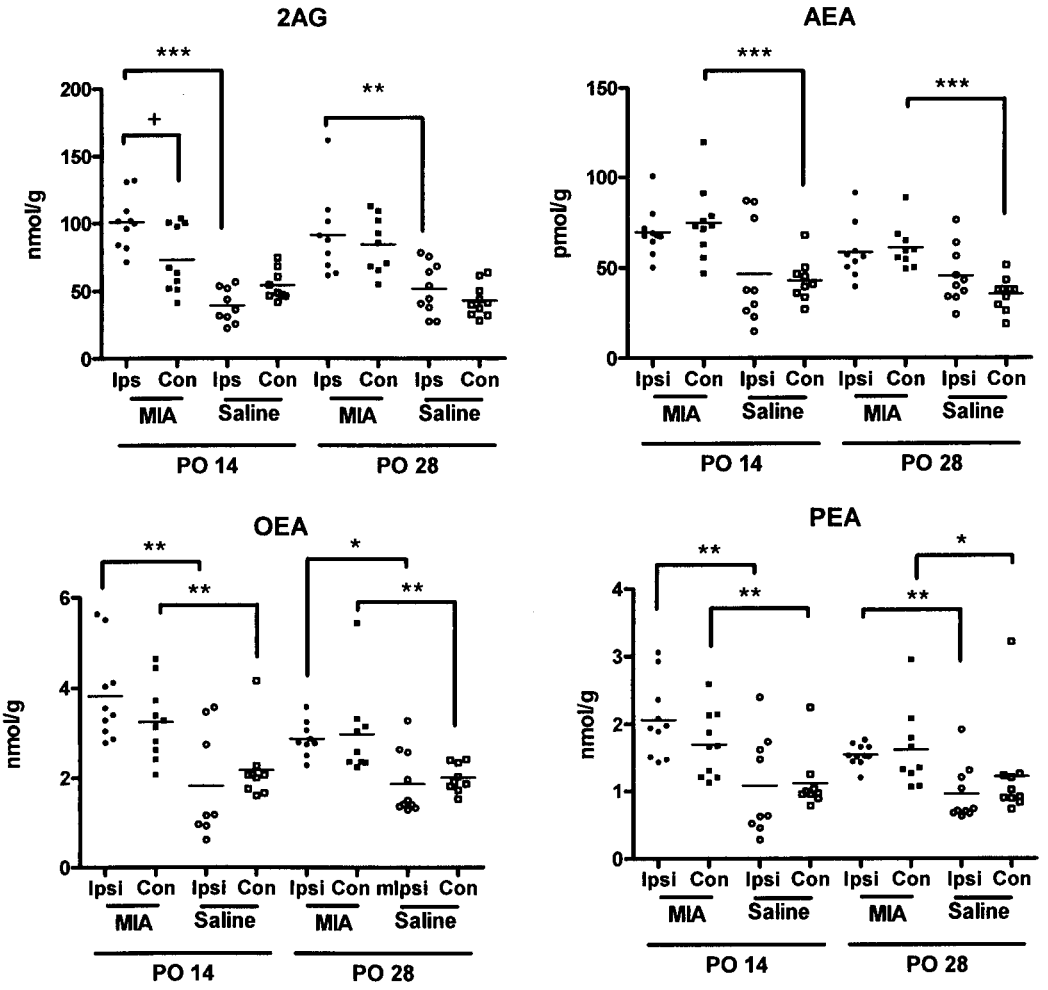

Figure 8.3 Levels of endocannabinoids and related compounds in the spinal cord of MIA-treated (1mg) and saline-treated rats 14 and 28 days after intra-articular injection. Statistical analysis comparing MIA-treated rats and saline-treated rats were carried out using a Mann Whitney test: *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. AEA, anandamide; 2-AG, 2-arachidonyl glycerol; OEA, oleoylethanolamide; PEA, palmitoylethanolamide.

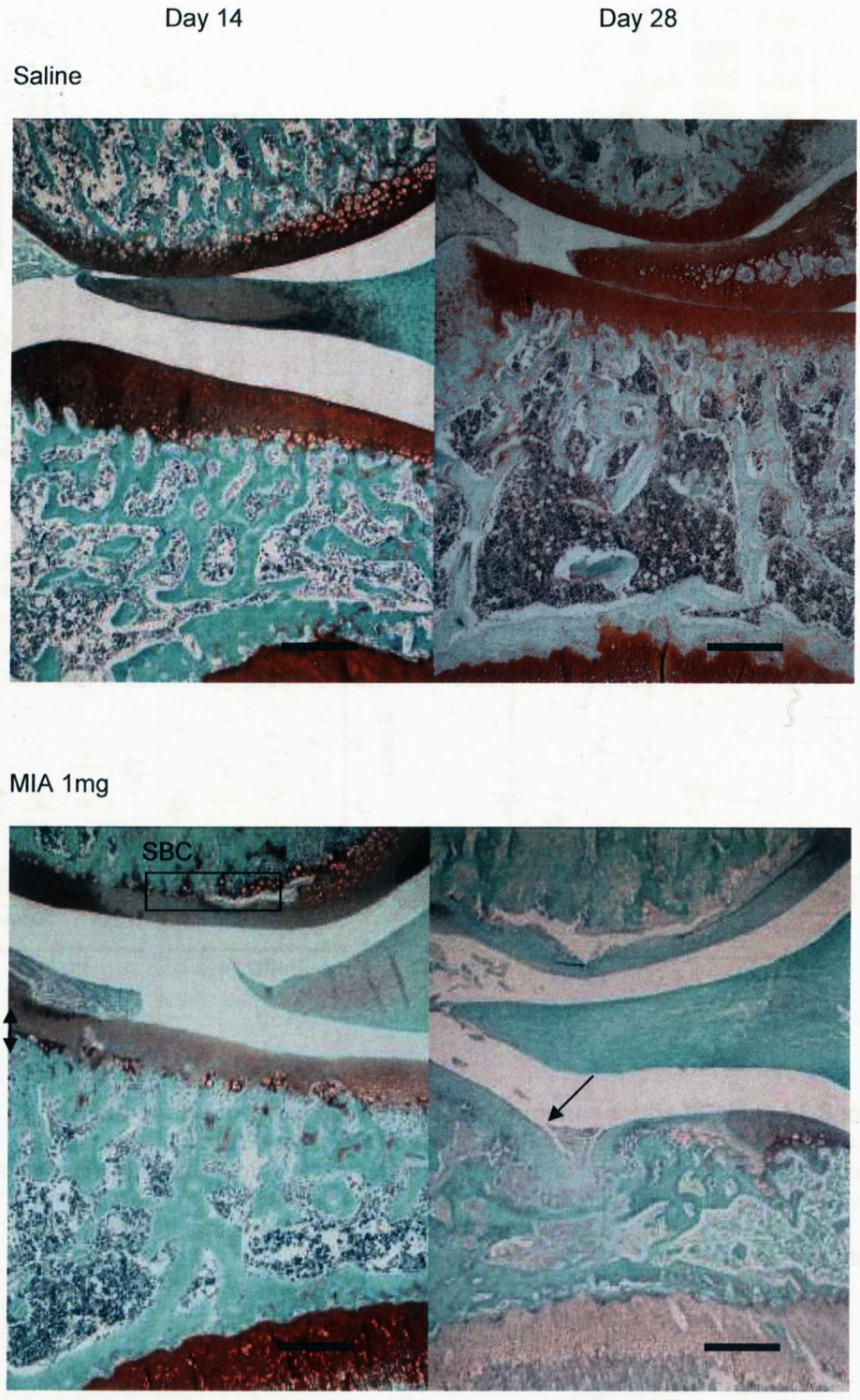

Figure 8.4 Coronal sections through knee joints of saline- and MIA (1mg)-treated rats, 14 and 28 days post injection. Decreased glycosaminoglycan content of cartilage evident through decreased Safranin-O (red) staining. Double-headed arrow (MIA, day 14) indicates reduced cartilage thickness; single-headed arrow (MIA, day 28) indicates cartilage delamination; SBC (MIA, day 14), subchondral bone cleft. Bar = 500μm.

Table 8.1 Scoring of OA-associated features of the cartilage, subchondral bone and synovium of the ipsilateral knee, 14 and 28 days following intra-articular injection of Saline, 0.3mg MIA, 1mg MIA or 3mg MIA.

	Day 14			Day 28			
	Saline n=4	**MIA 1mg n=6**	**MIA 3mg n=4**	**Saline n=4**	**MIA 0.3mg n=8**	**MIA 1mg n=8**	**MIA 3mg n=3**
Cartilage	0 (0)	7.75 (0-24)	11 (0-24)	0 (0)	6.5 (0.75-19)	6.13 (0-24)	24 (6-24)
Subchondral bone	0 (0-0.25)	1.42 (0-3)	1.50 (0-3.5)	0 (0)	0.83 (0.25-2.75)	1.0 (0-3.5)	3.75 (1.0-3.75)
Synovium	0 (0-0.25)	0.75 (0-2)	0.25 (0-2)	0 (0)	0.5 (0-1)	0 (0-2)	2.75 (0-3)

Intra-articular injection of 1mg MIA produced marked changes in the cartilage and subchondral bone at day 14 and day 28. The severity of the changes in cartilage, subchondral bone and synovium produced by intra-articular injection of 3mg MIA increased from day 14 to day 28. Synovitis was mild following either a 0.3mg or 1mg dose at day 28, and was also mild at day 14 following the 3mg dose. Synovitis was, however, evident 28 days following the 3 mg dose of MIA. n.b. since 0.3mg MIA produced no change in pain behaviour at day 14, joints were not collected at this timepoint and data are not available. Data are presented as median and, in brackets, the range.

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