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THE INVOLVEMENT OF THE CYCLOOXYGENASE PATHWAY IN SPINAL NOCICEPTIVE PROCESSING

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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Declaration.

All the work presented in this thesis is original unless otherwise acknowledged in the text or by references. The material contained in this thesis has not been submitted for another degree at this or any other University. The thesis is based on work conducted by myself mainly in the Department of Cell Physiology and Pharmacology at the University of Leicester during the period between October 1993 and January 1997. In addition, I have included results from antibody microprobe experiments conducted at the Physiology Institute, University of Würzburg, Germany. These experiments took place during a series of three visits to the University of Würzburg organised as a collaboration between Dr. Blair Grubb and Professor Hans-Georg Schaible, and I was fortunate to be able to take part in two of these. Data from the third visit, made by Dr. Grubb alone, is also included in calculations. I would like to thank Professor Schaible, firstly, for allowing me to visit his laboratory, and secondly for allowing me to include the data from these experiments in my thesis. Statistical analysis for the microprobe experiments was performed by Professor Schaible and is acknowledged in the text.

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This thesis is dedicated to the memory of my late step-father, Mike Statham.

Abstract.

In recent years evidence has accumulated which suggests that prostaglandins are involved in spinal nociceptive processing. Several studies have shown that spinally-administered prostaglandins evoke characteristic pain behaviour in rats. Non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, inhibit the enzyme cyclooxygenase (COX) and thus prevent the formation of prostaglandins. It has been hypothesised that a component of the analgesic properties of NSAIDs may be due to an action in the spinal cord, a theory which is supported by behavioural and electrophysiological studies. The current study shows by Western blotting that the two isoforms of COX, COX-1 and COX-2, are present in rat spinal cord tissue. Furthermore, using immunocytochemical techniques, we have localised COX-2-like immunoreactivity to regions of the spinal cord associated with nociceptive processing, namely the superficial and deep dorsal horn and around the central canal. Spinal COX-2, but not COX-1 is bilaterally upregulated in a time-dependent manner (< 3 days) following the induction of a unilateral inflammation around the ankle joint. An increase in spinal PGE_2 levels occurs over the same time course. The basal release of 4 species of prostaglandins was assessed by superfusing the spinal cord with artificial cerebrospinal fluid and analysing the superfusate by radioimmunoassay (PGD_2> PGE_2> 6-keto-$PGF_{1\alpha}$> $PGF_{2\alpha}$). Using this superfusion technique and an antibody-microprobe technique we detected increases in the spinal release of PGE_2 within 30 minutes following acute inflammation. The increased release of PGE_2 was maintained for at least 12 hours. Noxious mechanical stimulation also evoked the spinal release of PGE_2. In conclusion, we have shown that COX-2 is present in the spinal cord in locations commensurate with an involvement in nociceptive processing. The increase in spinal COX-2 and PGE_2 following inflammation suggests that prostaglandins (possibly synthesised by COX-2) may play an active role in modulating spinal nociceptive processing.

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List of Abbreviations.

aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
AP-1	activator protein-1
ATP	adenosine triphosphate
°C	degrees Celsius
C	cervical
cAMP	cyclic adenosine monophosphate
CGRP	calcitonin gene-related peptide
C/K	carrageenan/kaolin
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
cpm	counts per minute
CRE	cyclic AMP responsive element
CREB	cyclic AMP response element binding protein
CSF	cerebrospinal fluid
DRG	dorsal root ganglia
dpm	disintegrations per minute
FCA	Freund's Complete Adjuvant
FITC	fluorescein isothiocyanate
FLAP	5-Lipoxygenase activating protein
GRE	Glucocorticoid Response Elements
HRE	Hormone Response Elements
5-HPETE	5-hydroperoxy-6,8,11,14-eicosatetraenoic acid
5-HT	5-hydroxytryptamine (serotonin)
Hz	Hertz
IL	interleukin
i.p.	intra-peritoneal
i.t.	intra-thecal
i.v.	intra-venous
kDa	kiloDaltons
L	lumbar

LAN	lateral articular nerve
li	like immunoreactivity
LO	lipoxygenase
LPS	lipopolysaccharide
LT	leukotriene
MAN	medial articular nerve
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
$NF_{\kappa}B$	nuclear factor $_{\kappa}B$
NK	neurokinin
NMDA	*N*-methyl-*D*-aspartate
NOS	nitric oxide synthase
NPY	neuropeptide Y
NS	nociceptive-specific
NSAIDs	non-steroidal anti-inflammatory drugs
O.D	optical density
PAN	posterior articular nerve
PBS	phosphate buffered saline
pCREB	phosphorylated cAMP response element binding protein
PG	prostaglandin
PLA_2	phospholipase A_2
PPAR	Peroxisome Proliferator Activated Receptor
PPD	preprodynorphin
PPE	preproenkephalin
psi	pounds per square inch
r.p.m.	revolutions per minute
S	sacral
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gelelectrophoresis
S.E.M	standard error of the mean
SP	substance P
T	thoracic
TNF	tumour necrosis factor
TTBS	Tween-tris buffered saline
WDR	wide-dynamic-range

Chapter 1. General Introduction.

The Arthritis and Rheumatism Council estimates that between seven and eight million people in Britain currently suffer from a rheumatic disease. Approximately 88 million working days are lost every year due to rheumatic diseases, with arthritis being the main cause of disability in Britain. These diseases are very debilitating causing allodynia, hyperalgesia, chronic pain and impairment of joint function. Research into the disease process has uncovered many of the physiological and pathological changes which occur, and new treatments have been developed to control the progression of the disease and accompanying pain. However, no current therapy is entirely effective and/or without side-effects. It is essential that the changes which occur during the inflammation process are more fully understood in order to effectively treat arthritic pain.

Inflammation has been found to evoke profound changes in many systems, including the immune, vascular and nervous systems. The inflammation-induced changes which occur in the peripheral and central nervous systems contribute to the sensations of pain and hyperalgesia associated with inflammation.

This study investigates whether prostaglandins (known to be potent inflammatory mediators in the periphery) contribute to inflammation-induced changes within the central nervous system. It determines the location of prostaglandin synthesis within the spinal cord, and investigates the synthesis and release of a number of prostaglandins from the spinal cord of normal animals and animals with peripheral inflammatory lesions, with the aim to determine whether prostaglandins play a role in spinal nociceptive processing.

1.1 Experimental Models of Inflammation.

In order to study joint disease many experimental models of inflammation have been developed in animals. One of the most widely used models is carrageenan-induced inflammation. Carrageenan is a potent stimulator of the complement system, which, once activated triggers the release of inflammatory mediators and initiates an acute immune response (Willoughby *et al.*, 1969; Di Rosa *et al.*, 1970). The intraarticular injection of carrageenan, often administered in conjunction with kaolin, causes an acute inflammation in the joints of cats and rats (Schaible and Schmidt, 1985; Sluka and Westlund, 1993). The

inclusion of abrasive kaolin heightens the inflammatory response as the damage to the cells lining the synovial membrane causes additional release of inflammatory mediators. The injected joint rapidly becomes swollen due to synovial effusion and associated tissue oedema. Behavioural signs of hyperalgesia in conscious animals are observed between 1-3 hours following the injection (Schaible and Schmidt, 1985), and the inflammation can persist for 72 hours. This model is useful for studying early inflammatory responses, as the rapid changes can be followed for several hours during the development of inflammation (Santer *et al.*, 1983). However, as this model is not associated with long-term inflammatory changes, such as proliferation of synoviocytes and fibroblasts and the destruction of cartilage and bone tissue, another experimental model must be used in the study of chronic inflammation.

In 1959 Pearson and Wood characterised a chronic model of inflammation. Freund's Complete Adjuvant (FCA), a suspension of heat-killed *Mycobacterium tuberculosum* or *butyricum* in paraffin oil, was injected into the tail base or foot pad of rats. Initially, an acute local reaction occurred at the site of injection which was followed by a more widespread inflammation which developed in the distal joints. Secondary lesions developed at sites such as the eyes, nose and genitalia and the animal displayed profound weight loss. The inflammation of the joints lasted for approximately 4 weeks, but often recurred at later dates. This model of inflammation became known as FCA-polyarthritis, and has since been used by many researchers as a model of chronic inflammation (for example, Weihe *et al.*, 1988; Abbadie and Besson, 1992). This model of polyarthritis shares some characteristics with human rheumatoid arthritis including the proliferation of synoviocytes and fibroblasts, and the destruction of cartilage and bone (Nusbickel and Troyer, 1976; Rosenthale and Capetola, 1982; Rainsford, 1982; Pelegri *et al.*, 1995).

The inflammation has an immunological basis of action, an epitope contained on a bacterial heat shock protein (65kD) is crossreactive with an antigen present in human synovial fluid, chondrocyte culture medium, and cartilage proteoglycan (Van Eden *et al.*, 1985, 1989). The bacterial epitope is recognised by T-cells, which leads to an inflammatory reaction not only with the bacterial antigen but also with the cartilage antigen. It is thought that this model may mimic autoimmune arthritis in humans. (Van Eden *et al.*, 1985).

FCA-polyarthritis is a very severe model of inflammation, associated with chronic pain, stress and weight loss (Pearson and Wood, 1959; Colpaert, 1987). In the late 1980's, a less severe model of FCA-arthritis was developed which produced a discrete monoarthritis around an injected joint without the spread of secondary lesions (Grubb *et al.*, 1988; Iadarola *et al.*, 1988; Butler *et al.*, 1992; Donaldson *et al.*, 1993). This monoarthritis not only reduced the severe discomfort of the animals but also provided a model of chronic inflammation in which changes could be directly linked to the inflamed joint.

1.2 Joint Innervation.

Joints are supplied by nerves arising from the cutaneous and muscular branches of main nerve trunks. The majority of studies investigating joint innervation have concentrated on the knee joint of the cat. The cat knee joint is innervated by three nerves, the posterior articular nerve (PAN), the medial articular nerve (MAN) and the lateral articular nerve (LAN). The PAN is the largest of the three nerves, arising from the tibial nerve and innervating the posterior portions of the joint structure. The MAN arises as a branch of the saphenous and/or obturator nerve and innervates the medial aspects of the joint. The LAN is the smallest of the three nerves and arises from the peroneal nerve to supply the lateral areas of the joint (Skoglund, 1956; Freeman and Wyke, 1967).

Articular nerves consist of myelinated and unmyelinated primary afferent fibres and unmyelinated sympathetic efferent fibres. There are four types of primary afferents: thick myelinated group I afferents (Aα-fibres); thick myelinated group II (Aβ-fibres); thin myelinated group III (Aδ-fibres) and thin unmyelinated group IV (C-fibres). Afferents can be identified morphologically and/or electrophysiologically. Their properties are summarised on Table 1.1.

TABLE 1.1.

PRIMARY AFFERENT	FUNCTION	MYELINATED?	DIAMETER (1)	CONDUCTION VELOCITY (2)
Group I	Muscle Proprioception	Yes	>12μm	> 20 $m^{-1}sec^{-1}$
Group II	Joint Proprioception	Yes	6 - 12 μm	> 20 $m^{-1}sec^{-1}$
Group III	Joint Nociception	Yes	1 - 5 μm	2.5 -20 $m^{-1}sec^{-1}$
Group IV	Joint Nociception	No	0.3 - 1.5 μm	< 2.5 $m^{-1}sec^{-1}$

Table 1.1 Morphological and Electrophysiological Properties of Primary Afferents. This Table compares the properties of the small diameter, slow conducting group III and IV nociceptive primary afferents with the fast conducting group I and II proprioceptive primary afferents. Data shown for the diameter of primary afferents[1] was obtained from Heppelmann *et al.* (1988) and for conduction velocity[2] from Dorn *et al.* (1991).

Group I fibres are sometimes found in articular nerve bundles, but terminate in muscle spindle structures. They play an important role in muscular proprioceptive sensations but no role in joint innervation and therefore will not be discussed further.

Group II afferents have three types of receptive endings situated in the joint capsule, ligaments and connective tissue. The Ruffini-like capsule and Golgi-tendon organs are slowly adapting receptors activated at full extension and/or flexion of the joint (Burgess and Clark, 1969). The Pacini-like corpuscle is rapidly adapting and is activated by a wide range of joint movements, as described by Ferrell (1980) who found 18% of group II afferents from cat knee joint were activated by mid-range movement of the joint. Group II afferents may contribute to joint proprioceptive sensation (reviewed in Matthews, 1982). The injection of local anaesthetic into the intracapsular space of the human knee joint did not impede the subjects ability to detect changes in joint position (Clark *et al.*, 1979), which suggests the contribution of these afferents to the maintenance of proprioceptive sensation is relatively small compared to that of muscle spindles.

Group III and IV primary afferents terminate as free nerve endings, with no associated receptive structure, and have been found in the capsule, ligaments, menisci and periosteum of joints (Freeman and Wyke, 1967; Heppelmann *et al.*, 1990). These afferents are termed nociceptors and possess high activation thresholds. Nociceptors respond to damaging or potentially damaging stimuli and are therefore thought to play a role in processing painful sensations in the joint.

Most research of joint innervation has been conducted using the MAN and PAN of the cat as these are primarily articular nerves, relatively free from contamination by group I muscular or cutaneous afferents. Schaible and Schmidt (1983) electrophysiologically characterised the afferents of the MAN and PAN of the cat into 4 subtypes:

1) Low-threshold units activated by innocuous movements, with stronger responses to noxious movements;
2) Units weakly activated by innocuous movements, and strongly activated by noxious movements;
3) High threshold units activated only by noxious movements;
4) Units not activated by noxious or innocuous movements but activated by noxious pressure.

The majority of group II afferents (89%) responded to innocuous movements of the joint (subtypes 1 and 2), with the remaining 11% activated by noxious movement (subtype 3) (Dorn *et al.*, 1991), providing further evidence that these low-threshold may contribute to proprioception. The majority of group III and group IV fibres were activated only by noxious movements or pressure (subtype 3 and 4), only 33% of group III and 10% of group IV units responded to innocuous movements (Schaible and Schmidt, 1985). Later a further subtype of afferents were identified, which were insensitive to noxious movement and pressure but could be activated by electrical stimulation or by intraarticular injection of potassium chloride, bradykinin and prostaglandin (PG) E_2 (Grigg *et al.*, 1986; Schaible and Schmidt, 1988a; Neugebauer *et al.*, 1989). These afferents were termed 'silent nociceptors' (reviewed by Schaible and Grubb, 1993). Guilbaud *et al.* (1985) found similar response characteristics of afferents in the rat primary articulo-cutaneous ramus, a branch of the tibial nerve which supplies the ankle joint.

Heppelmann *et al.* (1988) investigated the fibre size and distribution of axons in the MAN and PAN of the cat knee joint using light and electron microscopy techniques. They found that 91% of afferents in the MAN, and 74% of the afferents in PAN were group III and IV nociceptors. The major role of joint innervation therefore appears to be the transmission of pain information to the central nervous system.

1.3 Peripheral Aspects of Joint Inflammation.

When a joint becomes inflamed, profound changes occur in the physical structure, the chemical medium, and the neuronal activity in afferent nerve fibres supplying the joint. One of the first physical changes is that there is a large synovial effusion. This increase in volume produces a physical distension of the joint. In patients with rheumatoid arthritis there is a large increase in the mean synovial pressure from subatmospheric (-12 to 0 mmHg) pressures in the normal joint to 19 mmHg in the arthritic joint (Jayson and Dixon, 1970). In 1986, Ferrell *et al.* studied the relationship between intraarticular pressure, joint angle and neuronal discharge by making whole nerve recordings from the dog MAN. In normal joints maximal discharge of group I and II fibres occurred at extension, smaller discharge occurred at flexion and minimal discharge was observed during mid-range movements of the joint. Following infusion of paraffin oil into the intraarticular space, which increases the volume and pressure within the joint, there was a reversal in the discharge pattern of the MAN. The maximal neuronal activity occurred at flexion with the neuronal activity at extension remaining similar to in the non-inflamed joint. Recordings from single units of the MAN indicated that this change in activity occurred due to recruitment of afferents, an increased rate of neuronal discharge, and an increase in activity during mid-range movements.

The relationship between intraarticular volume and nociceptor activity has not been similarly investigated. Schaible and Schmidt (1988a) reported a rapid increase in the activity of group III and IV afferents following the intraarticular injection of kaolin and carrageenan. The increase in volume may cause an increase in intraarticular pressure, particularly during movement of the joint, and this may evoke the mechanical activation of nociceptors. However, the increase in nociceptor activity is not entirely due to joint deformation but also due to inflammation-induced changes in the response characteristics of the nociceptors.

Dramatic changes occur in the activity of afferents on the induction and development of an experimental arthritis. Group III and group IV nociceptive fibres become 'sensitised' during inflammation, that is, there is a reduction in the threshold required for activation and afferents become spontaneously active (Coggeshall *et al.*, 1983). Schaible and Schmidt (1985) demonstrated that inflammation caused a general reduction in the threshold required to activate nociceptive fibres. In normal joints 33% of group III and 10% of group IV units of the MAN of the cat knee joint were activated by innocuous movements, whilst in inflamed joints there was a huge increase in the responsiveness of fibres (group III 89%, group IV 72%). In inflamed joints the majority of nociceptive units could be activated by movement of the joint, unlike normal joints in which 24% of group III and 36.5% of group IV fibres could not be activated by any joint movement (subtype 4). The number of nociceptive units exhibiting spontaneous activity and their discharge frequency doubled following the development of arthritis. The increase in resting activity was also noted by Guilbaud *et al.* (1985) who found that resting discharge of afferents innervating the rat ankle joint was absent in normal rats, but present in about 25% of units from FCA-polyarthritic rats. During the development of joint inflammation the mechanically-insensitive 'silent' group III and IV nociceptive units, described by Grigg *et al.* (1986) and Schaible and Schmidt (1988a), are activated by movements of the joint, indicating a possible role for these units in the transmission of nociceptive information.

The changes in joint afferent activity which follow the induction of inflammation result in increased afferent inflow to the spinal cord, and may contribute to the sensations of pain and primary hyperalgesia associated with joint disease. Nociceptors are sensitised by the actions of inflammatory mediators, which are produced in the inflamed joint tissue.

1.4 Inflammatory Mediators.

The synovial fluid of inflamed joints contains elevated levels of a variety of inflammatory mediators, such as histamine, prostaglandins, serotonin (5-hydroxytryptamine: 5-HT), bradykinin and nitric oxide, compared with normal joints. These compounds can elicit profound changes in the normal physiology of the joint. The inflammatory mediators are initially generated by endothelial cells, mast cells and macrophages present in the joint tissue. The release of these mediators causes vasodilation, which leads to increased blood flow around the damaged area. There is also an increase in the permeability of venule walls

which results in vascular cells, such as polymorphonuclear cells, monocytes, lymphocytes and platelets infiltrating the inflamed tissue. The accompanying plasma extravasation contributes to the formation of oedema around the site of inflammation. As well as these changes in vasculature, inflammatory mediators also evoke changes in the activity of primary afferents.

1.4.1 Histamine.

Histamine has an important role in the initial phase of the acute inflammatory response. It is a simple amine which is stored in intracellular granules within mast cells and circulatory basophils, usually found alongside blood vessels where they exert their main response. Elevated levels of histamine have been found in joint tissue following injury or the induction of inflammation. Following mast cell degranulation, histamine promotes oedema formation through vasodilation of blood vessels and plasma extravasation. Oedema formation is mediated by H_1 and H_2 receptors as pretreatment with receptor antagonists reduces vasodilation (Lam And Ferrell, 1990).

1.4.2 Serotonin.

Serotonin, (or 5-HT), is another amine stored in rodent mast cells, and is released during mast cell degranulation. Serotonin increases venule permeability, which contributes to oedema formation, but does not cause vasodilation. Serotonin can directly stimulate nociceptive afferents through 5-HT receptors. A number of serotonin receptors have been implicated in nociceptor activation, for example 5-HT_1 receptors (Rueff and Dray, 1993), 5-HT_2 receptors (Grubb *et al.*, 1988) and 5-HT_3 receptors (Richardson *et al.*, 1985; Grubb *et al.*, 1988), although the subtypes of these receptors responsible for nociceptor activation have not been identified. Approximately two-thirds of group III and IV fibres in the rat ankle joint were activated on stimulation with serotonin (Birrell *et al.*, 1990). Similarly in the MAN of the cat knee joint 43% of group III and 73% of group IV fibres were activated (Herbert and Schmidt, 1992).

1.4.3 Bradykinin.

Bradykinin is a nonapeptide formed by the action of the enzyme kallikrein on plasma protein substrates called kininogens. The inactive precursor of kallikrein, prekallikrein is also present in plasma and can be converted to the active enzyme by a variety of stimuli, for example by activated Hageman factor (factor XII of the blood clotting sequence). Increased levels of bradykinin have been found in inflamed joint tissues (Hargreaves *et al.*, 1988). The local increase in bradykinin results in increased vascular permeability at the site of inflammation, which leads to the extravasation of Hageman factor, prekallikrein and kininogens, their activation, and the subsequent formation of bradykinin. Bradykinin stimulates the production of nitric oxide and prostaglandins by endothelial cells, which further contribute to vasodilation.

Kanaka *et al.* (1985) found that intraarterial injection of bradykinin close to the cat knee joint caused activation of the majority of the group III and IV afferents but not group II afferents in the MAN. Grubb *et al.* (1991) found that bradykinin activated approximately 50% of the units supplying the rat ankle joint, and also sensitised 44% to movement. This sensitisation was also observed by Neugebauer *et al.* (1989) who reported 70% of group III and IV fibres became sensitised to innocuous movements, due to the lower stimulation threshold and the recruitment of previously mechano-insensitive units. The potent excitation and sensitisation of afferents by bradykinin indicates an important role in joint nociception. The actions of bradykinin are amplified in the presence of prostaglandins. The activation of primary afferents by bradykinin was enhanced by the presence of PGE_2 in 50% of group III and 75% of group IV fibres in the MAN of cat knee (Schaible and Schmidt, 1988b), and 90% of group III and IV fibres of rat ankle (Grubb *et al.*, 1991). Potentiation of the excitatory effects of bradykinin were also seen following the combined administration of bradykinin with PGI_2, and also the IP receptor agonist, cicaprost (Birrell *et al.*, 1993).

1.4.4 Neuropeptides.

As well as relaying sensory information from the site of inflammation to the spinal cord, nociceptors actually contribute to the inflammatory response by releasing neuropeptides from their peripheral terminals. This is referred to as neurogenic inflammation. Primary afferents contain a variety of neuropeptides such as substance P (SP), calcitonin gene-

related peptide (CGRP), galanin, neurokinin A (NKA) and neuropeptide Y (NPY). Using immunological techniques a number of groups have attempted to characterise the distribution of neuropeptides contained within primary afferents (Lawson *et al.*, 1984; Gulbenkian *et al.*, 1986; Hanesch *et al.*, 1991, 1992). It appears that the most predominant neuropeptides, SP and CGRP, may be colocalised in the peripheral terminals of a proportion of sensory afferents (Gulbenkian *et al.*, 1986; Hanesch *et al.*, 1992). These neuropeptides are released from peripheral terminals by the antidromic stimulation of adjacent terminals of the same nerve. Electrical stimulation of high threshold group IV afferents in the rat sciatic nerve caused the release of immunoreactive-SP into the knee joint (Yaksh, 1988). Increased levels of sensory neuropeptides have been discovered in synovial fluid and tissue from patients with joint disease (Devillier *et al.*, 1986; Larsson *et al.*, 1989) and animals with experimentally-induced arthritis (Levine *et al.*, 1984). The antidromic activation of group III afferents of rat saphenous nerve caused vasodilation (Jänig and Lisney, 1989) and the intrarticular injection of SP into the rat knee was shown to evoke plasma extravasation (Lam and Ferrell, 1989, 1990), possibly mediated through NK_1 receptors (Lam and Ferrell, 1991). SP mediates vasodilation and plasma extravasation indirectly, through mast cell degranulation and the subsequent release of histamine (Lembeck and Holzer, 1979) and by increasing the production of prostaglandins from synoviocytes (Lotz *et al.*, 1987).

1.4.5 Eicosanoids.

The eicosanoids are a group of 20-carbon unsaturated fatty acids consisting of a cyclopentane ring with two sidearms. The principal groups of eicosanoids are the prostaglandins, thromboxanes, and the leukotrienes. The eicosanoids are derived from a common precursor, arachidonic acid by the actions of the enzymes cyclooxygenase (COX) and lipoxygenase (LO). The biochemistry of the eicosanoids is discussed further in section 1.5. Elevated levels of prostaglandins and leukotrienes have been found in synovial fluid and tissue from patients with inflammatory joint disorders (Brodie *et al.*, 1980; McGuire *et al.*, 1982; Salmon *et al.*, 1983; Egg, 1984; Moilanen 1989) and animals with experimentally induced inflammations (Blackham *et al.*, 1974; Arai and Aizawa, 1978; Stichtenoth, 1995). Increased levels of COX have also been reported in inflamed tissue (Sano *et al.*, 1992; Seibert *et al.*, 1994; Vane *et al.*, 1994; Kang *et al.*, 1996). The major eicosanoid species found in inflamed tissue are leukotriene (LT) B_4, PGE_2 and PGI_2. LTB_4 is a powerful chemotactic agent which attracts neutrophils and macrophages to sites of inflammation

(Ford-Hutchinson *et al.*, 1980). PGE_2 and PGI_2 are potent vasodilators (Dick and Grennan, 1976, Williams, 1979) and although they do not themselves increase vascular permeability, they potentiate the actions of other inflammatory mediators (such as bradykinin) and maintain oedema around the site of inflammation (Komoriya *et al.*, 1978).

When prostaglandins are administered peripherally to conscious animals, the animals exhibit characteristic pain-behaviour (Ferreira *et al.*, 1978; Taiwo and Levine, 1989). It is thought that the pain-behaviour evoked by the administration of prostaglandins occurs not only due to direct activation of nociceptors but also as a result of sensitisation of the nociceptors. The mechanism by which prostaglandins lower the activation threshold of nociceptive fibres is not yet fully understood. However, it has been determined that prostaglandins modulate the activity of a number of receptors and associated ion channels. PGE_2 has a number of neuronal effects. For example: reducing the amplitude of voltage-gated potassium currents (I_K) in nodose ganglia cells (Fowler *et al.*, 1985) and sensory neurones (England *et al.*, 1996; Nicol *et al.*, 1997); increasing the amplitude of tetrodotoxin-resistant sodium currents (TTX-R I_{Na}) in small diameter sensory neurones (England *et al.*, 1996); enhancing hyperpolarisation-activated currents (I_h) (Ingram and Williams, 1996); and potentiating calcium currents in sensory neurones (Nicol *et al.*, 1992). These effects may increase the spontaneous activity of the neurones and/or facilitate neurotransmitter release which may potentiate the neurogenic component of the inflammatory response.

PGE_1 and PGE_2 have been shown to excite and sensitise articular group III and IV fibres, when administered intraarterially close to the cat knee joint (Heppelmann *et al.*, 1985; Schaible and Schmidt, 1988b). Mechanically-insensitive silent nociceptive units were excited. Afferents fired at an increased rate and the activation-threshold was lowered, the neurones therefore showed a greater responses to stimuli (Heppelmann *et al.*, 1985). PGE_2 excited approximately 60% of nociceptive afferents in a dose-dependent manner, and sensitised 64% of group III and 25% of group IV fibres to movement and 50% of group III and 75% of group IV fibres to the actions of bradykinin (Schaible and Schmidt, 1988b). A much smaller proportion of fibres were activated and/or sensitised by PGE_2 in rat (Grubb *et al.*, 1991). The action of PGE_2 on primary afferents may be mediated by the EP_1 receptor subtype (Taiwo and Levine, 1988), behavioural hyperalgesia was reduced by the administration of an EP_1 antagonist, SC19220. The action of PGE_1 may be mediated by another EP receptor, SC19220 had no effect on PGE_1-induced behavioural hyperalgesia

(Khasar *et al.*, 1994). Other prostaglandin receptors may be involved in the peripheral sensitisation and/or activation of nociceptors but these have not been characterised as very few selective receptor antagonists are available. Nociceptors are activated, in rat, to a much greater degree by PGI_2 and cicaprost, a stable IP receptor agonist. The intraarterial injection of prostacyclin close to the ankle joint excited 80% of group III and IV afferents whilst cicaprost excited 92% of the afferents. A similarly large proportion of afferents were sensitised by both PGI_2 and cicaprost (Birrell *et al.*, 1993).

The analgesia provided by non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, occurs due to inhibition of the enzyme COX (also known as prostaglandin G/H synthase) and the subsequent reduction in the synthesis of prostaglandins. NSAIDs have been shown to reduce the spontaneous and mechanically evoked activity of group III and IV fibres in animals with experimentally induced inflammation (Heppelmann *et al.*, 1986; Grubb *et al.*, 1991). The administration of PGE_2 to the inflamed knee joint of the cat, dose-dependently reversed the inhibitory effects of NSAIDs on group III and IV afferent activity (Heppelmann *et al.*, 1986). These experiments indicate that prostaglandins are important mediators of inflammation and play an integral role in maintaining nociceptor activity during inflammation.

1.5 Eicosanoid Biochemistry.

Due to their highly lipophilic nature, eicosanoids are not stored preformed within cells but are synthesised and released from cells in response to a variety of stimuli. The biosynthetic pathway of the eicosanoids has multiple steps, involving many enzymes. The precursor for eicosanoid formation is arachidonic acid, which is derived from essential fatty acids such as linoleic acid. These dietary fatty acids are desaturated and elongated to form 20-carbon fatty acids such as arachidonic acid. The amount of free arachidonic acid contained within cells is very low, however a large amount is stored in an esterified form mostly in the *sn*-2 position of membrane phospholipids and also, to a lesser degree, in lipid body triglycerides.

Arachidonic acid is liberated from membranes by the action of enzymes such as phospholipase A_2 (PLA_2), or the combined action of phospholipase C (PLC) and glyceride-lipases. Membrane phospholipids appear to be the major source of arachidonic acid used for prostaglandin production (Dennis, 1987; Dennis *et al.*, 1991; Smith, 1992) although there

may be some contribution from lipid body pools (Wellner *et al.*, 1991). There are three types of PLA_2, cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$) and Ca^{2+}-independent PLA_2, and individual cell types may contain more than one type of PLA_2. In kidney cells, following submicromolar increases in intracellular calcium concentration, a large (100 kDa) $cPLA_2$ has been shown to undergo a calcium-dependent translocation to cell membranes (Gronich *et al.*, 1988, 1990) where it is phosphorylated by mitogen-activated protein (MAP) kinases (Lin *et al.*, 1993; Schievella *et al.*, 1995). The phosphorylated $cPLA_2$ preferentially hydrolyses the *sn*-2 ester bond of phospholipids containing arachidonate, causing rapid increases in intracellular free arachidonic acid concentrations. Increased levels of $cPLA_2$ have been observed in human synovial cells following stimulation with interleukin-1, an inflammatory mediator (Angel *et al.*, 1994). The regulation of $cPLA_2$ is important since this is the rate limiting step in the generation of proinflammatory prostaglandins.

Long-term increase in prostaglandin production is often seen in conjunction with the induction of type-II $sPLA_2$ (Lyons-Giordano *et al.*, 1989; Nakazato *et al.*, 1991) which has been detected in large amounts in inflamed tissue (Scott *et al.*, 1991; Dennis, 1994; Murakami *et al.*, 1995). Type-II $sPLA_2$ differs from $cPLA_2$ as it requires millimolar calcium concentrations for activation. Once activated, it exhibits no specificity towards the *sn*-2 position of arachidonate-containing phospholipids, and releases a wide variety of fatty acids from phospholipids. There is some evidence to suggest that type-II $sPLA_2$ is linked to the delayed COX-2-mediated generation of prostaglandins (Murakami *et al.*, 1996).

The conversion of free arachidonic acid to prostaglandins and leukotrienes is controlled by the enzymes COX and LO respectively. Until recently only one form of COX had been described, then a second isoform was identified which could be rapidly upregulated by a variety of stimuli (Simmons *et al.*, 1989; Kujubu *et al.*, 1991, Yamagata *et al.*, 1993). The original isoform, COX-1, and the newly-discovered isoform, COX-2 have been cloned and characterised in a number of species, e.g. human (Yokayama and Tanabe, 1989; Hla and Neilson, 1992) and rat (Feng *et al.*, 1993; Kennedy *et al.*, 1993; Yamagata *et al.*, 1993).

In peripheral tissues, the predominant isoform of COX is COX-1 which is regarded as the 'housekeeping' isoform, responsible for the constitutive production of prostaglandins to maintain homeostasis. COX-1 is present in most tissues with large amounts of the mRNA transcript found in rat lung, liver, spleen, kidney, stomach and heart (Seibert *et al.*, 1994). A

similar widespread distribution of COX-1 mRNA has also been described in human tissue (O'Neill and Ford-Hutchinson, 1993). COX-2, however, is the predominant isoform in rat brain (Feng *et al.*, 1993; Seibert *et al.*, 1994). COX-2 is usually described as the inducible isoform, associated with the production of prostaglandins involved in the inflammatory response and cell differentiation processes.

Despite differences in regulation of expression, both isoforms of COX are membrane-bound proteins, found on the plasma and nuclear membranes and lumen of the endoplasmic reticulum. COX converts free arachidonic acid to the prostaglandin precursor PGH_2 in two consecutive steps, a cyclooxygenase-reaction followed by a peroxidase reaction. Both stages of the reaction are heme-dependent, with the heme group binding to the same pair of histidine residues, His388 and His207 in each isoform (Picot *et al.*, 1994). The active site of the cyclooxygenase reaction is a hydrophobic channel which extends from the membrane binding domain to the heme-binding site. The carboxyl group of free arachidonic acid binds to the Arg120 residue, and here the cyclooxygenase reaction takes place. A tyrosyl radical is liberated from both COX isoforms which is essential for catalysis (Shimokawa *et al.*, 1990). The tyrosyl radical is thought to arise from Tyr385 which lies in the hydrophobic channel of the enzyme (Picot *et al.*, 1994). The cyclooxygenase-catalysed reaction leads to two molecules of oxygen being inserted into arachidonic acid and the formation of the unstable intermediate PGG_2. The cyclooxygenase reaction is inhibited by NSAIDs such as aspirin and indomethacin. Most NSAIDs are not irreversible inhibitors of cyclooxygenase. They bind to various residues in the hydrophobic channel thus preventing the substrate from gaining access to the active site. Aspirin, on the other hand is an irreversible inhibitor as it permanently acetylates serine residues in the active site, which prevents the binding of arachidonic acid to the active site (DeWitt *et al.*, 1990; Picot *et al.*, 1994). The PGG_2 intermediate has a very short half-life and is rapidly reduced to PGH_2 by the peroxidase action of COX. It is thought that PGG_2 is released from the cyclooxygenase active site, then binds to the peroxidase active site which is situated opposite the heme group. The existence of two distinct active sites is supported by the observation that NSAIDs only inhibit the cyclooxygenase reaction, and not the peroxidase reaction (Meade *et al.*, 1993). PGH_2 is subsequently converted to prostaglandins by the action of specific prostaglandin-synthases/isomerases, the species of prostaglandins produced by a cell depends upon the type of synthase present in the cell. The COX pathway is summarised in Figure 1.1.

FIGURE 1.1.

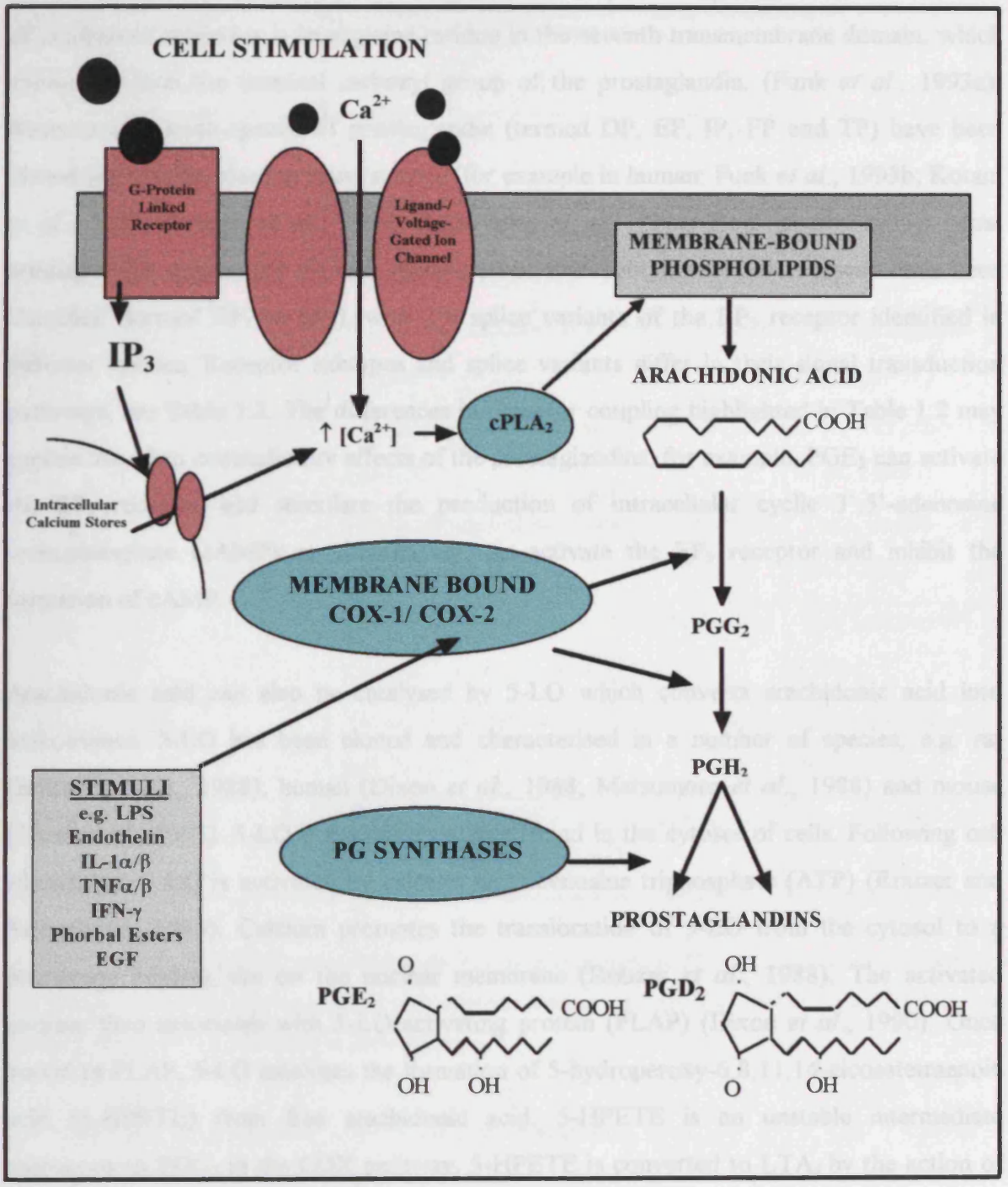


Figure 1.1. The COX Pathway. A highly simplified schematic diagram illustrating the liberation of free arachidonic acid from membrane phospholipids and subsequent formation of prostaglandins. COX-1 is constitutively present in most cells and COX-2 can be induced by a variety of stimuli including lipopolysaccharide (LPS), endothelin, interleukin (IL)-1, tumour necrosis factor (TNF) and phorbol esters (e.g. Fu *et al.*, 1990; Ristimaki *et al.*, 1994; Arias-Negrete *et al.*, 1995; Bakhle and Botting, 1996; Breder and Saper, 1996; Porreca *et al.*, 1996).

Prostaglandins act through specific cell-surface receptors belonging to the guanine-nucleotide-binding regulatory (G-protein)-coupled receptor family. The common feature of all prostanoid receptors is an arginine residue in the seventh transmembrane domain, which appears to bind the terminal carboxyl group of the prostaglandin. (Funk *et al.*, 1993a). Receptors for each species of prostaglandin (termed DP, EP, IP, FP and TP) have been cloned and characterised in many species (for example in human: Funk *et al.*, 1993b; Kotani *et al.*, 1995; Bastien *et al.*, 1994; Katsuyama *et al.*, 1994; Boie *et al.*, 1995). Most prostaglandin species act via one receptor, but four subtypes of EP receptor have been identified (termed EP_1 to EP_4), with 2-6 splice variants of the EP_3 receptor identified in different species. Receptor subtypes and splice variants differ in their signal transduction pathways, see Table 1.2. The differences in receptor coupling highlighted in Table 1.2 may explain the often contradictory effects of the prostaglandins, for example, PGE_2 can activate the EP_2 receptor and stimulate the production of intracellular cyclic 3',5'-adenosine monophosphate (cAMP) or alternatively can activate the EP_3 receptor and inhibit the formation of cAMP.

Arachidonic acid can also be catalysed by 5-LO which converts arachidonic acid into leukotrienes. 5-LO has been cloned and characterised in a number of species, e.g. rat (Balcarek *et al.*, 1988), human (Dixon *et al.*, 1988; Matsumoto *et al.*, 1988) and mouse (Chen *et al.*, 1995). 5-LO is a soluble enzyme found in the cytosol of cells. Following cell stimulation, 5-LO is activated by calcium and adenosine triphosphate (ATP) (Rouzer and Samuelsson, 1985). Calcium promotes the translocation of 5-LO from the cytosol to a membrane binding site on the nuclear membrane (Rouzer *et al.*, 1988). The activated enzyme then associates with 5-LO activating protein (FLAP) (Dixon *et al.*, 1990). Once bound to FLAP, 5-LO catalyses the formation of 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) from free arachidonic acid. 5-HPETE is an unstable intermediate analogous to PGG_2 in the COX pathway. 5-HPETE is converted to LTA_4 by the action of LTA_4 synthase. LTA_4 is then either hydrolysed to LTB_4, or converted to LTC_4 by a conjugation reaction with glutathione. LTC_4 can subsequently be converted to LTD_4 and LTE_4 (Samuelsson, 1983; Henderson, 1994).

TABLE 1.2.

LIGAND	RECEPTOR SUBTYPE	TRANSDUCTION SYSTEM	CLONED IN RAT?
PGD_2	DP	Via G_s: Stimulation of adenylate cyclase ↑cAMP	No, Hirata *et al.* (1994)
PGE_2	EP_1	Via G_q: Stimulation of phosphatidyl inositol ↑ intracellular Ca^{2+}	No, Watabe *et al.* (1993)
	EP_2	Via G_s: Stimulation of adenylate cyclase ↑cAMP	No, Katsuyama *et al.* (1995)
	EP_3 (splice varients)	Via G_i: Inhibition of adenylate cyclase ↓cAMP	Yes, Sugimoto *et al.* (1993)
	EP_4	Via G_s: Stimulation of adenylate cyclase and ↑cAMP	Yes, Sando *et al.* (1994)
$PGF_{2\alpha}$	FP	Via G_q: Stimulation of phosphatidyl inositol ↑ intracellular Ca^{2+}	Yes, Lake *et al.* (1994)
PGI_2	IP	Via G_s: Stimulation of adenylate cyclase and ↑cAMP	Yes, Sasaki *et al.* (1994)
TXA_2	TP	Via G_q: Stimulation of phosphatidyl inositol ↑ intracellular Ca^{2+}	No, Namba *et al.* (1992)

Table 1.2. Prostaglandin Receptors and their Mechanism of Action. This Table describes the G-protein coupling and transduction systems of rat prostaglandin receptor subtypes. The murine receptor is described if the rat receptor subtype has not yet been cloned.

1.6 Summary of Peripheral Aspects of Inflammation.

The development of inflammation in a joint is associated with profound changes in physiology. The joint becomes distended due to synovial effusion and swollen due to oedema of surrounding tissue. There are changes in the chemical medium in the joint, with the synthesis and/or release of inflammatory mediators such as histamine, bradykinin, and prostaglandins. These inflammatory mediators have dramatic effects on local vasculature and on nociceptive fibres. Some inflammatory mediators such as bradykinin, 5-HT, PGE_2 and PGI_2 have been shown to sensitise and/or stimulate high-threshold nociceptive

afferents. The nociceptors themselves contribute to neurogenic inflammation by releasing neuropeptides in the periphery on antidromic stimulation of the fibre. The inflammatory response is highly coordinated with individual inflammatory mediators complementing and/or potentiating the effects of other mediators. The peripheral inflammatory response causes significant changes in the activity of nociceptive fibres which become spontaneously active at rest and activated by innocuous stimulation, such as movement of the joint in the normal working range. This results in a dramatic increase in afferent inflow to the spinal cord where the nociceptive information is processed. These changes are summarised in Figure 1.2.

FIGURE 1.2.

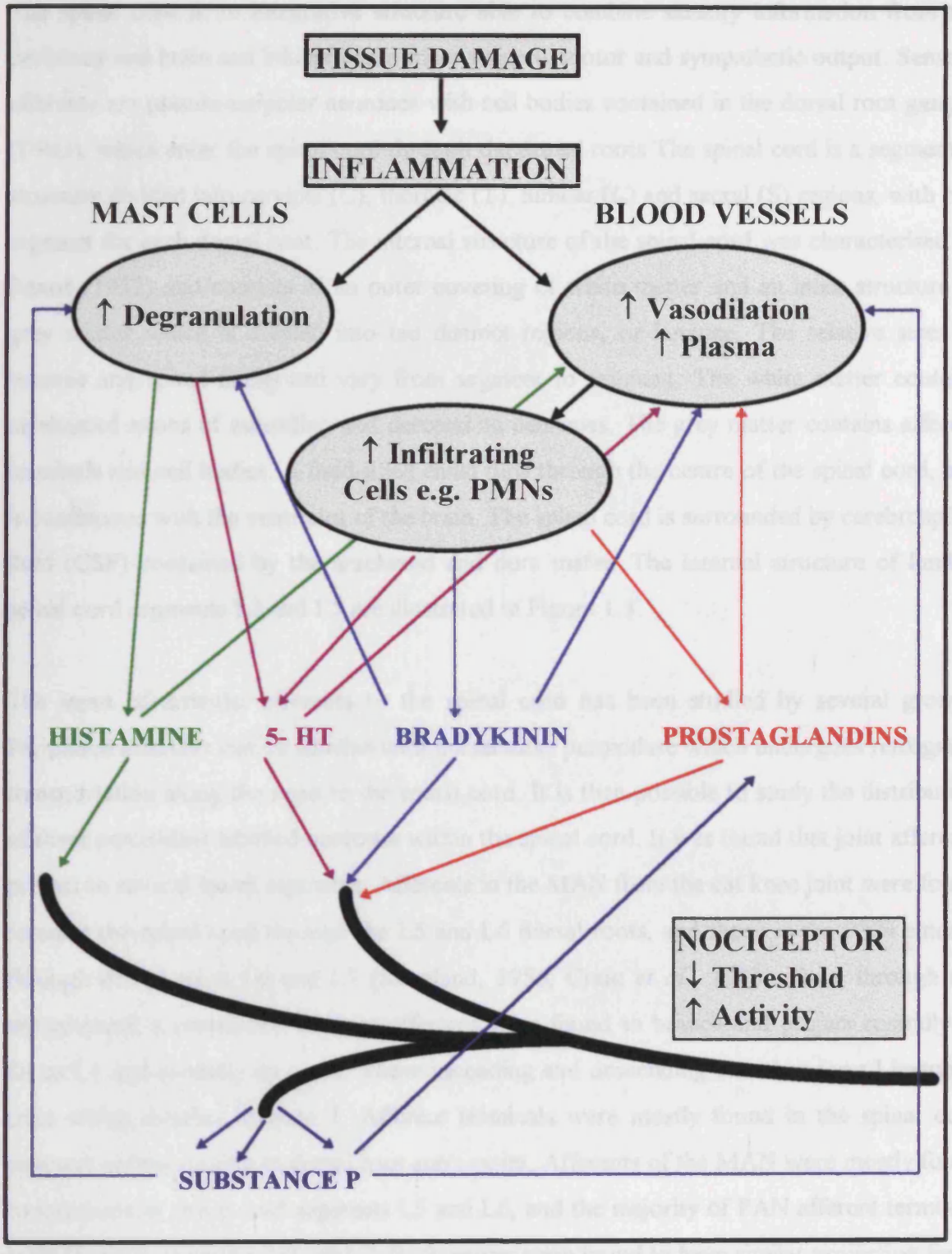


Figure 1.2. Peripheral Aspects of Joint Inflammation. This diagram summarises the cascade of events which are initiated during inflammation. It highlights the complex interactions which occur between inflammatory mediators causing activation and sensitisation of nociceptive afferents.

1.7 Input of Sensory Information to the Spinal Cord.

The spinal cord is an integrative structure able to combine sensory information from the periphery and brain and initiate appropriate sensory, motor and sympathetic output. Sensory afferents are pseudo-unipolar neurones with cell bodies contained in the dorsal root ganglia (DRG), which enter the spinal cord through the dorsal roots The spinal cord is a segmented structure divided into cervical (C), thoracic (T), lumbar (L) and sacral (S) regions, with one segment for each dorsal root. The internal structure of the spinal cord was characterised by Rexed (1952) and consists of an outer covering of white matter and an inner structure of grey matter which is divided into ten distinct regions, or laminae. The relative sizes of laminae and spinal nuclei can vary from segment to segment. The white matter contains myelinated axons of ascending and descending neurones. The grey matter contains afferent terminals and cell bodies. A fluid-filled canal runs through the centre of the spinal cord, and is continuous with the ventricles of the brain. The spinal cord is surrounded by cerebrospinal fluid (CSF) contained by the arachnoid and dura mater. The internal structure of lumbar spinal cord segments L2 and L5 are illustrated in Figure 1.3.

The input of articular afferents to the spinal cord has been studied by several groups. Peripheral afferents can be labelled with horseradish peroxidase which undergoes retrograde transportation along the axon to the spinal cord. It is then possible to study the distribution of these peroxidase labelled neurones within the spinal cord. It was found that joint afferents project to several spinal segments. Afferents in the MAN from the cat knee joint were found to enter the spinal cord through the L5 and L6 dorsal roots, and those in the PAN entered through dorsal roots L6 and L7 (Skogland, 1956; Craig *et al.*, 1988). Once through the dorsal roots, a proportion of these afferents were found to branch and project rostrally as far as L1 and caudally up to S2. These ascending and descending branches form Lissauer's tract which overlies laminae 1. Afferent terminals were mostly found in the spinal cord segment corresponding to dorsal root entry-point. Afferents of the MAN were mostly found to terminate in spinal cord segments L5 and L6, and the majority of PAN afferent terminals were found in segments L6 and L7. Both nerves were found to have similar projection fields within the spinal cord, namely to laminae I of the dorsal horn, laminae V and VI and the dorsal part of laminae VII of the deep dorsal horn, and to the medial portion of Clarke's column. No terminals were discovered in laminae II, III, and IV or the ventral horn (Craig *et al.*, 1988). Levine *et al.* (1984) found projections from the afferents supplying the ankle

joint in rat terminated in the superficial dorsal horn, laminae I and the substantia gelatinosa, of spinal segment L4.

FIGURE 1.3.

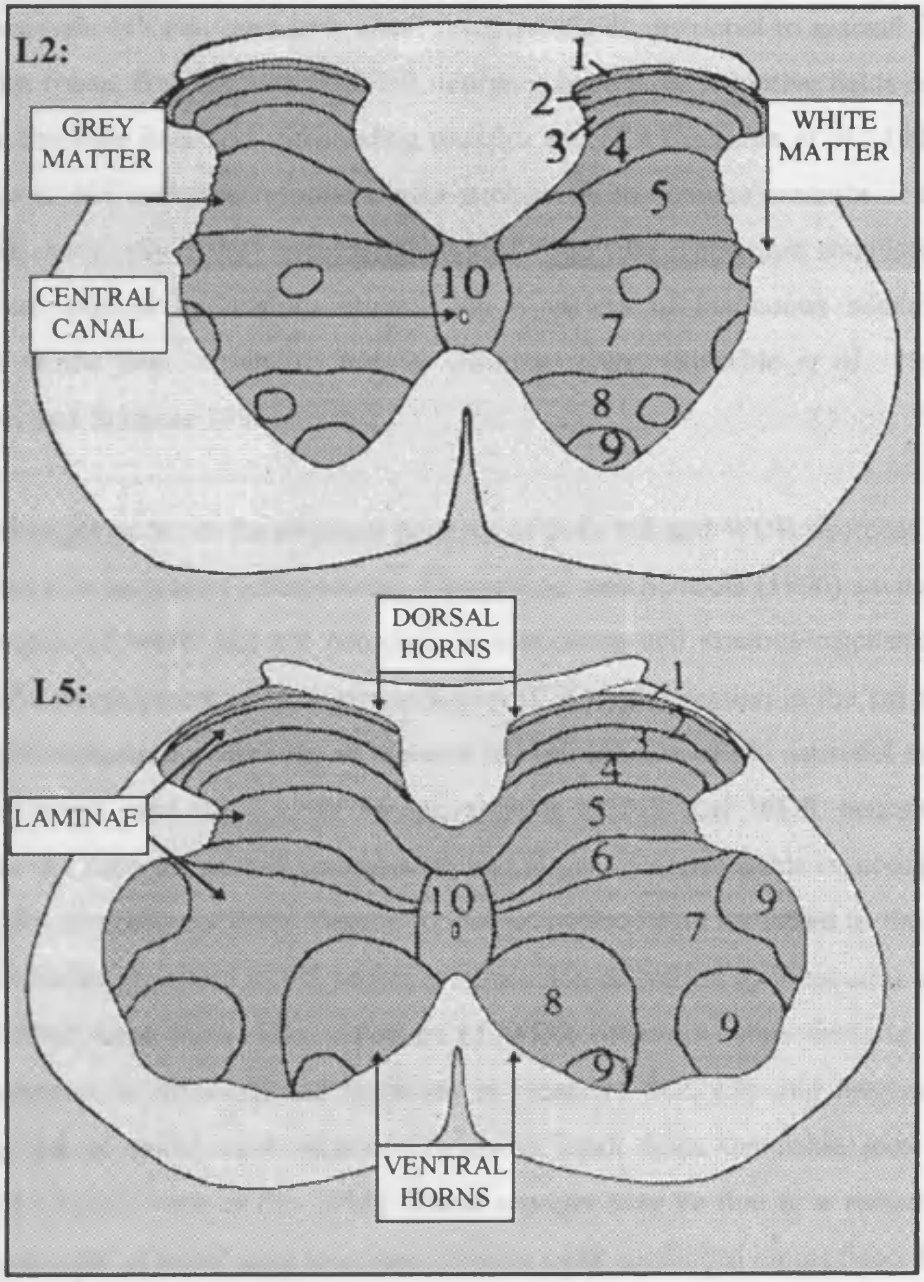


Figure 1.3. Internal Organisation of the Rat Spinal Cord. The internal structure of the spinal cord changes from segment to segment. Note the change in the actual size of the grey matter between L2 and L5, the larger grey matter in L5 reflects an increase in the amount of sensory information conveyed to the spinal cord from the hindlimbs. The distribution of laminae (numbered 1-10) within the spinal cord also varies, reflecting the type of sensory input processed by that segment.

1.8 Spinal Processing of Nociceptive Information.

Electrical stimulation of the PAN of the cat knee joint excites interneurones in laminae I, IV-VI, VII and VIII of spinal segments L4 to S1 (Schaible *et al.*, 1986, 1987). These interneurones are classified as either nociceptive-specific (NS) or wide-dynamic-range (WDR) neurones. NS neurones have small receptive fields restricted to a small focal point in deep joint tissue. By comparison, WDR neurones have large receptive fields and receive input from the knee joint and surrounding muscles and skin (Schaible *et al.*, 1987). In the normal animal, NS neurones respond almost exclusively to noxious pressure or movement of the joint, with only a few impulses generated following innocuous stimulation. WDR neurones are excited by noxious stimuli and a variety of innocuous stimuli such as movement of the joint within the normal working range, (Schaible *et al.*, 1987, 1991; Neugebauer and Schaible 1990).

Dramatic changes occur in the response patterns of both NS and WDR neurones following development of a peripheral inflammation. Neugebauer and Schaible (1990) studied changes in the response of WDR and NS neurones to innocuous and noxious mechanical stimuli following the development of carrageenan/kaolin (C/K) inflammation in the cat knee joint. Following inflammation, there was an increase in both the size of the neuronal response to mechanical stimuli and the size of receptive fields of NS and WDR neurones. 20/23 neurones in the deep dorsal and ventral horn had larger receptive fields involving both the ipsilateral and contralateral limbs, despite the inflammation being restricted to the ipsilateral knee. The lowered threshold of NS neurones resulted in activation by innocuous stimulation of the inflamed knee joint. The responses of WDR neurones were similarly enhanced. Similar decreases in threshold and increases in receptive field size and neuronal activity were observed in spinal cord neurones receiving input from the ankle joint of FCA-monoarthritic rats (Grubb *et al.*, 1993). These changes may be due to a reduction in the threshold potential of spinal cord neurones, so even weak subliminal inputs from the edge of the receptive field (e.g. the contralateral limb) become sufficient to depolarise the neurone, or due to depolarisation of the neurones moving the membrane potential closer to the threshold potential. This increase in sensitivity has been termed spinal hyperexcitability and leads to an amplification of spinal nociceptive processing.

Following development of an inflammation, the increase in spinal hyperexcitability corresponds with the increased nociceptive input to the spinal cord, although the 'stimulus-response' relationship is not a simple one. Low frequency electrical stimulation (0.1Hz) of group III and IV afferents initiated a stimulus-linked response (1 stimulus:1 discharge) in spinal WDR neurones. However, when high threshold group IV afferents were stimulated at high frequencies (0.5Hz) there was a dramatic change in the response pattern. The firing rate of WDR neurones rapidly increased until neurones were almost continuously firing (Davies and Lodge, 1987; Dickenson and Sullivan, 1990; Thompson *et al.*, 1990). This phenomenon is termed "wind-up" and is used to describe the successive increases in neuronal firing rate in response to repetitive stimulation of high threshold nociceptors.

Wind-up and associated central hyperexcitability is mediated in part by postsynaptic *N*-methyl-*D*-aspartate (NMDA) receptors. The intrathecal administration of specific NMDA receptor antagonists completely abolishes wind-up (Davies and Lodge, 1987; Dickenson and Sullivan, 1990), reduces the size of enlarged receptive fields (Ren *et al.*, 1991) and also reduces behavioural hyperalgesia (Yaksh, 1989). Conversely, the intrathecal administration of NMDA produces hyperalgesia in conscious rats (Malmberg and Yaksh, 1992a). These data indicate the importance of NMDA receptors in spinal nociceptive processing.

Glutamate, the endogenous NMDA receptor agonist, is released from nociceptive afferent terminals in the superficial dorsal horn of the rat (De Biasi and Rustioni, 1988). Increased levels of glutamate have been measured in the spinal cord following induction of inflammation (Sluka *et al.*, 1992; Malmberg and Yaksh, 1992b; Sorkin *et al.*, 1992). The release of glutamate from nociceptive afferents is enhanced by sensory neuropeptides such as SP and CGRP (Murase *et al.*, 1989; Kangra *et al.*, 1990). Increases in sensory neuropeptide release from spinal nociceptive terminals have been detected following noxious stimulation. Yaksh *et al.* (1980) demonstrated the release of SP from the spinal cord of anaesthetised cat following electrical stimulation of high threshold nociceptors. Noxious mechanical or thermal stimulation also evokes the release of spinal SP (Kuraishi *et al.*, 1985; Duggan *et al.*, 1988; Schaible *et al.*, 1992), NKA (Hope *et al.*, 1990) and CGRP (Schaible *et al.*, 1992). Using antibody microprobe techniques the site of SP release was localised to the superficial dorsal horn coinciding with the termination site of nociceptive afferents (Duggan *et al.*, 1992; Schaible *et al.*, 1992). Evidence suggests that increased

levels of neuropeptides are also released from nociceptive terminals in the dorsal horn following the development of inflammation (Oka *et al.* , 1987; Schaible *et al.*, 1990).

The increased release of sensory neuropeptides from the spinal cord following induction of inflammation may be due to increased synthesis of the peptides in response to inflammation. Using *in situ* hybridisation and immuncytochemical techniques a number of researchers (Minami *et al.*, 1989; Donaldson *et al.*, 1992; McCarson and Krause, 1994; Hanesch *et al.*, 1995) detected dramatic increases the expression of neuropeptides and their encoding genes in DRG. Donaldson *et al.* (1992) demonstrated increases in expression of preprotachykinin (the gene responsible for encoding SP and NKA) and CGRP mRNA in L5 DRG, following the development of FCA-monoarthritis. These increases occurred as early as 8 hours following the induction of inflammation and were maintained over the 14 days of the study. The expression of SP and CGRP neuropeptides were similarly increased in DRG following the development of joint inflammation (for example, Weihe *et al.* (1988) detected increased SP- and CGRP- like immunoreactivity (li); Hanesch *et al.* (1993) detected increased CGRP-li).

The activation of glutamate, tachykinin and CGRP postsynaptic receptors results in depolarisation of postsynaptic neurones and the development of spinal hyperexcitability. There are subsequent increases in the expression of a variety of second messengers, immediate early genes, neuromodulators and neurotransmitters in spinal cord neurones.

Activation of NMDA receptors promotes the transcription of immediate early genes such as *c-fos* and *c-jun.* Enhanced expression of these immediate early genes has been detected in dorsal horn neurones following inflammation or noxious stimulation (Draisci and Iadarola, 1989; Abbadie and Besson, 1992; Honore *et al.*, 1995). These immediate early genes encode proteins which bind to DNA recognition sites, such as AP-1, in the 5'-promoter region of target genes and initiate transcription of that gene. These immediate early genes may be responsible for the upregulation of a number of neuropeptides in the spinal cord following inflammation. For example, immunoreactive-Fos has been co-localised in dorsal horn neurones expressing preprodynorphin (PPD) and preproenkephalin (PPE) mRNA (Noguchi *et al.*, 1989, 1991). Several AP-1 binding sites have been found in the promoter regions of PPD and PPE, suggesting that Fos protein may be responsible for increased dynorphin and enkephalin transcription.

The upregulation of genes involved in spinal processing of nociceptive information, such as PPD (Iadarola *et al.*, 1988; Weihe *et al.*, 1988; Przewlocka *et al.*, 1992) and PPE (Weihe *et al.*, 1988; Draisci and Iadarola, 1989) has been studied in the dorsal horn following inflammation. Increased levels of immunoreactive dynorphin and enkephalin are found in laminae I, II, V and VI of dorsal horn neurones in segments that receive innervation from the inflamed joint (Millan *et al.*, 1988; Weihe *et al.*, 1988). These increases appear in neurones receiving direct input from nociceptive afferents (Cho and Basbaum, 1989). The upregulation of dynorphin is dependent on prolonged nociceptor activity, with the selective destruction of unmyelinated nociceptive afferents by capsaicin reducing inflammatory-induced increases of dynorphin (Hylden *et al.*, 1992).

1.9 A Role for Prostaglandins in Central Pain Processing?

Arachidonic acid and its metabolites may also be important mediators in the central nervous system. In the rodent central nervous system the predominant eicosanoids are PGD_2, PGE_2, PGI_2 (Abdel-Halim, *et al.*, 1977; Abdel-Halim and Änggård, 1979; Chaplin and Hillier, 1979), LTB_4 and LTC_4 (Dembinska-Kiéc *et al.*, 1984; Lindgren *et al.*, 1984; Shimizu *et al.*, 1987) with regional differences in the expression of these eicosanoids.

Using Northern blotting and *in situ* hybridisation techniques a number of prostanoid receptors have been detected in the central nervous system. In mouse, the mRNA of the PGE_2 receptor EP_3 is widely distributed in neurones of the brain and sensory ganglia, but not in the spinal cord (Sugimoto *et al.*, 1992, 1994), whilst other EP receptor mRNA is less detectable in brain (Watabe *et al.*, 1993; Katsuyama *et al.*, 1995; Honda *et al.*, 1993). The mRNA encoding EP_2 receptor has however been detected in the superficial laminae of rat spinal cord (Kawamura *et al.*, 1997) and recent work has determined the presence of mRNA encoding EP_2, EP_4 and DP, but not EP_3, receptors in normal rat spinal cord (Lucy Donaldson, personal communication, 1997).

The eicosanoids have been shown to be highly involved in specific neural functions. For example, PGD_2 is present at high concentrations in the preoptic area, an area associated with sleep-regulation. Injection of PGD_2 into this region rapidly induced sleep which was indistinguishable from physiological sleep (Hayaishi, 1988; Hayaishi, 1991). The expression of PGD_2 and PGD-synthase shows a circadian rhythm in parallel with the sleep-wake cycle

(Hayaishi *et al.*, 1993; Pandey *et al.*, 1995). These observations implicate PGD_2 to be an important mediator in sleep-regulation. PGE_2 has a role in fever regulation, and is detected in increased amounts in both hypothalamic microvasculature and neurones following peripheral administration of endotoxin (Van Dam *et al.*, 1993). PGD-synthase has been located in the superficial laminae of the rat spinal cord (Vesin *et al*, 1995).

Increasing evidence suggests that eicosanoids may play an important role in the central processing of nociceptive information, particularly at a spinal level. Iontophoretically-applied prostaglandins have been shown to influence the activity of brain stem neurones. PGE_2 excited 27.5% of neurones whilst $PGF_{2\alpha}$ excited 26% and inhibited 10% of neurones (Avanzino *et al.*, 1966). Similarly, iontophoretically-applied PGE_2 and $PGF_{2\alpha}$ increased the firing rate of 74% of hypothalamic neurones and decreased activity in 9% (Poulain and Carette, 1974). These contrasting effects may arise due to multiple receptor activation, for example activation of EP_2 contributes to neuronal excitability whereas activation of EP_3 is inhibitory.

Prostaglandins modulate the activity of a number of receptors and associated ion channels. In section 1.4.5 the excitatory actions of PGE_2 were described. Prostaglandins have been shown to evoke the release of a variety of excitatory neurotransmitters from neuronal tissue. CGRP is released from the spinal cord *in vitro* in response to stimulation with a number of prostaglandins, PGD_2 and PGE_2 were most effective in facilitating release, whereas PGI_2 and $PGF_{2\alpha}$ were ineffective (Andreeva and Rang, 1993). Stimulation of spinal cord slices and cultured sensory neurones with 10μM PGE_2 and PGD_2 (but not $PGF_{2\alpha}$) evoked a 1.6-fold increase in SP release (Vasko *et al.*, 1993). Prostaglandins may further contribute to spinal hyperexcitability by inhibiting the release of inhibitory neurotransmitters involved in descending inhibitory pathways, such as noradrenaline (Taube *et al.*, 1977).

When prostaglandins are administered via a central route in conscious animals they evoke characteristic pain-behaviour. It has been demonstrated that spinal administration of PGE_2 (1 pg-10 ng, i.t.) induced behavioural hyperalgesia which lasted up to 30 minutes following injection (Uda *et al.*, 1990; Minami *et al.*, 1994). Similarly, behavioural hyperalgesia was induced by intrathecal administration of PGD_2 (Horiguchi *et al.*, 1986; Uda *et al.*, 1990; Minami *et al.*, 1994) and $PGF_{2\alpha}$ (Taiwo and Levine, 1986). Both PGE_2-binding sites and PGD-synthase have been located in the superficial laminae of the rat spinal cord

(Matsumura *et al.*, 1992; Urade *et al*, 1989; Vesin *et al*, 1995) indicating possible roles for endogenous PGE_2, $PGF_{2\alpha}$ and PGD_2 in spinal nociceptive processing. Various groups have tried to identify the EP receptors through which PGE_2 may induce hyperalgesia and EP_1, EP_2 and EP_3 receptors have all been implicated. Intrathecally-administered AH6809 (an EP_1 antagonist; ≥500ng; Uda *et al.*, 1990) inhibited PGE_2-induced hyperalgesia, whilst butaprost (an EP_2 agonist: 10ng, i.t.) and sulprostone (an EP_3 agonist: 100pg, i.t.) mimicked PGE_2-induced hyperalgesia (Nishihara *et al.*, 1995). High levels of centrally-applied prostaglandins (intracerebroventricularly/intracisternally) have been found to elicit hypoalgesia (Bhattacharya, 1986; Horiguchi *et al.*, 1986) again illustrating the often contradictory properties of the eicosanoids.

The hyperalgesia induced by spinally-applied prostaglandins parallels the pain-behaviour observed in animals following noxious peripheral stimulation, indicating a possible role for prostaglandins in the generation and/or maintenance of hyperalgesia. If prostaglandins modulate spinal nociceptive processing, increased levels of prostaglandins would be expected to be found in the spinal cord following peripheral activation of nociceptors.

In 1966, Ramwell *et al.* discovered that high-intensity electrical stimulation of a hindlimb could increase the release of prostaglandins from the frog spinal cord. This provided the first evidence that peripheral stimulation could provoke the spinal production of prostaglandins. Following this observation, a variety of peripheral stimuli have been identified which can evoke the release of prostaglandins from the spinal cord. Noxious thermal stimulation (50°C) of the hindpaw of an anaesthetised rat increased the level of PGE_2, but not $PGF_{2\alpha}$, or 6-keto-$PGF_{1\alpha}$, in lumbar spinal cord perfusate samples, whereas innocuous stimulation (35°C) had no effect (Coderre *et al.*, 1990). The development of an acute inflammation was also found to increase the synthesis and release of spinal prostaglandins. The injection of formalin into the rat hindpaw evoked a biphasic release of PGE_2 from the spinal cord which paralleled the behavioural pain-response in conscious rats (Malmberg and Yaksh, 1995). Similarly, a C/K-induced knee joint inflammation evoked the release of PGE_2 from the spinal cord of anaesthetised rats (Yang *et al.*, 1996; Sorkin and Moore, 1996). Capsaicin, a neuroexcitant which activates unmyelinated nociceptive afferents, was found to induce a dose-dependent increase in PGE_2 release from a spinal cord slice preparation (Malmberg and Yaksh, 1994). The increased synthesis and release of

prostaglandins from the spinal cord corresponds with activation of high-threshold nociceptors.

Neurones do not synthesise arachidonic acid from linoleic acid, but instead take up arachidonic acid produced by other cells, such as endothelial and glial cells (Moore *et al.*, 1991) and incorporate it in its esterified form in cell membranes. As in the periphery, arachidonic acid is liberated from its membrane-bound state by the actions of phospholipases. Several forms of PLA_2, including $cPLA_2$, have identified in nervous tissue (Bonventre *et al.*, 1993; Kim *et al.*, 1995; Stella *et al.*, 1995). In neurones, the liberation of arachidonic acid from its membrane-bound state is closely related to activation of excitatory amino acid receptors, particularly NMDA receptors. Activation of NMDA receptors in striatal neurones was found to stimulate the release of arachidonic acid (through receptor-mediated calcium influx and subsequent activation of a Ca^{2+}-dependent PLA_2), whereas activation of kainate and quisqualte receptors produced no effect (Dumuis *et al.*, 1988). Similarly activation of NMDA receptors in cerebellar granule cells caused a dose-dependent release of arachidonic acid, whereas activation of other glutamate-receptors had little effect (Lazarewicz *et al.*, 1988). Stimulated arachidonic release by NMDA receptor activation has also been observed in hippocampal slice preparations (Pellerin and Wolfe, 1991).

In addition to prostaglandins having neuronal effects it has also been established that the precursor molecule arachidonic acid is also active. Liberated arachidonic acid sustains the activity of the NMDA receptor in several ways. Firstly, arachidonic acid potentiates the current through the NMDA channel by increasing the channel-open probability, by either increasing channel opening rate or time, or recruiting previously inactive channels. This potentiation augments the increase in intracellular calcium concentration (Miller *et al.*, 1992). Secondly, arachidonic acid has been shown to increase the release of glutamate from hippocampal synaptosome preparations (Lynch and Voss, 1990), and also to inhibit glutamate uptake by glial cells (Barbour *et al.*, 1989). The increase in glutamate concentration and potentiation of the current through the NMDA channel maintains neuronal excitability and arachidonic acid release.

Both isoforms of COX are present constitutively in the rat central nervous system (Breder *et al.*, 1992, 1995; Kaufmann *et al.*, 1996), although COX-2 appears to be the predominant isoform (Feng *et al*, 1993; Seibert *et al*, 1994). The expression of COX-2 can be rapidly

increased in response to a variety of stimuli, such as electrically-induced seizure (Yamagata *et al.*, 1993; Chen *et al.*, 1995; Adams *et al.*, 1996), ischemia (Planas *et al.*, 1995) and stress (Yamagata *et al.*, 1993). The induction of COX-2 appears dependent on NMDA receptor activation. Several groups have demonstrated that the induction of COX-2 can be attenuated by pretreatment with NMDA receptor antagonists, e.g. MK-801 (Yamagata *et al.*, 1993; Chen *et al.*, 1995; Adams *et al.*, 1996) and by lamotrigine a voltage-sensitive sodium channel blocker, which prevents pre-synaptic glutamate release (Adams *et al.*, 1996).

Some actions of prostaglandins may also be mediated through NMDA receptors. NMDA receptor antagonists block intrathecal PGE_2-induced hyperalgesia indicating a relationship between EP receptors and NMDA receptors in the nociceptive action of PGE_2 (Minami *et al.*, 1994; Nishihara *et al.*, 1995; Minami *et al.*, 1997). Two subunits of the NMDA receptor seem particularly important for the development of PGE_2-induced hyperalgesia. Knockout mice which lacked ε1, and/or ε4 subunits of the NMDA receptor showed no signs of PGE_2-induced hyperalgesia (Minami *et al.*, 1997). However, PGD_2-induced hyperalgesia is unaffected by NMDA receptor antagonists or in ε subunit knockout mice (Minami *et al.*, 1997). PGD_2-induced hyperalgesia may occur through interaction with the NK-1 receptor, as pretreatment with the NK-1 receptor antagonist CP 96,345 blocked PGD_2-induced hyperalgesia (Uda *et al.*, 1990; Minami *et al.*, 1997). The spinal administration of both SP and NMDA produces a dose-dependent thermal hyperalgesia mediated through NMDA and NK-1 receptors. This hyperalgesia was blocked by intrathecal administration of NSAIDs, indicating a role for spinal prostaglandins in the maintenance of SP- and NMDA-induced hyperalgesia (Malmberg and Yaksh, 1992).

Discrepancies were found to exist between the concentration of NSAIDs required to produce analgesia and the doses required to inhibit COX activity (McCormack and Brune, 1991; McCormack and Urquhart, 1995). This observation is effectively illustrated by the actions of two enantiomers (R- and S+) of flurbiprofen. Both forms of flurbiprofen are equally effective analgesics, despite the (R-)-enantiomer being a much less potent COX inhibitor (Brune *et al.*,1991). The intravenous administration of both enantiomers of flurbiprofen reduced the responses of rat spinal cord neurones, rendered hyperexcitable by carrageenan/kaolin-induced acute inflammation, to innocuous and noxious mechanical stimuli. The intraarticular administration of (S+)-flurbiprofen also reduced the responses of

spinal cord neurones, whereas the (R-)-enantiomer had no effect (i.a.) (Neugebauer *et al.*, 1995). These results suggest that (R-)-flurbiprofen does not act at the peripheral site of inflammation, but may act through systemic redistribution at a central site of action. A component of NSAIDs analgesic effects may therefore be due to an action in the central nervous system.

Evidence supporting this hypothesis that the analgesic potency of NSAIDs may not be related to the peripheral actions on COX enzymes comes from experiments which have demonstrated antinociceptive effects of spinally administered NSAIDs. Intrathecal delivery of many non-selective NSAIDs dose-dependently reduced paw flinching in the formalin model of behavioural hyperalgesia (Malmberg and Yaksh; 1992b, 1993). These spinally-applied NSAIDs were effective at much lower doses than those required to produce analgesia when delivered systemically. Furthermore, electrophysiological studies have indicated that spinally applied NSAIDs reduce the responsiveness of spinal cord neurones to high-threshold electrical stimulation of peripheral nerves (Jurna *et al*, 1992; Willingale and Grubb, 1996; Willingale *et al.*, 1997) and peripheral inflammation (Chapman and Dickenson, 1992). These data suggest that NSAIDs have a spinal site of action which is separate from the peripheral actions of COX inhibition. Evidence suggests that NSAIDs may only be effective at producing analgesia in models of inflammation, as they are not effective at reducing responses to noxious stimuli in the normal animal (Yaksh, 1982; Malmberg and Yaksh, 1992; Chapman and Dickenson, 1992; Willingale *et al.*, 1997). These data are supported by the observation that the selective COX-2 inhibitor NS-398 reduces the behavioural response to formalin injection, but not acute noxious thermal stimulation (Yamamoto and Nozaki-Taguchi, 1996). Prostaglandins, possibly generated by COX-2, may therefore be important in modulating nociceptive processing following inflammation, but not following acute noxious stimulation in the normal animal.

1.10 Project Aims.

Evidence suggests prostaglandins may play an important role in spinal nociception under conditions of sustained nociceptor activation, such as inflammation. In order to ascertain whether prostaglandins are involved in the spinal processing of nociceptive information they must meet several criteria:

- COX-1 and/or -2 must be present in the spinal cord and in a location consistent with an involvement in spinal nociceptive processing.
- The COX pathway must be active.
- Increased levels of prostaglandins must be released from the spinal cord in response to sustained nociceptor activity.
- The time-course of COX/PG upregulation must coincide with the period of behavioural hyperalgesia observed following inflammation.

The aim of this current study is to explore the relationship between spinally-generated prostaglandins and peripheral stimulation, and investigate the possibility that prostaglandins are important neuromodulators or mediators in the spinal cord.

Chapter 2. Materials and Methods.

FIGURE 2.1.

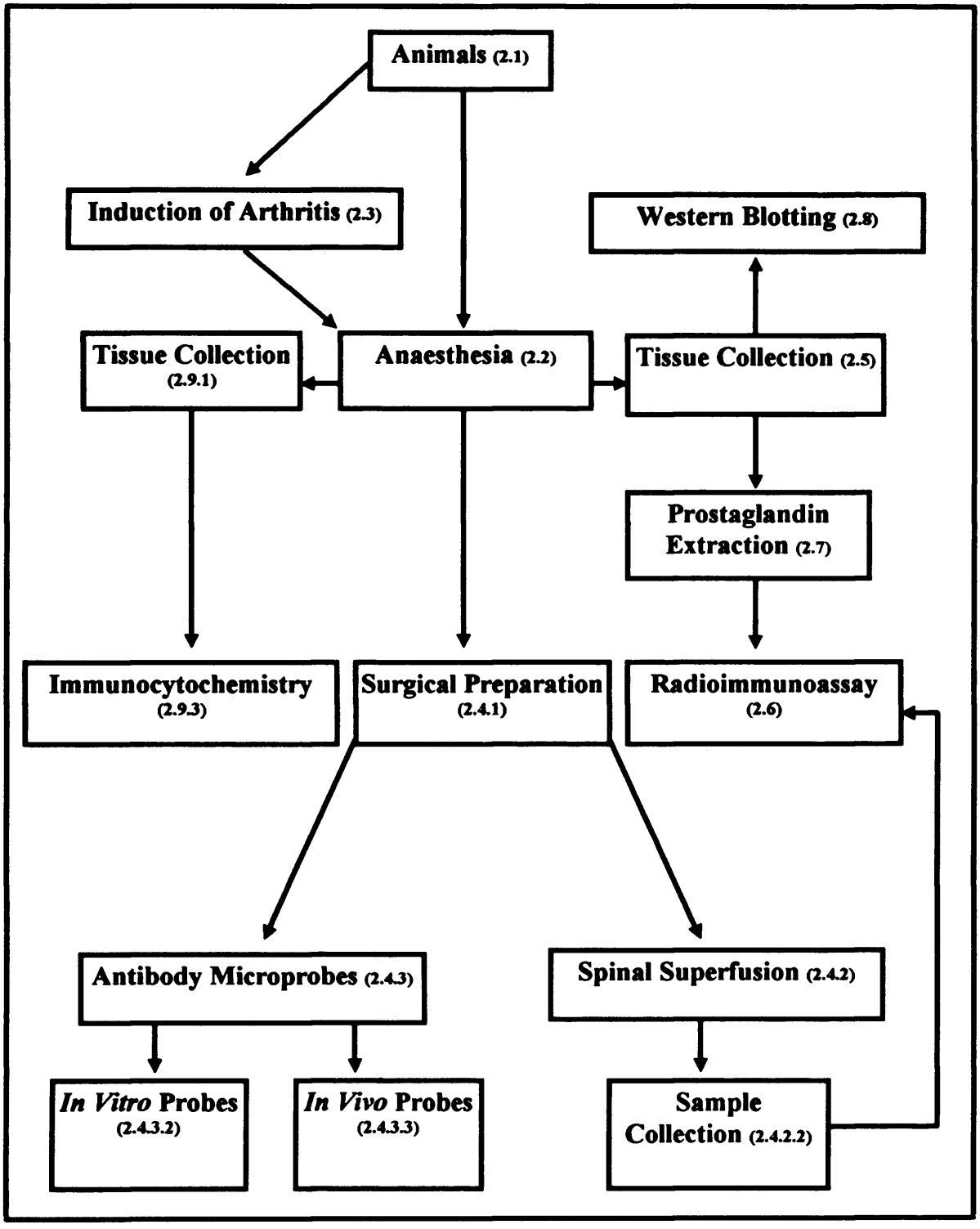


Figure 2.1. Methods. A schematic diagram illustrating the techniques used in this thesis. The numbers in parentheses refer to the relevant Chapter section.

This Chapter provides general information about the techniques used in this thesis (see Figure 2.1). Precise details pertaining to individual experiments (e.g. drug concentrations, antibody dilutions etc.) are described in the appropriate results chapters. Recipes for the solutions used in these experiments are given in Appendix 1.

2.1 Animals.

Male Wistar rats (200-600g) were used in all experiments. The animals were housed in a controlled environment (light: on 12hr, off 12hr; temperature: 18-20°C) and were allowed food and water *ad libitum*. All experimental procedures were conducted in accordance with 'The Animals (Scientific Procedures) Act, 1986.'

2.2 Anaesthesia.

Rats were anaesthetised with sodium thiopentone (120mg kg^{-1} i.p: Rhône Mérieux) or sodium thiobutabarbital (120mg kg^{-1} i.p: R.B.I.). Both barbiturates produced satisfactory surgical anaesthesia, although sodium thiobutabarbital was preferred due to its slower catabolism which resulted in a more stable level of anaesthesia. Additional intra-peritoneal injections were administered until corneal-blink and hindlimb-withdrawal reflexes were absent.

The depth of anaesthesia was carefully monitored throughout the experiment by testing: a) flexion withdrawal reflexes, which had to be absent; b) corneal blink reflexes, which had to be absent; and c) arterial blood pressure which was maintained between 100-120mmHg. Further anaesthetic was administered (i.p.) when necessary.

2.3 Induction of Arthritis.

Two models of joint inflammation were utilised in this thesis, a chronic model FCA-induced monoarthritis, described by Grubb *et al.* (1988), Iadorola *et al.* (1988) and Donaldson *et al.* (1993), and an acute model - C/K-induced arthritis described by Schaible and Schmidt (1985, 1988).

2.3.1 Freund's Complete Adjuvant (FCA)-Induced Monoarthritis.

Rats were briefly anaesthetised with 4% halothane until their hindlimb withdrawal reflex was absent. A total volume of 0.15ml FCA (*Mycobacterium Tubercolosis* 1mg/ml in paraffin; Sigma) was injected subcutaneously in 50µl volumes at 3 sites around the right tibio-tarsal joint to induce a unilateral inflammatory lesion. Animals were weighed daily and closely monitored for the development of primary and secondary inflammatory lesions. The circumferences of the injected and noninjected ankle joint were measured daily to determine the degree of swelling. Rats were also scored for signs of hyperalgesia and pain-behaviour (See Table 2.1). If any of the behavioural indices reached the maximum score, the animal was immediately culled. The monoarthritis was allowed to develop for a maximum of 14 days.

2.3.2 Carrageenan / Kaolin-Induced Arthritis.

An acute inflammation was induced by the intraarticular injection of 0.07ml 4% kaolin into the right knee joint of barbiturate anaesthetised rats (Sodium thiobutabarbital: 120mg kg^{-1}; i.p.). The knee joint was slowly flexed and extended for 15 minutes, then 0.07ml 2% carrageenan was injected into the knee joint. The joint was rhythmically manipulated for a further 30 minutes.

TABLE 2.1.

	BEHAVIOURAL SCORING SCHEME
A. Motility.	1 = Normal 2 = Responds when disturbed 3 = Very little activity.
B. Scratching.	1 = Normal 2 = Increased scratching 3 = Severe scratching, causing wounding.
C. Inflammatory signs (hind limbs and tail).	1 = Normal 2 = Some redness and swelling 3 = Severe swelling and wounds.
D. Inflammatory signs (snout and ears).	1 = Normal 2 = Some redness and swelling 3 = Severe swelling and wounds.
E. Stress on handling.	1 = Normal 2 = Increased heart rate/vocalisation/defecation 3 = Aggressive.
F. Gait.	1 = Normal 2 = Reduced weight bearing 3 = No weight bearing.

Table 2.1. Behavioural Scoring System for FCA-Monoarthritic Rats. Rats were scored daily for signs of hyperalgesia, stress and primary and secondary inflammatory lesions. If any score reached 3, the animal was culled.

2.4 Spinal Release of Prostaglandins.

2.4.1 Surgical Preparation.

These experiments were all conducted on barbiturate-anaesthetised rats as described in section 2.2. An incision was made through the skin overlying the ventral surface of the neck and was reflected back. The trachea was located and carefully dissected free from surrounding tissue. A horizontal cut was made between the cartilage rings below the larynx a cannula was inserted and tied in place. A jet of oxygen was directed at the cannula for the

duration of the experiment in order to aid blood oxygenation. A Portex cannula filled with heparinised saline (12.5 units heparin ml^{-1} 0.9% NaCl) was inserted in the left carotid artery. The cannula was attached via a three-way-tap to a pressure transducer (Druck Ltd) and blood pressure monitor (World Precision Instruments). A cannula filled with heparinised saline (1 unit heparin ml^{-1} 0.9% NaCl) was inserted into the left external jugular vein. Body temperature was monitored throughout the experiment by means of a rectal probe and maintained between 36°C and 38°C using a homeothermic blanket control unit (Harvard).

A longitudinal cut was made through the skin overlying the dorsal processes of the vertebrae between levels T11 to L3 and the vertebrae dissected free from surrounding tissue. The animal was transferred to a stereotaxic frame, supported by ear bars and swan-neck clamps placed under the lateral processes of the exposed vertebrae. The skin over the back was tied back with a series of sutures to form a pool, and exposed tissue covered with gauze dampened with 0.9% NaCl to prevent desiccation. In early experiments a minimal laminectomy was performed, but we found that blind insertion of subdural catheters often caused damage to blood vessels or the dura mater, making the efficient perfusion of the spinal cord impossible. A more extensive laminectomy was therefore found to be more appropriate. The dorsal surfaces of the vertebrae were carefully removed, exposing spinal cord segments T13 to L6. The exposed dura mater was covered with cotton wool dampened with 0.9% saline.

For experiments involving stimulation of the sural or tibial nerve, the right hindlimb of the rat was fixed and a cut made through the skin on the dorsal surface of the limb from the ankle to the knee. The skin was reflected and tied with a series of sutures to form a pool. The appropriate nerve was located under biceps femoris and carefully dissected free from surrounding tissue. The nerve was ligated and cut distally. The nerve was placed over silver wire stimulating electrodes. The pool was filled with warmed liquid paraffin to prevent tissue dehydration.

2.4.2 Spinal Cord Superfusion.

The highly diffusible nature of prostaglandins enables the release to be studied using spinal cord superfusion techniques. The spinal release of prostaglandins has previously been studied using this method by Coderre *et al.* (1990).

2.4.2.1 Perfusion System.

Polythene tubing (O.D 1.9mm; I.D 1.4mm; Portex) was flamed and gently stretched to form a fine filament 100-150μm (O.D.) in diameter. The tubing was inserted into Gilson Minipuls PVC tubing (I.D. 1.65mm) and glued in place. Once the glue had set, the internal surface of the catheters were coated with a siliconising reagent (Sigmacote, Sigma) and allowed to dry. Two catheters were attached to a Gilson Minipuls 3 peristaltic pump, and filled with warmed artificial cerebrospinal fluid (aCSF: NaCl 125mM, KCl 2.5mM, $NaHCO_3$ 26mM, NaH_2PO_4 1.25mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, gassed with 5% CO_2 / 95% O_2, pH 7.4). A small hole was cut cranially in the dura mater. The inflow catheter was sutured under the skin, before being inserted into the subdural space. The catheter was carefully advanced caudally, avoiding damage to blood vessels, until the tip was positioned over the lumbar enlargement. An additional piece of polythene tubing was inserted subdurally and placed between the central vein and the inflow catheter. The outflow catheter was then positioned over the polythene tubing, just caudal to the hole. The additional small piece of polythene tubing was found to restrict the movement of the central vein which can become labile during the course of the experiment. The perfusion system is summarised on Figure 2.2.

2.4.2.2 Sample Collection.

Warmed aCSF (37°C) was perfused over the surface of the spinal cord at a rate of approximately 225μl min^{-1}. Samples were collected from the outflow catheter at a rate of 200μl min^{-1} in 1ml aliquots in siliconised polypropylene tubes and placed on ice. Indomethacin (200μM) was added to each sample to prevent further prostaglandin synthesis. Any samples seen to contain blood elements were discarded. Samples were stored at -80°C until analysis by radioimmunoassay. The spinal cord was perfused for one hour prior to sample collection.

FIGURE 2.2.

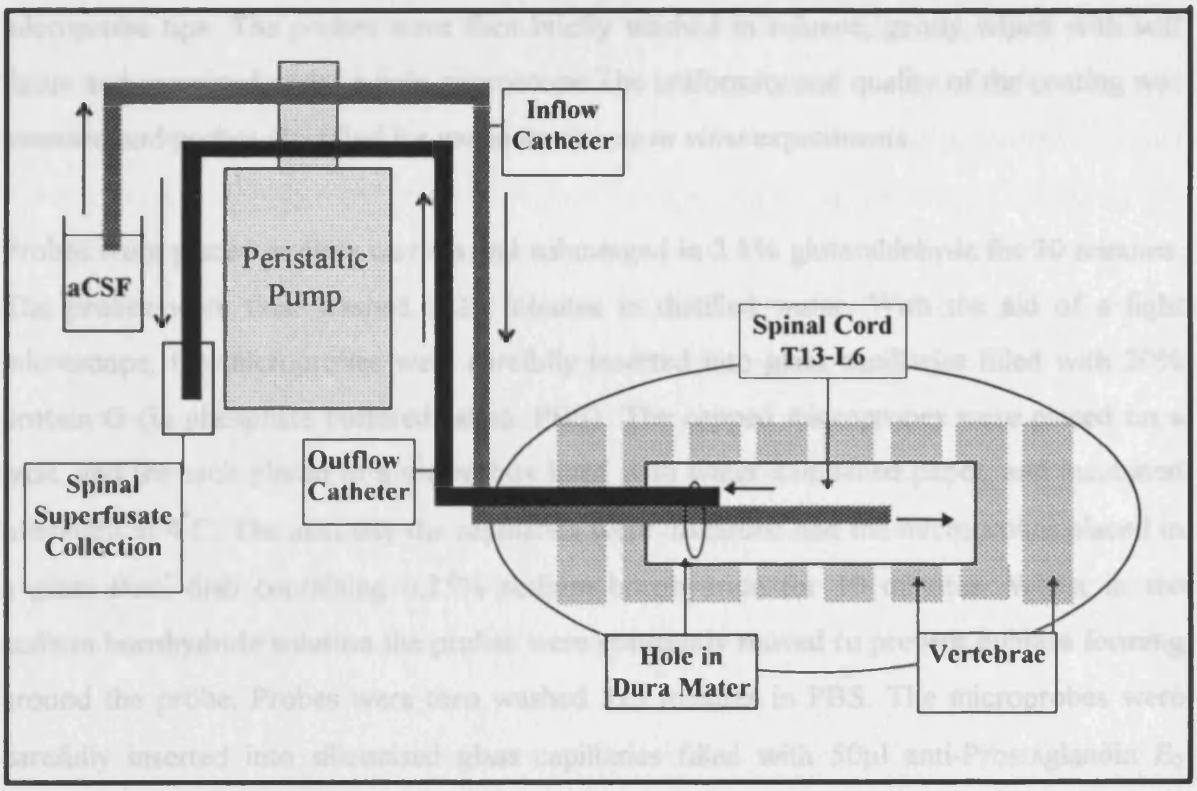


Figure 2.2. Spinal Cord Superfusion System. aCSF is perfused over the dorsal surface of the lumbar spinal cord at a rate of 225µlmin^{-1}. Superfusate samples are collected from the outflow catheter at a rate of 200µlmin^{-1} in 1ml aliquots into siliconised polypropylene tubes. Samples are placed on ice with 200µM indomethacin to prevent further prostaglandin synthesis.

2.4.3 Antibody Microprobes.

Antibody microprobes have been developed to study the spinal release of a number of peptides, such as SP (Duggan *et al.*, 1988, 1991). The generation of antibody microprobes against PGE_2 represents the first non-peptide compounds to be studied using this technique (Ebersberger *et al.*, 1997).

2.4.3.1 Preparation of Probes.

Fine gradually tapered microprobes were drawn from Pyrex glass capillaries by heating in a microelectrode puller. The probes were heat sealed at both ends, placed in a glass carrier and immersed in 50% nitric acid for 20 minutes. The probes were then washed 3 x 15 minutes in distilled water then dried overnight at 200°C. The following day, the glass carrier

containing the probes was placed in a solution of toluene:silane (67.5ml toluene:15ml silane) for 30 minutes, then centrifuged at 2000 r.p.m. for 60 minutes to remove liquid from the microprobe tips. The probes were then briefly washed in toluene, gently wiped with soft tissue and examined under a light microscope The uniformity and quality of the coating was assessed and probes classified for use in *in vivo* or *in vitro* experiments.

Probes were placed in glass carriers and submerged in 2.5% glutaraldehyde for 30 minutes. The probes were then washed 3x10 minutes in distilled water. With the aid of a light microscope, the microprobes were carefully inserted into glass capillaries filled with 20% protein G (in phosphate buffered saline: PBS). The capped microprobes were placed on a rack, and the rack placed in a sealed box lined with water-dampened paper, and incubated overnight at 4°C. The next day the capillaries were discarded and the microprobes placed in a glass Petri dish containing 0.25% sodium borohydride for 10 minutes. Whilst in the sodium borohydride solution the probes were constantly moved to prevent bubbles forming around the probe. Probes were then washed 3x5 minutes in PBS. The microprobes were carefully inserted into siliconised glass capillaries filled with 50µl anti-Prostaglandin E_2 (Sigma) and incubated, as before, overnight at 4°C.

2.4.3.2 *In Vitro* Probes.

The antibody-filled capillaries were removed and discarded from the microprobes, which were then washed for 5 minutes in PBS. Twenty-four siliconised capillaries were filled with either PBS or varying concentrations of PGE_2 (1.0×10^{-6} /1.0×10^{-7} M). The antibody-coated microprobes were carefully inserted into the capillaries and incubated at 37°C for 30 minutes. The probes were then washed in ice-cold, stirred PBS-Tween for 15 minutes, then inserted into siliconised capillaries containing 6000cpm iodinated-PGE_2 and incubated overnight at 4°C. The following day, probes were washed for 15 minutes in ice-cold, stirred PBS-Tween. The tips of the probes were then broken off, glued to pieces of paper and placed in counter tubes. The radioactivity was counted for 1 minute per tube. The inhibition of radioligand binding by unlabelled PGE_2 was then calculated.

2.4.3.3 *In Vivo* Probes.

Microprobes were removed from the antibody-filled capillaries, numbered and placed in PBS. Barbiturate-anaesthetised rats were surgically prepared as previously described (section 2.4.1). The dura mater was carefully removed using sterile surgical instruments and the spinal cord surface covered in 4% agar. The agar overlying the lumbar region of the laminectomy was removed and the exposed site perfused with warmed sterile Ringer's solution at a rate of 2ml min^{-1}. This irrigation system prevented the build-up of fibrin and inflammatory mediators which could damage the microprobe coating. Using a carbon filament extracellular micro-electrode, areas of the spinal cord receiving input from the knee joint were identified. Pairs of antibody microprobes were inserted into appropriate regions of the spinal cord to a depth of 2.5mm using a micromanipulator. The probes were present in the spinal cord for 20 minutes. Probes were washed in ice-cold PBS-Tween for 15 minutes, then incubated overnight at 4°C in radiolabelled PGE_2.

The following day the microprobes were washed in ice-cold PBS-Tween for 15 minutes. The tips were attached to a sheet of paper, which was placed in an X-ray film cassette and exposed to a sheet of X-ray film initially for 14 days. The films were developed and the images of the probes densitometrically analysed. Areas of reduced radiolabelled PGE_2 binding indicating the site of release of endogenous PGE_2.

2.4.4 Stimulation Protocols.

The existence of stimulus-dependent release of spinal prostaglandins was investigated using a range of mechanical and electrical stimuli. Stimulation periods typically lasted for 20 minutes. Electrical stimulation was applied to the sural or tibial nerve via silver wire electrodes in a square-wave pulse of 500μs duration using a programmable stimulator (Hi-Med). The frequency of stimulation ranged from 0.1-1Hz and intensity ranged from 0.2-15V. Innocuous and noxious mechanical stimulation were supplied by a pair of pneumatic callipers which applied reproducible and graded pressure stimuli to the ankle joint. Innocuous pressure was judged to be 25psi and noxious pressure 50psi, and these pressures were applied to the ankle joint for 10 seconds followed by 10 seconds with no stimulation. Other mechanical stimulation involved stroking the ankle joint with a soft-bristled brush at a frequency of 0.5Hz.

2.5 Tissue Collection.

Spinal cord samples for Western blotting or prostaglandin extraction procedures were obtained from barbiturate-killed rats (sodium thiopentone: 400mg kg^{-1}; i.p.) which were transcardially perfused with warmed 0.9% NaCl at a pressure of 100mmHg until all blood was removed. Lumbar spinal cord sections, L1 to S1 were rapidly exposed, excised and frozen in liquid nitrogen. Samples were stored at -80°C until extraction.

Plasma samples for prostaglandin extraction procedures were obtained from barbiturate-anaesthetised rats (section 2.2). Rats received an intravenous injection of heparin (0.15ml; 750 units heparin). After several minutes, blood was withdrawn from the arterial catheter using a siliconised, heparinised syringe and transferred to a siliconised vial. Indomethacin (200μM) was added to the blood sample to prevent further prostaglandin synthesis. The blood sample was immediately centrifuged at 3500 rpm for 10 minutes at 4°C, and the plasma supernatant removed and stored at -80°C until analysis.

2.6 Radioimmunoassay.

Prostanoids contained in plasma are known to freely associate with the carrier protein albumin (Gold and Edgar, 1978). Prostanoids are therefore traditionally processed using either solvent extraction procedures (Dray *et al.*, 1975; Morris *et al.*, 1981) or separation columns (Kelly *et al.*, 1989) prior to radioimmunoassay. However, Granström and Kindahl, (1982) have demonstrated that the presence of albumin in the assay system may actually protect prostaglandins from degradation, and does not interfere with antibody-antigen binding. Furthermore, impurities introduced into the assay during solvent extraction have been shown to reduce binding affinity (Morris *et al.*, 1981).

In early experiments we showed that the purification of spinal superfusate and plasma samples with Amprep C18 non-polar Minicolumns (Amersham) was not necessary. Unextracted samples were found to contain equal or greater amount of prostaglandins than corresponding extracted samples. This purification step was consequently omitted from the radioimmunoassay of spinal superfusate and plasma samples, although was used in the extraction of prostaglandins from spinal cord (see section 2.7).

2.6.1 Assay Protocol.

Radioimmunoassays for PGE_2, PGD_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ were adapted from an assay system developed by Granström and Kindahl, (1982). All plastic ware used throughout the assay procedure was coated with a siliconising agent, Sigmacote (Sigma).

A 100µl aliquot of either unknown sample, or prostaglandin standard (dilutions from 3pg/0.1ml to 2ng/0.1ml) was mixed with 100µl prostaglandin antibody (at a dilution which bound 50% of the tracer in the absence of unlabelled prostaglandin) in the presence of (0.15%) bovine-γ-globulin. All samples were assayed in duplicate. The tubes were incubated on ice for 30 minutes, then 100µl radiolabelled prostaglandin (containing 5000 dpm) was added to each tube. The tubes were vortexed and incubated overnight at 4°C. Antibody bound prostaglandins were separated by the addition of ice-cold polyethylene-8000 (final concentration 12.5% w/v) to each tube (Desbuquois and Aurbach, 1971). The tubes were vortexed for a total of 30 seconds, then centrifuged for 1 hour at 3,500 rpm at 4°C. The antibody bound prostaglandins were pelleted by the polyethylene glycol. When tritiated prostaglandins were used 1ml of the supernatant was removed, mixed with 1ml water and 4ml Ultima-Gold scintillation fluid (Packard) and counted for 3 minutes per tube. When iodinated prostaglandins were used, the supernatant was discarded and the pellet was counted for 1 minute per tube.

2.6.2 Calculation of Results.

The concentration of prostaglandins in the samples was calculated from a standard curve. The average counts (^{125}I) or disintegrations (^{3}H) per minute for each set of duplicates was calculated. When iodinated tracer was used, the net counts of each sample were determined by subtracting the non-specific binding cpm. The percent bound for each sample and standard was then determined as follows:

$$\%\text{Bound/Unbound} = \frac{\text{Net cpm/dpm of Standard or Sample}}{\text{Net cpm/dpm of total counts}} \times 100$$

Each assay system was optimised to give a maximal binding of 50% Bound/Unbound, to give the best balance between sensitivity and accuracy. A standard curve was generated

using Graphpad Prizm software, and the minimum assay sensitivity determined by calculating the amount of unlabeled prostaglandin required to shift zero binding by two standard deviations. The prostaglandin concentration in each sample were calculated from the graph. Superfusate samples were standardised to pg/ml/minute, plasma samples to pg/ml and spinal cord samples to pg/g wet weight.

2.7 Extraction of Prostaglandins from Spinal Cord.

In order to measure prostaglandin concentration in spinal tissue, it was necessary to extract prostaglandins using Amprep C18 non-polar Minicolumns (Amersham). The prostaglandins bind to the sorbent matrix in the column and are eluted by ethyl acetate. Lumbar spinal cord samples were weighed, finely chopped on dry-ice, then homogenised in ice-cold PBS, pH 7.4, (0.1g spinal cord /ml PBS) containing 200μM indomethacin. An aliquot of spinal cord homogenate was mixed with an equal amount of 75% ethanol and 10mM glacial acetic acid, incubated at room temperature for 5 minutes, then centrifuged at 3500 rpm for 5 minutes. A C18 Amprep minicolumn was primed with 2ml 10% ethanol then the sample supernatant applied to the column. The column was washed with 1ml water, followed by 1 ml hexane. The PGE_2 was then eluted from the column with 1.5ml ethyl acetate. The ethyl acetate fractions were collected in siliconised microfuge tubes, and evaporated under nitrogen. Once the ethyl-acetate had completely evaporated, the samples were rehydrated with Tris-HCl buffer, pH7.8, and stored at -80°C until analysis by radioimmunoassay.

2.8 Western Blotting.

2.8.1 Sample Preparation.

Frozen lumbar spinal cord sections were finely chopped on dry ice, transferred to ice-cold lysis buffer (NaCl 150mM, Tris pH 7.4 10mM, EDTA 1mM, EGTA 1mM, Triton-X-100 1%, PMSF 0.2mM, aprotinin 20μg ml^{-1}) and homogenised on ice. The homogenates were centrifuged at 20,000 rpm at 4°C for 10 minutes and the supernatant collected. The protein concentration in the supernatant was determined using the method of Lowry *et al.* (1952). The volume of each sample was corrected to ensure the protein concentration in each sample was approximately equal. Samples were mixed 1:1 with 2x denaturing sample buffer (see Appendix 1), and boiled for 5 minutes (Laemmli, 1970)

2.8.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE was performed using the Mini-Protean II electrophoresis system (Bio-Rad) and 0.75mm acrylamide gels. Glass plates were cleaned with ethanol and assembled using the gel casting apparatus. The resolving gel (acrylamide 10%, Glycine, 213mM, Tris base 27.5mM, SDS 3.9mM) was carefully poured between the plates, avoiding air bubbles, to a height of 5cm. The gels were immediately overlaid with 0.1% SDS (to exclude oxygen from the gel surface). Gels were allowed to polymerise for 20-30 minutes. Once the gels were set, the plates were inverted to remove the SDS and the gel surface was rinsed with distilled water. Excess water was blotted from the surface of the gel with filter paper. The stacking gel (4% acrylamide) was then poured on top of the resolving gel and the 10-well comb carefully inserted. The stacking gel was allowed to polymerise for 30-45 minutes, then the comb was removed and the wells washed with distilled water. The plates were clamped into place in the reservoir frame and positioned in the electrophoresis tank. The inner and outer reservoirs were filled with running buffer (Glycine 213mM, Tris base 27.5mM, SDS 3.9mM). Samples and pre-stained protein standards were warmed to 40°C for 5 minutes then centrifuged at 12,000 rpm for 5 minutes. Using flexible gel-loading tips the standards and samples were loaded into appropriate wells. The gels were run at 175-200V for 0.75-1.5 hours. Whilst the gel was running the level of buffer in the inner chamber was checked to ensure it covered the wells.

2.8.3 Gel Transfer Procedure.

Once SDS-PAGE was complete the stacking gel was separated from the resolving gel and discarded. The upper left corner of the resolving gel was marked to aid gel orientation. The gels were soaked in transfer buffer (Tris Base 48mM, Glycine 39mM, SDS 1.3mM, Methanol 20%) for 10-20 minutes. Four pieces of blotting paper and 1 piece of nitrocellulose paper (Schleicher and Schuell) were also soaked in transfer buffer for a similar time. Separated proteins were transferred from the gels to the nitrocellulose membrane using a semi-dry electrophoretic transfer cell (Bio-Rad). Two sheets of blotting paper were placed on the transfer apparatus followed by the nitrocellulose membrane. The gels were carefully positioned over the nitrocellulose, ensuring they were correctly orientated. Two more layers of blotting paper were carefully overlaid. It was important to ensure that no air bubbles were trapped between any layers. The proteins were then

transferred at 15 Volts for 30 minutes. After 30 minutes the two top layers of blotting paper were removed and the gel carefully peeled back to check the pre-stained markers have been transferred. The outline of the gel was cut on the nitrocellulose and the gel then discarded. The efficiency of the transfer procedure was checked by submerging the membrane in 1% Ponceau's reagent to visualise the protein bands.

2.8.4 Western Blotting Procedure.

The nitrocellulose membranes were incubated for at least 2 hours in TTBS (Tris Base 50mM, NaCl 150mM, Tween-20 0.1%, pH 8) containing 10% non-fat milk powder. The membranes were washed 3x5 minutes in TTBS before being transferred to primary antibody (diluted in TTBS containing 10% milk powder and 0.01% sodium azide) and incubated overnight. The following day the blots were washed in TTBS (3x5 minutes), then incubated in peroxidase-conjugated secondary antibody for 1.5 hours. The blots were then rewashed in TTBS (5x10 minutes). All the above incubations and washes were conducted at room temperature on a plate shaker.

The protein bands were visualised using an Enhanced Chemi-Luminescence (ECL)-detection kit (Amersham) according to the manufacturers instructions. The blots were exposed to blue-sensitive X-Ray film for appropriate periods of time and the films developed using an Amersham Hyperdeveloper.

2.8.5 Densitometric Analysis.

The films were scanned using a GS-70 scanning densitometer. Optical densities (OD) of the protein bands were determined using Molecular Analyst software (Bio-Rad). Briefly, equally-sized rectangles were carefully positioned over the image of protein bands and the mean OD calculated. An area of the film (not containing the bands) was also selected to act as a background control, and the mean OD of the background was automatically subtracted from the mean band OD.

2.9 Immunocytochemistry.

2.9.1 Tissue Preparation.

Rats were killed by barbiturate overdose (sodium thiopentone: 400mg kg^{-1} i.p.) and transcardially perfused with warmed 0.9% NaCl at a pressure of 100mmHg until all blood was removed. Lumbar spinal cord segments, L1 to S1, were carefully exposed and excised. The spinal cord section was embedded in Tissue-Tek (Miles Inc, Elkhart, IN) and frozen over dry ice/hexane (-65°C) and stored at -20°C.

2.9.2 Slide Preparation.

Glass slides were coated with a subbing solution (2% 3-aminopropyltriethoxysilane in acetone) to aid tissue section adhesion to the slide. Slides were soaked overnight in 5% Decon 90 (Decon Laboratories Ltd.), then washed for 30 minutes in hot running water. Slides were washed 5x5 minutes in distilled water then dried. Slides were then soaked in 2% 3-aminopropyltriethoxysilane for 1 minute. This was followed by 2x1 minute washes, in acetone and 2x1 minute washes in distilled water. Once dried, slides were ready to use.

2.9.3 Tissue Sectioning and Fixation.

Frozen sections (15μm) were cut from the rostral end of the tissue blocks using a freezing microtome (-18°C). Sections were mounted caudal end up onto pre-subbed slides and allowed to air dry. The tissue sections were then fixed in 2% paraformaldehyde (in Na_2HPO_4 80mM, NaH_2PO_4 20mM) for 10 minutes at room temperature, then washed in PBS pH 7.4 for 15 minutes.

2.9.4 Immunocytochemistry Protocol.

Sections were incubated in PBS containing 10% goat serum and 0.5% Triton-X-100 for 30 minutes at room temperature, then washed in PBS for 15 minutes. The sections were then incubated overnight at 4°C in primary antibody (diluted in PBS containing 10% goat serum). The next day, sections were washed 5x12 minutes in PBS before being incubated in fluorescein-conjugated secondary antibody for 2 hours at room temperature. Sections were

rewashed in PBS for 15 minutes. Propidium iodide (0.1% in PBS) was used as a nuclear counterstain in some sections. All incubations took place in a sealed box lined with dampened paper to maintain humidity. Excess fluid was removed from the slides prior to mounting the sections in Citifluor (U.K.C., Chem. Lab.), a mountant with low self-fluorescence. Sections were examined using epifluorescence or laser scanning confocal microscope.

2.10 Antisera and other Materials.

Primary antibodies: COX-1 (160110) and COX-2 (160106) antisera were purchased from Cayman Chemical. Manufacturer's instructions indicated that the monoclonal mouse anti-sheep COX-1 antibody bound sheep, rat, mouse and human COX-1 and showed little or no cross-reactivity with COX-2. The polyclonal rabbit anti-mouse COX-2 antibody bound sheep, rat, mouse, guinea pig and human COX-2 and showed little or no cross-reactivity with COX-1. The rabbit polyclonal 5-LO antisera (Merck Frosst) was raised against human leukocyte antigen. The mouse monoclonal $cPLA_2$ antisera was purchased from Santa Cruz (SV454). The PGI_2 synthase (PG23) and PGD_2 (PG30) antisera were purchased from Oxford Biomedical Research. Rabbit polyclonal anti-PGE_2 (P5164), anti-6-keto-$PGF_{1\alpha}$ (P1791), and anti-$PGF_{2\alpha}$ (P5539) were purchased from Sigma and specific for the target prostaglandin (with the exception of anti-PGE_2, which did not discriminate between PGE_1 and PGE_2).

Secondary antibodies: Fluorescein conjugated anti-rabbit (F9887), peroxidase-conjugated anti-mouse (A5906) and anti-rabbit (A6154) were purchased from Sigma.

All solutions described in this thesis were prepared using milliQ, deionised distilled water. Standard laboratory chemicals were purchased from Fisher or Sigma, unless stated otherwise.

Chapter 3. The Development of Freund's Complete Adjuvant (FCA)-Induced Monoarthritis: Characterisation of the Model.

3.1 Introduction.

FCA is a suspension of heat-killed *Mycobacterium tuberculosis* or *butyricum* in paraffin oil. In 1959 Pearson and Wood demonstrated that the intradermal administration of *Mycobacteria* to rat induced the development of an inflammatory disease which strongly resembled human rheumatoid arthritis.

The disease was characterised by areas of redness and swelling which appeared around distal joints approximately two weeks following inoculation. These inflammatory lesions appeared firstly around the hind paws then spread to the forepaws and tail. Non-articular lesions in the eyes, ears, skin and genitals developed in parallel with the articular lesions. The intensity of the inflammatory response was most marked between days 18 and 25 following inoculation, the ankle joints of the most severely affected animals were swollen 2-3 times their normal diameter. Histological examination of inflamed limbs revealed swollen hyperaemic tissue surrounding a joint distended by increased synovial effusion and proliferation of osteoblasts. The inflamed tissue was invaded by proinflammatory cells. During the acute stage of the disease the animals suffered profound weight loss and illness. The inflammatory response abated after approximately two months although lesions often spontaneously recurred. Animals retained lasting evidence of the disease, ankylosis restricted the normal movement of joints, the most severely affected animals suffered joints permanently fixed in an extended position.

The delayed onset of the disease, the accelerated inflammatory response to reinnoculation, the inability to induce the disease in juvenile rats, and the inhibition of the disease process by irradiation were factors which indicated the importance of the immune system in the development and maintenance of the disease. Pearson and Wood (1959) concluded that adjuvant-induced polyarthritis occurred due to an allergic response to *Mycobacteria* and commented on the similarity between the pathology of FCA-polyarthritis and human rheumatoid arthritis.

Adjuvant-induced inflammation and human rheumatoid arthritis have been found to share a number of pathological features. The main differences between these two diseases are the presence of rheumatoid factor in human rheumatoid arthritis and the presence of genital and skin lesions in FCA-polyarthritis. The two diseases progress at different rates and, unlike rheumatoid arthritis, adjuvant arthritis often resolves over time (Pearson, 1963; Nusbickel and Troyer, 1976; Rosenthale and Capetola, 1982; Rainsford, 1982; Pelegri *et al.*, 1995).

The supposition of Pearson and Wood (1959) that the adjuvant-induced disease was due to an immune response has proved to be well-founded. Whitehouse *et al.* (1969) demonstrated that the disease could be transmitted to healthy rats through lymphocytes obtained from polyarthritic rats. In 1985, a T-cell clone was identified in polyarthritic rats which responded not only to *Mycobacterium tuberculosis* but also to an antigen present in human synovial fluid, chondrocyte culture medium, and cartilage proteoglycan (Van Eden *et al.*, 1985). The mycobacterial epitope recognised by these T-cell clones is a 65kD heat-shock protein present on the bacterial coat (Van Eden *et al.*, 1988, 1989), although the cartilage antigen has not yet been identified. FCA-inflammation appears, therefore, to be an autoimmune response.

Polyarthritic rats exhibit features indicative of stress and chronic pain such as decreased motility, hypersensitivity of the inflamed tissue to stimulation, increased vocalisation and scratching, and reduced weight gain (Colpaert, 1987; De Castro-Costa *et al.*, 1987). Until the early 1990's this model was widely used to study physiological, biochemical and anatomical changes associated with chronic inflammation and pain. Inflammatory-induced changes in the physiology of the central and peripheral nervous systems have been studied using FCA-polyarthritis (for example, Weihe *et al.*, 1988; Guilbaud, 1991; Abbadie and Besson, 1992). It was difficult to attribute neuronal changes directly to the inflammatory response, since the disease process was so widespread. Modifications of the model were developed in which inflammation could be restricted to one hindpaw or ankle joint (Grubb *et al.*, 1988; Iadarola *et al.*, 1988; Stein *et al.*, 1988; Butler *et al.*, 1992; Donaldson *et al.*, 1993). The subdermal injection of a reduced dose of FCA around the tibio-tarsal joint or into the footpad induced an inflammation which could be localised to the injected joint, which was stable for up to six weeks (Butler *et al*, 1992). The severity of the inflammatory response could be manipulated by altering the dose of FCA. Low doses (<200μg) *Mycobacterium tuberculosis* induced a discrete monoarthritis of the injected joint, whereas

higher doses (>200μg) induced contralateral inflammatory lesions and development of mild secondary lesions (Donaldson *et al.*, 1993). Joint inflammation was associated with arthritic features such as cellular infiltration, synovial proliferation and destruction of cartilage tissue (Donaldson *et al.*, 1993).

FCA-monoarthritis has proved to be a more ethically acceptable model of arthritis, reducing the discomfort of animals and preventing the spread of secondary lesions. It has also provided the means for experimenters to directly link changes in peripheral and central neuronal function with increased afferent input from the inflamed joint.

FCA-monoarthritis has been shown to induce gene transcription and/or protein translation of a number of sensory neuropeptides in DRG (Weihe *et al.*, 1988; Donaldson *et al.*, 1992; Hanesch *et al.*, 1993) and spinal cord (Weihe *et al.*, 1988; Ohno *et al.*, 1990). The use of this model is, therefore, appropriate for the study of inflammation-induced changes in the arachidonic pathway in the spinal cord. The features of FCA-monoarthritis are discussed in this chapter.

3.2 Methods.

3.2.1 Induction of Freund's Complete Adjuvant-Induced Monoarthritis.

Rats were briefly anaesthetised with 4% halothane until their hindlimb withdrawal reflex was absent. A total volume of 0.15ml FCA or paraffin oil was injected subcutaneously in 50μl volumes at 3 sites around the right tibio-tarsal joint. Animals were weighed and monitored daily as described in section 2.3.1. Rats were scored for signs of hyperalgesia and pain-related behaviours (see Table 2.1). The monoarthritis was allowed to develop for a maximum of 14 days.

Statistical analysis of joint circumferences and behavioural indices was by ANOVA followed by Duncan's multiple range post-hoc test or by students t-test. Values are expressed as mean ± SEM.

3.3 Results

3.3.1 Development of a Monoarthritic Lesion.

The subcutaneous injection of 0.15ml FCA induced the development of a unilateral inflammation around the injected joint. Following inoculation there was a marked swelling around the injected ankle joint. Within six hours following inoculation the joint had significantly increased in circumference by 38% from 1.9 ± 0.04cm to 2.63 ± 0.08cm. The swelling was maximal 1 day following injection when there was a 67% increase in joint circumference to 3.18 ± 0.24cm. The swelling abated slightly by day 3, but the joint remained significantly swollen until day 14. The increases in the circumference of the ipsilateral tibio-tarsal joint were significant ($p<0.01$) at all time points studied over the fourteen day timecourse (Figure 3.1). There were no associated increases in the circumference of the noninjected contralateral joint at any point over the fourteen day timecourse.

The group of rats injected with 0.15ml paraffin vehicle showed a small non-significant 9% increase ($p>0.05$) in joint circumference compared to the circumference of the joint before injection (Figure 3.2).

FIGURE 3.1.

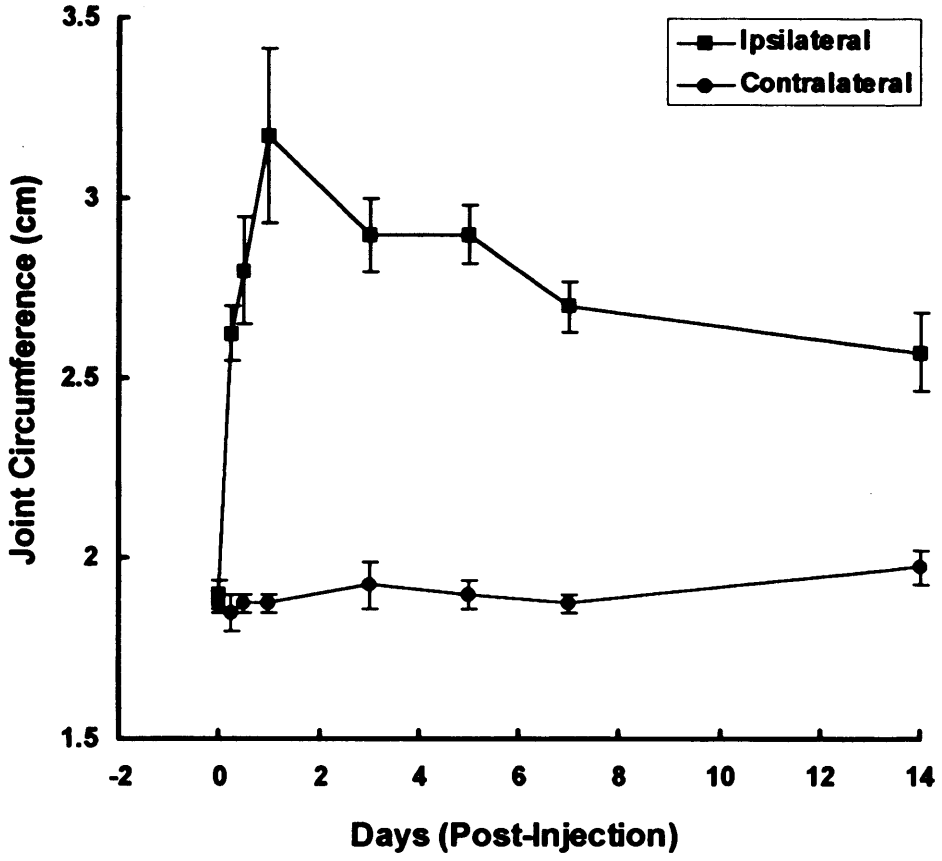


Figure 3.1 The Development of FCA-Monoarthritis. This figure shows the mean joint circumference of ipsilateral and contralateral tibio-tarsal joint circumference during the development of FCA-monoarthritis in rats. Data represent the mean circumference (cm) ± SEM obtained from 4 animals at each time point. The increases in the ipsilateral joint circumference are significant ($p<0.01$, ANOVA) at all time points over the 14 day time-course, when compared to pre-injection circumference. At no point was there a significant increase in the circumference of the contralateral joint.

FIGURE 3.2.

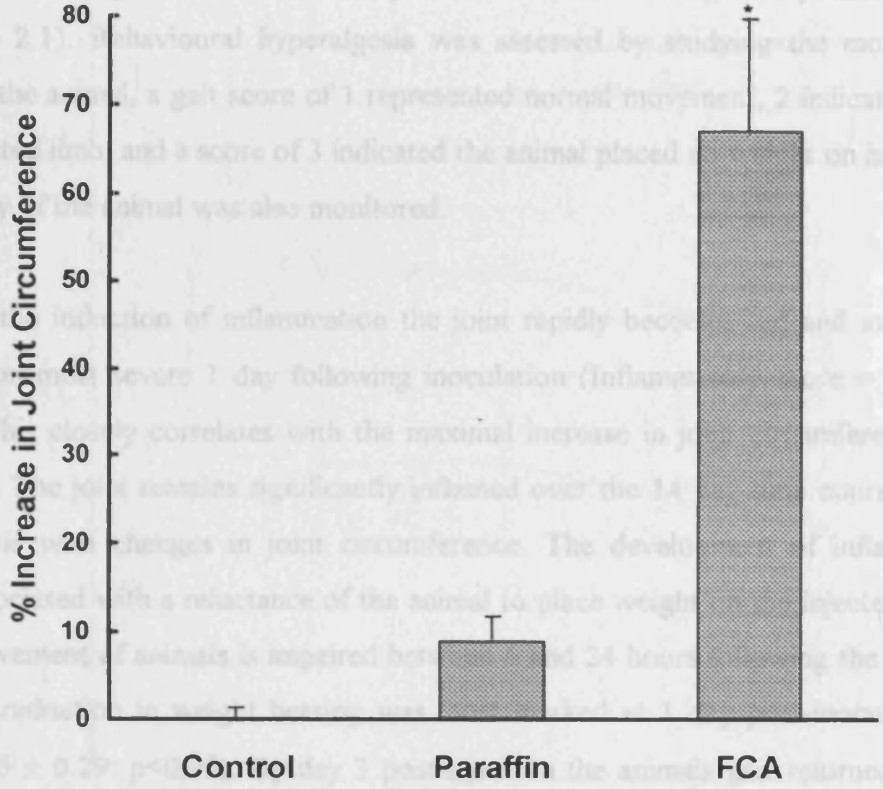


Figure 3.2 The Development of Inflammation Requires *Mycobacterium tuberculosis*. This figure compares the mean % increase in ipsilateral joint circumference of rats injected with either 0.15ml FCA or paraffin oil with the noninjected contralateral joint circumference (Control), 1 day post-injection. There is a significant increase (*$p<0.05$, t-test) in the circumference of the joint injected with FCA, but not in the joint injected with parraffin vehicle ($p>0.05$). Data represent the mean % increase in joint circumference ± SEM obtained from 4 animals at each time point.

3.3.2 Inflammation is Associated with Behavioural Hyperalgesia.

Inflammation of the injected joint was not only monitored by an increase in joint circumference but also using a scoring scheme in which inflammation was graded. An inflammation score of 1 represented no inflammation, 2 indicated the presence of some redness and swelling, and a score of 3 represented severe swelling with possible wounding (see Table 2.1). Behavioural hyperalgesia was assessed by studying the movement and activity of the animal, a gait score of 1 represented normal movement, 2 indicated guarding of the injected limb, and a score of 3 indicated the animal placed no weight on injected limb. The activity of the animal was also monitored.

Following the induction of inflammation the joint rapidly becomes red and swollen, with inflammation most severe 1 day following inoculation (Inflammatory score = 2.5 ± 0.29; $p<0.05$). This closely correlates with the maximal increase in joint circumference seen in Figure 3.1. The joint remains significantly inflamed over the 14 day time course ($p<0.05$), again in line with changes in joint circumference. The development of inflammation is closely associated with a reluctance of the animal to place weight on the injected limb. The normal movement of animals is impaired between 6 and 24 hours following the injection of FCA. The reduction in weight bearing was most marked at 1 day post-inoculation (Gait Score = 2.5 ± 0.29; $p<0.05$). By day 3 post-injection the animals gait returned to normal (Gait Score = 1.25 ± 0.25; $p>0.05$) despite the continuing inflammation of the joint (Figure 3.3). It was also observed that placing the measuring device around the ankle joint caused immediate flinching of the injected limb following inoculation, another indication of pain-related behaviour. Despite the development of this behavioural hyperalgesia, there was no significant reduction in the activity of the animals at any time point following injection of FCA (e.g. 1 day post-injection: Motility Score = 1.25 ± 0.25; $p>0.05$; data not shown).

FIGURE 3.3.

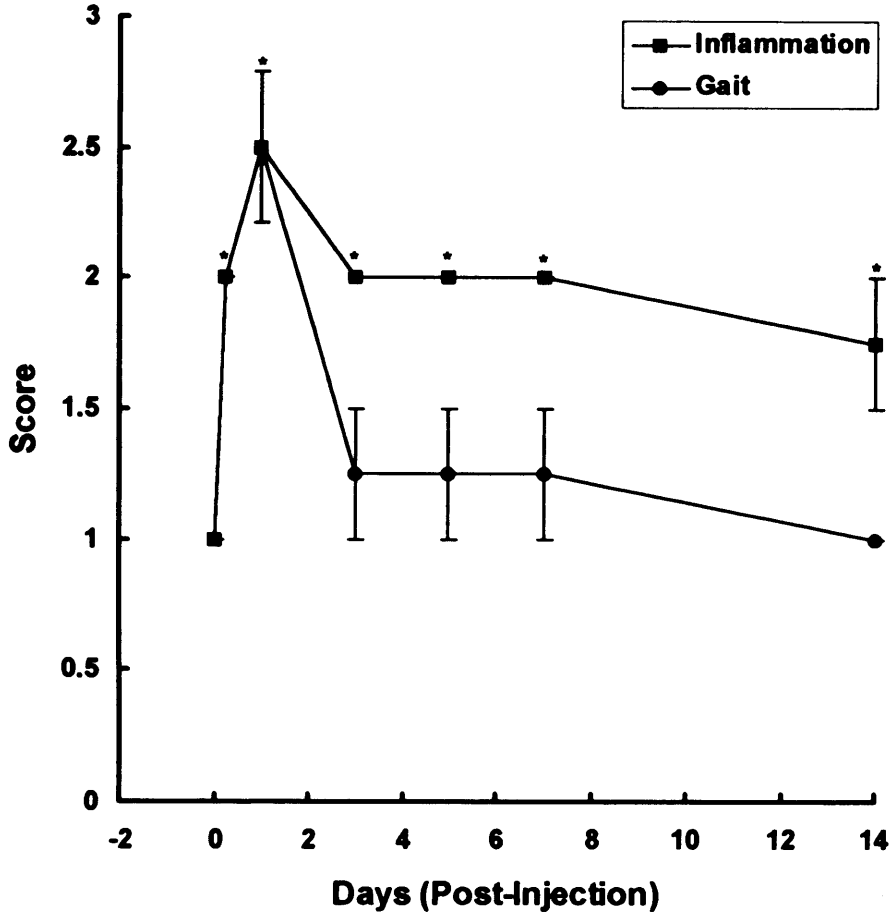


Figure 3.3 Inflammation of the Joint and Associated Behavioural Hyperalgesia. This graph shows the relationship between severity of inflammation and gait impairment (taken as an indicator of behavioural hyperalgesia) following subcutaneous injection of 0.15ml FCA around the tibio-tarsal joint. A score of 1 represents normal gait/no inflammation, a score of 3 indicates no weight bearing/severe inflammatory lesions. Data represent the mean score ± SEM (n=4) (*$p<0.05$, ANOVA).

3.3.3 FCA-Monoarthritis is not Accompanied by Weight Loss, Secondary Inflammations or Stress.

This model of FCA-monoarthritis appears to prevent the development of side-effects such as profound weight loss, secondary lesions, stress and general illness which accompany FCA-polyarthritis (Pearson and Wood, 1959; Colpaert, 1987; De Castro-Costa *et al*, 1987).

Figure 3.4 compares the weight gain of control, non-injected rats with the weight gain of FCA-monoarthritic rats. The normal gain in weight was not affected by the injection of FCA at any time point ($p>0.05$), indicating the animals were feeding as normal. These data are supported by the observation that the animals moved freely, albeit with a modified gait, and showed normal food-seeking behaviour. FCA-monoarthritic rats showed no signs of stress at any time point following the induction of arthritis, except during ankle measurement, nor did they develop any secondary lesions on the contralateral limb, eyes, nose or genitals (data not shown).

FIGURE 3.4.

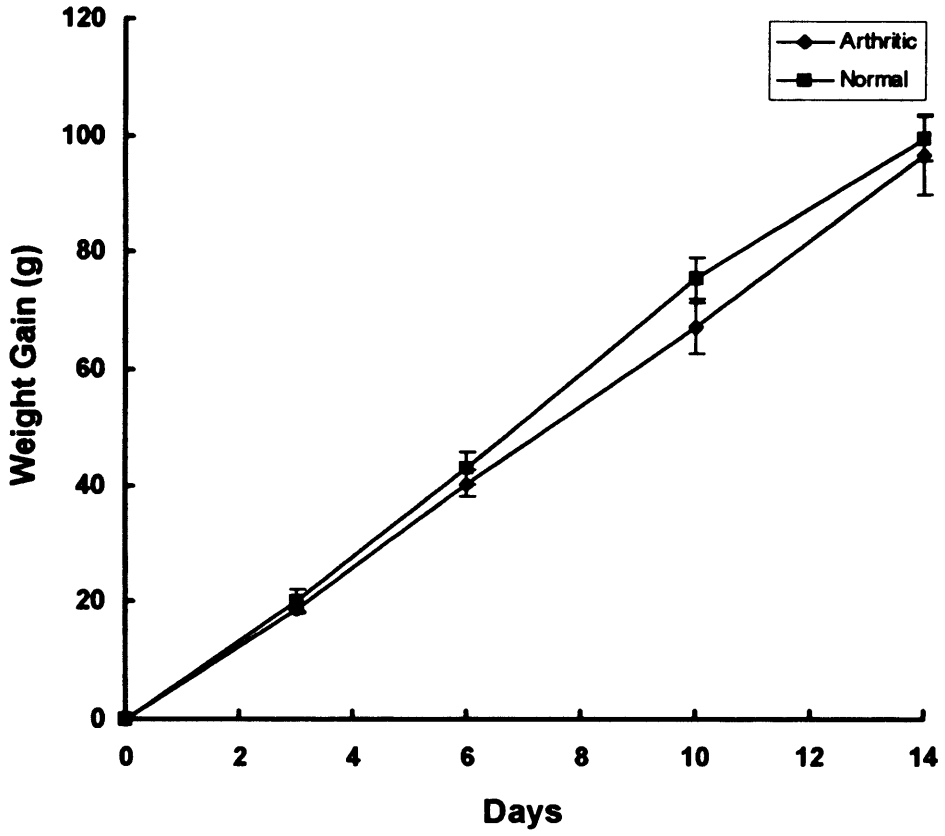


Figure 3.4 Weight Gain is Not Impaired following Induction of Arthritis. The graph compares weight gain of normal, non-injected rats with the weight gain of FCA-monoarthritic rats over a fourteen day time course. Data represent the mean gain in body weight (g) ± SEM (n=4). There is no significant difference between the pattern of weight gain of normal and arthritic rats.

3.4 Discussion.

The subcutaneous injection of 0.15ml FCA (150μg *Mycobacterium tuberculosis*) around the tibio-tarsal joint initiates the development of a discrete monoarthritis of the joint. The redness and swelling of the injected joint is sustained over the fourteen days of the study, indicating an ongoing local inflammatory response at the site of injection. The inflammation remains confined to the injected joint without the development of secondary inflammatory lesions. The development of inflammation required the presence of *Mycobacterium tuberculosis* in order to elicit the autoimmune response. The subcutaneous injection of 0.15ml paraffin caused a small non- significant increase in joint circumference, presumably due to the volume of vehicle distending the ankle, or possibly due to the development of skin inflammation at the sites of the multiple injections.

The animals did not show signs of stress or reduced motility and appeared in good general condition. The only signs of discomfort displayed by the animals was the reluctance to put weight on the injected limb during the acute phase (<3 days) of the inflammatory response and the aversive response to ankle measurement. This guarding of the limb indicated that placing of the foot and flexion of the inflamed joint within the normal working range had become painful. It was interesting to observe that pain-related behaviours abated whilst the joint was still very inflamed. This may indicate that the spinal cord is able to modify it's output within 3 days of inflammation to compensate for the increased nociceptor input from the injected limb.

Indices such as increased joint circumference and reduced nociceptive threshold are commonly used to describe the degree of inflammation (Grubb *et al*, 1988, 1991). The validity of using such indices was confirmed by Donaldson *et al.* (1993) who determined there was a close correlation between increases in joint circumference and inflammatory signs and the development of inflammation and destruction of joint tissue. It has been demonstrated that inflammation of the ipsilateral joint is associated with signs of tissue destruction including inflammatory cell infiltration, synovial effusion and proliferation, oedema and cartilage breakdown around the joint, no evidence of contralateral inflammation was observed using this dose of FCA (Donaldson *et al*, 1993). FCA-monoarthritis, unlike polyarthritis, was not accompanied by the development of ankylosis and severe bone destruction (Grubb *et al*, 1988; Donaldson *et al*, 1993).

FCA-monoarthritis therefore provides a model of inflammation associated with pain behaviour, which is less debilitating than FCA-polyarthritis and will be used in this thesis in order to study inflammation-induced changes in the arachidonic acid pathway in the spinal cord.

Chapter 4. Localisation of Cyclooxygenase Isoforms within the Rat Spinal Cord.

4.1 Introduction.

COX enzymes are responsible for the conversion of free arachidonic acid to prostaglandins and thromboxanes. Until recently only one form of COX had been described, then a second isoform was identified which could be rapidly upregulated by a variety of stimuli (Simmons *et al.*, 1989; Kujubu *et al.*, 1991, Yamagata *et al.*, 1993). The original isoform, COX-1, and the newly-discovered isoform, COX-2 have been cloned and characterised in a number of species, e.g. human (Yokayama and Tanabe, 1989; Hla and Neilson, 1992) and rat (Feng *et al.*, 1993; Kennedy *et al.*, 1993; Yamagata *et al.*, 1993).

As described in the introduction, COX-1 is the constitutive isoform found in peripheral tissues (O'Neill and Ford-Hutchinson, 1993; Seibert *et al.*, 1994). COX-1 is less prevalent in brain tissue where COX-2 is the predominant isoform (Feng *et al.*, 1993; Seibert *et al.*, 1994). Immunocytochemical studies have detected the presence of COX-2-like immunoreactivity within neurones in discrete regions of the brain (Yamagata *et al.*, 1993; Breder *et al.*, 1995; Kaufmann *et al.*, 1996). COX-2 appears to be constitutively expressed within the central nervous system.

In the early 1970's, NSAIDs, were found to inhibit the activity of COX and prevent the production of prostaglandins in inflamed tissue. The inhibition of COX at the site of inflammation was thought to confer the analgesic properties of these drugs (Vane, 1971). More recently, however, it has been hypothesised that the analgesic properties of NSAIDs may be, at least in part, separate to their anti-inflammatory properties. Discrepancies exist between the concentration of NSAIDs required to produce analgesia and the doses required to inhibit COX activity (McCormack and Brune, 1991; McCormack and Urquhart, 1995). NSAIDs may have several sites and mechanisms of action and a component of their analgesic effects may be due to an action in the central nervous system.

Evidence supporting this hypothesis comes from experiments which have demonstrated antinociceptive effects of spinally administered NSAIDs. Intrathecal delivery of NSAIDs dose-dependently reduced the second, but not the first, phase of paw flinching in the

formalin model of behavioural hyperalgesia (Malmberg and Yaksh; 1992b, 1993). The first phase of paw flinching is associated with the direct action of formalin on nociceptors. The second phase is associated with actions of proinflammatory mediators on nociceptors and the subsequent generation of spinal hyperexcitability. Spinally-applied NSAIDs were effective at reducing the second-phase of paw flinching at much lower doses than those required to produce analgesia when delivered systemically, which suggests the drugs do not act by systemic redistribution to the site of inflammation. The spinal NSAIDs were also effective within 3-4 minutes of injection suggesting a rapid diffusion to the active site. The intrathecal administration of the S(+) enantiomer of ibuprofen reduced paw flinching, whereas the R(-) enantiomer was ineffective, only the S(+) enantiomer of ibuprofen is effective as a COX inhibitor. This stereospecificity provides further evidence that NSAIDs have a spinal site of action (Malmberg and Yaksh, 1992b).

Experiments have shown that both COX-1 and COX-2 mRNA are present in normal rat spinal cord (Beiche *et al.*, 1996; Hay *et al.*, 1997). However, it is important to note that mRNA transcription and protein translation do not always coincide, and it is not yet known whether COX-1 and COX-2 proteins are present in the spinal cord.

This study investigates the expression and distribution of COX-1 and COX-2 proteins within the rat spinal cord using Western blotting and immunocytochemical techniques. Lumbar spinal cord segments L3-L5 were chosen since these segments receive the major terminations of the sural and tibial nerves which innervate the ankle joint. If centrally generated prostaglandins are involved in nociception, then COX-1 and/or COX-2 must be expressed within the spinal cord in sites consistent with an involvement in nociceptive processing.

4.2 Methods.

4.2.1 Western Blotting.

Spinal cord samples were obtained from male Wistar rats as described in section 2.5 and prepared for Western blotting (see section 2.8). Denatured samples were separated by SDS-PAGE using a 10% acrylamide resolving gel (200V for 1.5 hours). The membranes were pre-incubated for 2 hours in blocking buffer, then incubated overnight in either anti-COX-1 (1:1000) or anti-COX-2 (1:750). The following day, the blots were washed in TTBS then incubated in a peroxidase-conjugated secondary antibody (1:1000). The protein bands were then visualised using an ECL-detection kit according to the manufacturer's instructions (Amersham). Blots were placed against light-sensitive X-ray film for an appropriate period of time (usually < 5 minutes) and the film developed. In control experiments, primary antibody was omitted from the overnight incubation medium to determine the amount of non-specific binding.

4.2.2 Immunocytochemistry.

Spinal cord sections were prepared as described in section 2.9. Sections were incubated for 30 minutes at room temperature in blocking buffer, then overnight at 4°C in either anti-COX-1 (1:200) or anti-COX-2 (1:200). The following day, sections were washed in PBS and incubated for 2 hours at room temperature in FITC-conjugated secondary antibody (1:200). Sections were rewashed then mounted in Citifluor (UKC Chem Lab). Sections were examined either using an epifluorescence or a laser scanning confocal microscope. In control experiments primary antibody was omitted from the incubation medium in order to determine the amount of non-specific binding.

4.2.3 Antibodies and Standards.

COX-1 and COX-2 antisera were purchased from Cayman Chemical. Purified ovine COX-1 protein was obtained from Cayman Chemical, and purified ovine COX-2 protein was purchased from Oxford Biomedical Research Inc. Prestained SDS-PAGE molecular weight markers were bought from Bio-Rad. Peroxidase-conjugated and FITC-conjugated anti-mouse-/anti-rabbit-IgG were purchased from Sigma.

4.3 Results.

4.3.1 Characterisation of the Antibodies and Control Experiments.

The isoform-selectivity of the two COX antibodies used in this study was assessed using Western blot analysis. Panel A of Figure 4.1 shows a Western Blot obtained by incubating purified COX-1 and COX-2 proteins overnight with anti-COX-1 The antisera detected a single protein band in the lane loaded with COX-1 protein at approximately 75kD. No band was detected in the lane containing purified COX-2. Panel B shows a Western blot obtained by incubating purified COX-1 and COX-2 proteins overnight with anti-COX-2. The COX-2 antibody detected a single band at approximately 75kD in the band containing the purified COX-2, no band was detected in the lane containing COX-1. This indicates that the COX antibodies are specific for the appropriate isoforms.

The non-specific binding of the peroxidase-conjugated secondary antibodies to rat spinal cord proteins was assessed by omitting anti-COX-1 and anti-COX-2 from the overnight incubation medium. Two Western blots of rat spinal cord proteins were incubated overnight in blocking buffer, then for 2 hours with either peroxidase-conjugated anti-mouse or anti-rabbit IgG. The blots were processed and placed against light-sensitive X-ray film for 30 minutes, a relatively long exposure time. No peroxidase-labelling was observed in either Western blot (data not shown) indicating no non-specific binding occurs between the secondary antibodies and rat spinal cord proteins. The secondary antibodies are, therefore, selective for the target primary antibodies.

Similarly, the omission of primary antibody from the overnight immunocytochemistry incubation medium abolished FITC-labelling. Figure 4.2 shows a composite picture made from 4 confocal photomicrographs of a 15μm transverse section of rat lumbar spinal cord (L5) which was incubated overnight in blocking buffer, then for 2 hours in FITC-conjugated anti-rabbit-IgG. No regions of immunofluorescence were detected, indicating that no non-specific binding occurs between the FITC-conjugated secondary antibody and rat spinal cord proteins.

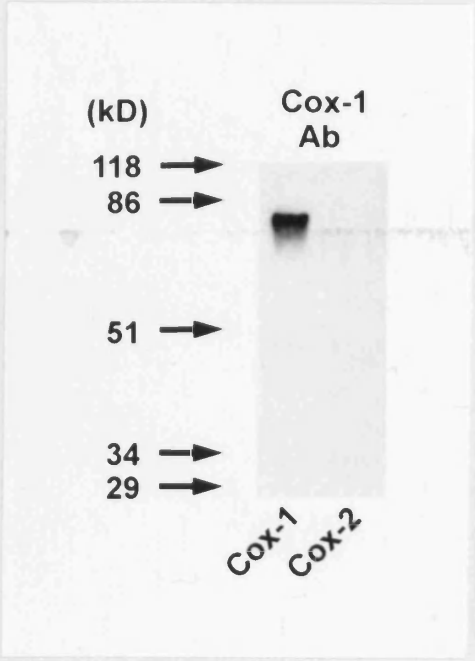
4.3.2 Cyclooxygenase Isoforms are Present in the Spinal Cord of Normal Rats.

Both isoforms of COX are present in protein samples obtained from rat lumbar spinal cord. Panel A of Figure 4.3 shows a Western blot obtained by incubating rat lumbar spinal cord protein (sc: left-hand lane) and purified COX-1 protein (COX-1: right-hand lane) overnight with anti-COX-1. A single protein band was detected in each lane at approximately 75kD, demonstrating the selectivity of the COX-1 antibody, and indicating the presence of COX-1 in the spinal cord of normal rats.

The COX-2 antisera detected a single band in the lane containing purified COX-2 at approximately 75kD (Figure 4.2, panel B). This band was also detected in the lane containing rat spinal cord protein (sc: left hand lane), indicating the presence of COX-2 in the spinal cord of normal rats. An additional band at approximately 68kD was also detected in the lane containing rat spinal cord protein (see discussion).

FIGURE 4.1.

A.



B.

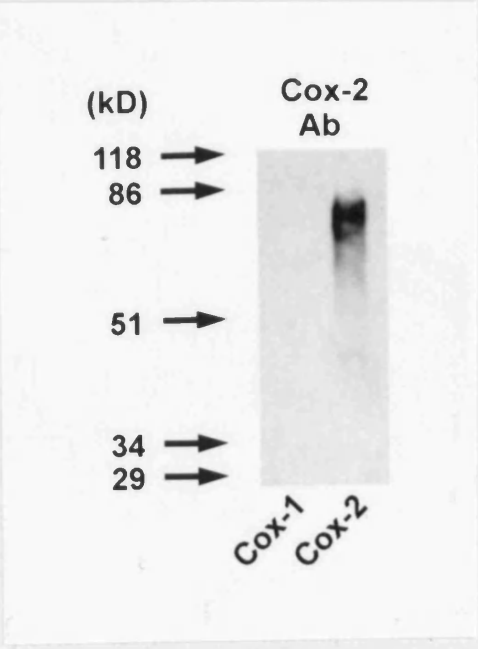


Figure 4.1 Primary Antibody Isoform Selectivity. This figure demonstrates the selectivity of anti-COX-1 (Panel A) and anti-COX-2 (Panel B) for purified COX-1 and COX-2 proteins respectively. A single protein band is detected at approximately 75kD in each blot. The antibodies therefore detect only the appropriate isoform.

FIGURE 4.2.

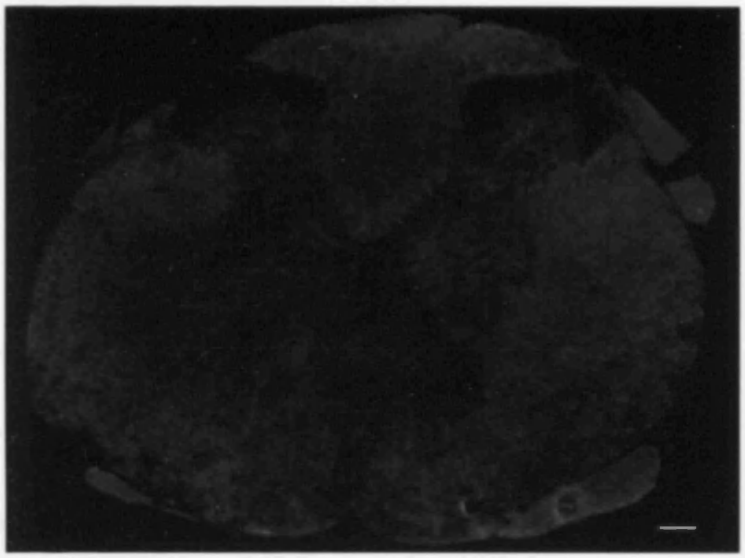


Figure 4.2 Secondary Antibody Specificity. A control composite confocal photomicrograph of a 15μm transverse section of rat L5 spinal cord that has been incubated overnight in blocking buffer then for 2 hours in FITC-conjugated anti-rabbit-IgG. The absence of immunofluorescence indicates no non-specific binding occurs between the secondary antibody and rat spinal cord proteins, the secondary antibody is, therefore, specific for the target primary antibody. Scale bar=200μm.

FIGURE 4.3

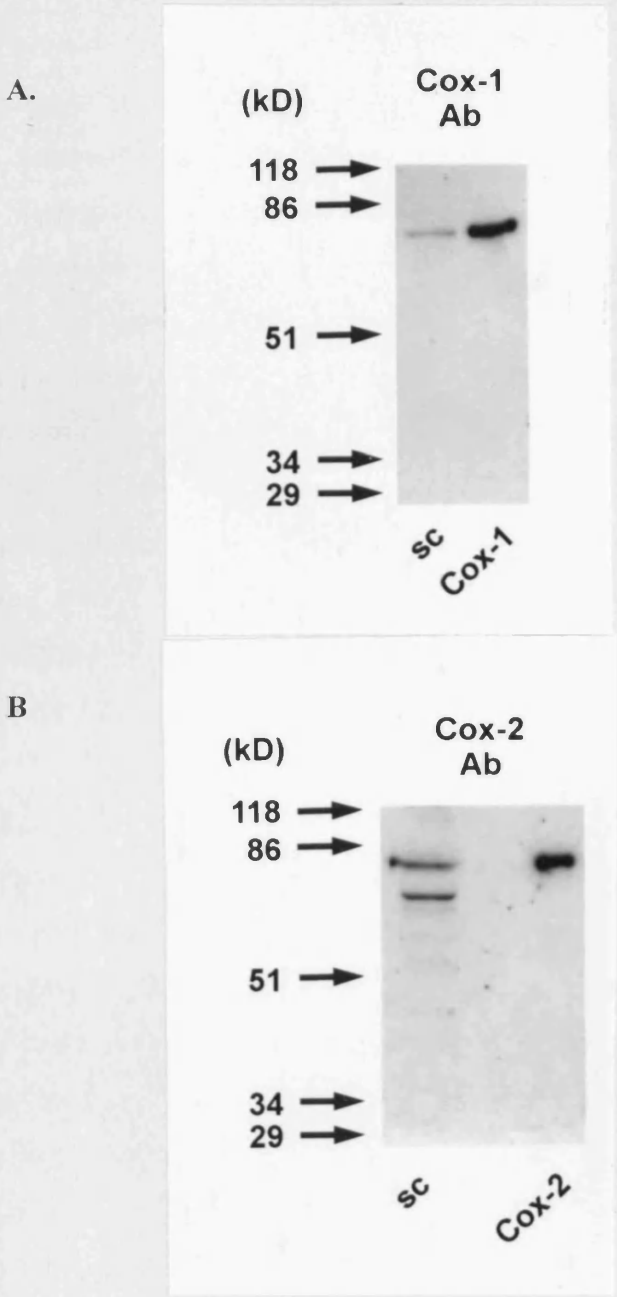


Figure 4.3. COX-1 and COX-2 are Present in Rat Spinal Cord. Panel A shows a Western blot obtained by incubating purified 1μg COX-1 to act as a marker (right lane) and 12.5μg spinal cord protein (SC, left lane) overnight with anti-COX-1 (1:1000). The anti-COX-1 recognises a single band in each lane at approximately 75kD, indicating the presence of COX-1 in normal rat spinal cord protein. Panel B shows a Western blot obtained by incubating 1μg purified COX-2 to act as a marker (right lane) and 12.5μg spinal cord protein (SC, left lane) with anti-COX-2 (1:750). The COX-2 antibody detects a single band in the left-hand lane and two bands in the lane containing rat spinal cord protein. These two bands indicate the presence of COX-2 in rat spinal cord, possibly in both glycosylated (75kD) and deglycosylated (68kD) forms (see discussion).

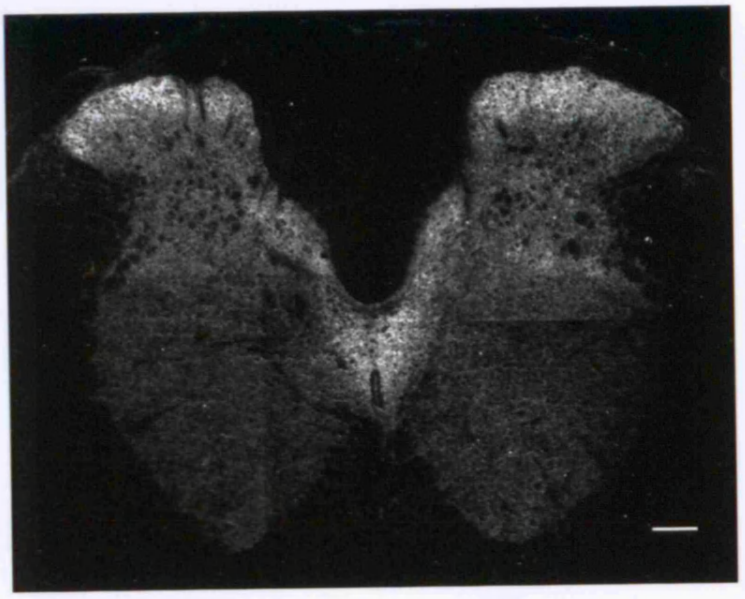
4.3.3 The Distribution of Cyclooxygenase-2-like-Immunoreactivity in the Lumbar Spinal Cord of Normal Rats.

Panel A of Figure 4.4 shows a composite confocal photomicrograph of a 15μm transverse section of rat spinal cord from spinal segment L3, which had been incubated overnight with anti-COX-2. Immunofluorescence from the FITC-conjugated secondary antibody appears white in this digitised image and is described as COX-2-like-immunoreactivity (COX-2-li). The internal structure of spinal segment L3 is shown, for reference, in panel B. Diffuse COX-2-li appears throughout the grey matter of the spinal cord and a number of immunoreactive axonal tracts can be observed radiating from the grey matter. With the exception of these processes, COX-2-li labelling in the white matter is similar to the background labelling observed in Figure 4.2. Several discrete regions of the grey matter exhibit intense immunoreactivity. Pronounced COX-2-li is present in the superficial dorsal horn (laminae I and II), the medial aspect of the deep dorsal horn (laminae IV and V) and around the central canal (lamina X). The antibody labelling is bilaterally symmetrical. The regions exhibiting COX-2-li are highlighted in panel B.

Panel A of Figure 4.5 shows a similar composite confocal photomicrograph of a 15μm transverse section of rat spinal cord obtained from rat spinal segment L5, which was incubated in anti-COX-2. The internal structure of the spinal cord changes shape from segment to segment, and the shape of L5 and the regions exhibiting COX-2-li in this section is highlighted in panel B. Diffuse COX-2-li was again observed throughout the grey matter of the section. Intense COX-2-li was detected in the superficial dorsal horn (laminae I-III). Less immunofluorescence can be seen in the laminae of the deep dorsal horn and around the central canal in this L5 section compared to the section obtained from L3 (Figure 4.4). In all sections examined, COX-2-li is consistently observed in the superficial dorsal horn of the lumbar spinal cord of normal rats. The additional immunofluorescence observed in Figure 4.4 in the laminae of deep dorsal horn and around the central canal is detected in some, but not all, sections.

FIGURE 4.4.

A.



B.

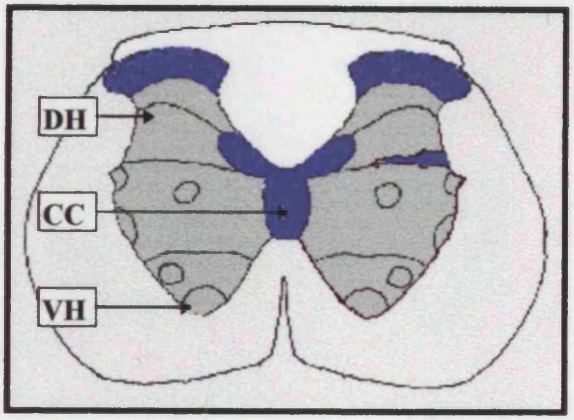
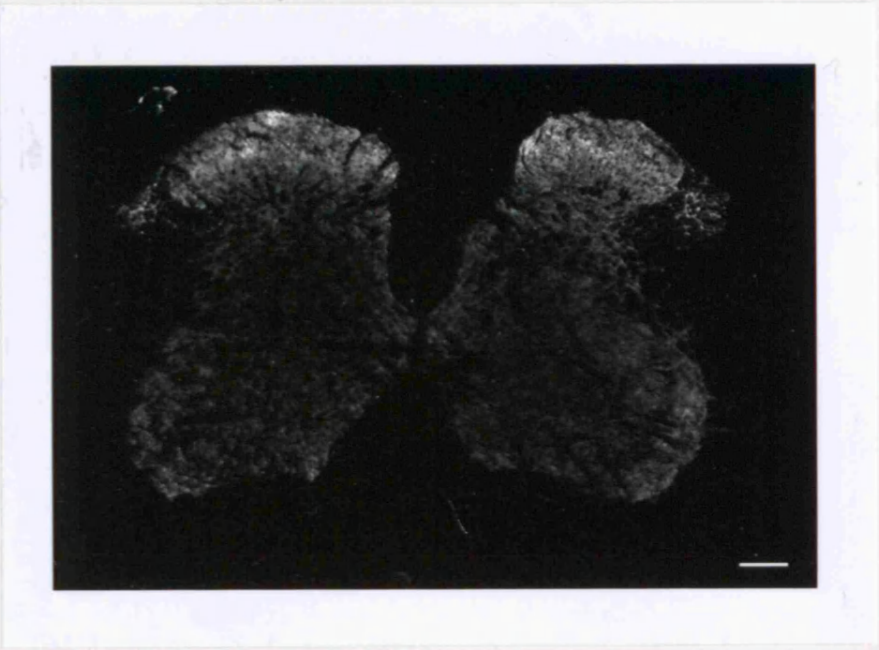


Figure 4.4 COX-2-li in L3 Rat Spinal Cord. Panel A shows a composite confocal photomicrograph of a 15μm transverse section of L3 rat spinal cord which has been incubated overnight with anti-COX-2. Diffuse COX-2-li is observed throughout the grey matter of the section with intense COX-2-li observed in lamina I-II, IV-V and X. These areas are highlighted on the diagram in panel B. For reference, the dorsal horn (DH), ventral horn (VH) and central canal (CC) are also labelled.

Scale bar = 200μm.

FIGURE 4.5

A.



B.

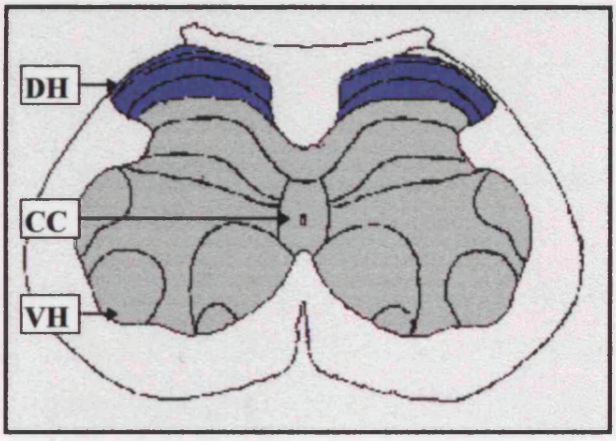


Figure 4.5 COX-2-li in L5 Rat Spinal Cord. Panel A shows a composite confocal photomicrograph of a 15μm transverse section of L5 rat spinal cord which has been incubated overnight with anti-COX-2. Diffuse COX-2-li is observed throughout the grey matter of the section with intense COX-2-li observed in lamina I-III. This region is highlighted in the diagram in panel B. For reference, the dorsal horn (DH), ventral horn (VH) and central canal (CC) are also labelled. Scale bar = 200μm.

4.3.4 The Regional Distribution of Cyclooxygenase-2-like-Immunoreactivity in the Lumbar Spinal Cord of Normal Rats.

The intense COX-2 li observed in the superficial dorsal horn of lumbar spinal cord is shown at higher magnification in panel A of Figure 4.6. Panel B is included to aid orientation. Diffuse COX-2-li is distributed throughout the grey matter. Intense COX-2-li is mainly localised in the superficial dorsal horn (laminae I and II). In this section, COX-2-li is observed in the medial aspects of laminae IV and V, and also in the lateral aspect of lamina V. It is difficult to identify individual immunoreactive cells in this region, as the neurones of the superficial laminae are small and tightly packed together.

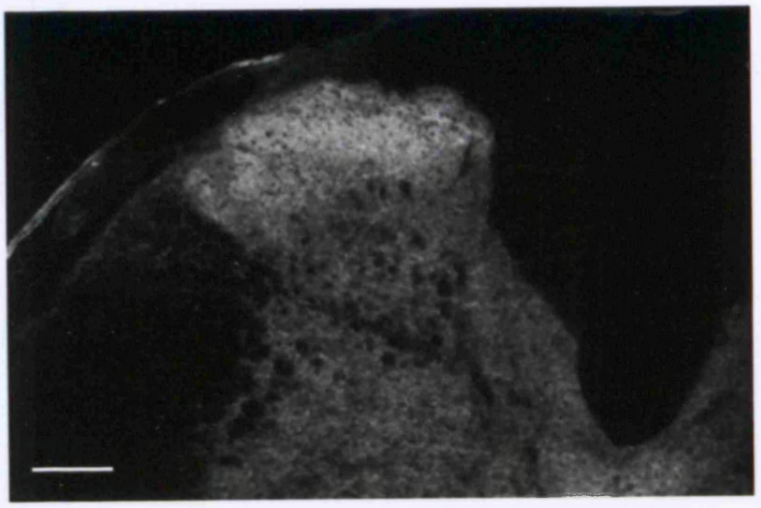
Panel A of Figure 4.7 shows the intense COX-2-li observed around the central canal in some spinal cord sections. COX-2-li encircles the central canal, and is most intense in the dorsal portion of lamina X. COX-2-li is absent from the ciliated ependymal cells lining the central canal.

In regions where the neurones are less closely packed such as in the ventral horn (Figure 4.8, Panel A), around the central canal (Figure 4.8, Panel B) and in the deep dorsal horn (Figure 4.8, Panel C) COX-2-li can clearly be observed in the somata of individual neuronal cell bodies (see arrows). The nuclear stain propidium iodide was used in some sections (Panels B and C) and appears as the high intensity punctate staining. The nuclei do not show COX-2-li which is consistent with the known distribution of COX-2-li in other cell types. Some motoneurones possess a distinct halo of COX-2-li around the cell bodies which may indicate that the plasma membrane of these neurones expresses COX-2.

The diffuse COX-2-li observed throughout the grey matter was not associated with either neuronal cell bodies or axonal processes, indicating that non-neuronal cell types also express COX-2-li. Using light microscopy it was not possible to determine the cell subtype.

FIGURE 4.6

A.



B.

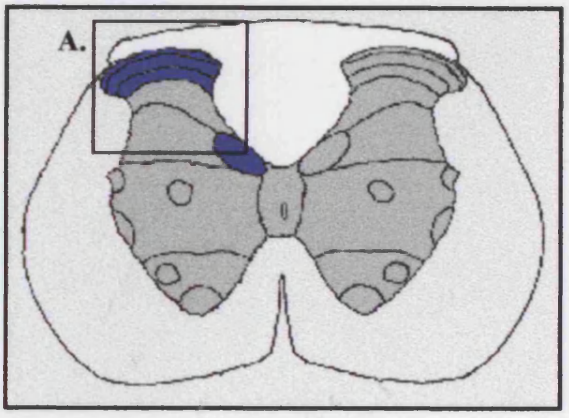
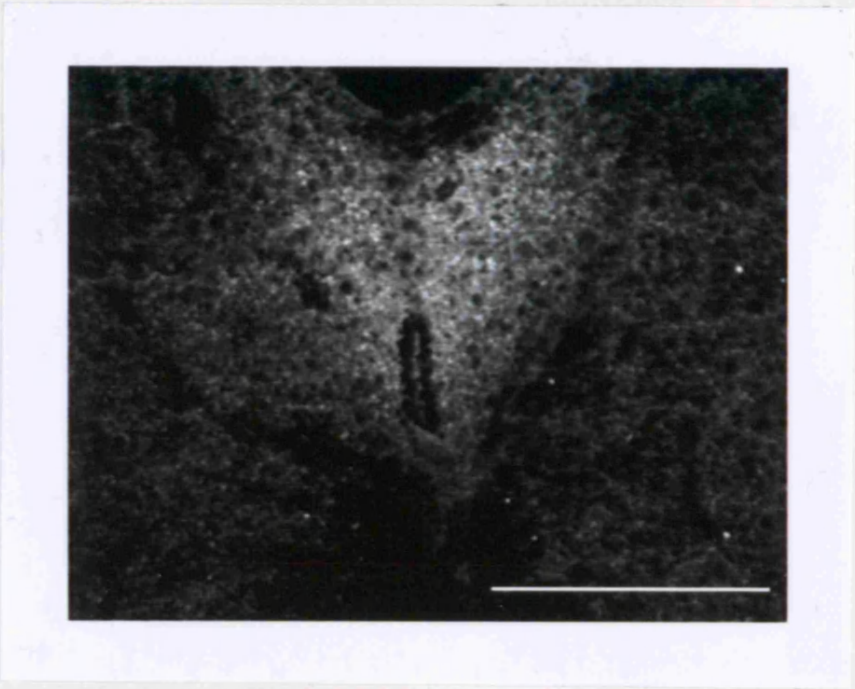


Figure 4.6 COX-2-li in the Superficial Dorsal Horn. Panel A shows a confocal photomicrograph of a 15μm transverse section through the dorsal horn of L3 spinal cord which has been incubated overnight in anti-COX-2. Diffuse COX-2-li is distributed throughout the grey matter of the dorsal horn. Intense COX-2-li is localised in laminae I and II, the medial aspects of laminae IV and V and the lateral aspect of lamina V. The insert (A) in panel B indicates the region of the spinal cord from which this section was taken and highlights the regions expressing intense COX-2-li. Scale bar = 250μm.

FIGURE 4.7

A.



B.

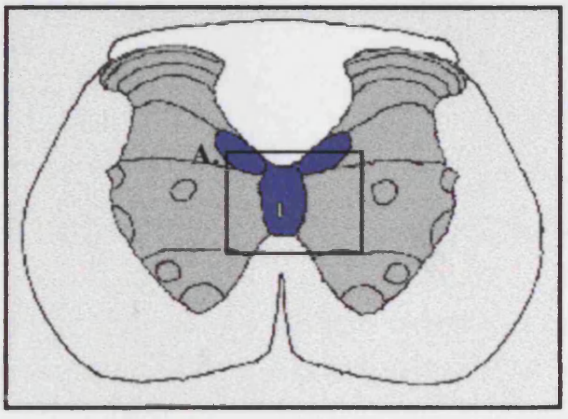
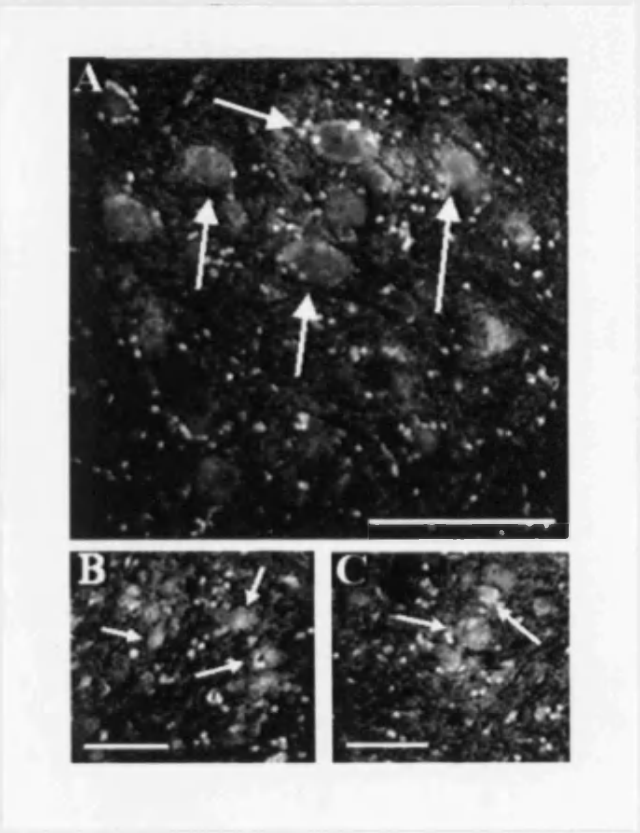


Figure 4.7 COX-2-li around the Central Canal. Panel A is a confocal photomicrographs of a 15μm transverse section through the region surrounding the central canal in L3 spinal cord which was incubated in anti-COX-2. The insert (A) in panel B indicates the region of the spinal cord from which this section was taken and highlights the regions expressing intense COX-2-li. COX-2-li encircles the central canal, but is absent from the ciliated ependymal cells lining the canal. Scale bar = 75μm.

FIGURE 4.8.



D.

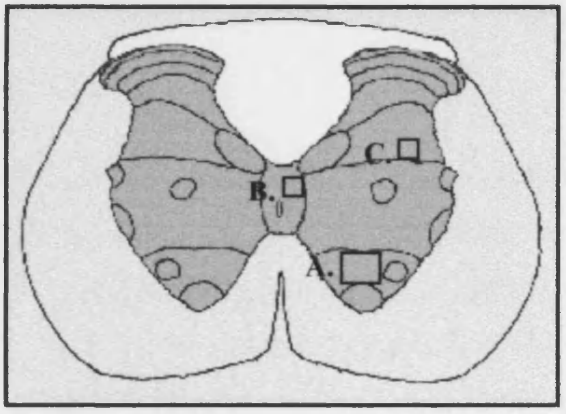


Figure 4.8 COX-2-li around the Central Canal, the Deep Dorsal Horn and the Ventral Horn. This figure shows confocal photomicrographs of 15μm transverse sections of L3 spinal cord which have been incubated overnight in anti-COX-2. COX-2-li can clearly be observed in the somata of individual neuronal cell bodies (see arrows) in the ventral horn (Panel A), the central canal (Panel B) and the deep dorsal horn (Panel C). The high intensity staining in panels B and C is the nuclear stain propidium iodide. COX-2-li is absent in nuclei. Some motoneurones possess a distinct halo of COX-2-li around the cell bodies, which may indicate that the plasma membrane of these neurones expresses COX-2. The inserts (A, B, C) in panel D indicate the regions of the spinal cord from which these sections were taken. Scale Bar = 25μm.

4.3.5 The Distribution of Cyclooxygenase-2-like-Immunoreactivity does not change in the Arthritic Rat Spinal Cord.

Whilst individual tissue sections showed a uniform distribution of COX-2-li, some variation was observed in the intensity of immunofluorescence between lumbar spinal cord sections (even those obtained from the same animal). This inherent variation in labelling made it difficult to determine whether differences in the intensity of immunofluorescence existed between spinal cord sections obtained from normal and monoarthritic rats. As a result of these technical considerations, no differences were observed between lumbar spinal cord sections obtained from normal rats and from rats with FCA-induced monoarthritis with respect to the level of COX-2-li. As in spinal cord sections obtained from normal rats, diffuse COX-2-li was expressed throughout the grey matter of the arthritic rat spinal cord. Intense COX-2-li was observed in the superficial dorsal horn (laminae I and II), some sections also showed intense COX-2-li in the medial aspect of the deep dorsal horn (laminae IV and V) and around the central canal (lamina X). COX-2-li remained bilaterally symmetrical in the spinal cord of the FCA-monoarthritic rat (data not shown).

4.3.6 Cyclooxygenase-1-like-Immunoreactivity is not Present in the Lumbar Spinal Cord of Normal Rats.

Incubation of rat lumbar spinal cord sections in anti-COX-1 produced a very different distribution of immunofluorescence than that seen following incubation in anti-COX-2, providing further evidence that the two antibodies are selective for appropriate COX isoforms. With the exception of some faintly immunoreactive fibres observed in the dorsal root entry zone and the superficial dorsal horn, no COX-1-li was observed in the rat lumbar spinal cord (data not shown). Intense COX-1-li was, however, observed in axons contained in the surrounding dorsal roots, this was further investigated by colleagues.

4.4 Discussion.

COX-1 (600-602 amino acids) and COX-2 (603-604 amino acids) proteins are highly homologous with 60% of the amino acid residues identical. The functionally relevant amino acid residues, such as heme-binding histidines, the catalytic tyrosine residue, the active site serine and the *N*-glycosylation sequences, are all conserved between the two isoforms (DeWitt *et al.*, 1990; Hla and Neilson, 1992). As a consequence of this homology, the isoforms possess similar enzymatic activities (V_{max}) and affinities for arachidonate (K_m) (Meade *et al.*, 1993). The two main differences between the isoforms occur at the amino-(N) and carboxyl-(C) terminals. COX-1 has a large hydrophobic sequence at the N-terminus whereas COX-2 has a much shorter sequence (17 less amino acids). COX-2 has an 18 amino acid insert close to the C-terminus which is absent in COX-1. These differences have been exploited during the manufacture of isoform-selective COX antibodies.

Selective antibodies can be used as probes to identify proteins that share similar structural and pharmacological characteristics. They are used in Western blotting to determine the apparent molecular mass of the protein and detect changes in the expression levels of the protein. They also localise the protein within tissues using immunocytochemical techniques. When conducting these immunodetection experiments it is important to ensure that the antibodies are selective.

A wide range of isoform-'selective' COX antibodies is available from commercial sources. The selectivity and specificity of COX antibodies from five different sources was assessed before choosing the antibodies from 'Cayman Chemical' described in this study. The manufacturer's instructions indicated that the monoclonal mouse anti-sheep COX-1 antibody detected sheep, rat, mouse and human COX-1 and showed little or no crossreactivity with COX-2 in three species tested (sheep, mouse, human). Similarly, the polyclonal rabbit anti-mouse COX-2 antibody detected sheep, mouse, rat guinea-pig and human COX-2, and showed little or no crossreactivity with COX-1 in three species tested (sheep, mouse, human).

The validity of this information was assessed by examining the cross-reactivity between the two antibodies and purified ovine COX-1 and COX-2. According to the manufacturer's instructions the COX-1 antibody should detect ovine COX-1 but not ovine COX-2, the

COX-2 antibody should detect ovine COX-2 but not ovine COX-1. Figure 4.1 (Panels A and B), illustrates that the manufacturers correctly described the absence of isoform cross-reactivity in sheep, and the antibodies were selective for the appropriate isoform. It would have been desirable to repeat this experiment using rat COX-1 and COX-2 standards, but this was not possible as purified rat COX proteins were not commercially available. However, given that the manufacturers state that no cross-reactivity occurs between rat isoforms, and also that there is a high degree of sequence homology between species (for example 17/18 amino acids in the C-terminus insert are identical in chick, human, rat and mouse COX-2: Otto and Smith, 1995), it is not unreasonable to assume that the COX antibodies are equally selective for the appropriate rat isoforms. This hypothesis is supported by the very different distributions of immunolabelling in the rat spinal cord observed using the two antibodies. The selectivity of the secondary antibodies strongly suggest that the immunoreactivity observed in these experiments represents the presence and/or distribution of spinal COX-1 or COX-2.

COX-1 and COX-2 mRNA have previously been detected in rat spinal cord tissue (Beiche *et al.*, 1996; Hay *et al.*, 1997), but these studies provided no information about the expression or distribution of the two enzymes within the spinal cord. This current study is the first to localise COX proteins within the spinal cord.

Measurable amounts of COX-1 were detected in rat spinal cord tissue using Western blotting techniques (Figure 4.3, Panel A). A single protein band was detected at approximately 75kD by anti-COX-1, which corresponded to the position of the purified ovine COX-1 protein. Immunocytochemical analysis indicated that, with the exception of some weakly labelled COX-1-li fibres in the dorsal root entry zone and superficial dorsal horn, very little COX-1-li was present in rat spinal cord. It may have been argued that the antiserum was unsuitable for immunocytochemistry, were it not for the intense COX-1-li observed in surrounding dorsal roots, which had not been removed during tissue processing. This clearly illustrates the importance of using several techniques to examine a system. These data indicate that little or no COX-1-li is present in the rat lumbar spinal cord but is present in axons contained within the dorsal roots. A different conclusion may have been reached if only one of these techniques had been used.

Breder *et al.* (1992) described an extensive distribution of neuronal COX in the ovine brain. COX-li was most intense in structures concerned with integrative processing such as the cerebral cortex, hippocampus and amygdala. This distribution was later attributed to COX-1-li (Breder *et al.*, 1995), although it may be possible that their antibody detected both COX isoforms as subsequent experimenters have found little or no COX-1-li or COX-1 mRNA in brain tissue (Yamagata *et al.*, 1993; Seibert *et al.*, 1994; Marchesselli and Bazan, 1996).

The presence of intense COX-1-li in dorsal roots and faint immunoreactivity in fibres of the dorsal root entry zone and superficial dorsal horn in the present study indicates that COX-1 may be present in sensory neurones. Colleagues studied the distribution of COX-1-li in DRG cell bodies and found intense COX-1-li in the cytoplasm, nuclear membrane and axonal processes of small to medium sized ($<1000\mu m^2$) DRG cell bodies. No COX-2-li was observed in these cells (Grubb *et al.*, 1997; Willingale *et al.*, 1997). Small diameter neuronal cell bodies often contain neuropeptides involved in nociceptive processing and this is one method commonly often used to classify them as nociceptors (Lawson *et al.*, 1993; Hanesch *et al.*, 1993). For example, 70% of DRG cells which express SP-li are small diameter neuronees which have conduction velocities in the group III and IV range (McCarthy and Lawson, 1989). PGD synthase-li has been localised to these small diameter cell bodies, indicating that the prostaglandin biosynthetic pathway in these neurones is operational (Vesin and Droz, 1993). The function of COX-1, and prostaglandins, in these nociceptive neurones is unknown although the presence of immunoreactive axonal processes indicates that prostaglandins may be released from axon terminals. Since very few COX-1-li fibres were observed entering the dorsal horn, it is unlikely that prostaglandins are released centrally. In the periphery proinflammatory prostaglandins are generally thought to originate from damaged cells or invading immunocompetent cells. It will be intriguing to discover whether nociceptive afferents have the capability to synthesise and release prostaglandins at a site of inflammation and thus 'auto-sensitise' their own peripheral terminals.

Measurable amounts of COX-2 protein were detected in the rat spinal cord protein sample using Western blotting techniques (Figure 4.3, Panel B). Two protein bands were detected by anti-COX-2, one at approximately 75kD, which corresponded to the position of purified ovine COX-2, and one at approximately 68kD. Western blots of protein samples derived from cell or tissue extracts typically produce a double or triple band when incubated with anti-COX-2 (O'Banion *et al.*, 1991; Feng *et al.*, 1993; Habib *et al.*, 1993; Percival *et al.*,

1994; Kaufmann *et al.*, 1996). One explanation for this multiple banding pattern may be partial degradation of the COX protein during sample preparation. A number of precautions were taken during the sample preparation process to prevent such degradation. The spinal cord was rapidly removed from the animal and immediately frozen in liquid nitrogen (<5 minutes), the tissue was homogenised in ice-cold lysis buffer in the presence of various proteinase inhibitors, which should have minimised COX-2 degradation.

Another possible explanation for the multiple banding pattern of COX-2 Western blots is deglycosylation of the enzyme. COX-2 has 5 sites available for *N*-glycosylation (Aspartane (Asn)-x-Serine/Threonine) at Asn68, Asn144, Asn410 and at 2 Asn residues within the 18 amino acid C-terminus insert (Otto *et al.*, 1993; Hoff *et al.*, 1994). The theoretical molecular mass of COX-2 is 67kD, whereas the apparent molecular mass is 74kD. These molecular masses are very similar to those of the two protein bands seen in the COX-2 Western blot (Figure 4.3, Panel B). The protein band at approximately 68kD may therefore be the deglycosylated form of COX-2, which may be naturally present in the spinal cord or generated during sample preparation.

The distribution of COX-2-li in the rat brain was extensively catalogued by Breder *et al.* (1995). They found that COX-2-li was widely distributed throughout the brain and localised in discrete regions associated with the processing of nociceptive, visceral and sensory input such as the cortex, hippocampus, amygdala hypothalamus and thalamic periaqueductal grey matter. COX-2-li was detected in neurones primarily in the cytoplasm and also in some dendritic processes. No COX-2-li was observed in glial cells (Yamagata *et al.*, 1993; Kawasaki *et al.*, 1993; Breder *et al.*, 1995; Kaufmann *et al.*, 1996).

It is now possible to describe the distribution of COX-2-li in the rat spinal cord. Diffuse COX-2-li is present throughout the grey matter of the spinal cord, which may indicate that, in contrast to brain tissue, both neuronal and non-neuronal cell types express COX-2 in the spinal cord. This observation is supported by the observation that both neuronal and non-neuronal cell types, such as astrocytes and microglia, have the capability to synthesise prostaglandins (Bishai and Coceani, 1992). COX-2 has also been detected in stimulated rat microglial cultures (Bauer *et al.*, 1997). Several spinal cord regions exhibit intense COX-2-li. Immunofluorescence is strongest in the superficial dorsal horn (laminae I-II), the medial

aspects of the deep dorsal horn (laminae IV-V) and around the central canal (lamina X) of lumbar spinal segments (Figure 4.4, 4.5).

It was difficult to identify individual immunoreactive structures within the dorsal horn (Figure 4.6) due to the high density of cells, but in regions where the neurones were less closely packed such as in the ventral horn (Figure 4.8), it was possible to distinguish individual labelled neurones. COX-2-li was clearly located in the somata of neurones which was consistent with the pattern of neuronal labelling described in other studies (Yamagata *et al.*, 1993; Breder *et al.*, 1995 Kaufmann *et al.*, 1996). COX-2-li was absent from the spinal white matter, with the exception of a number of immunoreactive axonal processes which radiated from the grey matter (Figure 4.7). In some cells a halo of COX-2-li was observed around the cell bodies which may indicate the labelling of the plasma membrane, or alternatively may be an artefact created during tissue sectioning (Figure 4.8).

The areas exhibiting intense COX-2-li in the spinal cord are consistent with regions known to be involved in the processing of nociceptive input. Nociceptors terminate primarily in the superficial dorsal horn (Craig *et al.*, 1988; Levine *et al.*, 1984), but also in the deep dorsal horn, the medial portion of Clarke's column (Craig *et al.*, 1988) and around the central canal in lamina X (Light and Perl, 1979). Extracellular recordings from interneurones in the dorsal horn and lamina X indicate they primarily respond to noxious stimuli and have characteristically discrete receptive fields (Nahin *et al.*, 1983; Schaible *et al.*, 1986, 1987, 1991; Neugebauer and Schaible 1990). These regions also contain sensory neuropeptides and opioids known to mediate nociception such as SP, CGRP, dynorphin and enkephalin (Gibson *et al.*, 1981; Ruda, 1986; Weihe *et al.*, 1988; Ribeiro-da-Silva *et al.*, 1989; Marlier *et al.*, 1991). Therefore, localisation of COX-2-li in these regions indicates a potential role for COX-2, or prostaglandins generated by COX-2, in spinal nociceptive processing.

Immunocytochemical studies have shown that prostaglandin-D-synthase-li is present and active in the superficial laminae of rat and chick spinal cord (Urade *et al.*, 1989; Vesin *et al.*, 1995). The genes encoding the PGD_2 receptor DP and the PGE_2 receptors EP_2 and EP_4 have been recently detected in rat spinal cord tissue (Lucy Donaldson, personal communication, 1997), Furthermore, EP_2 mRNA has been localised to the superficial dorsal horn (Kawamura *et al.*, 1997).

The localisation of COX-2-li in the regions of the spinal cord associated with nociceptive processing and the overlap between COX-2-li, PGD-synthase-li, and EP_2 mRNA indicates a potential role for PGD_2 and PGE_2, possibly generated by COX-2, in spinal nociceptive processing. The development of sensitive molecular probes for the newly cloned prostaglandin receptors will provide valuable information as to how prostaglandins mediate their spinal effects.

Chapter 5. Cyclooxygenase Expression in the Spinal Cord of FCA-Monoarthritic Rats.

5.1 Introduction.

The development of a peripheral inflammatory lesion has been shown to initiate profound functional changes in the peripheral and central nervous systems. Phenotypic changes occur in spinal cord neurones which receive nociceptive input from the inflamed limb (Cho and Basbaum, 1989), resulting in the increased production of a variety of neurotransmitters and neuromodulators known to be involved with spinal nociceptive processing.

There is a rapid increase in the transcription of immediate early genes such as *c-fos, c-jun* and *zif268* in dorsal horn neurones following the induction of inflammation (Draisci and Iadarola, 1989; Abbadie and Besson, 1992; Honore *et al.*, 1995). Immediate early genes encode transcription factors (e.g. Fos and Jun) which bind to DNA recognition sites, such as AP-1, in the 5'-promoter region of target genes and regulate transcription of that gene.

The promoter regions of PPD and PPE mRNA contain several AP-1 recognition sites and Fos-like immunoreactivity coexists in 80% of dorsal horn neurones which express PPD and PPE mRNA (Noguchi *et al.*, 1989, 1991). This evidence suggests that upregulation of Fos may increase the spinal expression of dynorphin and enkephalin, opioid peptides known to be involved in nociceptive processing. Indeed, following inflammation, increases have been detected in the levels of PPD and PPE mRNA and dynorphin-li and enkephalin-li in laminae I, II, V and VI of the spinal cord (Iadarola *et al.*, 1988; Weihe *et al.*, 1988; Millan *et al.*, 1988; Draisci and Iadarola, 1989; Przewlocka *et al.*, 1992). The expression of sensory neuropeptides, such as SP, CGRP and somatostatin (Weihe *et al.*, 1988; Ohno *et al.*, 1990; Hanesch *et al.*, 1993), and excitatory amino acids, such as glutamate (Sluka *et al.*, 1992; Malmberg & Yaksh, 1992; Sorkin *et al.*, 1992) are also upregulated in the dorsal horn following inflammation. The transcription of immediate early genes therefore initiates a cascade of events, which results in phenotypic changes occurring within spinal cord neurones.

The COX-2 gene shares a number of similarities with immediate early genes. The size of the COX-2 gene (8kb) is consistent with its characterisation as an immediate early gene which

are typically less than 8kb (Herschman *et al.*, 1991; Kraemer *et al.*, 1992; Fletcher *et al.*, 1992). The 5'-promoter region of COX-2 mRNA contains a 'TATA' box, a motif common in highly inducible genes (Fletcher *et al.*, 1992), and multiple transcription factor binding sites, such as nuclear factor $_{\kappa}$B ($NF_{\kappa}B$), AP-1 binding sites, and cAMP responsive elements (CRE) (Sirois *et al.*, 1993; Otto and Smith, 1995). This indicates that transcription of COX-2 is rapid and highly regulated. The 3'-untranslated region of rat COX-2 mRNA contains 14 copies of the Shaw-Kamens (AUUUA) destabilisation sequence (Feng *et al.*, 1993). The half-life of COX-2 mRNA is estimated to be between 30-90 minutes (Hamasaki *et al.*, 1993; Ristimaki *et al.*, 1994), COX-2 protein is also unstable with a half-life of approximately 30 minutes (DeWitt and Meade, 1993), indicating that when active the cell will need to produce more enzyme in order to maintain prostaglandin synthesis.

Prostaglandins are not generally thought of as transcription factors. The discovery that a large proportion of intracellular COX-2 is located on the inner membrane of the nuclear envelope (Morita *et al.*, 1995) indicates a potential role for COX-2 generated prostanoids in nuclear transcriptional control. In fact, PGD_1, PGD_2 and PGJ_2, the breakdown product of PGD_2, have been shown to bind to and activate all isoforms (α, δ and γ) of nuclear Peroxisome Proliferator Activated Receptor (PPAR) (Yu *et al.*, 1995). This soluble receptor associates with the 9-*cis*-retinoic acid receptor then binds to Hormone Response Elements (HRE) in the promoter region of target genes. Transcription of the gene is initiated following activation of the two receptors (Forman *et al.*, 1995).

The rapid induction and degradation of COX-2 mRNA and the short half-life of COX-2 protein contribute to the rather transient expression of COX-2 protein. Most tissues do not express COX-2-li under normal conditions, with the exception of vas deferens (Zhang *et al*, 1997), brain (Yamagata *et al.*, 1993; Breder *et al.*, 1995; Kaufmann *et al.*, 1996) and spinal cord (see Chapter 4; Gardiner *et al.*, 1997; Willingale *et al.*, 1997). COX-2 can be rapidly induced in tissues in response to a variety of inflammatory and pathological stimuli. For example, COX-2 mRNA is undetectable in the normal rat paw, but is induced following intraplantar injection with 1% carrageenan (Seibert *et al.*, 1994).

The constitutive expression of COX-2 in discrete regions of brain indicates a central role for prostanoids under normal conditions. In addition, COX-2 expression is upregulated in brain by a number of experimental manipulations, such as ischemia, stress, bacterial endotoxin,

electroconvulsive-induced seizure, and NMDA-receptor-dependent neuronal activation (Yamagata *et al.*, 1993; Chen *et al.*, 1995; Planas *et al.*, 1995; Breder *et al.*, 1996) suggesting an enhanced role for COX-2 products in activity-dependent plasticity in the central nervous system. Prostanoids may be important mediators of the spinal hyperexcitability and neuronal plasticity observed following inflammation. Evidence supporting this theory has come from a number of behavioural and electrophysiological studies which have suggested that prostanoids play a greater role in spinal nociceptive processing in rats with a peripheral inflammatory lesion than in normal rats (Yaksh, 1982; Malmberg and Yaksh, 1992; Chapman and Dickenson, 1992)

Recently, (during the course of this work), a number of groups have described increases in COX-2 mRNA levels in the rat spinal cord following the induction of FCA-induced monoarthritis (Beiche *et al.*, 1996; Hay *et al.*, 1997). In the previous Chapter it was demonstrated that COX-2 protein is present in the dorsal horn of normal rats in a location consistent with processing nociceptive input. If COX-2 is involved with spinal nociceptive processing then we might expect an increase in spinal COX-2 protein levels following the development of a peripheral inflammatory lesion.

In this chapter, COX-2 protein levels are examined in the spinal cord of normal and FCA-monoarthritic rats using Western blotting techniques. Other enzymes in the arachidonic acid pathway ($cPLA_2$, COX-1, 5-LO, and PGI_2-synthase) are also examined in the spinal cord of normal and FCA-monoarthritic rats.

5.2 Methods.

5.2.1 Induction of FCA-Monoarthritis.

Thirty-two male Wistar rats were briefly anaesthetised with 4% halothane until their hindlimb withdrawal reflex was absent. A total volume of 0.15ml FCA or paraffin oil was injected subcutaneously in 50µl volumes at 3 sites around the right tibio-tarsal joint. Animals were monitored daily as described in section 2.2. Rats were killed by barbiturate overdose (sodium thiopentone, 500mg/kg, i.p.) between 6 hours at 14 days following inoculation, then transcardially perfused with prewarmed 0.9% NaCl. Spinal cord segments L1 to S1 were rapidly excised and frozen in liquid nitrogen as described in section 2.5.

5.2.2 Western Blotting.

Whole or hemisected lumbar spinal cord samples from perfused normal and monoarthritic rats were prepared for Western blotting (section 2.8.1). Denatured protein samples were separated (10-12.5μg protein/lane) by SDS-PAGE using a 10% acrylamide resolving gel (200V for 1.5 hours) and transferred to a nitrocellulose membrane using a semi-dry transfer unit. The membranes were pre-incubated for 2 hours in blocking buffer, then incubated overnight in primary antibody. The following day, blots were washed in TTBS then incubated in a peroxidase-conjugated secondary antibody (1:1000). All incubations were conducted at room temperature on a plate shaker. The protein bands were then visualised using an ECL-detection kit following the manufacturer's instructions (Amersham). The optical densities of the protein bands were determined using a GS-70 scanning densitometer and analysed using Molecular Analyst software (Bio-Rad) as described in section 2.8.5.

Statistical analysis of optical densities was by 1-way ANOVA, followed by Duncan's multiple range post-hoc test. Values are expressed as mean O.D. ± SEM.

5.2.3 Antibodies and Standards.

COX-1 and -2 antisera and purified protein standards were purchased from Cayman Chemical, and were described and characterised in Chapter 4. The rabbit polyclonal 5-lipoxygenase antiserum was raised against purified human leukocyte antigen and was a kind gift from Dr J. Evans (Merck Frosst, Canada). The $cPLA_2$ antisera was purchased from Santa Cruz and the anti-PGI_2-synthase was purchased from Oxford Biomedical Research Inc.

5.3 Results.

5.3.1 Cyclooxygenase-2, but not Cyclooxygenase-1, is upregulated in the Spinal Cord of FCA-Monoarthritic Rats.

In Chapter 4 the presence of both COX-1 and COX-2 in lumbar spinal cord protein extracts obtained from normal rats was determined by Western blot analysis using specific COX-1 and COX-2 antisera (Figure 4.3).

The development of FCA-induced monoarthritis is associated with an increase in the amount of COX-2 protein present in the spinal cord. Figure 5.1 shows portions of 2 Western blots obtained by incubating lumbar spinal cord protein obtained from normal rats and from monoarthritic rats at various time-points following the induction of arthritis with anti-COX-1 (panel A) and anti-COX-2 (panel B).

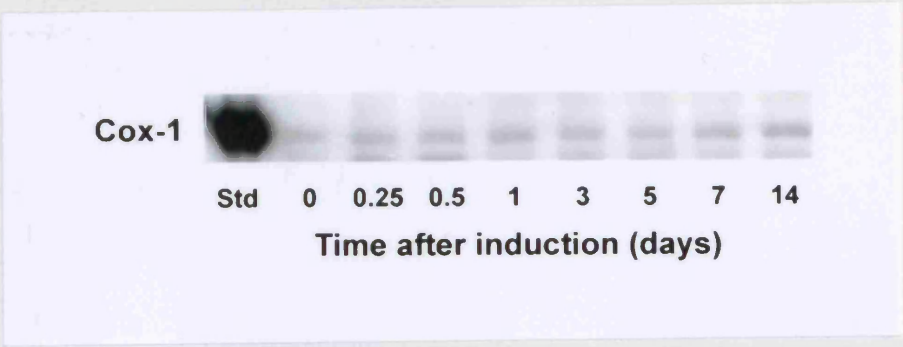
Spinal COX-1 protein levels (panel A) did not change over the 14 day time-course of the experiment, whereas marked increases in spinal cord COX-2 protein levels were observed between 6 and 24 hours following the induction of the arthritic lesion (panel B). Spinal COX-2 protein returned to basal levels by day 3 and stayed at this basal level for the remainder of the experiment.

The observations made in these blots were confirmed using scanning densitometry. The graph in Figure 5.2 quantifies COX-1 and COX-2 protein levels in the spinal cord of normal and monoarthritic rats. Each time-point represents the mean optical density of COX-1 and COX-2 protein bands from 4 animals. There was a rapid increase in the amount of COX-2 protein in the spinal cord. Spinal COX-2 protein levels were significantly elevated at 6 hours following the induction of arthritis ($p<0.05$). COX-2 levels were maximally elevated (3.5 fold increase) between 12 and 24 hours ($p<0.01$), then returned to a basal level of expression by day 3. COX-1 protein levels did not significantly ($p>0.05$) change over the time-course of the experiment.

There was a small (5%), non-significant ($p>0.05$) increase in spinal cord COX-2 protein levels 1 day following the injection of 0.15ml paraffin vehicle (data not shown).

FIGURE 5.1.

A. COX-1.



B. COX-2.

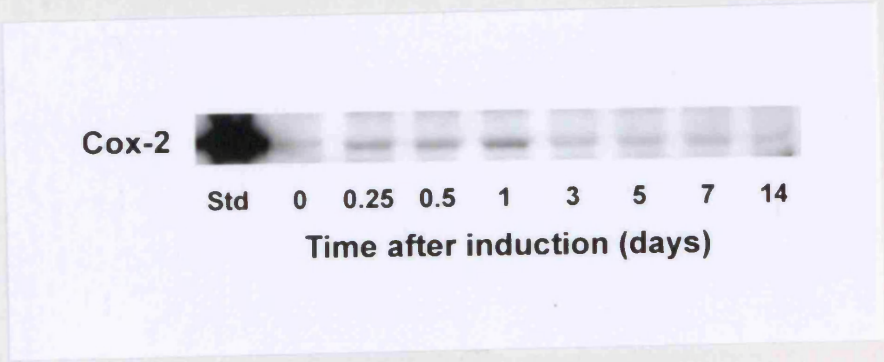


Figure 5.1 COX-2, but not COX-1, is Upregulated in the Spinal Cord of FCA-Monoarthritic Rats. This figure shows two representative Western blots obtained by incubating rat lumbar spinal cord samples (12.5μg protein/lane) obtained from normal and monoarthritic rats with anti-COX-1 (panel A) or anti-COX-2 (panel B). Purified COX-1 and COX-2 (1μg protein/lane) have been included in the left hand lanes to act as markers. Spinal COX-1 protein levels (panel A) did not change over the 14 day time course of the experiment, whereas marked increases in spinal cord COX-2 protein levels (panel B) can be seen 0.25, 0.5, and 1 day following the induction of FCA-monoarthritis at the ankle joint.

FIGURE 5.2.

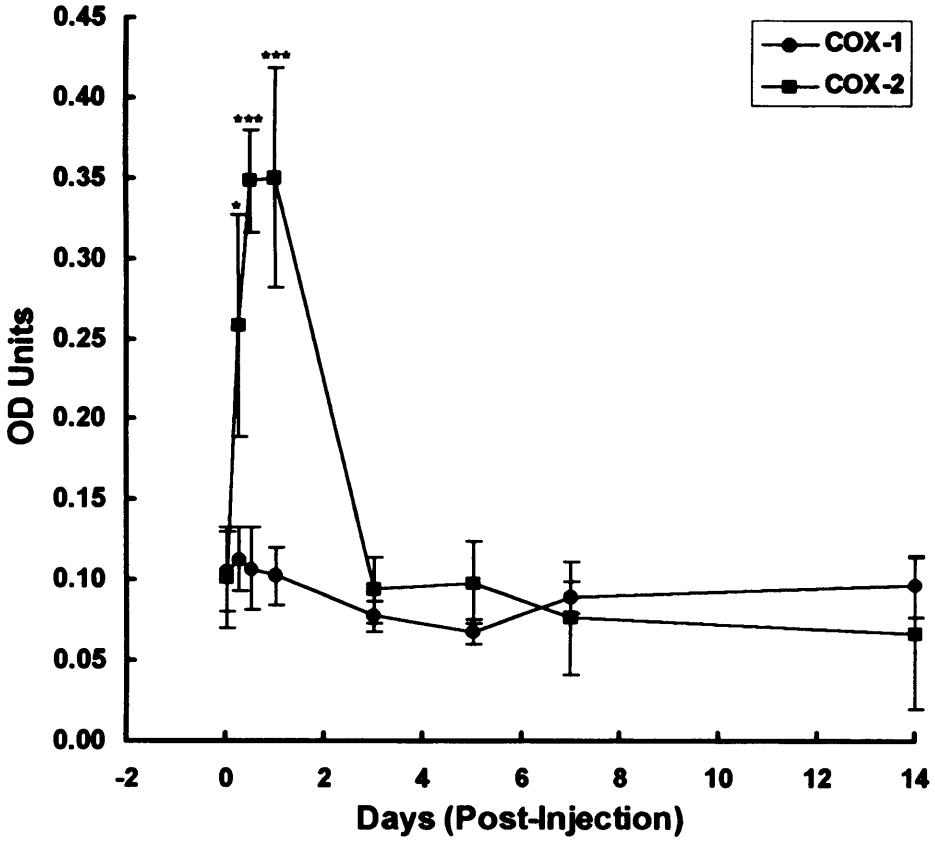


Figure 5.2 Upregulation of COX-2 in the Spinal Cord of FCA-Monoarthritic Rats. This graph shows the mean optical density (± SEM) of COX-1 and COX-2 protein bands in Western blots of spinal cord protein samples obtained from normal and monoarthritic rats (n=4). Significant increases in spinal COX-2 levels can be seen during the acute phase of the inflammatory response at 6, 12 and 24 hours (*** $p<0.01$; * $p<0.05$, ANOVA). No significant change in COX-1 protein levels were observed during this time course ($p>0.05$).

5.3.2 The Time-course of Spinal Cyclooxygenase-2 Upregulation Mirrors the Time-course of Behavioural Hyperalgesia.

As discussed in Chapter 3, the development of FCA-induced monoarthritis is associated with behavioural hyperalgesia. Animals guard the injected limb for 24 hours following the injection of FCA. The reduction in weight bearing was most marked at 1 day post-inoculation (Gait Score = 2.5 ± 0.29; $p<0.05$), a time-point which coincided with maximal inflammation of the joint. Weight bearing returned to normal by 3 days following the induction of arthritis, despite the persistence of inflammation at the ankle joint (see Figures 3.1 & 3.3).

This time-course of behavioural hyperalgesia mirrors the time-course of COX-2 upregulation in the spinal cord of monoarthritic rats (Figure 5.3). Both spinal COX-2 protein levels and gait scores were significantly elevated at 6 hours, peaked at 24 hours and returned to normal by 3 days following the development of inflammation.

FIGURE 5.3.

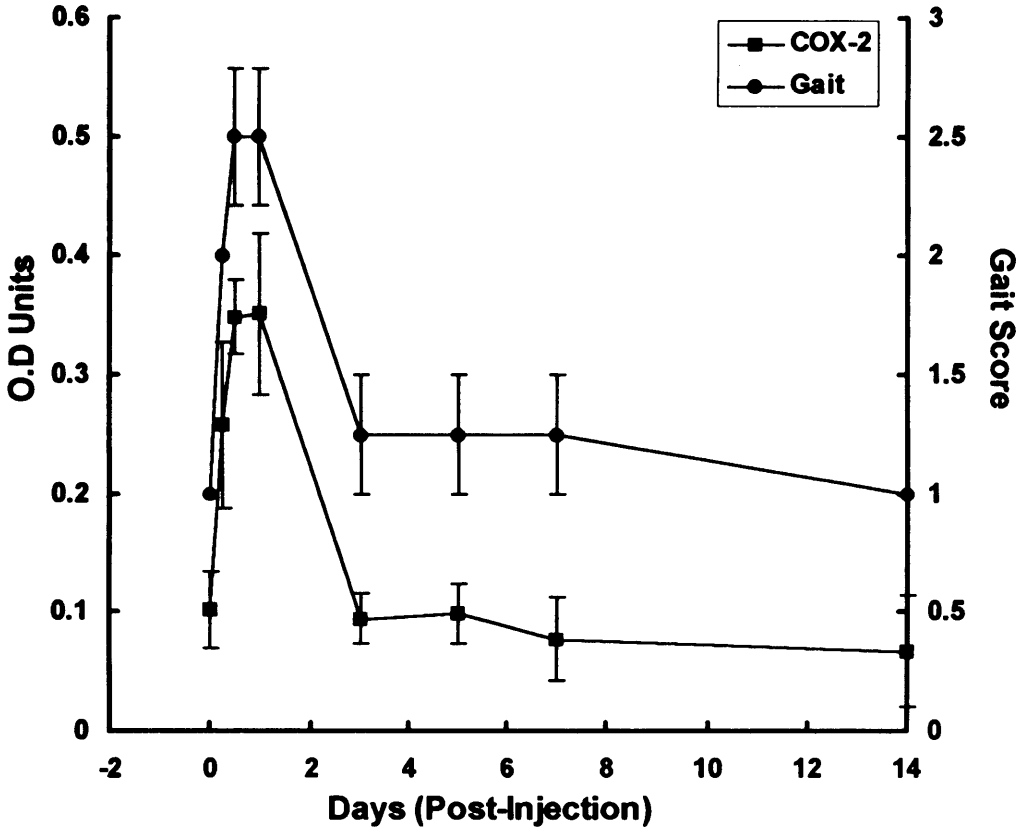


Figure 5.3 The Time-course of Behavioural Hyperalgesia Mirrors the Time-course of COX-2 Induction in the Spinal Cord of Monoarthritic Rats. This figure compares the mean optical density ± SEM of COX-2 protein bands in Western blots of spinal cord protein samples obtained from normal and monoarthritic rats (n=4) with the mean gait score ± SEM of normal and monoarthritic rats (n=4). A gait score of 1 indicates normal movement, whereas a gait score of 3 indicates no weight bearing on the injected limb. COX-2 induction in the spinal cord occurs during a time when monoarthritic rats exhibit characteristic pain behaviour, i.e guarding of the injected limb.

5.3.3 Cyclooxygenase-2 Upregulation occurs Bilaterally in the Lumbar Spinal Cord of FCA-Monoarthritic Rats.

Afferents from the rat ankle joint terminate predominantly in the mid to lower spinal cord segments (L3-L6). COX-2 protein levels were compared between different spinal segmental levels in samples obtained from normal rats and rats one day following induction of FCA-monoarthritis.

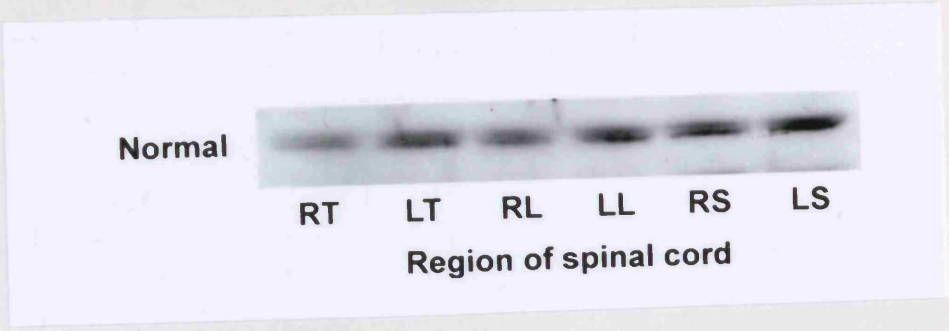
In the normal rat spinal cord COX-2 protein appeared equally distributed throughout the thoracic, lumbar and sacral regions (Figure 5.4, panel A). In the 1 day monoarthritic rat, more COX-2 protein was present in the lumbar spinal cord than in the thoracic or sacral regions (Figure 5.4, panel B). These observations were quantified using scanning densitometry. In the normal rat spinal cord, no significant differences were detected between COX-2 protein levels in different regions of the spinal cord ($p>0.05$; ANOVA, Figure 5.5, panel A). No significant differences were detected between COX-2 protein levels in different regions of the FCA-monoarthritic spinal cord ($p>0.05$; ANOVA, Figure 5.5, panel B). However, a direct comparison between thoracic and lumbar regions revealed significantly more COX-2 was present in the lumbar region than in the thoracic region ($p<0.05$, students t-test).

No differences were observed between COX-2 protein levels in the ipsilateral (left) and contralateral (right) spinal cord. Figure 5.6 shows two portions of Western blots obtained by incubating bisected lumbar spinal cord samples from normal and 1 day monoarthritic rats with anti-COX-2. Ipsilateral spinal cord samples obtained from monoarthritic rats contain more COX-2 than spinal cord samples obtained from normal rats (Panel A). Similarly, contralateral spinal cord samples obtained from monoarthritic rats also contain more COX-2 protein than samples obtained from normal rats (Panel B).

This bilateral increase was quantified by scanning densitometry (Figure 5.7). Monoarthritic rats were found to contain significantly more COX-2 protein in both the ipsilateral (3.2 fold; $p<0.01$) and contralateral (2.3 fold; $p<0.05$) lumbar spinal cord than normal rats.

FIGURE 5.4.

A. Normal Rats.



B. FCA-Monoarthritic Rats.

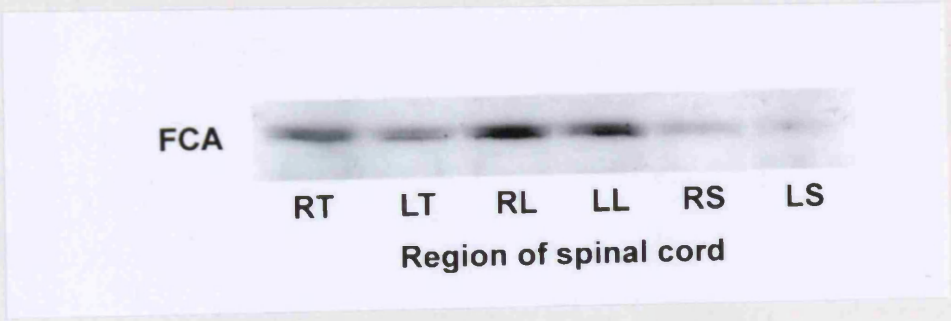
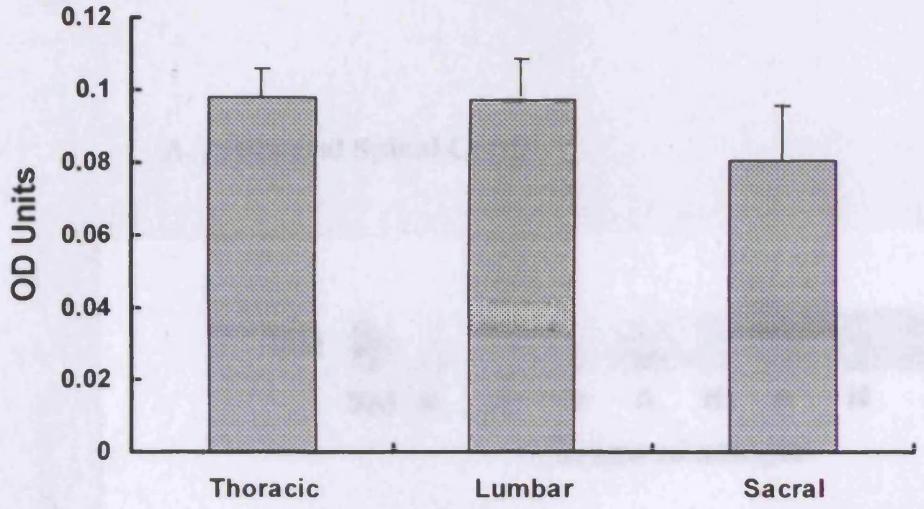


Figure 5.4 Upregulation of COX-2 is Localised to the Lumbar Spinal Cord of Monoarthritic Rats This figure shows two representative Western blots obtained by incubating rat hemisected spinal cord protein samples obtained from different segmental levels (10μg protein/lane) from normal (Panel A) and 1 day monoarthritic rats (Panel B) with anti-COX-2. In panel A, COX-2 protein appears equally distributed throughout the thoracic (RT, LT), lumbar (RL, LL) and sacral (RS, LS) regions of the normal rat spinal cord. The lumbar region of the monoarthritic rat spinal cord (Panel B) contains more COX-2 protein than the thoracic and sacral regions. No differences in COX-2 protein levels can be seen between the ipsilateral (left) and contralateral (right) spinal cord.

FIGURE 5.5.

A. Normal Rats.



B. FCA-Monoarthritic Rats.

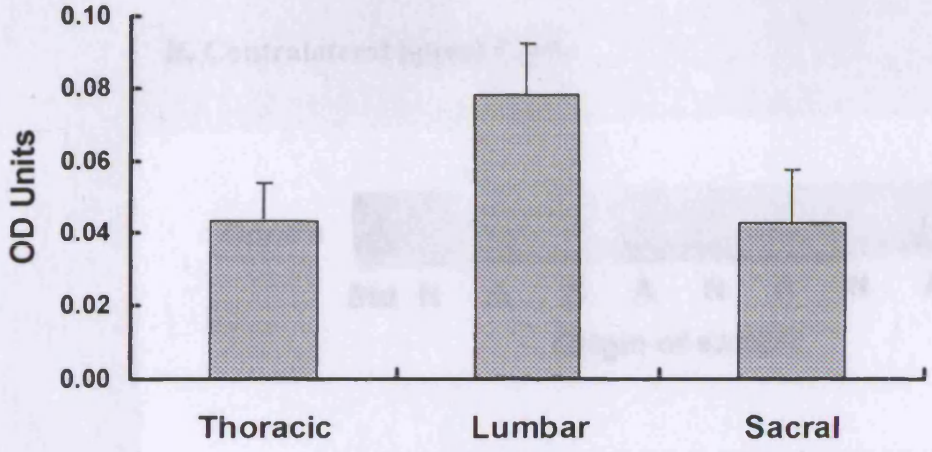
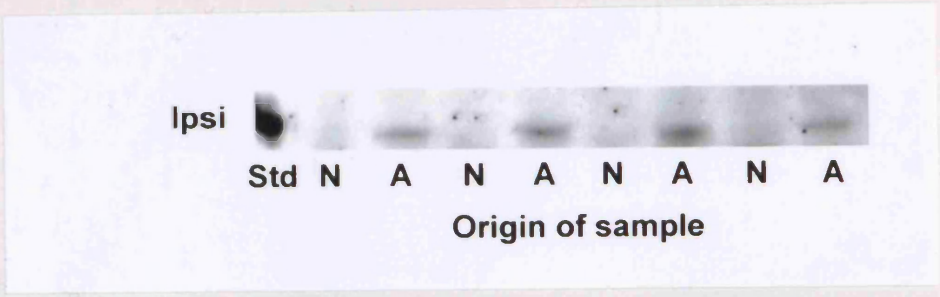


Figure 5.5 COX-2 Protein Levels in the Lumbar Spinal Cord of FCA-Monoarthritic Rats are not Significantly Greater than Thoracic and Sacral Levels. This figure shows the densitometric analysis of COX-2 Western blots of hemisected spinal cord protein samples obtained from different segmental levels from normal and 1 day monoarthritic rats. Samples obtained from ipsilateral and contralateral samples were combined. COX-2 protein was equally distributed throughout the thoracic, lumbar and sacral regions of the normal rat spinal cord. The levels of COX-2 protein in the lumbar spinal cord of the FCA-monoarthritic rat were not significantly greater than the levels of COX-2 thoracic and sacral segments ($p>0.05$, ANOVA).

FIGURE 5.6.

A. Ipsilateral Spinal Cord.



B. Contralateral Spinal Cord.

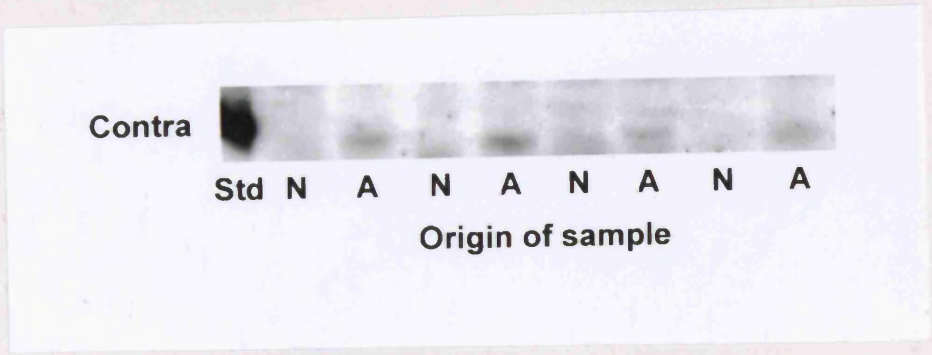


Figure 5.6 The Bilateral Upregulation of COX-2 in the Lumbar Spinal Cord. This figure shows two Western blots obtained by incubating ipsilateral (Panel A) and contralateral (Panel B) lumbar spinal cord samples (10μg protein/lane) obtained from normal and monoarthritic rats with anti-COX-2. Purified COX-2 (1μg protein/lane) has been included to act as a marker. Both ipsilateral and contralateral spinal cord samples obtained from monoarthritic rats contain more COX-2 than spinal cord samples obtained from normal rats.

FIGURE 5.7.

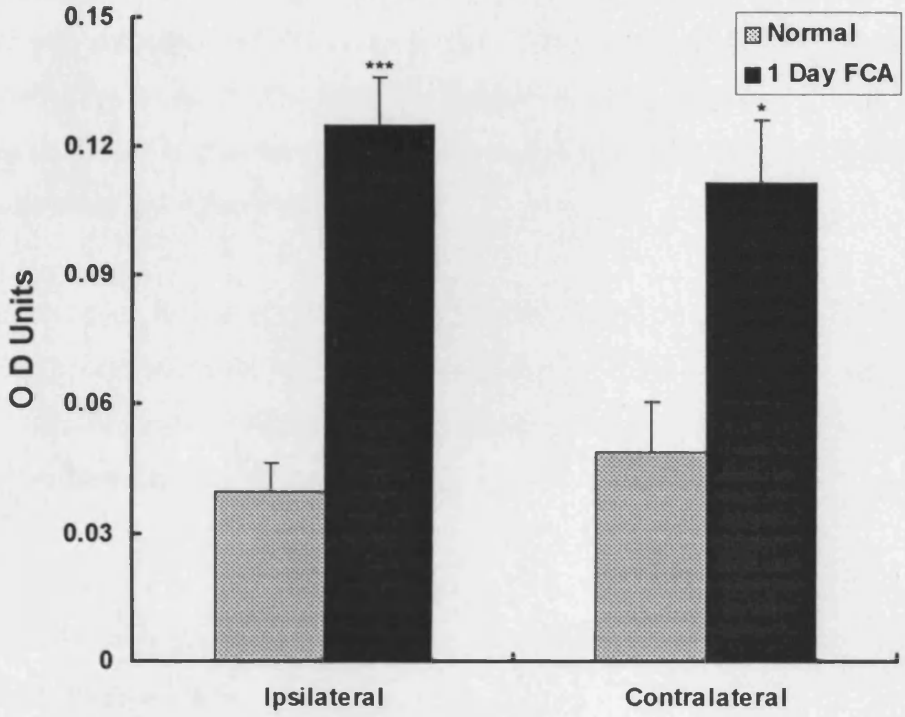


Figure 5.7 The Bilateral Upregulation of COX-2 in the Lumbar Spinal Cord. This figure shows the mean optical density (± SEM) of COX-2 protein bands in Western blots of bisected lumbar spinal cord protein samples obtained from normal and 1 day monoarthritic rats (n=4). Both ipsilateral (3.2 fold) and contralateral (2.3 fold) spinal cord samples were found to contain significantly more COX-2 protein than spinal cord samples obtained from normal rats (* $p<0.05$, *** $p<0.01$, students t-test).

5.3.4 Spinal Cyclooxygenase-2 Upregulation is not Associated with a Concomitant Increase in Spinal Cytosolic Phospholipase A_2.

The increase in COX-2 protein is not associated with an increase in $cPLA_2$ synthesis in the spinal cord of monoarthritic rats. Panel A (Figure 5.8) shows a representative Western blot obtained by incubating lumbar spinal cord protein obtained from normal and monoarthritic rats with anti-$cPLA_2$ (1:250). The antisera detected a single protein band at approximately 110kD. The levels of $cPLA_2$ did not change following inflammation over the 14 day time-course. $cPLA_2$ is therefore present in rat spinal cord but is not upregulated in response to the development of inflammation.

This observation was verified using scanning densitometry. Panel B (Figure 5.8) compares COX-2 protein levels with $cPLA_2$ protein levels in the spinal cord of normal and monoarthritic rats (n=4). No significant changes in $cPLA_2$ protein levels were detected over the fourteen day time-course.

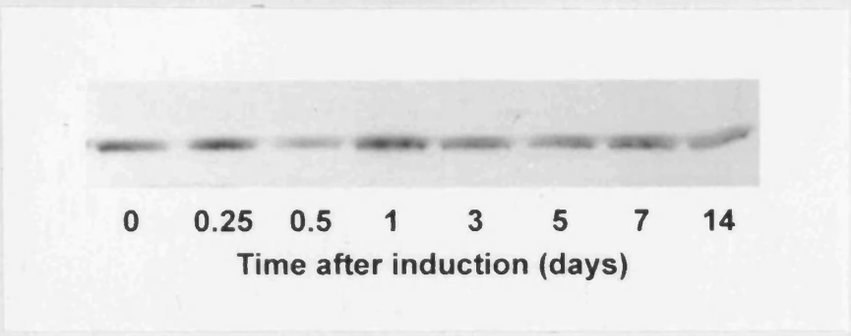
5.3.5 Prostaglandin I_2-Synthase is Upregulated in the Spinal Cord of FCA-Monoarthritic Rats.

Western blots obtained by incubating lumbar spinal cord samples obtained from normal and monoarthritic rats with anti-PGI_2-synthase (1:500) indicate that spinal PGI_2-synthase protein is rapidly upregulated following the development of FCA-monoarthritis. A single protein band was detected by the anti-PGI_2-synthase at approximately 60kD (Figure 5.9, panel A). A biphasic increase in spinal cord PGI_2-synthase protein levels was observed between 6 and 12 hours and 3 and 5 days following the induction of arthritis.

The observations made in this blot were confirmed using scanning densitometry. Figure 5.9 (panel B) shows densitometric analysis of PGI_2-synthase in the spinal cord of normal and monoarthritic rats. There was a rapid increase in the amount of PGI_2-synthase protein in the spinal cord, levels were significantly elevated 6 hours following the induction of arthritis ($p<0.05$), levels returned to normal by 1 day then the increases at 12 hours, 3 and 5 days proved not significant at the 5% level.

FIGURE 5.8

A.



B.

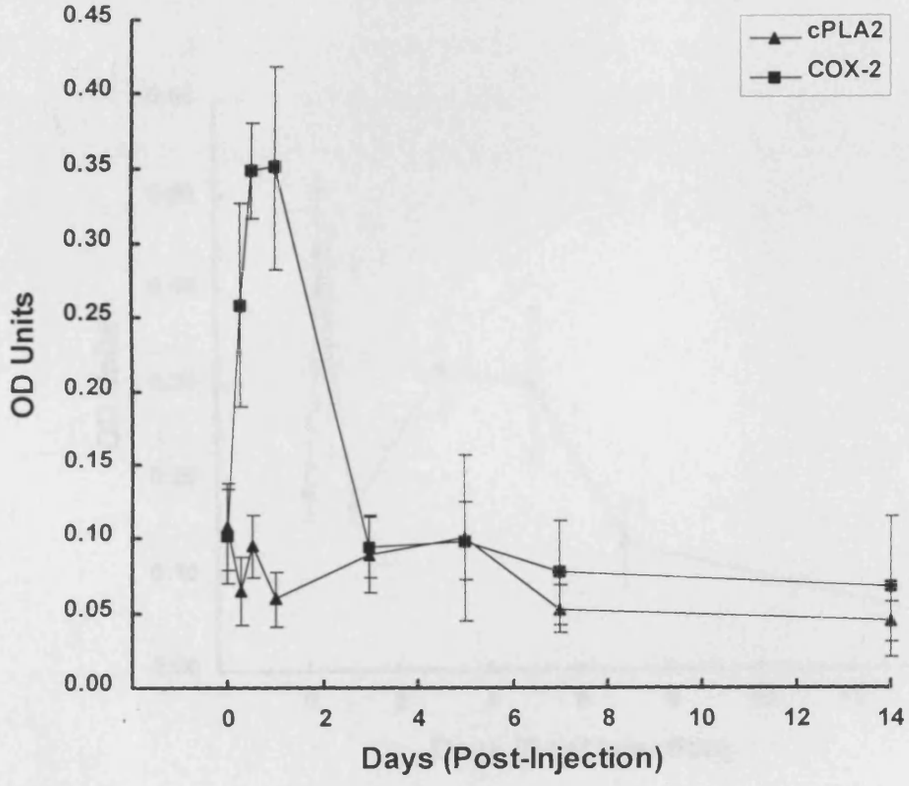
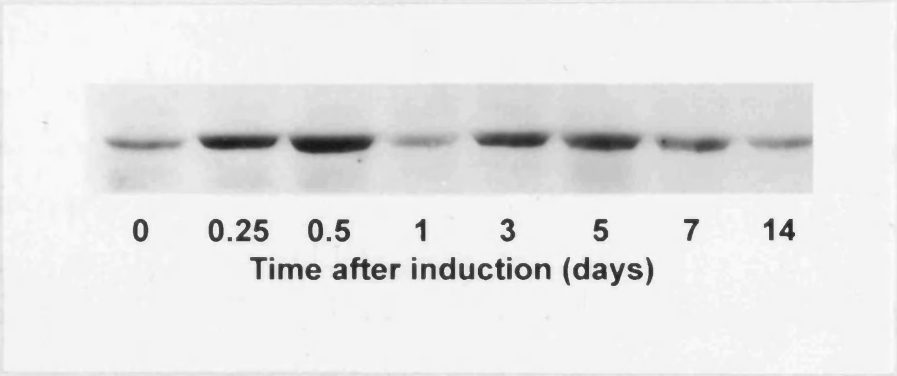


Figure 5.8 Spinal COX-2 Upregulation is not Associated with $cPLA_2$ Upregulation. Panel A shows a representative Western blot obtained by incubating rat spinal cord samples (12.5μg protein/lane) obtained from normal and monoarthritic rats with anti-$cPLA_2$. Spinal $cPLA_2$ protein levels did not change over the 14 day time-course of the experiment Panel B compares the mean optical density (± SEM) of $cPLA_2$ and COX-2 protein bands in Western blots of spinal cord protein samples obtained from normal and monoarthritic rats (n=4). It confirms that COX-2 upregulation is not associated with significant changes in $cPLA_2$ protein levels in the spinal cord of monoarthritic rats.

FIGURE 5.9.

A.



B.

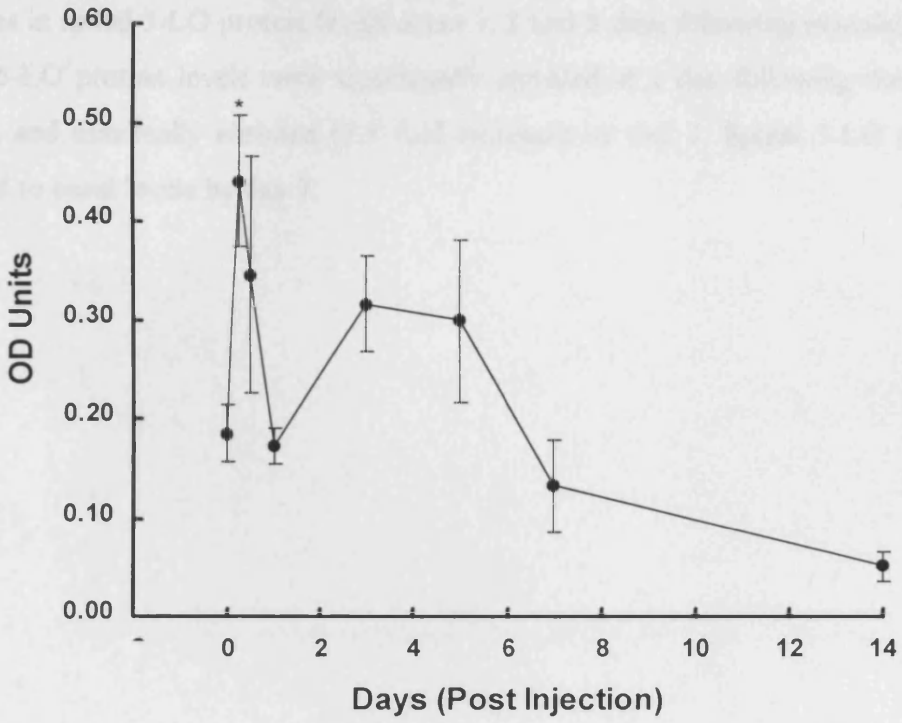


Figure 5.9 Upregulation of PGI_2-Synthase in the Spinal Cord following Induction of FCA-Monoarthritis. Panel A shows a representative Western blot obtained by incubating lumbar spinal cord protein samples (12.5µg protein/lane) from normal and monoarthritic rats with anti-PGI_2-synthase. Panel B shows the mean optical density (± SEM) of PGI_2-synthase protein bands (n=4). A significant increase in PGI_2-synthase protein levels occurs 6 hours following the induction of arthritis ($p<0.05$). The increases observed at 12 hours, 3 and 5 days were not significant ($p>0.05$).

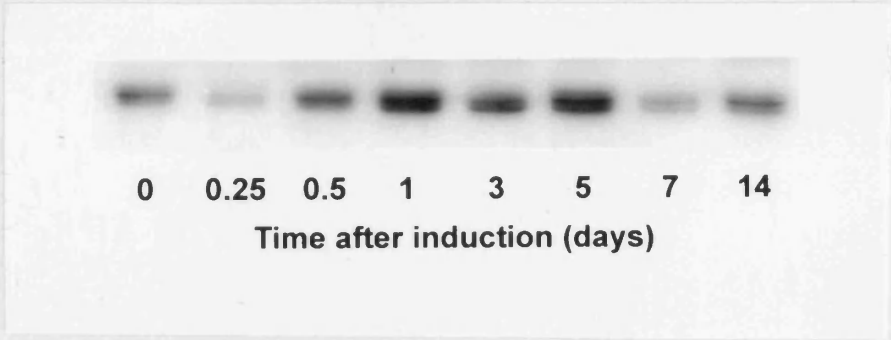
5.3.6 5-Lipoxygenase is upregulated in the Spinal Cord of FCA-Monoarthritic Rats.

The development of FCA-induced arthritis is associated with an increase in the amount of 5-LO, as well as COX-2 protein, in the spinal cord. Figure 5.10 shows a Western blot obtained by incubating lumbar spinal cord protein obtained from normal rats and from monoarthritic rats with anti-5-LO (panel A). Increases in spinal 5-LO levels were observed between 1 and 5 days following the induction of the arthritic lesion.

The observations made in this blot were quantified using scanning densitometry. Panel B of Figure 5.10 show a densitometric analysis of 5-LO protein in the spinal cord of normal and monoarthritic rats. The increase in 5-LO occurs at a later time-point to the increase in spinal COX-2, as COX-2 levels return to normal there is an increase in 5-LO levels. Significant increases in spinal 5-LO protein levels occur 1, 3 and 5 days following inoculation ($p<0.05$). Spinal 5-LO protein levels were significantly elevated at 1 day following the induction of arthritis and maximally elevated (1.8 fold increase) by day 3. Spinal 5-LO protein levels returned to basal levels by day 7.

FIGURE 5.10.

A.



B.

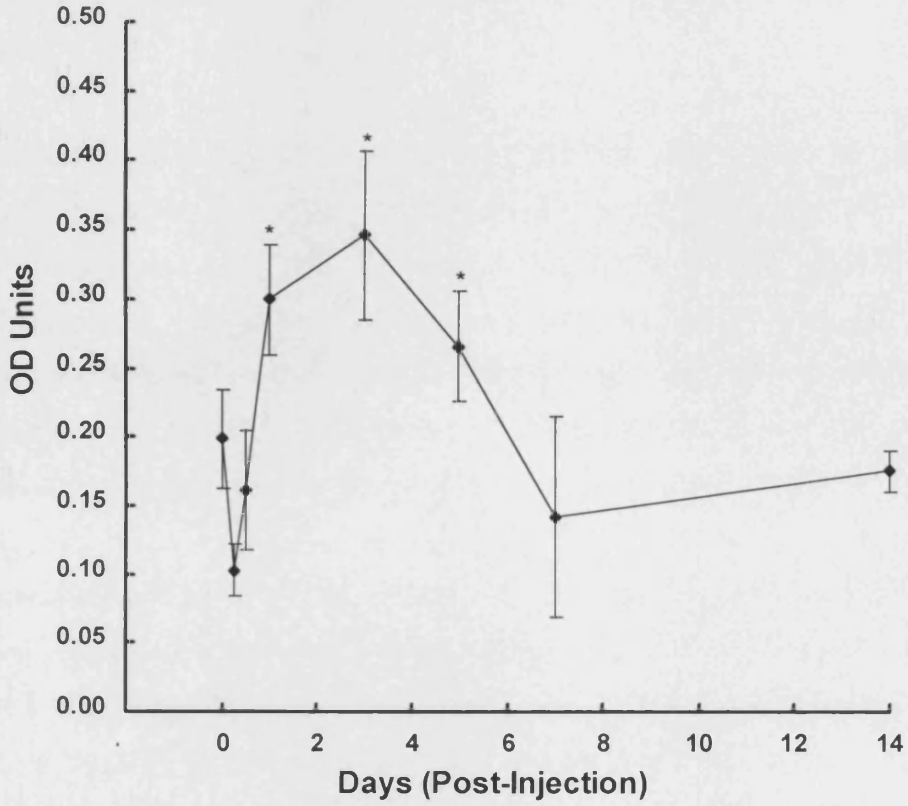


Figure 5.10 Upregulation of 5-LO in the Spinal Cord of FCA-Monoarthritic Rats. Panel A shows a representative Western blot obtained by incubating lumbar spinal cord protein samples (12.5μg protein/lane) from normal and monoarthritic rats with anti-5-LO. Panel B shows the mean optical density (± SEM) of 5-LO protein bands obtained from normal and monoarthritic rats (n=4). Significant increases in the levels of 5-LO protein are seen 1, 3 and 5 days following the induction of arthritis ($p<0.05$).

5.4 Discussion.

These data provide the first demonstration that COX-2 and 5-LO protein levels increase in a time-dependent manner in the rat lumbar spinal cord following the development of peripheral inflammation. Significant increases in spinal COX-2 protein levels were observed between 6 and 24 hours, with the maximal increase (3.5 fold) occurring between 12 and 24 hours following inflammation. Spinal COX-2 protein returned to basal levels by day 3 and was sustained at this level for the remainder of the time course. As spinal COX-2 returned to basal levels, spinal 5-LO protein levels increased. Significant increases in 5-LO levels occurred between 1 and 5 days following inoculation and were maximally elevated at day 3 (1.8 fold increase). COX-1 protein levels did not change over the time-course of the experiment.

The activation of $cPLA_2$ is generally regarded as the rate-limiting step in the COX pathway as it controls the liberation of free arachidonic acid. In the spinal cord, however, COX-2 protein upregulation is not accompanied by an increase in $cPLA_2$ protein levels. Existing intracellular $cPLA_2$ may be phosphorylated in response to inflammation, which will lead to increased liberation of arachidonic acid. Alternatively, there may be activation and/or upregulation of a different phospholipase, for example, activated PLD is involved in arachidonic acid liberation in rat peritoneal mast cells (Ishimoto *et al.*, 1994).

The time-course of COX-2 protein upregulation in the spinal cord agrees with studies which examined COX-2 mRNA levels using a similar model of FCA-induced monoarthritis (Beiche *et al.*, 1996; Hay *et al.*, 1997). Beiche *et al.* (1996) analysed the levels of COX-2 mRNA in the rat lumbar spinal cord using the reverse transcription-polymerase chain reaction (RT-PCR) technique. They demonstrated that COX-2 mRNA levels were maximally raised at 6 hours (2-fold increase) and returned to basal levels 3 days following a unilateral intraplantar injection of FCA. Using the same model of inflammation and Northern blot analysis of COX-2 mRNA, Hay *et al.* (1997) reported a 3-fold increase in COX-2 mRNA levels between 2 and 4 hours following inflammation. COX-2 mRNA levels returned to basal levels by 1 day. The time-course of spinal COX-2 mRNA upregulation associated with FCA-monoarthritis at the ankle joint has not yet been fully determined. Lucy Donaldson (personal communication, 1997) reports a marked increase in COX-2 mRNA levels (9-fold, compared with control rats) one day following inflammation, which

may indicate our model of inflammation induces longer-term changes in the spinal cord than intraplantar injection of FCA.

The induction of COX-2 in the spinal cord of FCA-monoarthritic rats coincides with the development of behavioural hyperalgesia. Pain behaviour subsides at the same time that spinal COX-2 levels return to normal, despite the continuing inflammation at the joint. This indicates a potential role for prostaglandins synthesised by spinal COX-2 in the generation and/or maintenance of behavioural hyperalgesia. Indeed it has been demonstrated that spinal administration of PGE_2 (Minami *et al.*, 1994), PGD_2 (Horiguchi *et al.*, 1986; Uda *et al.*, 1990; Minami *et al.*, 1994) and $PGF_{2\alpha}$ (Taiwo and Levine, 1986) to conscious animals evoked characteristic pain-behaviour. Furthermore, PGE_2-binding sites and PGD-synthase have been located in the superficial laminae of the rat spinal cord (Matsumura *et al.*, 1992; Urade *et al.*, 1989; Vesin *et al.*, 1995) indicating possible roles for endogenous PGE_2, $PGF_{2\alpha}$ and PGD_2 in spinal nociceptive processing. The mRNA encoding the PGE_2 receptor EP_3 has been localised in neurones of the brain and sensory ganglia but was not present in the spinal cord (Sugimoto *et al.*, 1992, 1994). EP_2 mRNA, however, has been detected in the superficial laminae of rat spinal cord (Kawamura *et al.*, 1997). Recent work has determined the presence of mRNA encoding EP_2, EP_4 and DP, but not EP_3, receptors in normal rat spinal cord (Lucy Donaldson, personal communication, 1997) These three prostaglandin receptors are all coupled to the G-protein 'G_s', activation of G_s stimulates adenylate cyclase and increases the level of intracellular cAMP. Increases in intracellular cAMP cause activation of protein kinases, and subsequent protein phosphorylation. This may be one mechanism by which prostaglandins promote spinal hyperexcitability. Indeed, inhibition of adenylate cyclase has been shown to inhibit PGE_2-induced potentiation of the response to bradykinin in sensory neurones (Cui and Nicol, 1995).

Yamagata *et al.* (1993) were the first to describe upregulation of COX-2 in the central nervous system. They measured COX-2 mRNA by Northern blot analysis in the hippocampus and cortex following electroconvulsive seizure. COX-2 mRNA levels were induced within 30 minutes, peaked between 1 and 2 hours, remained elevated over 8 hours and returned to basal levels by 24 hours. COX-2 protein levels also increased and peaked between 3 and 4 hours following seizure. COX-2-li was found in neuronal, but not glial, cells in the somata and proximal dendrites. No nuclear labelling was observed. A similar time-course and distribution of COX-2 induction in the brain has also been described

following transient focal ischemia (Planas *et al.*, 1995), kainate injection (Chen *et al.*, 1995; Adams *et al.*, 1996; Marcheselli and Bazan, 1996) and the administration of bacterial endotoxin (Breder and Saper, 1996). Yamagata *et al.* (1993) discovered that pretreatment with the NMDA receptor antagonist MK801 (1mg/kg., i.p., administered 1 hour prior to seizure) completely blocked the induction of COX-2 and so hypothesised that neuronal COX-2 expression was induced by NMDA-dependent synaptic activity.

Activation of NMDA receptors in hippocampal dentate gyrus cells evoked a dramatic increase in the levels of the immediate early genes *c-fos* and *NGFI-A* in approximately 90% of neurones (Lerea *et al.*, 1995). There was also a 2-fold increase in the levels of arachidonic acid in stimulated neurones 10 minutes following activation, which further increased to 6-fold at 90 minutes (Lerea *et al.*, 1997). The transcription of both *c-fos* and *NGFI-A* were reduced by direct and indirect PLA_2 inhibitors, which prevent the liberation of arachidonic acid from membrane phospholipids, indicating a role for the arachidonic acid pathway in mediating NMDA-receptor-dependent regulation of gene expression (Lerea *et al.*, 1995). Pretreatment with NSAIDs reduced the induction of *c-fos* but not *NGFI-A* mRNA. Aspirin inhibited the NMDA-receptor-mediated increase in *c-fos* mRNA by 58% (Lerea *et al.*, 1995). The inhibition of NMDA-receptor-dependent transcription may be mediated by COX-2, or prostaglandins produced by COX-2, as the selective COX-2 inhibitor NS-398 reduced the number of spinal cord neurones expressing *c-fos* mRNA by 56% following carrageenan-induced unilateral inflammation (Buritova *et al.*, 1996). By contrast, inhibition of LO activity in dentate gyrus neurones reduced the induction of *NGFI-A* but not c-*fos* mRNA transcripts (Lerea *et al.*, 1995). The different effects of COX and LO inhibitors indicate they act at separate sites downstream of the NMDA receptor. The NMDA-receptor-dependent transcription of *c-fos* and *NGFI-A* may therefore be mediated by separate intracellular pathways: *c-fos* transcription via the COX pathway and *NGFI-A* transcription via the LO pathway.

Prostaglandins, synthesised by COX-2, may play a role in the development of wind-up, an NMDA receptor mediated event. The administration of the selective COX-2 inhibitor SC58125 dose-dependently reduced the wind-up of a spinal nociceptive reflex, evoked by repetitive high-threshold electrical stimulation of the sural nerve (Willingale *et al.*, 1997). This suggests that prostaglandins contribute to nociceptive processing once the spinal cord has been rendered hyperexcitable, i.e. following NMDA receptor activation. The liberation

of free arachidonic acid following NMDA-receptor activation is associated with the increased synthesis and release of $PGF_{2\alpha}$ (230%), PGE_2 (180%) but not PGD_2 from dentate gyrus neurones (Lerea *et al.*, 1997). As previously described, NMDA-induced transcription of c-*fos* was significantly reduced by NSAIDs such as aspirin and the selective COX-2 inhibitor NS-398 (Lerea *et al.*, 1995 Buritova *et al.*, 1996). Transcription of *c-fos* was restored in a dose-dependent manner by the addition of $PGF_{2\alpha}$, but not PGE_2 or PGD_2, in presence of NMDA. The COX-2 mediated NMDA-receptor dependent transcription of *c-fos* in dentate gyrus neurones may therefore be mediated, at least in part, by $PGF_{2\alpha}$ (Lerea *et al.*, 1997). The mechanism by which $PGF_{2\alpha}$ induces *c-fos* transcription has not yet been determined. The mRNA encoding the $PGF_{2\alpha}$ receptor (FP) has been detected in high levels in rat brain, activation of the FP receptor stimulates IP_3 turnover, increases intracellular calcium levels and phosphorylates several protein kinases (Watanabe *et al.*, 1994). Activated protein kinases are then able to phosphorylate and activate transcription factors such as cAMP response element binding protein (CREB) which may possibly initiate the transcription of *c-fos* mRNA. A protein which colocalises with the FP receptor has recently been cloned and sequenced, and has been termed 'Prostaglandin $F_{2\alpha}$ Receptor Regulatory Protein' (FPRP). FPRP is thought to have a negative regulatory function, the 3'-untranslated region of the gene contains 7 copies of the Shaw-Kamens (ATTTA) destabilisation sequence which suggests the mRNA is highly-regulated with a short half-life, although the presence of this protein in the central nervous system has not been examined (Orlicky and Nordeen, 1996). The induction of FPRP and subsequent inhibition of FP receptor activity may possibly be one mechanism by which the activity of the NMDA receptor is moderated following inflammation.

The discovery that COX-2 is located on the inner surface of the nuclear membrane (Morita *et al.*, 1995) makes prostaglandins ideal candidates to either act directly as transcription factors or bind to and activate other transcription factors. This is one area of research that has not been widely studied. Yu *et al.* (1995) found that PGD_1, PGD_2, and PGJ_2 (but not PGE_2, $PGF_{2\alpha}$, or PGI_2) activated the transcription factor PPAR (α, δ and γ). PPAR is a soluble nuclear receptor that once associated with the 9-*cis*-retanoic acid receptor binds to HRE sites in the promoter regions of target genes and initiates transcription of that gene. This could be one possible mechanism by which *c-fos* transcription is mediated by COX activity. It is likely that prostaglandins may activate other, as yet unidentified, transcription

factors and this information might provide a valuable key to understanding the role of prostaglandins in the dynamic gene regulation observed during spinal hyperexcitability.

The expression of *c-fos*, like COX-2, is rapidly upregulated in a time-dependent manner in the spinal cord following the development of inflammation. The intraplantar administration of carrageenan or formalin evokes a high level of expression of spinal *c-fos* mRNA by 30 minutes which remains at maximal levels for at least 2 hours, then returns to basal levels by 8 hours following injection (Draisci and Iadarola, 1989; Ji and Rupp, 1997). Following the intraplantar administration of FCA there is an initial increase in the numbers of neurones expressing Fos-li in the superficial dorsal horn which later extends to the deeper dorsal horn. The number of neurones expressing Fos-li is significantly raised at 1.5 hours and remains elevated over a 4 day time-course (Ma and Woolf, 1996). Neurones which express Fos-li are predominantly found in the superficial (laminae I-II) and deep dorsal horn (laminae III-VI) and around the central canal (lamina X) (Buritova *et al.*, 1996), a distribution which closely resembles the distribution of COX-2-li (see Chapter 4) and coincides with regions which receive direct nociceptive input. This has lead to Fos-li being commonly used as a marker of nociceptive neuronal activity. The similar nociceptive-specific distribution and induction of COX-2-li may therefore make COX-2 a valid marker of nociceptive activity.

The major difference between the induction of *c-fos* and COX-2 following unilateral inflammation is that *c-fos* is initially induced in ipsilateral spinal cord neurones, contralateral expression of *c-fos* occurs at a later time point (Draisci and Iadarola, 1989; Buritova *et al.*, 1996; Ji and Rupp, 1997). The bilateral induction of spinal COX-2 mRNA (Beiche *et al.*, 1996; Ichitani *et al.*, 1997; Donaldson, personal communication, 1997) and protein may occur concommitantly, although earlier time points following inflammation need to be examined. The bilateral induction of COX-2 is surprising as gene activation and/or protein upregulation following unilateral inflammation is predominantly restricted to the ipsilateral spinal cord. The sensory neuropeptide SP is upregulated in the ipsilateral dorsal root ganglia (Noguchi *et al.*, 1988; Smith *et al.*, 1992; Donaldson *et al.*, 1992; Hanesch *et al.*, 1993, 1995) and the ipsilateral spinal cord (Sluka *et al.*, 1993; Galeazza *et al.*, 1995) following the development of a unilateral inflammation. The mRNA encoding the NK-1 receptor is also upregulated in the ipsilateral dorsal horn following unilateral inflammation (McCarson and Krause, 1994; Abbadie *et al.*, 1996). A number of studies have shown that there is a

delayed contralateral upregulation of SP mRNA and SP-li in dorsal root ganglia (Donaldson *et al.*, 1995) and spinal cord (Mapp *et al.*, 1993) but this contralateral upregulation occurs at a much later time-point than the contralateral induction of COX-2. It will be intriguing to discover the mechanism for this rapid bilateral upregulation of COX-2 protein.

A number of bilateral changes in the spinal cord have been described following peripheral unilateral stimulation. Porro *et al.* (1991) examined the metabolic activity of the rat spinal cord following a unilateral injection of 5% formalin. Radiolabelled 2-deoxyglucose was injected intravenously, then rats were killed at various time points, and the spinal cord removed and sectioned. Transverse spinal cord sections were placed against X-ray film with known concentrations of ^{14}C, in order to quantify the amount of isotope present in various regions. They discovered that there was a rapid increase in the rate of glucose utilisation in both the ipsilateral and contralateral dorsal horn between 2 and 20 minutes following injection. The increases in metabolic activity were observed primarily in laminae I-II, but also in laminae IV-VI and X of the spinal cord.

Enzymatic activity has also been shown to increase bilaterally following unilateral inflammation. Nicotinamide adenine dinucleotide phosphate (NADPH) is an electron donor for many enzymes, including nitric oxide synthase (NOS) and cytochrome P-450 reductase (Bredt *et al*, 1991). Several of these enzymes can reduce soluble nitroblue tetrazolium dye to an insoluble product, labelling neurones with NADPH-diaphorase activity. Following the unilateral injection of carrageenan, there was a bilateral time-dependent increase in NADPH-diaphorase labelling in laminae I-III, IV-VII and X of the lumbar spinal cord between 2 and 24 hours (Traub *et al.*, 1994). The development of FCA-monoarthritis was also associated with an increase in spinal NADPH-d labelling, although the lateralisation of the labelling was not examined, the labelling followed an identical time-course to the induction of spinal COX-2 protein (Blair Grubb, personal communication, 1997). Neuronal NOS (nNOS) is upregulated bilaterally following the injection of formalin into the rat hind paw. Bilateral increases in the number of nNOS-li neurones were reported 1, 2, 4 and 7 days following injection, no results obtained from earlier time points were described. The number of nNOS-li was consistently higher on the ipsilateral side than on the contralateral side (32 vs. 25 nNOS-li neurones at 1 day following inflammation) (Lam *et al.*, 1996). The regulation of the NOS pathway is similar to the regulation of the COX pathway, and it is interesting that NOS is also upregulated bilaterally. Nitric oxide (NO) has been shown to

modulate COX-2 activity, possibly through binding to the heme complex in COX (Salvemini *et al.*, 1993) and prostaglandins modulate NOS activity, possibly indirectly through increases in levels of cAMP. In the periphery, both inducible NOS (iNOS) and COX-2 are upregulated following inflammation (Vane et al., 1994).

CREB is a transcription factor which, once phosphorylated, binds to CRE in the promoter region of many genes. The consensus sequence for CRE (T/G T/A CGTCA) is present in the promoter regions of many genes involved in nociception, including *c-fos*, somatostatin and dynorphin. Interestingly, the rat COX-2 gene also contains a modified form of the CRE sequence (Sirois *et al.*, 1993) indicating COX-2 transcription may be regulated, at least in part, by phosphorylated CREB (pCREB). In the normal rat spinal cord unphosphorylated CREB-li is present in neurones throughout the grey matter whereas only a few pCREB-li neurones are present. Following the uniplantar injection of 5% formalin, marked increases in the number of pCREB-li neurones were detected. The phosphorylation of CREB was maximal between 10 and 30 minutes and levels returned to normal by 2 hours following injection. Intense neuronal labelling was observed in regions associated with nociception, particularly in the medial aspect of laminae I-II, lamina V and around the central canal (especially in the dorsal region of lamina X and the medial part of lamina VI). Some weaker pCREB-li was observed in the lateral aspect of laminae I-II, laminae III, IV and VII. Labelling was not restricted to the ipsilateral spinal cord but again appeared equally in the contralateral spinal cord (Ji and Rupp, 1997). The activation of pCREB, like *c-fos* and COX-2, may be partly due to NMDA receptor activation as pretreatment with MK801 (3.5mg/kg, i.p., administered 20 minutes prior to formalin injection) reduced the number of pCREB-li neurones by approximately 50%.

These bilateral changes all involve post-translational modifications, i.e. increased enzymatic activity and transcription factor signalling, rapid contralateral increases in gene transcription have not been described. Ipsilateral spinal cord neurones receive direct monosynaptic inputs from nociceptive afferents from the inflamed limb, whereas contralateral spinal cord neurones receive only indirect input from the limb, through connecting interneurones and descending spinal tracts. Ji and Rupp (1997) suggested that inflammation-induced synaptic input to the contralateral side was sufficient to evoke post-translational effects, such as the phosphorylation and activation of constitutive transcription factors such as CREB, but was not sufficient to induce contralateral transcription of genes.

The question still remains as to how COX-2 is induced bilaterally, while *c-fos* and other nociceptive-specific factors are predominantly induced ipsilaterally following development of unilateral inflammation. COX-2 is constitutively expressed in the normal rat spinal cord, which indicates that COX-2 mRNA transcription is operational under normal conditions, whereas Fos-li is virtually absent in normal rat spinal cord (Abbadie and Besson, 1992). The promoter region of rat COX-2 mRNA has a number of regulatory elements including CRE (Sirois *et al.*, 1993). Small contralateral increases in the levels of activated transcription factors (such as pCREB) may therefore be sufficient to evoke an increase in COX-2 mRNA transcription, but not sufficient to induce transcription of *c-fos*. Following the development of FCA-monoarthritis there is a marked bilateral increase in COX-2 mRNA levels in the spinal cord (Lucy Donaldson, personal communication, 1997), which may demonstrate a bilateral increase in transcription of COX-2 mRNA. A recent paper also reported a bilateral increase in spinal COX-2 mRNA levels within 6 hours following inflammation (Ichitani *et al.*, 1997). This may indicate the increase in COX-2 mRNA levels occurs concomitantly in the ipsilateral and contralateral spinal cord, although these results may not be reliable as the authors did not fix the spinal cord tissue prior to *in situ* hybridisation. Alternatively, the increase in COX-2 mRNA levels may be a result of enhanced post-transcriptional stabilisation of COX-2 mRNA which is normally rapidly degraded due to the high number of destabilisation sequences (AUUUA) in the 3'-untranslated region (Feng *et al.*, 1993). It has been shown that COX-2 mRNA levels are upregulated by translation inhibitors such as cycloheximide (Kujuba *et al.*, 1991; O'Banion *et al.*, 1992; Ristimäki *et al.*, 1994), pactamycin and puromycin (Ristimäki *et al.*, 1994), which are thought to act by inhibiting mRNA degradation factors. The contralateral induction of COX-2 may therefore be due to post-translational inactivation of COX-2 mRNA degradation factors, which would extend the half-life of COX-2 mRNA and thus increase the levels of COX-2 protein. It would be interesting to identify the factors responsible for COX-2 mRNA degradation and determine whether they are rapidly inactivated following inflammation.

The development of FCA-monoarthritis is accompanied by an increase in the levels of circulating corticosterone (Donaldson *et al.*, 1994). Glucocorticoids inhibit COX-2 activity, by acting both directly through binding to Glucocorticoid Response Elements (GRE) in 5'-promoter region of COX-2, and indirectly through the induction of lipocortin-1 which inhibits arachidonic acid release (via inhibition of PLA_2 activity or phospholipid binding). Yamagata *et al.* (1993) found that pretreatment with the glucocorticoids dexamethasone

(4mg/kg, i.p.) or hydrocortisone (50mg/kg, i.p.) reduced the induction of cortical COX-2 mRNA following electroconvulsive seizure. The increase in endogenous glucocorticoids may therefore inhibit COX-2 and return spinal levels back to baseline values. COX-2 is an auto-inactivating enzyme which spontaneously inactivates after approximately 13,000 cycles (Marshall *et al.*, 1987). Sustained synthesis of COX protein is therefore required in order to maintain high levels. COX activity decreases as intracellular calcium levels increase (Volterra *et al.*, 1995) and the ratio between COX and LO metabolism of arachidonic acid is modified. In hippocampal astroglial cultures, COX activity is three times higher than LO activity in calcium free medium, but as calcium concentration increases the activity of COX falls to 60% of LO activity. Sustained increases in intracellular calcium levels may therefore redistribute arachidonic acid metabolism by decreasing COX and increasing LO activity.

There is an increase in the levels of spinal 5-LO protein between 1 and 5 days following the induction of FCA-monoarthritis. This increase in the levels of 5-LO may indicate increased production of leukotrienes during this period. Comparatively little is known about the actions of 5-LO and leukotrienes in the central nervous system. Following cell stimulation, 5-LO is translocated to a nuclear membrane binding site (Rouzer *et al.*, 1988) where it associates with FLAP (Dixon *et al.*, 1990). The activated enzyme catalyses the formation of 5-HPETE from free arachidonic acid which is then converted to LTA_4, LTB_4, LTC_4, LTD_4 and LTE_4 (Samuelsson, 1983; Henderson, 1994). The major leukotrienes present in the rat central nervous system are LTB_4 and LTC_4 (Dembinska-Kiéc *et al.*, 1984; Lindgren *et al.*, 1984; Shimizu *et al.*, 1987). It has recently been demonstrated that LTB_4 directly binds to and activate PPARα. Activated PPARα can regulate the expression of genes involved in the oxidation of fatty acids (Devchand *et al.*, 1996). The upregulation of spinal 5-LO following inflammation may ultimately result in the transcription of genes involved in eicosanoid oxidation and inactivation, returning spinal fatty acid levels back to basal levels. This dynamic self-regulatory mechanism may therefore control the duration of the eicosanoid-mediated spinal response to inflammation.

Chapter 6. Stimulus-Evoked Release of Prostaglandins from the Rat Spinal Cord.

6.1 Introduction.

The predominant prostaglandins in the rat central nervous system are PGD_2, PGE_2 and PGI_2, as measured by gas chromatography and/or mass spectrometry of brain homogenates (Abdel-Halim, *et al.*, 1977; Abdel-Halim and Änggård, 1979; Chaplin and Hillier, 1979). It has been demonstrated that these prostaglandins have the ability to modulate the activity of a number of receptors and associated ion channels and thus increase neuronal excitability and facilitate the release of other neurotransmitters. PGD_2 and PGE_2 (but not PGI_2 or $PGF_{2\alpha}$) facilitate the release of CGRP from the spinal cord *in vitro* (Andreeva and Rang, 1993). Similarly, stimulation of spinal cord slices and cultured sensory neurones with either PGD_2 or PGE_2 (but not $PGF_{2\alpha}$) increased the release of SP (Vasko *et al.*, 1993), presumably through potentiation of calcium currents (Nicol *et al.*, 1992) and suppression of potassium currents (Fowler *et al.*, 1985; Nicol *et al.*, 1997).

The spinal administration of prostaglandins to conscious animals evokes characteristic behavioural hyperalgesia, such as that seen following inflammation (Horiguchi *et al.*, 1986; Taiwo and Levine, 1986; Uda *et al.*, 1990; Minami *et al.*, 1994). Evidence suggests that spinally applied NSAIDs may only be effective at producing analgesia when the spinal cord has been rendered hyperexciTable, for example following inflammation, as they are not effective at reducing behavioural or neuronal responses to acute noxious stimuli in the normal animal (Yaksh, 1982; Malmberg and Yaksh, 1992; Chapman and Dickenson, 1992; Willingale *et al.*, 1997). These data suggest prostaglandins may modulate nociceptive processing in rats with peripheral inflammatory lesions, but not following noxious stimulation in normal non-arthritic rats.

The *in vivo* release of neurotransmitters/modulators from the spinal cord has been studied using a variety of techniques, including:

- perfusion of the surface of the spinal cord with aCSF and analysing superfusate samples (e.g. Yaksh, 1984; Coderre *et al.*, 1990).

- infusion of aCSF into microdialysis tubes implanted in the spinal cord and analysing dialysate samples (e.g. Malmberg and Yaksh, 1995; Yang *et al.*, 1996).
- insertion of antibody microprobes into the spinal cord in order to localise the site of release (e.g. Duggan *et al.*, 1988, 1991).

Each technique has advantages and disadvantages. For example, spinal superfusion cannot be used to study the release of compounds which are not lipophilic nor can it provide information about the site of release. It is, however, a relatively non-invasive technique and may cause less neuronal damage than the insertion of microdialysis tubing and antibody microprobes into the spinal cord. Microdialysis tubing is inserted into the spinal cord, under anaesthesia, usually 2-7 days prior to sample collection, in order to minimise surgical trauma. Sample collection can therefore be performed on conscious animals, thus reducing any interference from anaesthesia or surgery. The disadvantages of microdialysis, is that it can only be used to study the release of small molecules which are readily exchanged through the membrane, and the percentage recovery of even small compounds is often very low. The insertion of the relatively large tubing (outer diameter: 300 μm) is likely to cause substantial neuronal damage and may cause long-term alterations in cell phenotype, which may lead to artificial results. Antibody microprobes are much finer in diameter than microdialysis tubing and insertion will not cause as much tissue damage. The main advantage of using antibody microprobes is that it is possible to localise the site of release and detect very small stimulus-evoked release of the compound.

Using these techniques, the levels of a number of neurotransmitters/modulators have been shown to increase in response to peripheral inflammation and/or noxious stimulation. Increased levels of glutamate, and other excitatory amino acids, have been measured in the spinal cord following inflammation (Sluka *et al.*, 1992; Malmberg and Yaksh, 1992b; Sorkin *et al.*, 1992). Yaksh *et al.* (1980) demonstrated the release of SP from the spinal cord of anaesthetised cat following electrical stimulation of high threshold nociceptors. Noxious mechanical or thermal stimulation also evokes the release of SP (Kuraishi *et al.*, 1985; Duggan *et al.*, 1988; Schaible *et al.*, 1990, 1992) and CGRP (Schaible *et al.*, 1992) from the spinal cord.

If prostaglandins modulate spinal nociceptive processing then increased levels should be released from the spinal cord following peripheral activation of nociceptors. Ramwell *et al.*

(1966) demonstrated that high-intensity electrical stimulation of a hindlimb increased the release of prostaglandins from the frog spinal cord. A number of other stimuli have been identified which can also evoke the release of prostaglandins from the spinal cord, such as noxious thermal stimulation (Coderre *et al.*, 1990) and acute inflammation (Malmberg and Yaksh, 1995; Yang *et al.*, 1996; Sorkin and Moore, 1996).

The sites of sensory neuropeptide release in the spinal cord have been localised to the superficial dorsal horn a region coinciding with the termination site of nociceptive afferents (Duggan *et al.*, 1992; Schaible *et al.*, 1990, 1992). The localisation of COX-2 in these regions (Chapter 4) indicates that prostaglandins may also play an important role in spinal nociceptive processing. Furthermore, the dynamic upregulation of COX-2 following the development of a peripheral inflammation (Chapter 5) indicates an enhanced role for spinal prostaglandins under these conditions.

This chapter examines the relationship between peripheral stimulation and the associated synthesis and release of spinal prostaglandins in normal rats and rats with peripheral inflammatory lesions. Prostaglandin release is studied using spinal cord superfusion and radioimmunoassay of superfusate samples and antibody microprobe techniques.

The basic principle of radioimmunoassay is the competition between radiolabelled and non-labelled prostaglandins for specific antibody binding sites. The concentration of antibody and radiolabelled tracer is known and fixed, whereas the concentration of unlabelled prostaglandin is known and varied (standards) or unknown (samples). In the absence of unlabelled prostaglandin (zero dose) a large proportion of the tracer binds to the antibody. As the concentration of the unlabelled prostaglandin increases, the tracer is displaced from the antibody. The degree of displacement can then be quantified by separating the antibody-bound fraction from the free fraction, and determining the radioactivity of either fraction. A standard curve is then constructed and the concentration of the prostaglandin in a sample determined.

The principle of the antibody microprobe technique is similar to that of the immunoassay, namely exploitation of the competition between radiolabelled and unlabelled prostaglandins for specific antibody binding sites. Anti-PGE_2 is immobilised on fine glass microprobes which are then inserted into the rat spinal cord for 20 minutes. The probes are then

incubated in radiolabelled PGE_2, which binds to unoccupied antibody-binding sites on the probe. The probes are placed against X-ray film for an appropriate length of time, and the film then developed and the images of the probes densitometrically analysed. Regions of reduced radioligand binding are taken to represent sites of endogenous PGE_2 binding.

6.2 Methods.

6.2.1 Surgical Preparation.

Male Wistar rats (200-300g) were anaesthetised with sodium thiobutabarbital (120mg kg^{-1} i.p., R.B.I., section 2.2) and surgically prepared as described in section 2.4.1. Anaesthesia was closely monitored throughout the experiment by testing: a) flexion withdrawal reflexes, which had to be absent; b) corneal blink reflexes, which had to be absent; and c) arterial blood pressure which was maintained between 100-120mmHg. Further anaesthetic was administered (i.p.) when necessary. A laminectomy exposed spinal cord segments T13 to L6. The exposed dura mater was covered with cotton wool dampened with 0.9% saline.

For experiments involving electrical stimulation of the tibial or sural nerve, a cut was made through the skin on the dorsal surface of the right hindlimb between the ankle and the knee. The skin was reflected and tied with a series of sutures to form a pool. The tibial or sural nerve was located under biceps femoris, carefully dissected free from surrounding tissue, then ligated and cut distally. The nerve was carefully placed over silver wire stimulating electrodes. The pool was filled with warmed liquid paraffin to prevent tissue dehydration.

6.2.2 Spinal Cord Superfusion.

Siliconised polythene catheters (100-150μm: O.D.) were attached to a Gilson Minipuls 3 peristaltic pump and filled with warmed aCSF. A small hole was cut rostrally in the dura mater and the inflow catheter carefully inserted into the subdural space until the tip was positioned over the lower lumbar enlargement (L6). The outflow catheter was then inserted into the subdural space just caudal to the hole in the dura mater. aCSF was perfused over the surface of the spinal cord at a rate of approximately 225μl min^{-1}. The spinal cord was perfused for one hour prior to sample collection. Samples were then collected from the outflow catheter at an approximate rate of 200μl min^{-1} in 1ml aliquots into siliconised

polypropylene tubes and placed on ice with 200μM indomethacin. The collection rate for 1ml superfusate was recorded for every sample. Samples containing blood were discarded. Samples were stored at -80°C until analysis by radioimmunoassay.

6.2.3 Radioimmunoassay.

Radioimmunoassays were developed for PGE_2, PGD_2, $PGF_{2\alpha}$ and 6-Keto-$PGF_{1\alpha}$ as described in section 2.6.1., and used to determine the concentration of prostaglandins in spinal superfusate samples, spinal cord homogenates or plasma samples (see section 2.5). Fixed amounts of radiolabelled prostaglandin (5000 d.p.m.) and prostaglandin antisera (at a dilution which bound 50% of the tracer in the absence of unlabelled prostaglandin) were incubated overnight at 4°C with either known concentrations of unlabelled prostaglandin (serial dilutions, from 3 pg to 2 ng) or unknown samples. All samples were assayed in duplicates. The antibody-bound fraction was then separated from the unbound fraction by the addition of polyethylene glycol 8000 (final concentration 12.5% w/v). The tubes were centrifuged for 1 hour at 3,500 r.p.m. at 4°C. When tritiated prostaglandin tracer was used (^{3}H-PGD_2, -$PGF_{2\alpha}$ and -6-Keto-$PGF_{1\alpha}$), 1ml of the supernatant was removed, mixed with 1ml water and 4ml 'Ultima-Gold' scintillation fluid (Packard) and counted for 3 minutes. When iodinated prostaglandin tracer was used (^{125}I-PGE_2), the supernatant was discarded, excess fluid removed from the tube, and the pellet counted for 1 minute. The mean radioactivity (cpm/dpm) of the standard and sample duplicates were calculated. The percentage bound for each sample and standard was then determined. A sigmoidal standard curve was generated and the prostaglandin content of samples determined using 'Graphpad Prizm' software.

Spinal superfusate samples were standardised to pg PG ml^{-1} min^{-1}, plasma samples to ng PG ml^{-1} plasma and spinal cord samples to ng PG g^{-1} wet weight spinal cord. The minimum sensitivity of each assay determined by calculating the amount of unlabeled prostaglandin required to shift zero binding by two standard deviations.

Statistical analysis of samples in individual experiments was by ANOVA. A statistical comparison of mean control versus stimulated samples was by student's t-test. Significance is $p<0.05$. Values are expressed as mean ± SEM.

6.2.4 Antibody Microprobes.

Prostaglandin E_2 antibody microprobes were prepared as described in section 2.4.3. The competition between labelled and unlabelled PGE_2 and the antibody binding sites on the microprobe was assessed using *in vitro* microprobes. Microprobes were inserted into siliconised capillaries containing PBS or PGE_2 (0, 1 x 10^{-6}, 1 x 10^{-7} M) and incubated at 37°C for 30 minutes. The probes were washed in ice-cold PBS-Tween, then inserted into siliconised capillaries containing ^{125}I-PGE_2 (6000cpm) and incubated overnight at 4°C. The following day probes were rewashed for 15 minutes. The tips of the probes were broken off and placed in counter tubes. The radioactivity was counted for 1 minute per tube. The inhibition of radioligand binding by unlabelled PGE_2 was then calculated.

Barbiturate-anaesthetised rats (120mg kg^{-1} i.p., R.B.I.), were surgically prepared as previously described (section 2.4.1 and 2.4.3.3). Areas of the ipsilateral spinal cord receiving input from the knee joint were identified using a carbon filament extracellular micro-electrode. A pair of antibody microprobes were then inserted to a depth of 2.5mm into these regions for 20 minutes. Probes were then removed from the spinal cord, washed, then incubated overnight at 4°C in siliconised capillaries containing ^{125}I-PGE_2 (6000cpm). Microprobes were then rewashed. The tips of the probes were attached to a sheet of paper and exposed to X-ray film for 14 days at room temperature.

The films were developed and the images of the probes densitometrically analysed by Professor H.-G. Schaible (University of Würzburg, Germany) using an analysis program developed by Dr. G. McConnell (University of Edinburgh). Every 10μm of the microprobe is scanned and the grey scale value determined. The values are then averaged for each group of probes to give a mean (± S.E.M.) grey scale value for each point. Since the probes are tapered, the thick shaft of the probe has a high grey scale value, whilst the tips of the probes have a lower value. This results in a typically shaped graph with a low grey scale value at the right hand side of the graph, reflecting the faint tip of the microprobe, and a high grey scale value at the left hand side, reflecting the denser shaft of the probe. Increased release of the ligand results in an upward deviation of the line (indicating reduced radioligand binding).

A statistical comparison of the control versus treatment groups of probes was performed using multiple student's t-tests between corresponding points on each set of probes. Significance is $p<0.05$.

6.2.5 Stimulation Protocols.

The stimulus-dependent release of spinal prostaglandins was investigated using a range of electrical, mechanical and inflammatory stimuli. Stimulation periods typically lasted for 20 minutes and were separated by 1 hour periods of no stimulation.

In initial superfusion experiments mechanical stimuli were applied in an order of increasing intensity. Firstly, the ankle was brushed with a soft-bristled brush at a frequency of 0.5Hz, then innocuous (25 psi) and noxious pressures (50 psi) were applied to the joint a pair of pneumatic callipers which generated reproducible, graded pressure. These stimuli were applied to the ankle joint for 10 seconds, followed by 10 seconds with no stimulation over the 20 minute period. In antibody microprobe experiments, and later superfusion experiments, only noxious pressure (50 psi) was applied to the ankle joint.

Similarly, in initial superfusion experiments electrical stimuli were applied to the sural nerve in order of increasing intensity and frequency. Firstly, a low intensity, low frequency (0.2V at 0.1Hz) stimulus was applied to the sural nerve via silver wire electrodes in a square-wave pulse of 500μs duration using a programmable stimulator (Hi-Med). The second period of electrical stimulation involved the use of a higher intensity stimulus at the same low frequency (10-20V at 0.1Hz). The intensity of stimuli used was one which elicited a nociceptive reflex from biceps femoris. The final stimulation period involved high intensity stimulation at a higher frequency of stimulation (10-15V at 1Hz). In antibody microprobe experiments, and later superfusion experiments, only high intensity high frequency (10-15V at 1Hz) electrical stimuli were applied to either the sural or tibial nerve.

An acute inflammation was induced by the intraarticular injection of 0.07ml 4% kaolin followed by the injection of 0.07ml 2% carrageenan into the right knee joint of barbiturate anaesthetised rats (Sodium thiobutabarbital: 120mg kg^{-1}; i.p). FCA-monoarthritic rats were also used in the spinal superfusion experiments 1 day post-injection (when COX-2 levels

were maximally elevated and 3 days post-injection (when COX-2 levels had returned to basal levels).

6.2.6 Tissue Collection and Purification.

Spinal cord samples for prostaglandin extraction procedures were obtained from barbiturate-killed rats (sodium thiopentone: 400mg kg^{-1}; i.p.) which were transcardially perfused with warmed 0.9% NaCl at a pressure of 100mmHg until all blood was removed. Lumbar spinal cord sections, L1 to S1 were rapidly exposed, excised and frozen in liquid nitrogen. Samples were stored at -80°C until sample purification using Amprep C18 Minicolumns (Amersham). Spinal cord samples were processed and purified as described in section 2.7. then analysed by radioimmunoassay.

Plasma samples for prostaglandin extraction procedures were obtained from heparinised (0.15ml; 750 units heparin) barbiturate-anaesthetised rats (section 2.2). Indomethacin (200μM) was added to the blood sample to prevent further prostaglandin synthesis. The blood sample was immediately centrifuged at 3500 rpm for 10 minutes at 4°C, and the plasma supernatant removed and stored at -80°C until analysis by radioimmunoassay.

6.2.7 Antisera and Radioligands.

Anti-PGE_2, Anti-$PGF_{2\alpha}$, and anti-6-keto-$PGF_{1\alpha}$ were all purchased from Sigma. All were rabbit polyclonal antisera developed using prostaglandin-BSA (bovine serum albumin) as the immunogen. According to the manufacturer's instructions all antisera are specific for the target antigen, with the exception of anti-PGE_2 which does not discriminate between PGE_1 and PGE_2. Anti-PGD_2 was purchased from Oxford Biomedical Research, Inc. Radiolabelled prostaglandins (^{3}H-PGD_2, ^{3}H- $PGF_{2\alpha}$ and ^{3}H-6-keto-$PGF_{1\alpha}$ and ^{125}I-PGE_2) were all purchased from N.E.N.

6.3 Results.

6.3.1 Characterisation of Radioimmunoassay and Antibody Microprobe Techniques.

In preliminary experiments, spinal superfusate samples were analysed using commercial enzyme immunoasay kits (Amersham/Biogenesis). Assays were performed on 96 well microtitre plates which permitted the construction of one standard curve and the assay of 37 duplicate samples. As spinal cord superfusion experiments generated up to 70 samples, the use of these enzyme immunoassay kits was clearly not feasible.

Radioimmunoassays for four prostaglandin species (PGD_2, PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$- the stable metabolite of PGI_2) were designed and developed. These radioimmunoassays were much cheaper and less restrictive than commercial enzyme immunoassay kits. A range of antibody dilutions were tested in order to determine the optimal working titre of the antibody. A dilution which bound between 40 and 60% of the tracer in the absence of unlabelled prostaglandin (zero dose) was used in these experiments in order to give the optimum balance between sensitivity and accuracy (Granström and Kindahl, 1982). By manipulating the antibody dilution it was possible to shift the standard curve to the left or right, and thus establish an antibody titre which produced a suitable standard curve for each prostaglandin radioimmunoassay. Table 6.1 shows the working titres for the antibodies used in these experiments.

The displacement of radiolabelled prostaglandin from the antibody by unlabelled prostaglandin was quantified by incubating fixed amounts of tracer (5000 dpm) and antibody (optimal dilution) overnight with known amounts of unlabelled prostaglandin (serial dilutions from 3pg to 2ng). The following day, the antibody-bound fraction was separated from the unbound fraction. The radioactivity was determined in either the bound fraction - when iodinated prostaglandin tracer was used (PGE_2: Figure 6.1) or the free fraction - when tritiated tracer was used (e.g. 6-keto-$PGF_{1\alpha}$: Figure 6.2). As the concentration of unlabelled prostaglandin increased there was a graded, reproducible decrease in the amount of antibody-bound tracer (Figure 6.1) as it was displaced from the antibody into the free fraction (Figure 6.2).

TABLE 6.1.

	Anti-PGD_2 (O.B.R)	Anti-PGE_2 (Sigma)	Anti-$PGF_{2\alpha}$ (Sigma)	Anti-6-Keto-$PGF_{1\alpha}$ (Sigma)
Final Antibody Dilution.	1:87.5	1:700	1:100	1:100
% Tracer Binding (zero dose)	56.7 ± 1.7	49.7± 1.7	60.9 ± 0.5	52.6 ± 1.6

Table 6.1. Optimal Antibody Titres for Prostaglandin Radioimmunoassays. This table details the optimal final dilutions of prostaglandin antibodies for use in the radioimmunoassay of spinal superfusate samples, and the associated mean percentage binding of total tracer to the antibody in the absence of unlabelled prostaglandin (zero dose). Data represent mean % tracer binding ± S.E.M. (n=5).

The sensitivity of each radioimmunoassay was determined by calculating the amount of unlabelled prostaglandin required to shift zero dose binding by two standard deviations, samples which fell below these levels were said to fall below the minimum detection limit of the assay system (Table 6.2). Commercial enzyme and radioimmunoassay kits claim to be highly sensitive, with minimum detectable limits occurring between 0.8 and 2 pg/tube (for example, ^{125}I-PGE_2 and ^{125}I-6-keto $PGF_{1\alpha}$ radioimmunoassay systems, Amersham). Although our assay systems may appear less sensitive then the commercial systems, they are more than adequate for our purposes, as (unlike the commercial kits) the optimised antibody titre ensures that the mean levels of prostaglandins in superfusate samples fall in the sensitive midrange of the standard curves (Table 6.3)

TABLE 6.2.

	PGD_2	PGE_2	$PGF_{2\alpha}$	6-Keto-$PGF_{1\alpha}$
Assay Sensitivity (pg prostaglandin/ tube).	12.0 ± 1.5	3.7 ± 0.9	4.7 ± 0.9	3.6 ± 0.7

Table 6.2 Radioimmunassay Sensitivity. This table shows mean pg prostaglandin ± S.E.M. required to shift zero dose binding by two standard deviations (n=6). Samples which fall below these values were said to fall below the minimum detection levels of the assay system.

FIGURE 6.1.

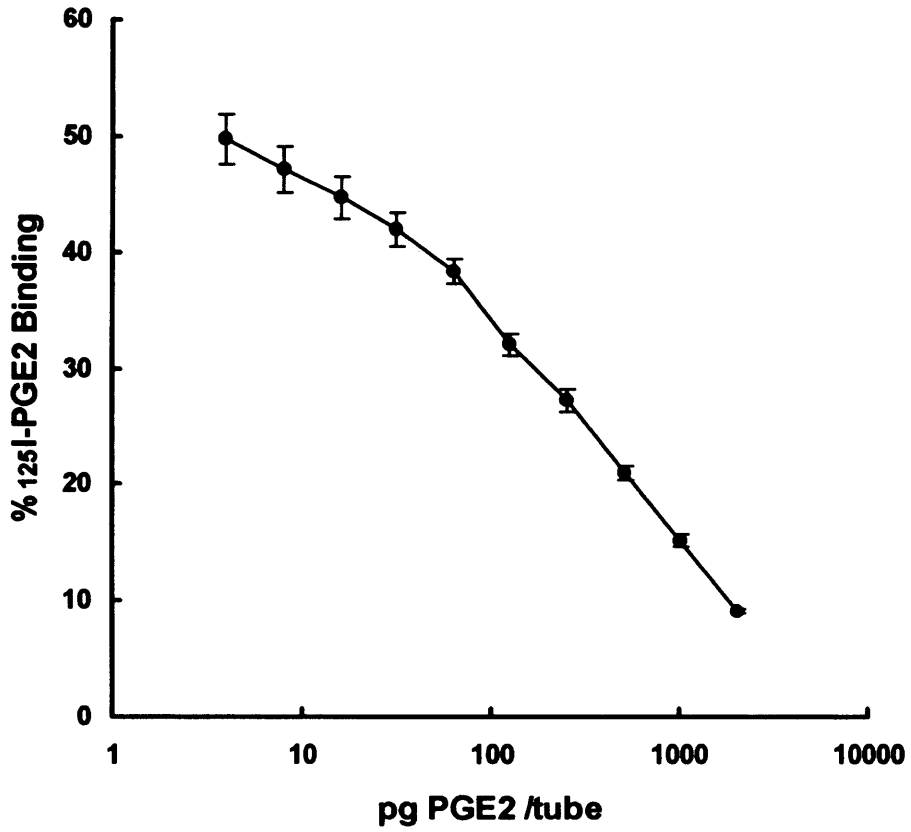


Figure 6.1. PGE_2 Standard Curve. This graph shows the competition between labelled and unlabelled PGE_2 for anti-PGE_2 binding sites. The data represent the mean percent of antibody-bound tracer ± S.E.M. (n=6). As the concentration of unlabelled PGE_2 increases, there is an associated decrease in the amount of antibody-bound ^{125}I-PGE_2 (as measured in the pellet). The concentration (pg PGE_2/tube) of the unknown samples can be calculated from this graph.

FIGURE 6.2.

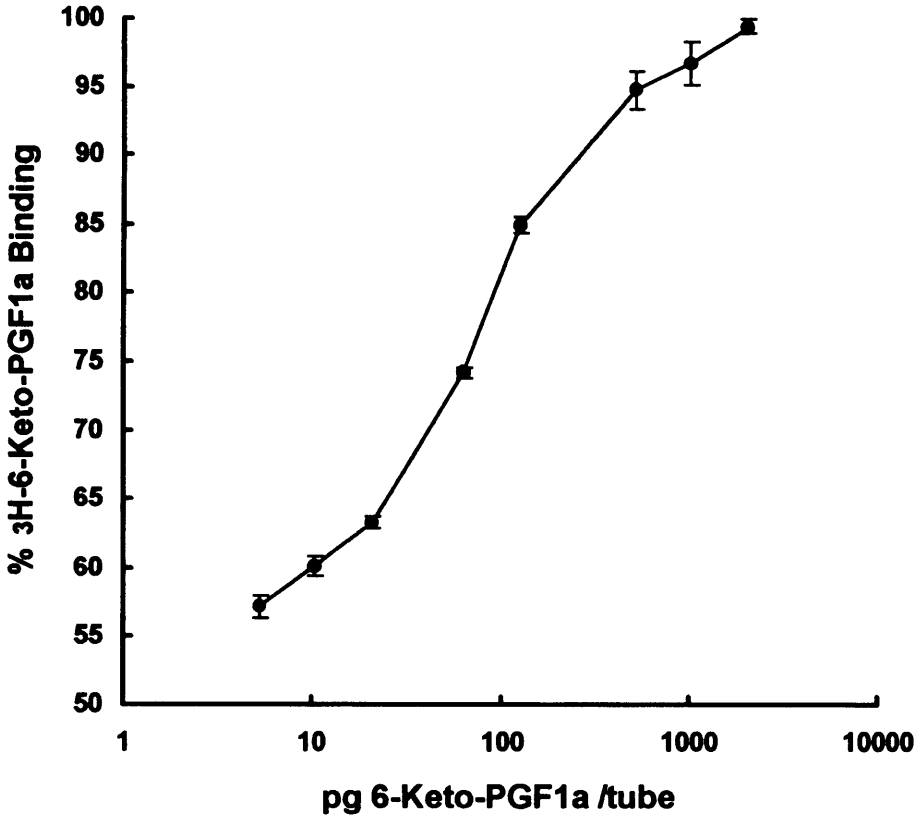


Figure 6.2. 6-Keto-$PGF_{2\alpha}$ Standard Curve. This graph shows the competition between labelled and unlabelled 6-keto-$PGF_{1\alpha}$ for anti-6-keto-$PGF_{1\alpha}$ binding sites. The data represent the mean percent antibody-bound tracer ± S.E.M. (n=6). As the concentration of unlabelled of 6-keto-$PGF_{1\alpha}$ increases, there is an associated decrease in antibody-bound ^{3}H-6-keto-$PGF_{1\alpha}$ and an increase in the amount of ^{3}H-6-keto-$PGF_{1\alpha}$ in the free fraction (as measured in the supernatant). The concentration (pg PGE_2/tube) of the unknown samples can be calculated from the graph.

During the development of the radioimmunoassays it became apparent that prostaglandins were 'sticking' to polypropylene tips and tubes, despite polypropylene being the recommended handling material (Amersham). This 'stickiness' was abolished by coating all plasticware with a siliconising reagent ('Sigmacote': Sigma). Figure 6.3 demonstrates the binding of PGE_2 to unsiliconised tubes. A known concentration of PGE_2 (diluted in aCSF) was divided between siliconised and unsiliconised polypropylene tubes and stored for 2 months at -80°C. Approximately 25% of PGE_2 was lost from the sample following storage in unsiliconised tubes, whereas there was no significant decrease in PGE_2 concentration following storage in siliconised tubes. This demonstrates the importance of siliconising every piece of plastic ware which comes into contact with prostaglandins, failure to do so will lead to inaccurate results. This experiment also demonstrates that PGE_2 is stable in aCSF at -80°C for at least 2 months.

The basic principle of antibody microprobe techniques is identical to that of immunoassays, namely exploitation of the competition between radiolabelled and non-labelled prostaglandins for specific antibody binding sites. Anti-PGE_2 is immobilised on fine glass microprobes which were then inserted into the rat spinal cord. The probes are then incubated in radiolabelled PGE_2, which binds to unoccupied antibody-binding sites. The probes are placed against X-ray film for an appropriate length of time, and the film then developed and the images of the probes densitometrically analysed. Figure 6.4 shows with *in vitro* probes that as the concentration of unlabelled PGE_2 increases there is a graded reduction in antibody-bound radiolabelled PGE_2, indicating that both unlabelled and labelled PGE_2 are able to bind to the immobilised anti-PGE_2. Hence, areas of reduced tracer binding, *in vivo*, may legitimately represent regions of endogenous PGE_2 binding.

FIGURE 6.3.

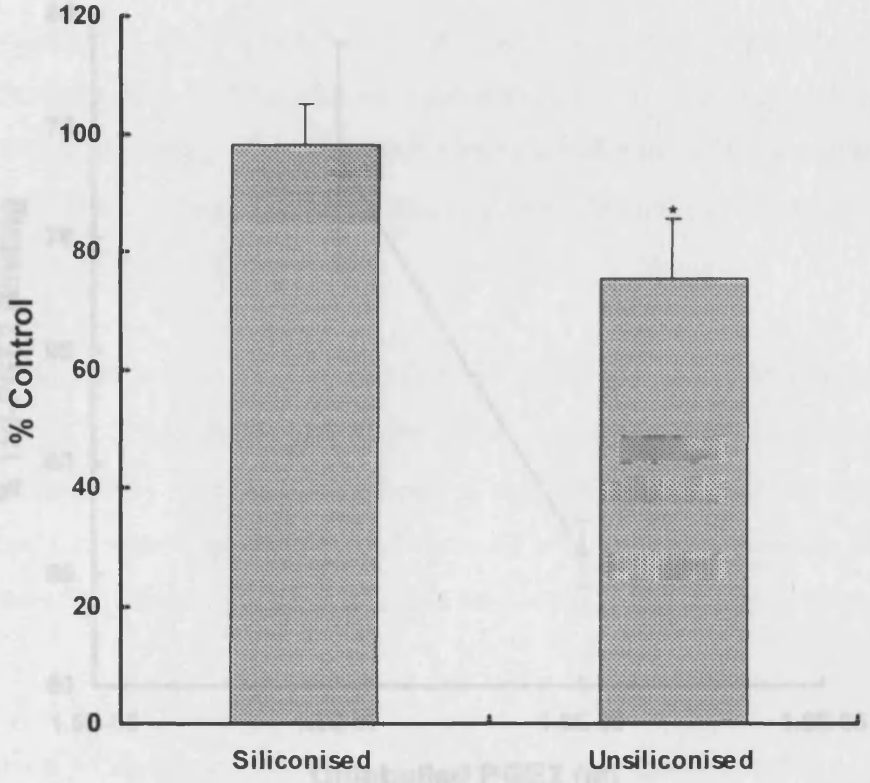


Figure 6.3. Prostaglandins Bind to Unsiliconised Tubes. This graph demonstrates the need to siliconise all plastic/glassware used in prostaglandin assays. The data represents the mean percent recovery of PGE_2 from siliconised and unsiliconised tubes ± S.E.M. (n=12). There is a significant (* $p<0.05$, t-test) decrease in the recovery of PGE_2 from samples stored in unsiliconised tubes, whereas there is almost 100% recovery of PGE_2 from samples stored in siliconised tubes. This also indicates PGE_2 is stable at -80°C for at least 2 months in siliconised tubes.

FIGURE 6.4.

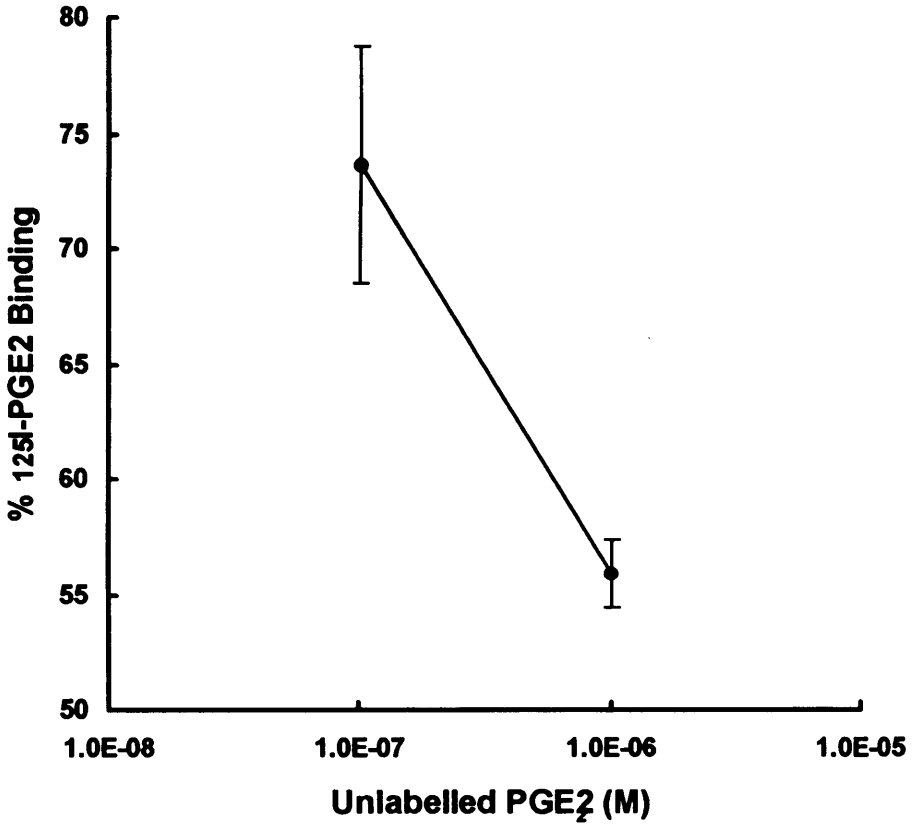


Figure 6.4. Inhibition of Radioligand Binding to Antibody Microprobes. This graph shows the competition between labelled and unlabelled PGE_2 for anti-PGE_2 binding sites on antibody microprobes. As the concentration of unlabelled PGE_2 increases, there is an associated decrease in antibody-bound ^{125}I-PGE_2. The data represent the mean percent bound ^{125}I-PGE_2/zero dose binding of ^{125}I-PGE_2 ± S.E.M. (n=4).

6.3.2 Prostaglandin E_2 in the Plasma and Spinal Cord of Normal and FCA-Monoarthritic Rats.

Changes in circulating PGE_2 levels were measured by analysing plasma samples obtained from anaesthetised normal and FCA-monoarthritic rats. Samples were assayed directly, as plasma purification using C18 Amprep minicolumns (Amersham) was found to be unnecessary (Crichton Lang, University of St. Andrews, personal communication, 1997). Following the induction of FCA-induced monoarthritis there was a significant ($p<0.01$) increase in circulating PGE_2. Three days following the induction of inflammation there was an increase from 5.5 ± 1.3 ng PGE_2 ml^{-1} plasma in the normal uninjected rat to 26.5 ± 3.6 ng PGE_2 ml^{-1} plasma (Figure 6.5).

There was a significant ($p<0.01$) increase in spinal PGE_2 one day following inflammation, from 2.0 ± 0.3 ng PGE_2 g^{-1} spinal cord in the normal uninjected rat to 6.1 ± 0.9 ng PGE_2 g^{-1} spinal cord (Figure 6.6). The maximal increase in spinal PGE_2 occurs at the same timepoint as the maximal increase in spinal COX-2 (Figure 5.3), 1 day post-inoculation. Spinal PGE_2 was still elevated in some rats but not in others and overall the increase was not significant ($p>0.05$).

By day 3, spinal PGE_2 has returned towards basal levels, whereas circulating PGE_2 is still markedly elevated. This may indicate that whilst circulating prostaglandins play a role in maintaining inflammation of the injected limb (see Figures 3.1, 3.3), they do not cross the blood-brain barrier and influence spinal processing. The increase in spinal PGE_2 one day following inflammation may indicate a role for COX-2 generated PGE_2 in spinal nociceptive processing during this acute phase of the inflammatory response

FIGURE 6.5.

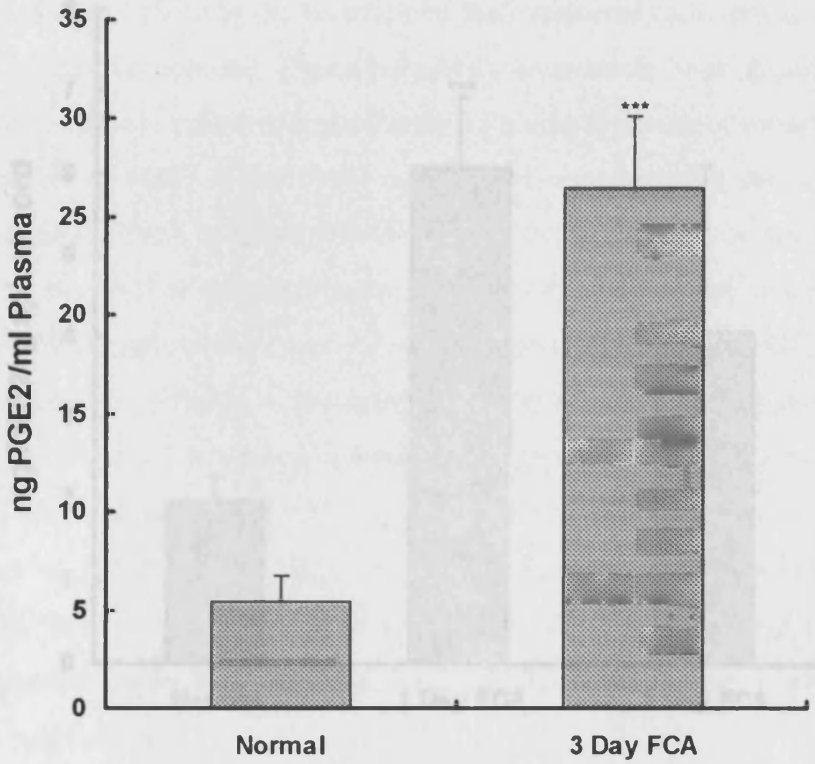


Figure 6.5. Increase in Circulating PGE_2 Following FCA-induced Monoarthritis. This graph illustrates the significant (*** $p<0.01$, t-test) increase in the levels of PGE_2 in the plasma of rats with peripheral inflammatory lesions (3 day FCA). Arterial blood samples were taken from anaesthetised rats which had been pretreated with heparin (750 units, i.v.) to prevent clotting. Indomethacin (200μM) was added to the blood sample to prevent further prostaglandin synthesis. Blood was centrifuged at 3500 rpm for 10 minutes at 4°C. The plasma supernatant removed and stored at -80°C until analysis by radioimmunoassay. Data represent the mean ng PGE_2 /ml plasma ± S.E.M. (n=6).

FIGURE 6.6.

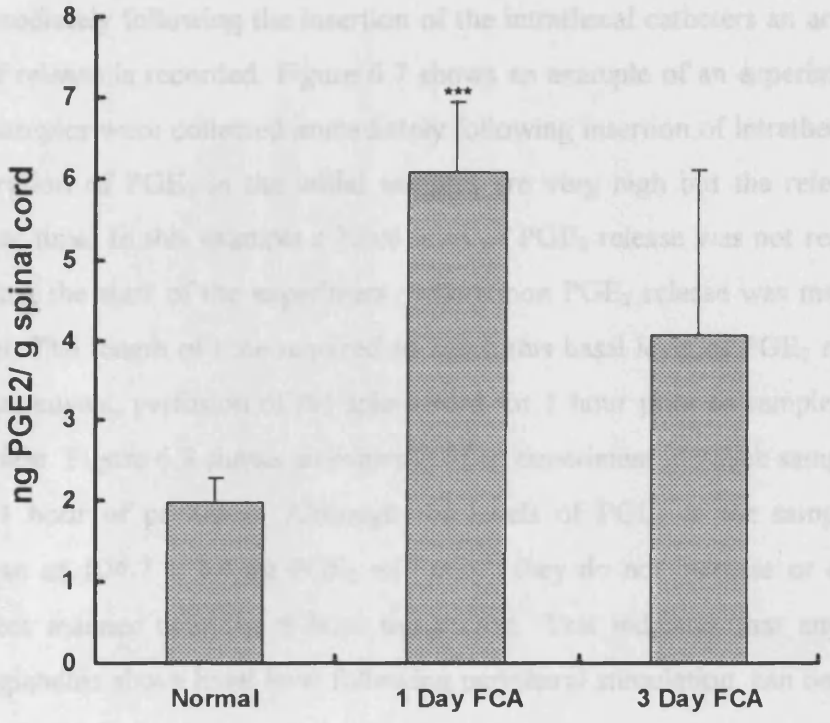


Figure 6.6. Increase in Spinal PGE_2 Following FCA-induced Monoarthritis. This graph illustrates the increase in PGE_2 in the spinal cord of rats with peripheral inflammatory lesions (1 and 3 day FCA). Lumbar spinal cord samples were obtained from barbiturate-killed rats, which had been transcardially perfused with warmed saline in order to remove blood. Spinal cord samples were processed and purified using C18 Amprep minicolumns (Amersham) and analysed by radioimmunoassay. Data represent the mean ng PGE_2 / g wet weight spinal cord ± S.E.M. (n=6). The increase in spinal PGE_2 was significant (*** $p<0.01$, t-test) one day post-inoculation.

6.3.3 Basal Release of Prostaglandins from the Spinal Cord of Normal and FCA-Monoarthritic Rats.

Prostaglandins are synthesised and released in response to tissue injury. It is therefore likely that the insertion of catheters into the subdural space may cause some trauma to the surface of the spinal cord and evoke the release of prostaglandins. If spinal superfusate samples are collected immediately following the insertion of the intrathecal catheters an artificially high basal level of release is recorded. Figure 6.7 shows an example of an experiment in which superfusate samples were collected immediately following insertion of intrathecal catheters. The concentration of PGE_2 in the initial samples are very high but the release of PGE_2 decreases over time. In this example a basal level of PGE_2 release was not reached until 3 hours following the start of the experiment, whereupon PGE_2 release was maintained at a constant level. The length of time required to reach this basal level of PGE_2 release in this experiment is unusual, perfusion of the spinal cord for 1 hour prior to sample collection is usually sufficient. Figure 6.8 shows an example of an experiment in which sample collection began after 1 hour of perfusion. Although the levels of PGE_2 in the samples fluctuate around a mean of 104.7 ± 2.4 pg PGE_2 ml^{-1} min^{-1}, they do not increase or decrease in a time-dependent manner over the 5 hour timecourse. This indicates that any increase in spinal prostaglandins above basal level following peripheral stimulation, can be described as a stimulus-evoked release.

FIGURE 6.7.

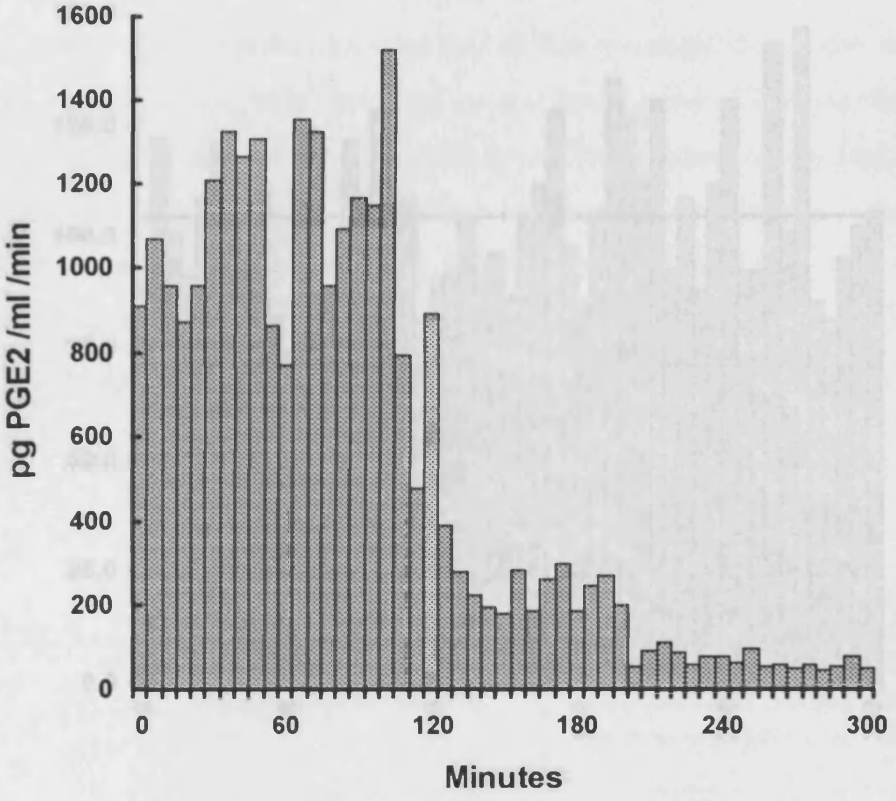


Figure 6.7. Release of PGE_2 from the Rat Spinal Cord Following the Insertion of Intrathecal Catheters. This figure shows the release of PGE_2 ml^{-1} min^{-1} from the rat spinal cord immediately following the insertion of intrathecal catheters over a five hour time course. The levels of PGE_2 in the samples gradually decrease over time. A basal level of release was not reached until 3 hours following the start of the experiment, after this point PGE_2 was maintained at a constant level. This indicates that the basal prostaglandin release from the spinal cord must not be measured for some time following surgery.

FIGURE 6.8.

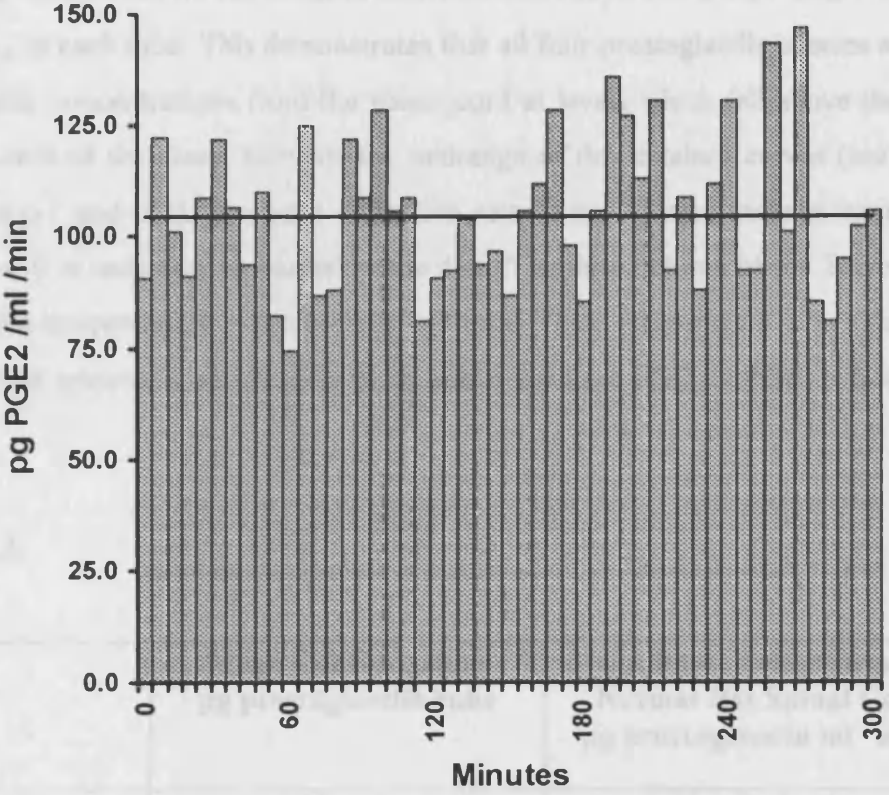


Figure 6.8. Basal Level of PGE_2 Released from the Rat Spinal Cord is Stable over Five Hours. This figure shows the release of PGE_2 from the spinal cord of a normal rat. Sample collection began following 1 hour of perfusion. The release of PGE_2 from the rat spinal cord fluctuates around a mean level of 104.7 ± 2.4 pg PGE_2 ml^{-1} min^{-1} (shown as solid line), but does not increase or decrease in a time-dependent manner over the 5 hour time course.

The spinal cord is therefore routinely perfused for at least one hour before sample collection. Superfusate samples are then collected for a further hour in order to determine the level of basal prostaglandin release. Occasionally, the first few samples in the collection period have higher levels than later samples, these are discarded. Table 6.3 shows the results of radioimmunoassays of basal spinal cord superfusate samples obtained from normal rats. The second column shows the mean absolute concentration of PGD_2, PGE_2, $PGF_{2\alpha}$ and 6-Keto-$PGF_{1\alpha}$ in each tube. This demonstrates that all four prostaglandin species are released in measurable concentrations from the spinal cord at levels which fall above the minimum detectable limit of the assay, towards the midrange of the standard curves (see Table 6.2, and Figures 6.1 and 6.2). Since the collection rate of superfusate samples varies between experiments, it is necessary to normalise the data. The third column of the Table shows the prostaglandin concentration expressed per unit time. These experiments show that the major prostaglandins released from the normal rat spinal cord are PGD_2 > PGE_2 > 6-Keto-$PGF_{1\alpha}$ > $PGF_{2\alpha}$.

TABLE 6.3.

	Mean Concentration pg prostaglandin/tube	**Mean Basal Release from the Normal Rat Spinal Cord pg prostaglandin ml^{-1} min^{-1}**
PGD_2 (n=4)	142.9 ± 18.2	294.1 ± 22.1
PGE_2 (n=14)	84.5 ± 9.4	169.0 ± 16.4
6-Keto-$PGF_{1\alpha}$ (n=6)	22.5 ± 4.4	47.6 ± 6.7
$PGF_{2\alpha}$ (n=4)	14.7 ± 1.9	23.6 ± 2.1

Table 6.3. Basal Prostaglandin Release from the Spinal Cord of Normal Rats. The spinal cord was perfused for one hour prior to sample collection, which continued for a further hour. Samples were analysed by radioimmunoassay. The first column indicates the prostaglandin species, the number in parentheses indicates the number of animals from which the samples were obtained. The second column shows mean ± S.E.M. absolute concentration of the prostaglandin species in each tube. The third column shows the normalised mean basal release of the prostaglandin species expressed per unit time ± S.E.M. The principle prostaglandins released from the normal rat spinal cord are PGD_2 > PGE_2 > 6-Keto-$PGF_{1\alpha}$ > $PGF_{2\alpha}$.

Table 6.4 compares the basal release of PGD_2, PGE_2, $PGF_{2\alpha}$ and 6-Keto-$PGF_{1\alpha}$ from the spinal cord of normal rats with the basal release from the spinal cord of rats with FCA-induced monoarthritis. There were no significant differences in the mean prostaglandin release from the spinal cord of normal and FCA-monoarthritic rats of any prostaglandin species tested. This finding is surprising since previous experiments showed that significantly more PGE_2 is present in spinal cord samples obtained from 1 day FCA-monoarthritic rats (Figure 6.6).

TABLE 6.4.

	Normal Rats	**1 Day FCA-Monoarthritic Rats**	**3 Day FCA-Monoarthritic Rats**
pg PGD_2 ml^{-1} min^{-1}	294.1 ± 22.1 (n=4)	-	229.9 ± 48.5 (n=4)
pg PGE_2 ml^{-1} min^{-1}	169.0 ± 16.4 (n=14)	91.7 ± 13.0 (n=7)	154.0 ± 30.0 (n=10)
pg 6-Keto-$PGF_{1\alpha}$ ml^{-1} min^{-1}	47.6 ± 6.7 (n=6)	53.0 ± 8.0 (n=6)	31.3 ± 6.8 (n=6)
pg $PGF_{2\alpha}$ ml^{-1} min^{-1}	23.3 ± 2.1 (n=14)	-	26.7 ± 3.4 (n=15)

Table 6.4. Basal Prostaglandin Release from the Spinal Cord of Normal and FCA-Monoarthritic Rats. The spinal cord was perfused for one hour prior to sample collection, which continued for a further hour. Samples were analysed by radioimmunoassay. The second column indicates prostaglandin release from the spinal cord of normal rats. The third column indicates prostaglandin release from the spinal cord of FCA-monoarthritic rats 1 day following inoculation. The fourth column indicates prostaglandin release from the spinal cord of FCA-monoarthritic rats 3 day following inoculation. The numbers in parentheses indicate the number of animals from which the samples were obtained. No significant differences were detected in the mean prostaglandin release from the spinal cord following inflammation of any prostaglandin species tested.

The discrepancy between the amount of PGE_2 contained in spinal cord tissue and the amount of PGE_2 released from the spinal cord of FCA-monoarthritic rats may arise due to the manner in which the data was collected. Spinal cord samples were obtained from rats which had been killed by barbiturate overdose, whereas the rats used in superfusion experiments were firstly anaesthetised to a surgical level ('inactin': sodium thiobutabarbitol, i.p.) then surgically prepared. Figure 6.9 shows the amount of anaesthetic required to induce a surgical level of anaesthesia in normal and FCA-monoarthritic rats. Significantly ($p<0.05$) more anaesthetic was required to anaesthetise 1 day FCA-monoarthritic rats, from 219.0 ± 10.4 mg inactin/kg in normal rats to 257.0 ± 8.9 mg inactin/kg in FCA-monoarthritic rats, 1 day following inoculation. It is therefore possible that the increased concentration of anaesthetic may interfere with the mechanism by which prostaglandins are released from the spinal cord.

FIGURE 6.9.

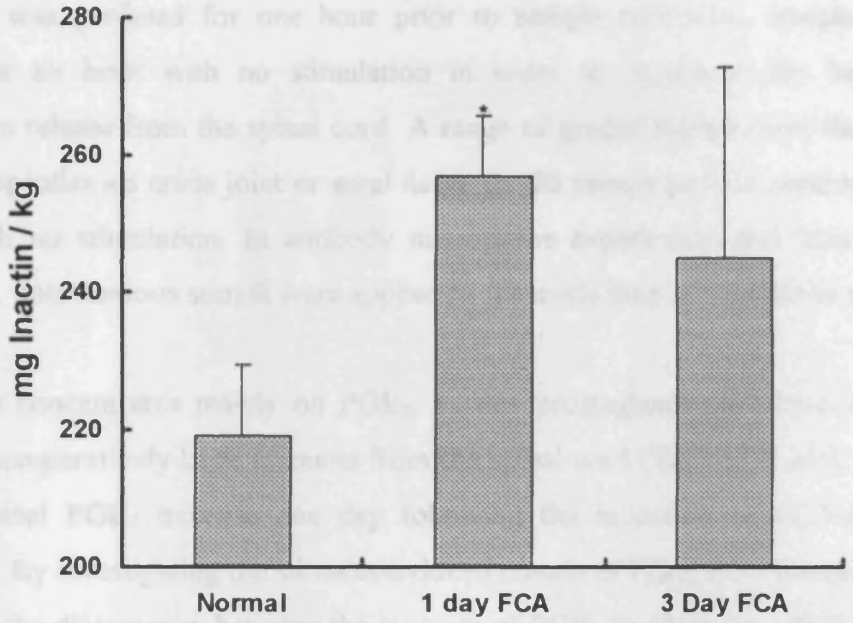


Figure 6.9. The Levels of Anaesthetic Required to Induce Surgical Anaesthesia in Normal and FCA-Monoarthritic Rats. This graph shows the mean amount of inactin (sodium thiobutabarbitol, i.p.) ± S.E.M. required to induce a surgical level of anaesthesia (n=6) in normal and FCA-monoarthritic rats. Surgical anaesthesia was assessed by testing: a) flexion withdrawal reflexes, which had to be absent; and b) corneal blink reflexes, which had to be absent. Significantly (* $p<0.05$, t-test) more anaesthetic was required to anaesthetise rats 1 day following induction of inflammation.

6.3.4 Mechanical and Electrical Stimulus-Evoked Release of Prostaglandins from the Spinal Cord of Normal and FCA-Monoarthritic Rats.

The release of prostaglandins from the spinal cord of normal and FCA-monoarthritic rats were examined following the application of mechanical stimulation to the ankle joint and electrical stimulation to the sural or tibial nerve. In initial spinal superfusion experiments, the spinal cord was perfused for one hour prior to sample collection, samples were then collected for an hour with no stimulation in order to determine the basal level of prostaglandin release from the spinal cord. A range of graded stimuli were then applied to the normal or inflamed ankle joint or sural nerve for 20 minute periods separated by 30-60 minutes with no stimulation. In antibody microprobe experiments and later superfusion experiments, only noxious stimuli were applied to the ankle joint or sural/tibial nerve.

This section concentrates mainly on PGE_2, as this prostaglandin has been shown to be released in comparatively large amounts from the spinal cord (Table 6.3) and, furthermore, levels of spinal PGE_2 increase one day following the induction of FCA-monoarthritis (Figure 6.6). By investigating the stimulus-induced release of PGE_2 from the spinal cord, we may explain the discrepancy between the increase of PGE_2 levels in the spinal cord (Figure 6.6) and the absence of any change in the levels of PGE_2 released from the spinal cord (Table 6.4) one day following FCA-inoculation. Unfortunately, the release of PGD_2, is less well characterised than the release of PGE_2, due to the termination of anti-PGD_2 production by the supplier and the absence of any alternative sources.

Figure 6.10 shows the release of PGE_2 from the spinal cord of a normal rat following mechanical stimulation of the ankle joint. The black bars represent periods of mechanical stimulation in order of increasing intensity (light brushing, innocuous pressure, noxious pressure). There was no distinct stimulus-dependent release of PGE_2 from the spinal cord in association with any of the mechanical stimuli tested. This experiment was repeated in a further four rats and again no stimulus-evoked release of PGE_2 from the spinal cord was detected. Figure 6.11 demonstrates the mean release of PGE_2 from the normal rat spinal cord following innocuous and noxious mechanical stimulation. No significant increases or decreases in the release of spinal PGE_2 were detected compared to the basal release of PGE_2.

FIGURE 6.10.

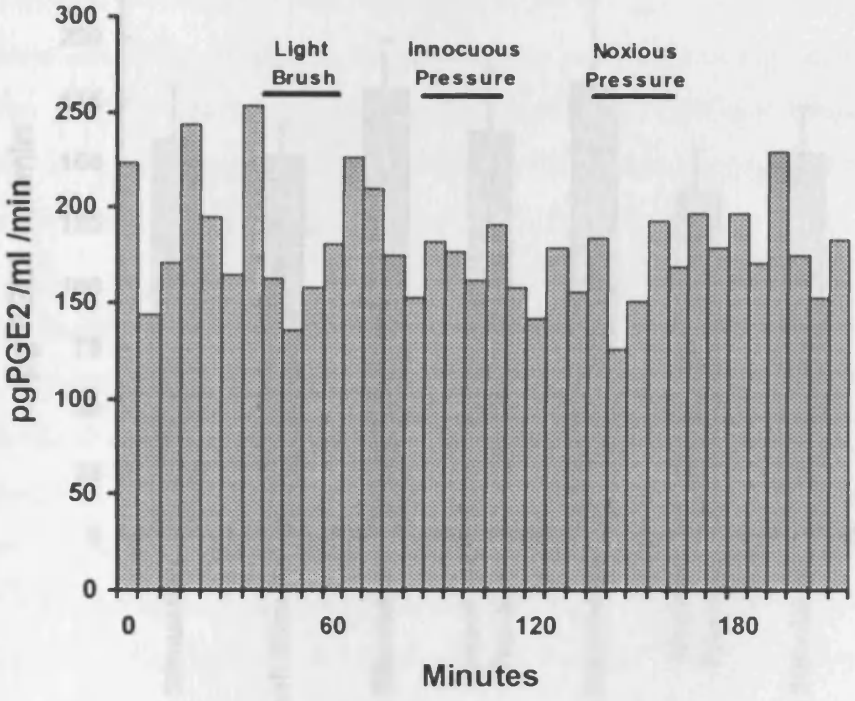


Figure 6.10. The Basal Release of PGE_2 from the Spinal Cord of a Normal Rat is Not Affected by Mechanical Stimulation of the Ankle Joint. This Figure shows the release of PGE_2 from the spinal cord of a normal rat. Sample collection began following 1 hour of perfusion. Mechanical stimuli were applied to the ankle joint in order of increasing intensity (light brushing, innocuous pressure, and noxious pressure). Periods of stimulation typically lasted for 20 minutes and are indicated by the solid lines above the x-axis. The release of PGE_2 from the rat spinal cord does not increase or decrease in a stimulus-dependent manner.

FIGURE 6.11.

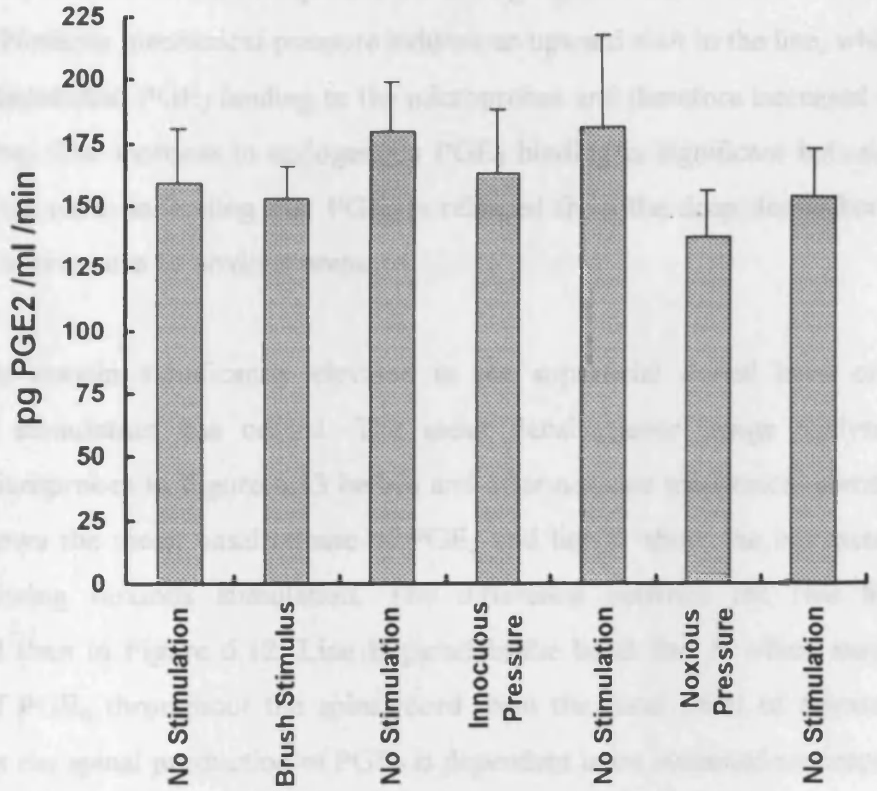


Figure 6.11 Mechanical Stimulation of the Ankle Joint does not Influence the Mean Basal Release of PGE_2 from the Spinal Cord. This Figure shows that the mean basal release of PGE_2 from the spinal cord of normal rats does not significantly ($p>0.05$, ANOVA) change following innocuous or noxious mechanical stimulation of the ankle joint. Data represents the mean levels of PGE_2 pg $ml^{-1}min^{-1}$ ± S.E.M. measured in samples collected before, during and following stimulation (n=5).

A noxious mechanical stimulus-induced release of PGE_2 from the rat spinal cord was however detected using antibody microprobes, which may be more sensitive than spinal superfusion techniques. Figure 6.12 shows the mean densitometric image analysis of PGE_2 antibody microprobes before and during noxious mechanical stimulation. Line A of Figure 6.12 shows the mean basal release of PGE_2 from the spinal cord, and line B shows the mean level of PGE_2 released from the spinal cord during noxious mechanical stimulation of the ankle joint. Noxious mechanical pressure induces an upward shift in the line, which indicates reduced radiolabelled PGE_2 binding to the microprobes and therefore increased endogenous PGE_2 binding. The increase in endogenous PGE_2 binding is significant between 1-1.5mm into the spinal cord, indicating that PGE_2 is released from the deep dorsal horn of the rat spinal cord in response to noxious pressure.

PGE_2 levels remain significantly elevated in the superficial dorsal horn once noxious mechanical stimulation has ceased. The mean densitometric image analysis of PGE_2 antibody microprobes in Figure 6.13 before and after noxious mechanical stimulation. Line A again shows the mean basal release of PGE_2 and line B show the increased release of PGE_2 following noxious stimulation. The difference between the two lines is less pronounced than in Figure 6.12. Line B parallels the basal line A which may reflect the diffusion of PGE_2 throughout the spinal cord from the focal point of release. This may indicate that the spinal production of PGE_2 is dependent upon sustained nociceptive input to the spinal cord.

The reliability of these results must now be questioned:

- The total binding of the radioligand to the *in vitro* microprobes in this series of experiments (in the absence of unlabelled PGE_2) was reduced to 14%, compared to 20% binding in other experiments. The assay depends upon a balance between sensitivity and accuracy, this reduced level of radioligand binding clearly leaves the experiment open to artefacts (false positives).
- As a result of this low level of radioligand binding the *in vivo* microprobes were exposed to X-ray film for longer than the usual 14 days. Despite this longer exposure period the images of the microprobes were still faint and often uneven.

When all probes from the experiments were analysed, the results shown in Figures 6.12 and 6.13 were obtained. However, following removal of probes with uneven images from calculations, the result obtained was not significant. Further experiments are required in

order to validate or refute these results and determine the relationship between acute noxious stimulation and spinal PGE_2 release.

Using spinal superfusion techniques, it was not possible to detect any mechanical stimulus-induced release of other prostaglandin species from the spinal cord of normal rats. Figure 6.14 demonstrates that there were no significant changes in the release of PGD_2 (panel A) or $PGF_{2\alpha}$ (panel B) from the normal rat spinal cord following mechanical stimulation.

Similarly, it was not possible to detect any mechanical stimulation-induced changes in the mean release of prostaglandins from the spinal cord of FCA-monoarthritic rats either one (PGE_2: n=5) or three days following inoculation (PGE_2: n=4; PGD_2: n=4; $PGF_{2\alpha}$: n=4) (data not shown).

FIGURE 6.12.

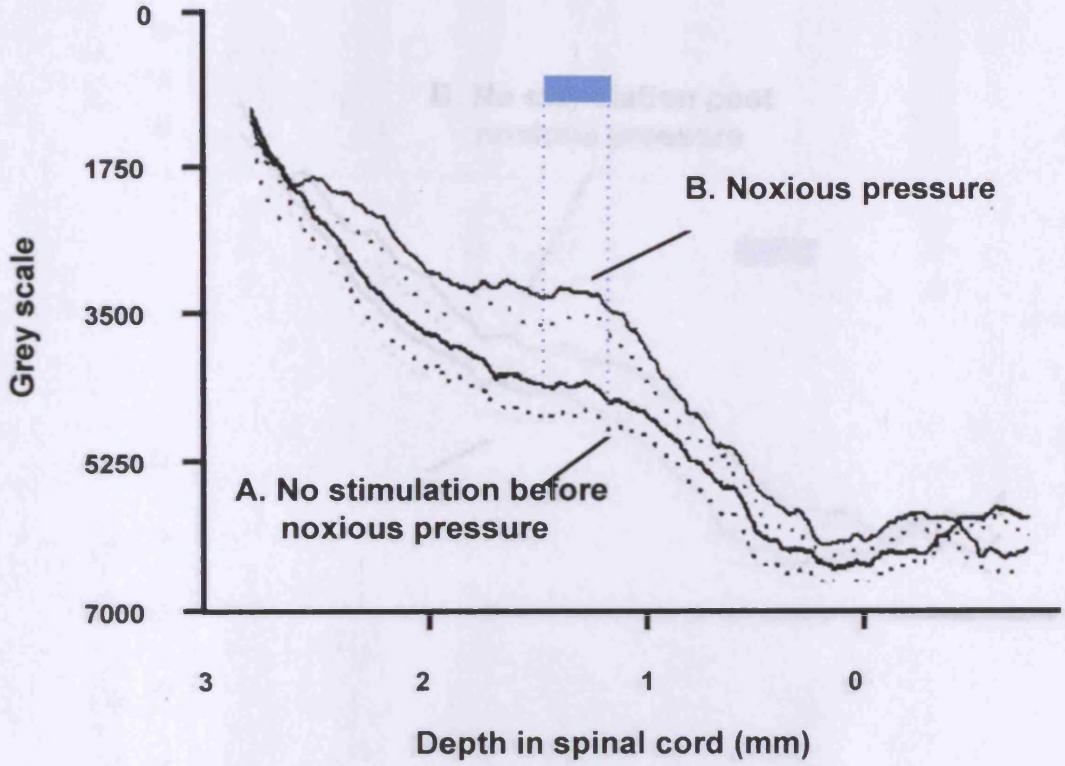


Figure 6.12. Mechanically Evoked Release of PGE_2 from the Normal Rat Spinal Cord. This figure shows mean densitometric image analysis of PGE_2 antibody microprobes before and during noxious mechanical stimulation of the ankle joint. Line A shows the mean basal release of PGE_2 from the spinal cord (n=34 probes) and line B shows the mean level of PGE_2 released from the spinal cord during noxious mechanical stimulation (n=22 probes). Data represent the mean image density of the probes (solid lines) ± S.E.M. (dotted lines). Noxious mechanical pressure induces a significant increase in the release of endogenous PGE_2 from the deep dorsal horn ($p<0.05$, represented by the blue shaded region, t-tests). However, the validity of this data has been questioned (refer to text).

FIGURE 6.13.

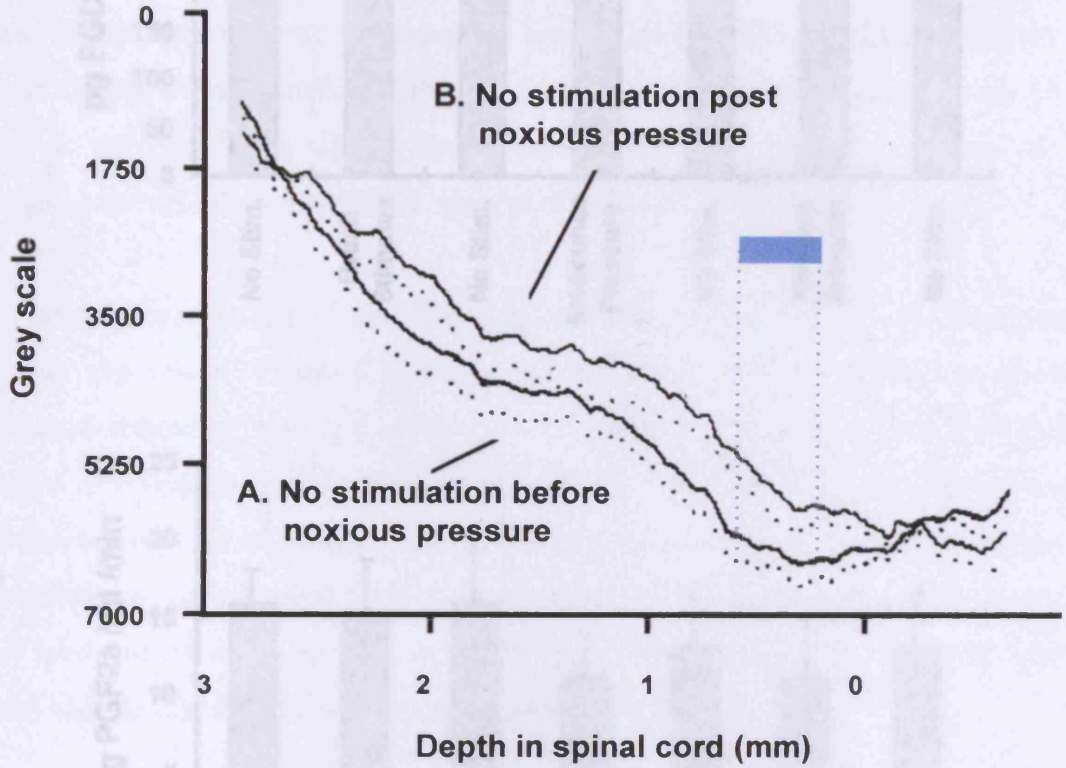
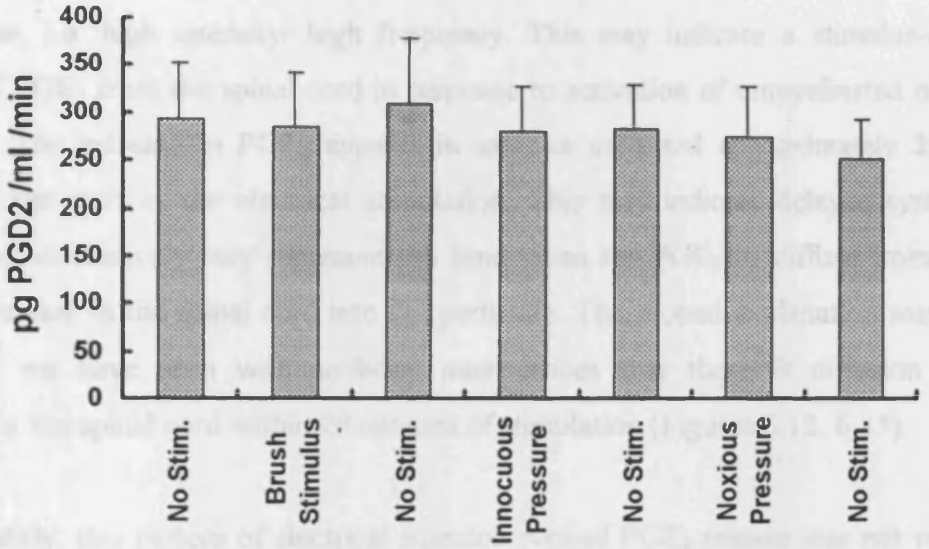


Figure 6.13. The Increase in Spinal PGE_2 is Sustained following Noxious Mechanical Stimulation. This figure shows mean densitometric image analysis of PGE_2 antibody microprobes before and following noxious mechanical stimulation of the ankle joint. Line A shows the mean basal release of PGE_2 from the spinal cord (n=34 probes) and line B shows the mean level of PGE_2 released from the spinal cord following noxious mechanical stimulation (n=24 probes). Data represent the mean image density of the probes (solid lines) ± S.E.M. (dotted lines). The levels of PGE_2 remain significantly elevated in the superficial dorsal horn ($p<0.05$, represented by the blue shaded region, t-test). Line B parallels line A (mean basal release) which may indicate that the PGE_2 produced during the period of stimulation diffuses throughout the spinal cord. However, the validity of these experiments has been questioned (refer to text).

FIGURE 6.14.

A.



B.

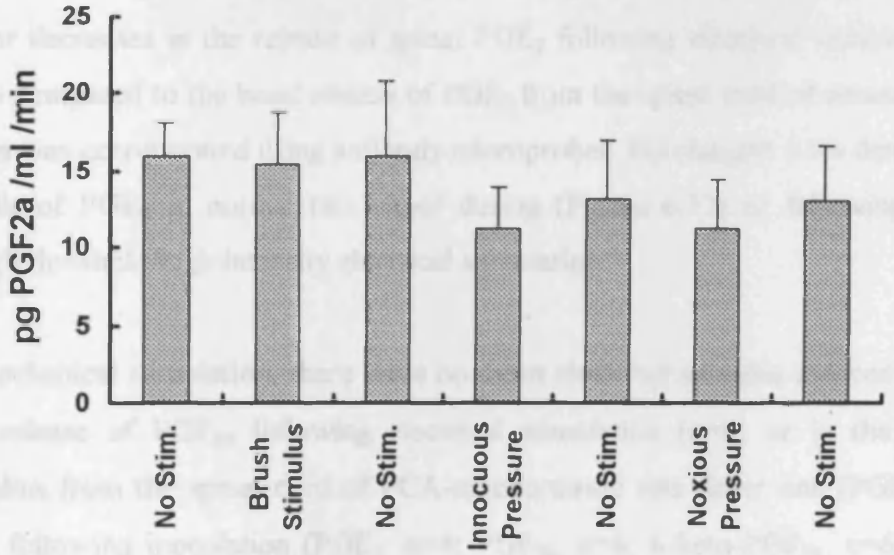


Figure 6.14 Mechanical Stimulation of the Ankle Joint does not Affect the Mean Basal Release of PGD_2 or $PGF_{2\alpha}$ from the Spinal Cord. Of the Normal Rat. This figure shows that the mean basal release of PGD_2 (Panel A) and $PGF_{2\alpha}$ (Panel B) from the spinal cord of normal rats does not significantly ($p>0.05$, ANOVA) change following mechanical stimulation of the ankle joint. Data represents the mean levels of prostaglandin pg $ml^{-1}min^{-1}$ ± S.E.M. measured in samples collected before, during and following mechanical stimulation (Panel A:n=4; Panel B: n=5).

Figure 6.15 shows the release of PGE_2 from the spinal cord of a normal rat following electrical stimulation of the sural nerve. The black bars represent 20 minute periods of mechanical stimulation in order of increasing intensity (0.2V at 0.1Hz; 10-20V at 0.1Hz; 10-20V at 1Hz). There is a small increase in PGE_2 levels following the second stimulation period, followed by a dramatic increase in PGE_2 in samples following the third period of stimulation, i.e. high intensity/ high frequency. This may indicate a stimulus-dependent release of PGE_2 from the spinal cord in response to activation of unmyelinated nociceptive afferents. The increase in PGE_2 appears in samples collected approximately 25 minutes following the start of the electrical stimulation. This may indicate delayed synthesis and release, or alternatively may represent the time taken for PGE_2 to diffuse from the focal point of release in the spinal cord into the perfusate. The second explanation may be more likely, as we have seen with antibody microprobes that there is diffusion of PGE_2 throughout the spinal cord within 20 minutes of stimulation (Figures 6.12, 6.13).

Unfortunately, this pattern of electrical stimulus-evoked PGE_2 release was not repeated in any other experiment. Figure 6.16 shows the mean release of PGE_2 from the normal rat spinal cord following electrical stimulation of the sural nerve. There are no significant increases or decreases in the release of spinal PGE_2 following electrical stimulation of the sural nerve compared to the basal release of PGE_2 from the spinal cord of normal rats. This observation was corroborated using antibody microprobes. No changes were detected in the spinal levels of PGE_2 in normal rats either during (Figure 6.17) or following (data not shown) high threshold/high intensity electrical stimulation.

As with mechanical stimulation, there were no mean electrical stimulus-induced changes in the basal release of $PGF_{2\alpha}$ following electrical stimulation (n=6) or in the release of prostaglandins from the spinal cord of FCA-monoarthritic rats either one (PGE_2: n=5) or three days following inoculation (PGE_2: n=4; $PGF_{2\alpha}$: n=6; 6-keto-$PGF_{1\alpha}$: n=6) (data not shown).

FIGURE 6.15

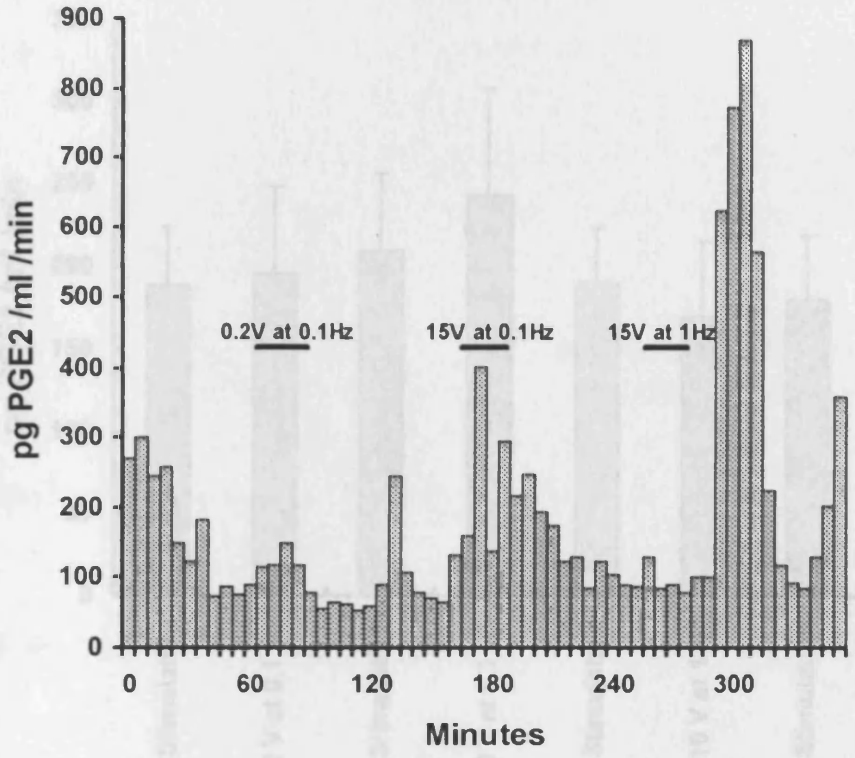


Figure 6.15. The Release of PGE_2 from the Spinal Cord is Increased by High Threshold, High Frequency Electrical Stimulation of the Sural Nerve of one Normal Rat. This figure shows the release of PGE_2 from the spinal cord of one normal rat. Electrical stimuli were applied to the sural nerve in order of increasing intensity (0.2V at 0.1Hz; 15V at 0.1Hz; 15V at 1Hz). Periods of stimulation lasted for 20 minutes and are indicated by the solid lines. The release of PGE_2 from the rat spinal cord increases

FIGURE 6.16

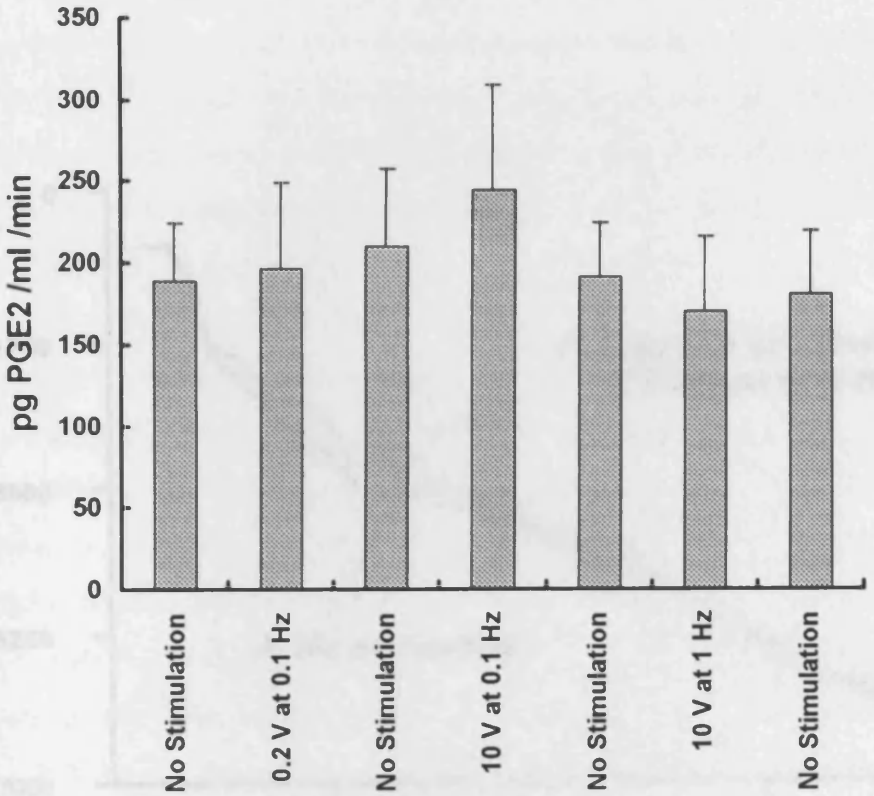


Figure 6.16 Electrical Stimulation of the Sural Nerve does not Affect the Mean Basal Release of PGE_2 from the Spinal Cord. This figure shows that the mean basal release of PGE_2 from the spinal cord of normal rats does not significantly ($p>0.05$, ANOVA) change following electrical stimulation of the sural nerve. Data represents the mean levels of PGE_2 pg $ml^{-1}min^{-1}$ ± S.E.M. measured in samples collected before, during and following stimulation (n=5).

FIGURE 6.17.

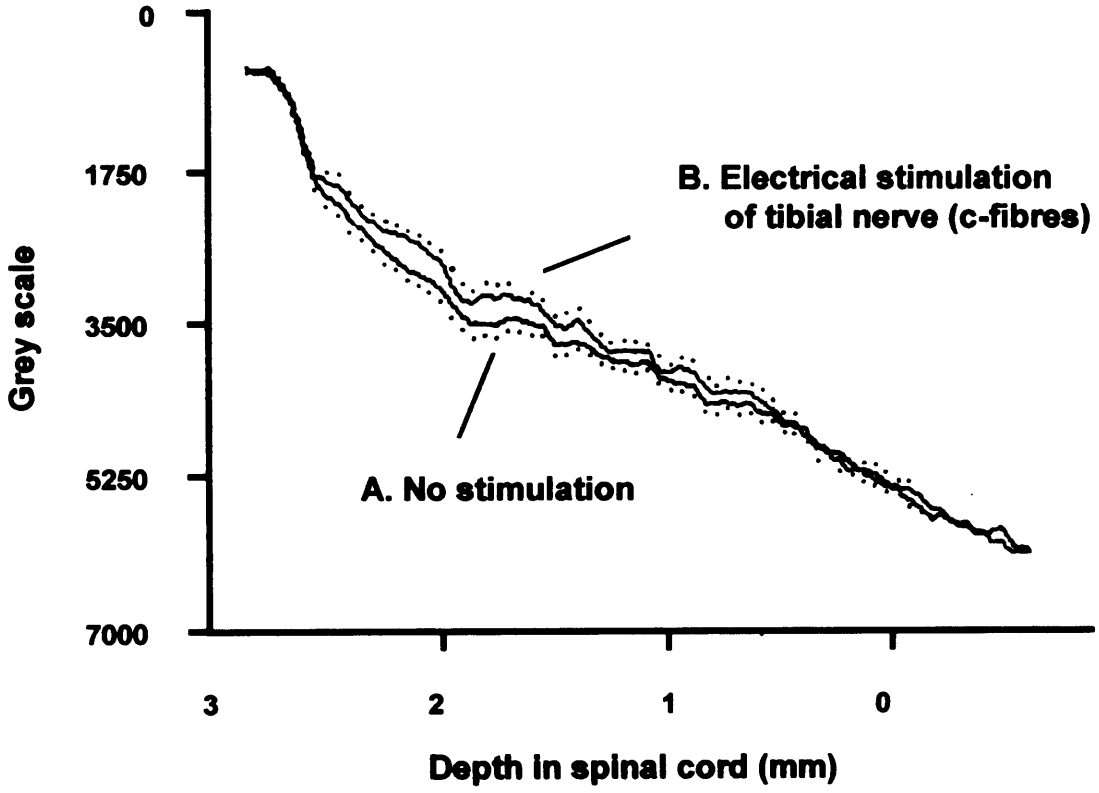


Figure 6.17 Electrical Stimulation of the Tibial Nerve does not Affect the Mean Basal Release of PGE_2 from the Spinal Cord. This figure shows mean densitometric image analysis of PGE_2 antibody microprobes before and during high threshold/high intensity (20V at 1 Hz) electrical stimulation of the tibial nerve. Line A shows the mean basal release of PGE_2 from the spinal cord (n=31 probes) and line B shows the mean level of PGE_2 released from the spinal cord during electrical stimulation of the tibial nerve (n=40 probes). Data represent the mean image density of the probes (solid lines) ± S.E.M. (dotted lines).This Figure shows that the basal release of PGE_2 from the spinal cord of normal rats does not change following electrical stimulation of the tibial nerve.

6.3.5 Acute Inflammation-Evoked Release of PGE_2 from the Rat Spinal Cord.

The release of PGE_2 from the spinal cord was examined following the development of an acute inflammation using both spinal cord superfusion and antibody microprobe techniques. In superfusion experiments, the spinal cord was perfused for one hour prior to sample collection, samples were then collected for an hour in order to determine the basal level of PGE_2 release from the spinal cord. An acute inflammation was induced by the intraarticular injection of 0.07ml 4% kaolin into the right knee joint which was then slowly flexed and extended. Fifteen minutes later, 0.07ml 2% carrageenan was injected into the knee joint which was rhythmically manipulated for a 30 minutes. Superfusate samples were collected for 5 hours following the induction of inflammation. Following approximately 5 hours of sample collection 100μM indomethacin was included in the aCSF. Figure 6.18 shows the release of PGE_2 from the spinal cord of a normal rat following the intrarticular injection of kaolin and carrageenan. Within 30 minutes of the kaolin injection (and 15 minutes of the carrageenan injection) there is a dramatic increase in the levels of PGE_2 in the superfusate samples. These pulsatile increases in PGE_2 are sustained over a period of 4.5 hours. Mechanical stimulation of the joint did not evoke further PGE_2 release. Within 6 minutes of the addition of 100μM indomethacin into the perfusate there was a reduction in spinal PGE_2 to below basal levels. This spinal action of indomethacin indicates that PGE_2 is being synthesised at a spinal site, and the increase in PGE_2 is not due to circulating prostaglandins, generated at the site of inflammation, crossing the blood-brain barrier.

Figure 6.19 shows the mean PGE_2 levels in spinal superfusate samples obtained from 4 rats between 0 to 3 and 3 to 5 hours following the induction of inflammation and the inclusion of 100μM indomethacin in the aCSF. There are significant ($p<0.01$) increases in the release of PGE_2 from the spinal cord between 0-3 hours (4.9 fold) and 3-5 hours (5.3 fold) following carrageenan/kaolin-induced inflammation. The spinal administration of 100μM indomethacin significantly ($p<0.01$) reduced PGE_2 to below basal levels.

There was no increase in 6-keto-$PGF_{1\alpha}$ levels in spinal superfusate samples following the intrarticular injection of kaolin and carrageenan within 5 hours of the induction of inflammation (data not shown).

FIGURE 6.18.

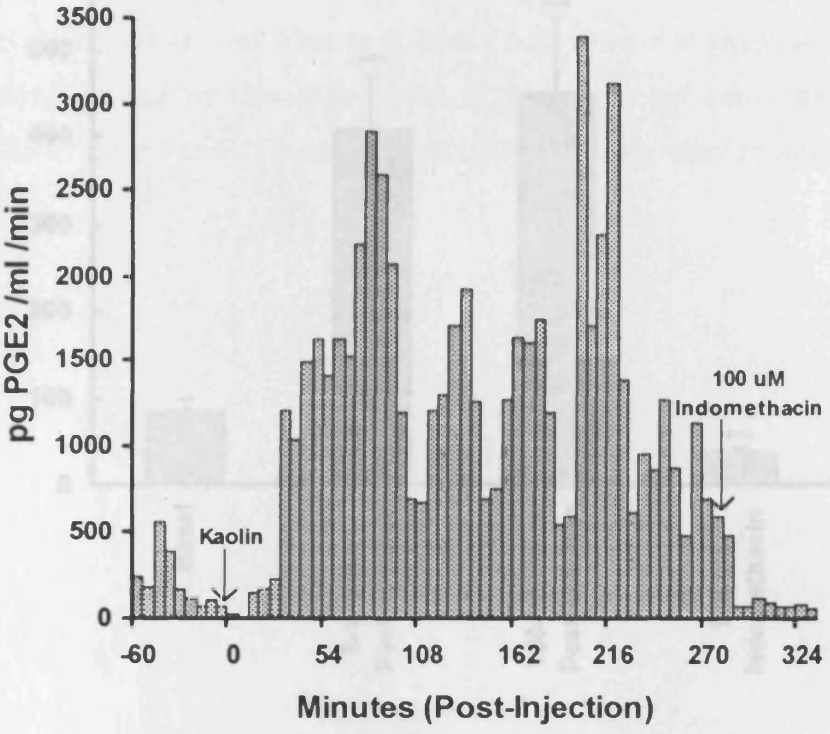


Figure 6.18. The Intraarticular Injection of Kaolin and Carrageenan Evokes a Rapid Release of PGE_2 from the Rat Spinal Cord. This figure shows the release of PGE_2 from the spinal cord of a normal rat following the intrarticular injection of kaolin (at 0 minutes) and carrageenan (at 15 minutes-not shown on graph). Within 30 minutes of the kaolin injection there is a dramatic increase in pg PGE_2 $ml^{-1}min^{-1}$ in the superfusate samples. The increase in PGE_2 levels is sustained over 4.5 hours. Within 6 minutes of the inclusion of 100μM indomethacin in the aCSF there is a reduction in PGE_2 to basal levels.

FIGURE 6.19.

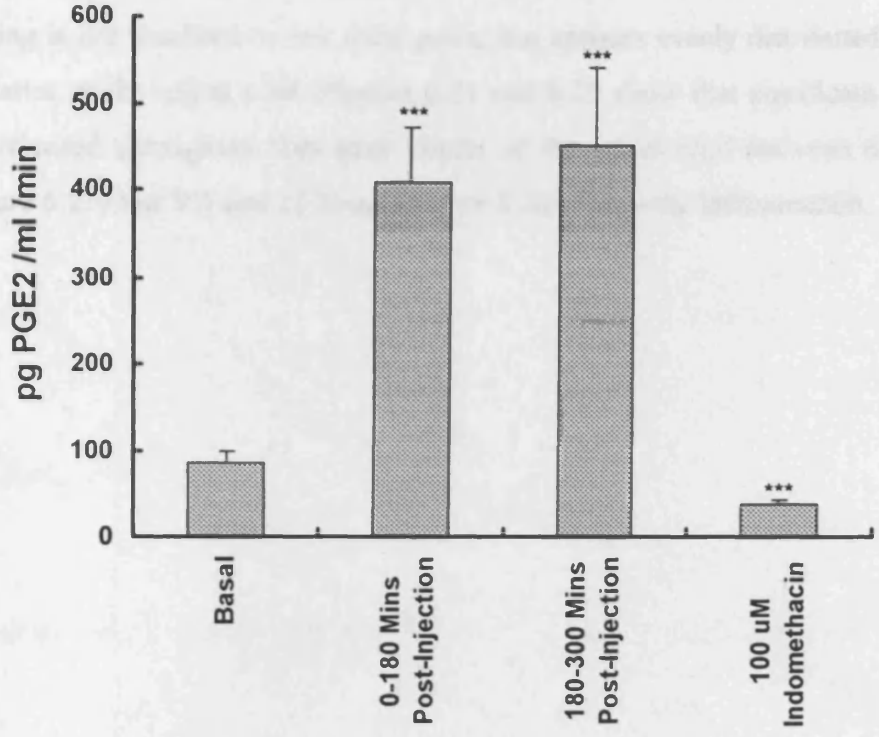


Figure 6.19 Intraarticular Kaolin and Carrageenan Evokes an Increase in the Release of PGE_2 from the Rat Spinal Cord. This figure compares the mean basal release of PGE_2 from the spinal cord of a normal rat (n=52 superfusate samples) with the increase in PGE_2 release between 0-3 (n=100 superfusate samples) and 3-5 hours following the intrarticular injection of kaolin and carrageenan (n=60 superfusate samples). There are significant (*** $p<0.01$) increases in pg PGE_2 $ml^{-1}min^{-1}$ in the superfusate samples following inflammation. The inclusion of 100μM indomethacin in the aCSF significantly (*** $p<0.01$, t-test) reduces the levels of PGE_2 to below basal levels (n=25 samples). Data were obtained from 4 animals.

Figure 6.20 shows mean densitometric image analysis of PGE_2 antibody microprobes obtained before and during carrageenan/kaolin-induced inflammation. Line A shows the mean basal release of PGE_2 from the spinal cord, and line B shows the mean level of PGE_2 released from the spinal cord between 3 and 5 hours following inflammation. No significant increases in PGE_2 release were observed during this period. However, after this time, significant amounts of PGE_2 are released from the spinal cord. The increase in endogenous PGE_2 binding is not localised to any focal point, but appears evenly distributed throughout the grey matter of the spinal cord. Figures 6.21 and 6.22 show that significant amounts of PGE_2 are released throughout they grey matter of the spinal cord between 6.5 and 8.75 hours (Figure 6.21) and 9.5 and 12 hours (Figure 6.22) following inflammation.

FIGURE 6.20.

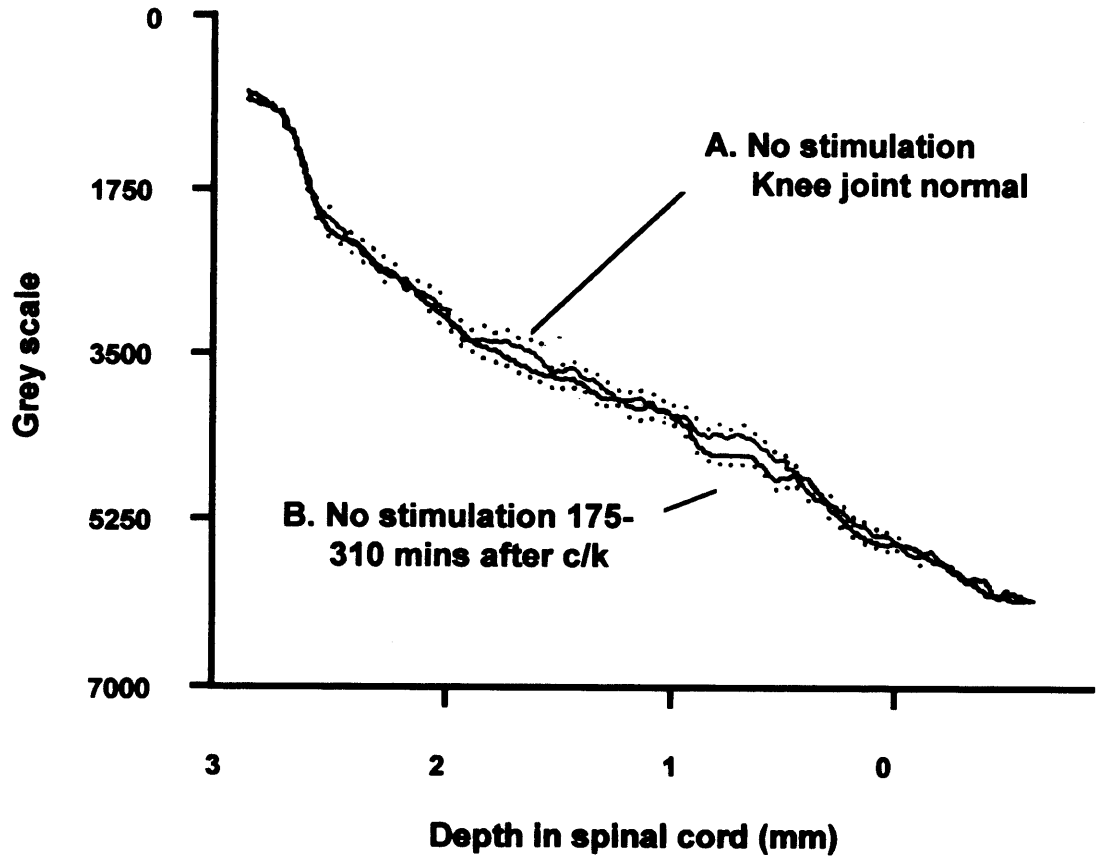


Figure 6.20 Antibody Microprobes Do Not Detect an Increase in Spinal PGE_2 Between 3-5 Hours Following the Intraarticular Injection of Kaolin and Carrageenan. This figure compares mean densitometric image analysis of PGE_2 antibody microprobes obtained before (Line A, n=39 probes) and following kaolin/ carrageenan inflammation. Line B shows the mean level of PGE_2 released from the spinal cord between 3 and 5 hours following inflammation (n=40 probes). No significant increase in intraspinal PGE_2 levels are observed during this period. Data represent the mean image density of the probes (solid lines) ± S.E.M. (dotted lines).

FIGURE 6.21.

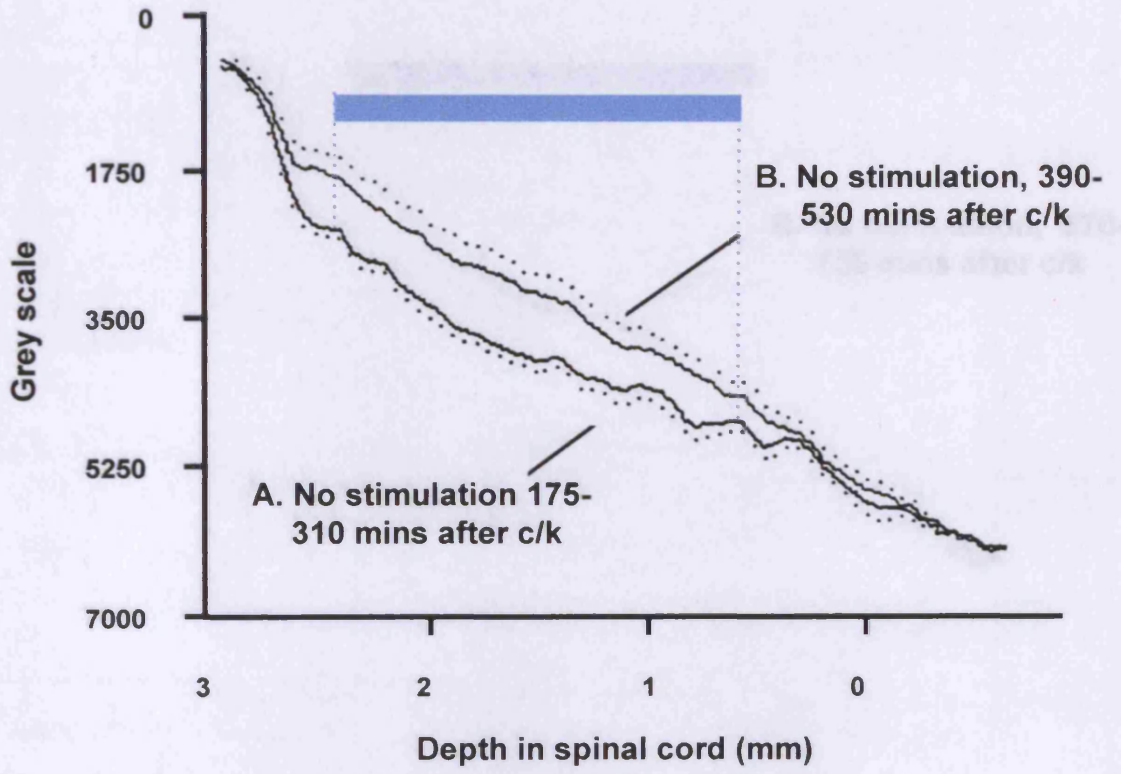


Figure 6.21. Antibody Microprobes Detect An Increase in Spinal PGE_2 Between 6.5 and 8.75 Hours Following the Intraarticular Injection of Kaolin and Carrageenan. This figure compares mean densitometric image analysis of PGE_2 antibody microprobes obtained between 175 and 310 minutes and microprobes obtained between 390 and 530 minutes following the intraraticular injection of kaolin and carrageenan. Line A shows the mean level of PGE_2 released from the spinal cord between 175-310 minutes following inflammation (n=20 probes), line B shows the mean level of PGE_2 released from the spinal cord between 390 and 530 minutes following inflammation (n=21 probes). Data represent the mean image density of the probes (solid lines) ± S.E.M. (dotted lines). The reduction in radiolabelled PGE_2 binding indicates that there is an increase in spinal PGE_2. Significant amounts of PGE_2 ($p<0.05$, represented by the blue shaded region, t-test) are released throughout the grey matter of the spinal cord.

FIGURE 6.22.

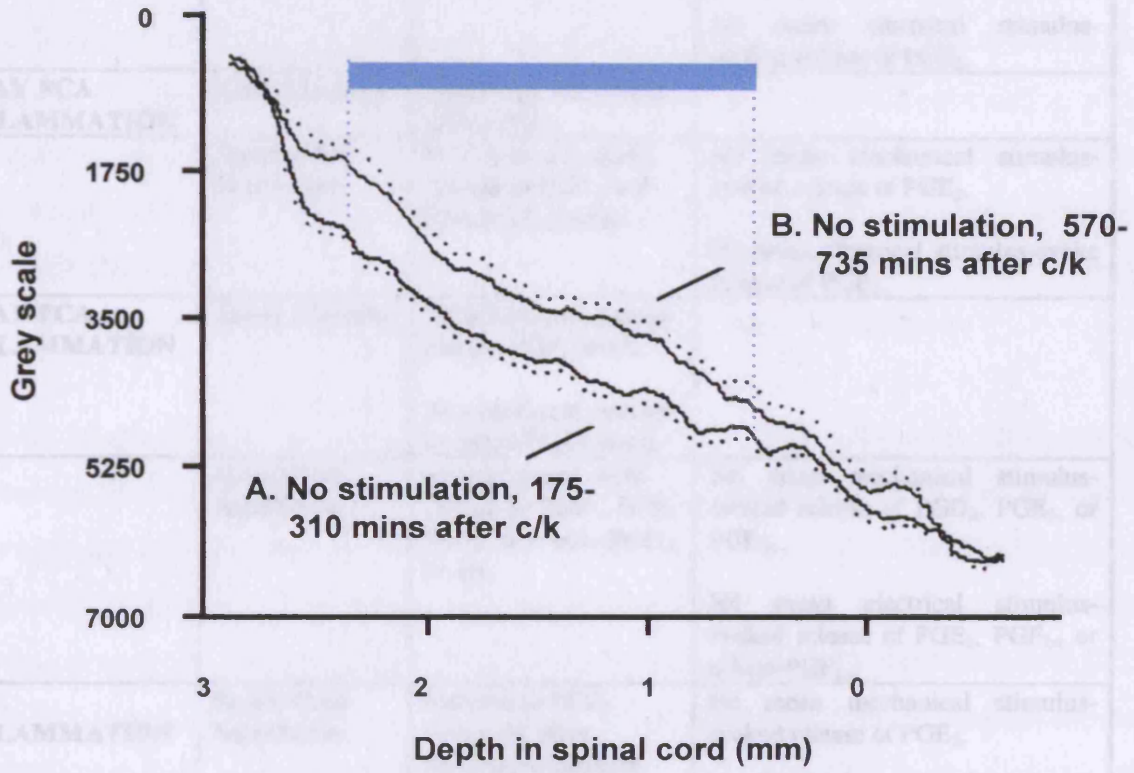


Figure 6.22. Spinal PGE_2 Levels are Increased up to 12 Hours Following the Intraarticular Injection of Kaolin and Carrageenan. This Figure compares mean densitometric image analysis of PGE_2 antibody microprobes obtained between 175 and 310 minutes and microprobes obtained between 570 and 735 minutes following the intraraticular injection of kaolin and carrageenan. Line A shows the mean level of PGE_2 released from the spinal cord between 3 and 5 hours following inflammation (n=20 probes), line B shows the mean level of PGE_2 released from the spinal cord between 570 and 735 minutes following inflammation (n=16 probes). Data represent the mean image density of the probes (solid lines) ± S.E.M. (dotted lines). Spinal PGE_2 levels remain significantly elevated ($p<0.05$, shown by blue shaded region, t-test) for up to 12 hours following the development of an acute inflammation.

TABLE 6.6.

RAT	PROCEDURE	BASAL RELEASE	STIMULUS-EVOKED RELEASE
NORMAL	Spinal Cord Superfusion	PGD_2>PGE_2> 6-keto-$PGF_{1\alpha}$>$PGF_{2\alpha}$	No mean mechanical stimulus-evoked release of PGE_2, PGD_2 or $PGF_{2\alpha}$. No mean electrical stimulus-evoked release of $PGF_{2\alpha}$ or PGE_2.
	Antibody Microprobes	-	No mean mechanical stimulus-evoked release of PGE_2 *. No mean electrical stimulus-evoked release of PGE_2.
1 DAY FCA INFLAMMATION	Tissue Analysis	Significant increase in spinal PGE_2.	-
	Spinal Cord Superfusion	No significant mean change in PGE_2 or 6-keto-$PGF_{1\alpha}$ levels.	No mean mechanical stimulus-evoked release of PGE_2. No mean electrical stimulus-evoke release of PGE_2.
3 DAY FCA INFLAMMATION	Tissue Analysis	Significant increase in plasma PGE_2 levels. No significant increase in spinal PGE_2 levels.	-
	Spinal Cord Superfusion	No significant mean change in PGD_2, PGE_2 $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ levels.	No mean mechanical stimulus-evoked release of PGD_2, PGE_2, or $PGF_{2\alpha}$ No mean electrical stimulus-evoked release of PGE_2, $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$.
C/K INFLAMMATION	Spinal Cord Superfusion	Increase in PGE_2 within 30 mins. Significant increase between 180-300 mins.	No mean mechanical stimulus-evoked release of PGE_2.
	Antibody Microprobes	Increase in PGE_2 at 6.5 hours, sustained for at least 12 hours.	No mechanical stimulus-evoked release of PGE_2.

Table 6.6. Summary of Results. This Table highlights the main results from this Chapter. There is a significant increase in the spinal levels of PGE_2 following the development of inflammation, as measured by tissue analysis following FCA-induced monoarthritis, and spinal cord superfusion and antibody microprobes following C/K induced inflammation. There is little evidence to suggest a relationship between acute noxious stimulation (mechanical and electrical) and spinal prostaglandin release.

* There was some evidence to suggest that noxious mechanical stimuli may induce the release of spinal PGE_2, but further experiments need to be conducted in order to determine whether these results were valid.

6.4. Discussion.

The development of inflammation is associated with changes in spinal nociceptive processing. For example, there is an increase in the size of the neuronal response to mechanical stimuli and an increase in the size of neuronal receptive fields following the development of inflammation (Neugebauer and Schaible, 1990; Grubb *et al.*, 1993). Neurotransmitters known to be involved in nociceptive processing are upregulated in the dorsal horn following inflammation (Iadarola *et al.*, 1988; Weihe *et al.*, 1988; Millan *et al.*, 1988; Draisci and Iadarola, 1989; Ohno *et al.*, 1990; Przewlocka *et al.*, 1992; Sluka *et al.*, 1992; Hanesch *et al.*, 1993). In Chapters 4 and 5 of this thesis, COX-2 was found to be present in the superficial and deep dorsal horn of the normal rat spinal cord and is also upregulated in response to inflammation. This indicates that prostaglandins may be synthesised and released from the spinal cord of the normal rat and that increased levels of prostaglandins may be generated following the development of inflammation. The increased levels of spinal prostaglandins may be involved in spinal nociceptive processing.

Analysis of spinal superfusate samples has indicated that the COX pathway is active in the normal rat spinal cord, and of the prostaglandins studied, PGD_2 and PGE_2 are the major prostaglandin species released from the rat spinal cord (Table 6.3). This finding is in agreement with previous studies which have shown that these are the predominant prostaglandins present in rat brain (Abdel-Halim, *et al.*, 1977; Abdel-Halim and Änggård, 1979; Bishai and Coceani, 1992). It is of interest to note that both PGD-synthase (Vesin *et al*, 1995) and PGE_2-binding sites have been localised in the superficial laminae of the rat spinal cord (Matsumura *et al.*, 1992), indicating both PGE_2 and PGD_2 are synthesised and active in regions of the spinal cord associated with spinal nociceptive processing.

The evidence obtained in the present studies also suggests there is a relationship between the development of inflammation and the spinal release of PGE_2. An increase in the level of spinal PGE_2 was also measured in whole spinal cord homogenates obtained from normal rats and rats with FCA-induced monoarthritis. There was a significant increase in the levels of spinal PGE_2 one day following FCA-induced inflammation (Figure 6.6). Spinal PGE_2 was still elevated above basal levels by day 3 but this increase was not significant. The increase in PGE_2 levels mirrored the time course of the induction of COX-2 in the spinal cord following FCA-induced inflammation. Both PGE_2 (3-fold increase) and COX-2 (3.5 fold

increase, Figure 5.3) were maximally elevated 1 day post-inoculation and returned to near basal levels by day 3. This may indicate that PGE_2 generated, at least in part, by COX-2 may be an important mediator of nociceptive processing following inflammation.

The intraarticular injection of carrageenan/kaolin evokes an increase in the synthesis and release of PGE_2 from the spinal cord. The increase in basal levels were detected using spinal cord superfusion and antibody microprobe techniques (Figures 6.20-6.22). Within 30 minutes of the kaolin injection there was a dramatic increase in the levels of PGE_2 in spinal superfusate samples. The increase was sustained in a rather pulsatile manner for at least 5 hours following inflammation. Spinally-applied indomethacin (100μM) reduced PGE_2 release to below basal levels within 6 minutes. This suggests that indomethacin acts by rapid diffusion to an active site in the spinal cord not by systemic redistribution to the site of inflammation. This provides evidence that the increase in PGE_2 levels in spinal superfusate samples following inflammation is due to increased COX activity in the spinal cord, not due to circulating prostaglandins entering the CSF. Indeed the blood-brain barrier largely prevents this (Jones *et al.*, 1994). Using antibody microprobes, an increase in the level of spinal PGE_2 was detected between 6.5 and 12 hours following inflammation.

The inflammation-induced release of PGE_2 from the spinal cord has been described by several other groups. Malmberg and Yaksh (1995) used chronically implanted intrathecal microdialysis catheters to study the release of PGE_2 from the spinal cord of conscious rats following the subcutaneous injection of 5% formalin into a hindpaw. Samples were collected every 10 minutes over a one hour period following formalin injection. They found a biphasic increase in PGE_2 levels in dialysate samples collected between 0-10 minutes and 20-30 minutes, which corresponded to the time course of the biphasic behavioural hyperalgesia observed following formalin injection. Yang *et al.* (1996) used an identical method to measure the spinal release of PGE_2 following an intrarticular injection of carrageenan/kaolin. Rats were anaesthetised with halothane and 2 x 10 minute dialysate samples were collected in order to determine the basal level of prostaglandin release. A solution of 3% kaolin and 3% carrageenan was then injected into the right knee joint. Animals were allowed to recover and 10 minute samples were collected at varying timepoints following the injection (10, 30, 60, 120, 180, 240, and 300 minutes). A further two dialysate samples were collected the following day (24 hours post-injection).

Significant increases in PGE_2 were detected in samples collected 10 minutes and 24 hours following injection, no significant increases were observed at any other time point.

There are a number of problems with the protocol described by Yang *et al.* (1996) which may explain the differences between their results and the results obtained by spinal superfusion techniques. The main advantage of using chronically implanted microdialysis is that experiments can be performed on conscious animals. Yang *et al.* (1996) analysed samples obtained from halothane anaesthetised rats in order to determine basal PGE_2 levels, and compared these samples with samples obtained from conscious rats following inflammation. Halothane is known to have spinal effects (see later in discussion), and PGE_2 basal levels should have been determined using conscious non-injected rats. Interestingly, the only two samples which showed increased levels of PGE_2 were collected at 10 minutes and 24 hours (points at which anaesthesia ceased and animals recovered). It has been shown that PGE_2 may be released in a pulsatile manner (Figure 6.18) and the infrequent collection of dialyslate samples by Yang *et al.* (only seven 10 minute samples in a five hour period) may mean that they missed periods of PGE_2 release. This was the primary reason why we developed a radioimmunoassay system, to ensure that all samples from an experiment could be analysed. The recovery rate of prostaglandins using microdialysis is very small. Malmberg and Yaksh (1995) estimated a 30% recovery of PGE_2 *in vitro* and only a 10% recovery of PGE_2 from the spinal cord *in vivo*. It is also worth noting that both Malmberg and Yaksh (1995) and Yang *et al.* (1996) stored their dialysate samples in unsiliconised polypropylene tubes before analysis by radioimmunoassay which may lead to inaccurate results (Figure 6.3).

The increases in spinal PGE_2 associated with C/K-induced inflammation, FCA-induced monoarthritis and formalin-induced inflammation (Malmberg and Yaksh, 1995) occur at times associated with the development of behavioural hyperalgesia in conscious animals. The intraarticular injection of carrageenan/kaolin induces behavioural hyperalgesia between 1-3 hours following injection (Schaible and Schmidt, 1985) and induces thermal hypersensitivity within 1 hour (Yang *et al.*, 1996). The development of behavioural hyperalgesia following FCA-induced monoarthritis peaks one day post-inoculation (Figure 3.3).

A number of studies have implicated PGE_2 in the development of behavioural hyperalgesia. The spinal administration of PGE_2 (1 pg-10 ng i.t.) induced behavioural hyperalgesia which lasted up to 30 minutes following injection (Uda *et al.*, 1990; Minami *et al.*, 1994). and various groups have tried to pharmacologically identify the receptors through which PGE_2 may induce hyperalgesia. EP_1, EP_2 and EP_3 receptors have all been implicated. Intrathecally-administered butaprost (an EP_2 agonist) and sulprostone (an EP_3 agonist) mimicked PGE_2-induced hyperalgesia (Nishihara *et al.*, 1995) whilst AH6809 (an EP_1 antagonist) inhibited PGE_2-induced hyperalgesia (Uda *et al.*, 1990). The prostaglandin receptors have recently been cloned and characterised. As pharmacological prostaglandin receptor agonists and antagonists are relatively non-specific, work is currently underway to examine the distribution of prostaglandin receptors in the rat spinal cord using molecular techniques. Recent work in our laboratory has determined the presence of mRNA transcripts encoding EP_2 and EP_4 but not EP_3, receptors in normal rat spinal cord (Lucy Donaldson, personal communication, 1997). The mRNA encoding the EP_2 receptor has been detected in the superficial laminae of rat spinal cord (Kawamura *et al.*, 1997) indicating a potential role of the activated EP_2 receptor in nociceptive processing.

Prostaglandin-evoked behavioural hyperalgesia may be mediated through NMDA and/or NK-1 receptors. PGD_2-induced behavioural hyperalgesia may occur through interaction with the NK-1 receptor, pretreatment with the NK-1 receptor antagonist CP 96,345 blocked PGD_2-induced hyperalgesia (Uda *et al.*, 1990; Minami *et al.*, 1997). The spinal administration of SP produces a dose-dependent thermal hyperalgesia mediated through NK-1 receptors, which was blocked by intrathecal administration of NSAIDs (Malmberg and Yaksh, 1992). PGD_2-induced hyperalgesia was unaffected by NMDA receptor antagonists (Minami *et al.*, 1997). The spinal actions of PGE_2, however, are intrinsically linked with the activation of NMDA receptors. The spinal administration of NMDA produces a dose-dependent thermal hyperalgesia which is blocked by intrathecal administration of NSAIDs (Malmberg and Yaksh, 1992). Conversely, NMDA receptor antagonists block PGE_2-induced behavioural hyperalgesia (Minami *et al.*, 1994; Nishihara *et al.*, 1995; Minami *et al.*, 1997). The development of wind-up in spinal cord neurones is mediated in part by NMDA receptors (Davies and Lodge, 1987; Thompson et al., 1990). Intrathecal administration of specific NMDA receptor antagonists completely abolishes wind-up (Davies and Lodge, 1987; Dickinson and Sullivan, 1990; Willingale et al., 1997). The selective COX-2 inhibitor SC58125 dose-dependently reduces the wind-up of a spinal

reflex evoked by high threshold electrical stimulation of the sural nerve, but has no effect on neuronal responses to acute noxious mechanical stimulation (Willingale and Grubb, 1996; Willingale et al., 1997). These data suggest that prostaglandins regulate the processing of nociceptive input when the spinal cord has been rendered hyperexcitable, for example, following inflammation or high threshold stimulation-induced wind-up of a spinal reflex.

The increase in the level of spinal PGE_2 measured in whole spinal cord homogenates obtained from normal rats and rats with FCA-induced monoarthritis was not accompanied by an increase in the levels of PGE_2 released from the spinal cord into superfusate samples (Table 6.4). This finding is in contrast to studies which have found that CGRP (Collin *et al.*, 1993), Met-enkephalin-Arg^6-Gly^7-Leu^8 (a peptide derived from proenkephalin) and α-neoendorphin (a peptide derived from prodynorphin) (Przewlocka *et al.*, 1992) increase in the spinal cord following the development of FCA-induced mono/polyarthritis and are also released in increased amounts from the rat spinal cord. Whilst PGE_2 levels increase in the spinal cord (as measured by whole spinal cord homogenate analysis and antibody microprobes), PGE_2 may be broken down before it can diffuse out of the spinal cord into the perfusate, due to an increased turnover of PGE_2 following inflammation. Another possibility is that the additional anaesthetic ('inactin': sodium thiobutabarbitol, i.p.) required to surgically anaesthetise 1 day FCA-monoarthritic rats may interfere with the mechanism by which prostaglandins are synthesised and released from the spinal cord.

Barbiturates act by reducing the activity of excitatory synapses and enhancing pre- and post-synaptic inhibition. There are many examples of barbiturates modulating neurotransmitter release. They have been shown to dose-dependently reduce noxious mechanical stimulus-induced responses in dorsal horn neurones (Collins and Ren, 1987). In our laboratory, it has been noted that administration of additional sodium thiobutabarbital during electrophysiological experiments caused an inhibition of wind up of a spinal nociceptive reflex, which is mediated, in part, by COX activity (Hilary Willingale and Blair Grubb, personal communication, 1997). Large amounts of barbiturates may therefore inhibit the basal release of prostaglandins from the rat spinal cord.

Barbiturates reduce the increase in spinal metabolic activity evoked by the subcutaneous injection of formalin. Rats were surgically anaesthetised with sodium pentobarbital (40mg/kg, i.p.), 5% formalin was injected into a forepaw, then [^{14}C]-2-deoxyglucose was

injected intravenously. Forty-five minutes later the rats were killed by barbiturate overdose, and the spinal cord removed and sectioned. Transverse spinal cord sections were placed against X-ray film with known concentrations of ^{14}C, in order to quantify the amount of isotope present in various regions. In unanaethetised rats, there was a bilateral increase in the rate of local glucose utilisation (LGU) in the spinal cord following the injection of formalin. Anaesthesia abolished the contralateral increase in LGU and significantly reduced the ipsilateral increase (Porro et al., 1991).

Sodium pentobarbital also reduces the induction of *c-fos* in the spinal cord following noxious thermal stimulation. Rat were anaesthetised with sodium pentobarbitone and the hindpaw immersed in 52°C water for 20 seconds. If the animals were allowed to recover from anaesthetic, an increase in Fos-li neurones was observed in the ipsilateral superficial dorsal horn 2 hours following stimulation whereas animals maintained under anaesthesia showed less neuronal Fos-li. (Williams *et al.*, 1990).

Barbiturates are not the only anaesthetics with spinal effects. Rats anaesthetised with 2% halothane have significantly fewer *c-fos*-positive neurones in the ipsilateral superficial dorsal horn than unanaesthetised rats. Halothane reduced the number of *c-fos*-positive neurones by 74.4%. Pretreatment with halothane also suppressed the phosphorylation of CREB in the ipsilateral and contralateral dorsal horn and around the central canal (Ji and Rupp, 1997).

Despite the potential inhibitory actions of barbiturates, a noxious thermal induced release of PGE_2 from the spinal cord of barbiturate (sodium pentobarbital, 65mg/kg, i.p.) anaesthetised rats has been described (Coderre et al., 1990). Immersion of the hindpaw in 35°C water for 40 minutes did not affect the levels of PGE_2 in spinal superfusate samples, whereas immersion in 50°C water (noxious stimulus) for 40 minutes caused an approximate 2-fold increase in PGE_2 levels in spinal superfusate samples. There were no significant stimulus-induced changes in the levels of $PGF_{2\alpha}$ or 6-keto-PGF_1. This evidence indicates that a longer stimulation period may be required in order to measure noxious mechanical stimulus-induced release of PGE_2 using spinal superfusion techniques.

Electrical stimulation of the sural (superfusion experiments) and tibial (antibody microprobe experiments) nerves did not evoke the release of PGE_2 from the spinal cord, despite using high intensity stimuli (10-20V) in order to activate unmyelinated nociceptive afferents at a

frequency (1Hz) known to induce spinal wind-up. Sensory neuropeptides such as CGRP (Andreeva and Rang, 1993), SP (Duggan and Hendry, 1986) and NKA (Hope et al., 1990) are released from the spinal cord in response to such electrical stimuli. Although Andreeva and Rang (1993) indicated that a frequency of 10Hz was required to induce a measurable release of CGRP from the spinal cord *in vitro*. A higher frequency of stimulation may therefore be required to initiate the spinal release of PGE_2. Alternatively, whole nerve stimulation may be a rather crude stimulus which prevents the induction of PGE_2 release from the rat spinal cord. The application of local anaesthetic to the sciatic nerve following noxious thermal stimulation increases the number of neurones expressing Fos-li in the rat spinal cord. This suggests that under normal conditions the activation of primary afferents exert a tonic inhibition which controls the induction of *c-fos* (Williams *et al.*, 1990). The induction of COX-2 in the spinal cord shares some similarities with the induction of *c-fos* (see Chapter 5), whole nerve electrical stimulation may inhibit the release of PGE_2 from the rat spinal cord.

In summary, we have shown that the COX biosynthetic pathway is active in the normal rat spinal cord and the basal release of PGE_2 is rapidly upregulated in response to peripheral inflammatory stimuli. There is no evidence to suggest that a relationship exists between acute noxious stimuli and spinal prostaglandin release.

Chapter 7. Discussion.

The aims of this project were to investigate whether the COX pathway is involved in spinal nociceptive processing and explore the relationship between spinally-generated prostaglandins and peripheral stimulation. In Chapter 1 it was argued that the following criteria would have to be met in order to establish whether prostaglandins are involved in spinal nociceptive processing:

- COX-1 and/or -2 must be present in the spinal cord and in a location consistent with an involvement in spinal nociceptive processing.
- The COX pathway must be active.
- Increased levels of prostaglandins must be released from the spinal cord in response to sustained nociceptor activity.
- The time-course of COX/PG upregulation must coincide with the period of behavioural hyperalgesia observed following inflammation.

The current study has advanced our knowledge with respect to the presence of COX isoforms within the spinal cord, where they are located, their activity (i.e. which prostaglandins they synthesise) and regulation. The data presented in this thesis shows that the machinery required for prostaglandin biosynthesis is present and active in the normal rat spinal cord. Using Western blotting techniques, both COX-1 and COX-2 were detected in protein extracts obtained from normal rat lumbar spinal cord. Using immunocytochemical methods we identified intense COX-2-li in regions of the rat spinal cord associated with processing nociceptive input. The COX pathway is active and measurable amounts of PGD_2 and PGE_2 (and also, to a lesser extent 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$) are released from the normal rat spinal cord. Furthermore, following the development of a peripheral inflammatory lesion there is a rapid dynamic upregulation of spinal COX-2, but not COX-1, in the lumbar spinal cord. The increase in COX-2 is accompanied by a concomitant increase in intraspinal PGE_2 levels. The spinal release of PGE_2 is also increased by the development of an acute inflammatory lesion. These results support the hypothesis that prostaglandins, possibly synthesised by COX-2, play an important role in modulating spinal nociceptive processing following inflammation.

The observation that spinal COX-2 protein levels and PGE_2 released from the spinal cord are upregulated in response to inflammation suggest that they play a dynamic role in the development of spinal nociceptive processing and neuronal hyperexcitability. Until recently, it was thought that the analgesic properties of NSAIDs arise due to their peripheral anti-inflammatory properties, i.e. the inhibition of COX and subsequent prevention of prostaglandin production at the site of inflammation (Vane, 1971). Evidence has now accumulated to suggest that a component of the analgesic properties of NSAIDs may be due to an action in the spinal cord (Brune *et al.*,1991; McCormack and Brune, 1991; McCormack and Urquhart, 1995), a theory supported by a number of behavioural and electrophysiological studies (Malmberg and Yaksh; 1992b, 1993; Chapman and Dickenson, 1992; Jurna *et al.*, 1992; Willingale and Grubb, 1996). During the course of this study, two groups identified COX-1 and COX-2 mRNA transcripts in homogenised rat spinal cord tissue (Beiche *et al.*, 1996; Hay *et al.*, 1997), but provided no information about the distribution of the two enzymes.

Using immunocytochemical techniques, COX-1-li was detected in dorsal roots surrounding the spinal cord. Further investigation revealed that COX-1-li is restricted to small to medium sized cell bodies in the DRG of normal rats (Grubb *et al.*, 1997; Willingale *et al.*, 1997). Neurones of this size are thought to be nociceptors as many contain SP and CGRP (Lawson *et al.*, 1993). The function of COX-1, and prostaglandins released from these nociceptive neurones, is unknown although it is conceivable that prostaglandins are released from axon terminals. Since very few COX-1-li fibres were observed entering the dorsal horn, it is unlikely that prostaglandins are released from nociceptors centrally. It would be interesting to determine whether nociceptive afferents have the capability to synthesise and release prostaglandins at peripheral terminals. This 'auto-sensitisation' may have a priming function. Diffuse COX-2-li was detected throughout the grey matter of the spinal cord, which may indicate that both neuronal and non-neuronal cell types express COX-2. In regions where the neurones were less densely packed, such as the deep dorsal and ventral horn, it was possible to distinguish COX-2-li in the somata of individual neurones, which is consistent with the pattern of neuronal labelling described in other studies (Yamagata *et al.*, 1993; Breder *et al.*, 1995 Kaufmann *et al.*, 1996). Intense COX-2-li was detected in the superficial dorsal horn (laminae I-II), and also, less consistently, in the medial aspects of the deep dorsal horn (laminae IV-V) and around the central canal (lamina X) of lumbar spinal cord segments. Nociceptors terminate primarily in these regions of the spinal cord (Light

and Perl, 1979; Craig *et al.*, 1988; Levine *et al.*, 1984) and furthermore, these regions contain primary afferent and intrinsic spinal neuropeptides known to be involved in nociceptive processing (Gibson *et al.*, 1981; Ruda, 1986; Weihe *et al.*, 1988; Ribeiro-da-Silva *et al.*, 1989; Marlier *et al.*, 1991; Zhang *et al.*, 1993). The localisation of COX-2-li in these regions is commensurate with COX-2, or prostaglandins synthesised by COX-2, being involved in processing nociceptive input.

Like other neurotransmitters and modulators involved in spinal nociceptive processing, spinal COX-2 is rapidly upregulated in response to peripheral inflammation. COX-2 protein levels increased between 6 and 24 hours following induction of FCA-monoarthritis and returned to basal levels by day 3. This time course is in agreement with studies examining COX-2 mRNA upregulation using a similar model of inflammation (Beiche *et al.*, 1996; Hay *et al.*, 1997).

The bilateral induction of COX-2 is surprising as gene activation and/or protein upregulation following unilateral inflammation is usually initially restricted to the ipsilateral spinal cord. A number of questions immediately spring to mind. Why is COX-2 upregulated bilaterally? Do the bilateral increases occur concomitantly, or is a lag phase between the ipsilateral and contralateral increases? If there is a lag phase, does this occur due to signals from the ipsilateral spinal cord moving through ascending and descending spinal tracts to the contralateral spinal cord? Alternatively, do direct neuronal connections between the two sides of the spinal cord induce COX-2 gene transcription on the contralateral side? The increase in lumbar spinal COX-2 suggests that the induction mechanism is related to an increase in nociceptive afferent input from the inflamed limb. This observation excludes the possibility that circulating cytokines cross the blood-brain barrier and upregulate spinal COX-2.

In order to determine whether the induction of spinal COX-2 is related to increased nociceptive afferent input, a series of experiments should be performed in which FCA-monoarthritis is induced in rats with capsaicin-induced lesions to the sciatic nerve. Capsaicin causes preferential degeneration of unmyelinated nociceptive afferents. If the spinal increase in COX-2 protein levels is due to increased nociceptor activity, then we would not expect to see COX-2 upregulation in capsaicin-lesioned rats. In order to dissect the spinal mechanism of COX-2 upregulation we could use specific receptor (e.g. glutamate, tachykinin)

antagonists to prevent the induction of COX-2. As discussed in Chapter 5, spinal COX-2 appears to be functionally linked with the NMDA receptor, indeed prostaglandins generated by COX-2 modulate the wind-up of a nociceptive spinal reflex (Willingale and Grubb, 1996; Willingale *et al.*, 1997).

PGE_2 and PGD_2 were the two most abundant species (of the four species tested) released from the spinal cord of normal rats. These data agree with studies which have shown these prostaglandins to be the predominant species in brain tissue (Abdel-Halim, *et al.*, 1977; Abdel-Halim and Änggård, 1979). PGE_2-binding sites and PGD-synthase have been localised to the superficial dorsal horn of the rat spinal cord (Matsumura *et al.*, 1992; Urade *et al*, 1989; Vesin *et al*, 1995; Kawamura *et al.*, 1997), a region which overlaps with COX-2-li. Furthermore, mRNA transcripts encoding EP_2, EP_4 and DP receptors have been detected in normal rat spinal cord tissue (Lucy Donaldson, personal communication, 1997). This suggests a potential role for PGE_2 and PGD_2 in nociceptive processing.

Spinal PGE_2 levels increased following the development of FCA-monoarthritis with the same time course as COX-2 upregulation which also coincides with development of behavioural hyperalgesia. Pain behaviour subsides at the same time that spinal COX-2 levels return to normal, despite the continuing inflammation at the joint. Prostaglandins synthesised by spinal COX-2 may, therefore, be important in the generation and/or maintenance of behavioural hyperalgesia and spinal hyperexcitability. Spinal COX-2, however, cannot be wholly responsible for the generation of hyperalgesia, as it is induced bilaterally, whereas behavioural hyperalgesia occurs unilaterally.

A number of studies have shown that intrathecal NSAIDs reduce the responsiveness of spinal cord neurones in circumstances where spinal hyperexcitability has been intentionally induced e.g. reflex wind-up, inflammation-induced hyperexcitability (Malmberg and Yaksh, 1992a; Neugebauer *et al.*, 1994; Willingale *et al.*, 1997) but do not reduce responses to acute noxious stimuli in the normal animal (Yaksh, 1982; Malmberg and Yaksh, 1992; Chapman and Dickenson, 1992; Willingale *et al.*, 1997).

The role of spinal prostaglandins is likely to be neuromodulatory, and since they are highly diffusible they are likely to influence neuronal activity at and around the site of synthesis. Prostaglandin receptors EP_2, EP_4 and DP are all coupled to the G-protein 'G_s', activation of

G_s stimulates adenylate cyclase and increases the level of intracellular cAMP, and this may be one mechanism through which PGE_2 and PGD_2 may exert their effects. Inhibition of adenylate cyclase inhibits PGE_2-induced potentiation of the response to bradykinin in sensory neurones (Cui and Nicol, 1995). PGE_2 increases neuronal activity by modulating the tetrodotoxin-resistant sodium current (TTX-R I_{Na}) in DRG cells via a cAMP cascade (England *et al.*, 1996). The firing threshold of neurones was reduced by 1μM PGE_2, to such an extent that a current injection converted a single evoked action potential to a train of action potentials. These sodium channels are predominantly found in small diameter, capsaicin-sensitive DRG neurones (Arbuckle and Docherty, 1995). It is conceivable that the spinal prostaglandins synthesised by COX-2 in response to inflammation diffuse to the presynaptic nociceptor terminal, where they bind to their specific receptor, increase intracellular cAMP levels and facilitate the release of excitatory neurotransmitters, such as SP (Nicol *et al.*, 1992; Vasko *et al.*, 1993) and CGRP (Andreeva and Rang, 1993). This may be one mechanism by which prostaglandins generated by COX-2 may, indirectly, contribute to the generation of spinal wind-up (Willingale *et al.*, 1997) an event primarily mediated by activation of postsynaptic NMDA receptors (Davies and Lodge, 1987; Thompson *et al.*, 1990). Alternatively, prostaglandins may, in some way, directly interact with and enhance the activity of the NMDA receptor.

If the facilitatory actions of prostaglandins are mediated through spinal prostaglandin receptors intrathecal receptor antagonists should block the effects. The absence of selective pharmacological receptor antagonists makes neurophysiological assessment of the role of the receptor subtypes impractical. The use of intrathecal antisense oligodeoxynucleotides directed against specific receptors may provide valuable information about the role of prostaglandins in spinal pain processing. This technique has been used successfully in the study of the role of opioid receptors (Bilsky *et al.*, 1996; Narita *et al.*, 1997) and G protein alpha subunits (Standifer *et al.*, 1996) in nociceptive processing. It may also be possible to generate knockout animals which lack specific prostaglandin receptors. This technique has already been used to demonstrate that mice lacking the IP receptor show altered pain responses in models of neuropathic pain (Murata *et al.*, 1997).

As well as their extracellular effects, spinally generated prostaglandins may also influence neuronal physiology through acting intracellularly as transcription factors and promoting upregulation of other genes. PGD_1, PGD_2 and PGJ_2 bind to and activate all isoforms (α, δ

and γ) of PPAR (Yu *et al.*, 1995), which once associated with the 9-*cis*-retinoic acid receptor binds to HRE in the promoter region of target genes and initiates transcription. Prostaglandins have been shown to influence the transcription of *c-fos*, pretreatment with NSAIDs inhibited NMDA-receptor mediated induction of *c-fos* mRNA in dentate gyrus neurones (Lerea *et al.*, 1995), other genes may also be regulated by prostaglandins. It is probable that prostaglandins activate other, as yet unidentified, transcription factors and this information might provide a valuable key to understanding the role of prostaglandins in the dynamic gene regulation.

This study has provided a new insight into the importance of COX and prostaglandins in the processing of nociceptive input within the spinal cord of normal and monoarthritic rats. The development of spinal hyperexcitability is thought to underlie aspects of post-operative pain and hyperalgesia (Wall, 1988). The observations that COX-2 is located in regions of the spinal cord associated with nociception, and prostaglandins play a role in the development of spinal hyperexcitability (Willingale *et al.*, 1997) may be important factors in understanding and improving the clinical management of pain. Spinal delivery of selective COX-2 inhibitors prior to operations may inhibit the synthesis of spinal prostaglandins and inhibit the development of neuronal hyperexcitability.

Traditionally, NSAIDs are thought to produce analgesia by inhibiting COX at the site of inflammation. However, it may be possible that a component of the analgesia arises through an action in the spinal cord. In order to exert this spinal effect drugs must be able to penetrate the blood brain barrier. Rice *et al.* (1993) demonstrated that systemically administered keterolac penetrated the blood brain barrier in humans and was detectable in CSF, albeit in concentrations approximately 1000 times less than plasma concentrations. This seemingly low concentration of keterolac may be sufficient to inhibit spinal COX and contribute to analgesia. This hypothesis is supported by the studies of Malmberg and Yaksh (1993b) who demonstrated that intrathecally applied NSAIDs were effective at reducing behavioural hyperalgesia at doses up to a 1000 times lower than the systemic dose required to exert the same effect. Systemically applied NSAIDs by therefore provide analgesia, at least in part, by redistribution to a spinal site of action. If this is the case a more effective method of analgesia may be provided through the development of specific COX-2 inhibitors which readily penetrate the blood brain barrier. These drugs would have the advantage of

no gastrotoxic effects, arising through inhibition of COX-1 in the stomach, and could be administered in relatively lower doses.

This results from this study provide a new insight into the role of COX and prostaglandins in nociceptive processing, and this advance in our knowledge may require a reworking of the theory of NSAID induced analgesia in inflammatory pain.

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Appendix 1. Solutions.

1. General Laboratory Solutions.

- PBS.

For 1 litre: mix 8.0g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 and 0.2g KH_2PO_4. pH to 7.2.

- Heparinised Saline.

For 1 litre: Stock solution of 12.5units/ml, add 2,5 ml heparin (from stock of 5000units/ml) to 0.9% saline. For Stock Solution of 1unit/ml, add 0.2ml heparin (from stock of 5000units/ml) to 0.9% saline.

- aCSF.

For 1 litre: mix 7.29g NaCl, 0.19g KCl, 0.2g Na_2PO_4, 2.18g $NaHCO_3$, 2ml 1M $CaCl_2$ and 1ml $MgCl_2$. (Note: add $CaCl_2$ last). Gas with 5% O_2/95% CO_2 to give a pH of 7.4.

2. Radioimmunoassay Solutions.

- Tris-HCl, pH 7.8.

For 1 litre: mix 7.02g Tris-HCl, 0.67g Tris Base and 0.372g EDTA.

- Bovine-γ-Globulin.

For 100mls: gently dissolve 0.5g Bovine-γ-Globulin into Tris-HCl.

- Polyethylene Glycol 6000.

For 1 litre: slowly mix 250g into distilled water.

3. Western Blotting:

- Sample Denaturing Buffer.

For 10 ml: mix 2.5 ml 3X Running Buffer, 1.7 ml Glycerol, 0.171 g SDS, 0.27 g Sucrose, 0.29 ml 100 mM EDTA, 0.007 g Bromophenol Blue, 0.01 g Tris Base and 0.048g Imidazole. Freeze in 1ml aliquots until use, before use add 50µl 1M dithriothreitol.

- 3X Running Buffer.

For 1 litre: mix 10 g Tris Base, 3.33 g SDS and 48 g Glycine. Filter before use.

- Imidazole Stacking Buffer.

For 100 ml: mix1.29 g Tris-HCl, 4.75 g Imidazole and 0.3 g SDS. pH to 6.8 with HCl.

- SDS-PAGE Transfer Buffer.

For 1 litre: mix 5.82g Tris Base, 3.0 g Glycine, 0.39 g SDS and 200 ml Methanol.

- TTBS

For 1 litre: mix 6.05 g Tris Base,8.75 g NaCl and 1 ml Tween 20. pH solution to 8 with HCl.

- Blocking Buffer.

For 100mls: mix 10g *Marvel* non-fat milk powder with TTBS.

- Resolving Gel

For 4 Minigels: mix10 ml 30% Acryl, 10 ml Water, 10 ml 3X Running Buffer. Then add 40 µl TEMED and 160 µl 10% Ammonium Persulphate. Swirl gently to mix and use immediately.

- Stacking Gel

For 4 Minigels: mix 2.4 ml Acryl, 6.0 ml Imidazole Stacking Buffer, 9.6 ml Water. Then add 24µl TEMED and 150 µl 10% Ammonium Persulphate. Swirl gently to mix and use immediately

4. Antibody Microprobes.

- PBS-Azide.

For 1 litre: mix 100mg Na-azide with PBS pH 7.2.

- PBS-Tween.

For 1 litre: mix 5ml 10% Tween-80 with PBS-Azide.

- Borate Buffer.

For 1 litre: mix 12.36g sodium borate with distilled water. PH to 8.5 with 1M NaOH.

5. Immunocytochemistry.

- Slide Subbing Solution.

For 100mls: dilute 2ml 3-aminopropyltriethoxysilane in acetone.

- Tissue Fixation.

  For 200mls: mix 4g paraformaldehyde with 80mls 0.2MNa_2HPO_4, heat gently until dissolved. When cool, add 20ml NaH_2PO_4 and 100ml distilled water.

- Blocking Solution.

For 100mls: mix 10ml goat serum and 0.5ml Triton-X-100 in PBS pH 74.

Appendix 2. Publications arising from this thesis.

- **Abstracts.**

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