A ROLE FOR PROSTANOIDS IN SPINAL NOCICEPTIVE PROCESSING

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by

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Increasingly, evidence suggests that non-steroidal anti-inflammatory drugs (NSAIDs) have central actions within the spinal cord in addition to the peripheral actions that have traditionally been associated with them. NSAIDs are commonly used in pain relief and inhibit cyclooxygenase activity (cox) that converts arachidonic acid to prostaglandins (PGs). This study addressed the hypothesis that prostaglandins within the spinal cord play a role in pain processing.

The presence of two isoforms of cox, cox-1 and cox-2, within the spinal cord of rats was identified for the first time using Western blotting techniques. Cox-2, but not cox-1, was shown to be upregulated during the development of an acute arthritis. The active biosynthetic pathway for PG production was therefore shown to be present and active in the spinal cord.

Extracellular recordings of wide dynamic range neurons within the lumbar spinal cord were made to study their responses to nociceptive mechanical stimuli in non-arthritic, acutely arthritic and chronically arthritic rats. Intrathecal NSAIDs had no effect on the responses recorded in non-arthritic rats. The responses of hyperexcitable, but not non-hyperexcitable neurons, in arthritic rats were concentration-dependently reduced by intrathecal NSAIDs. Hyperexcitable cells were defined subjectively as those with bursting activity and were found in arthritic but not non-arthritic rats. The C-fibre wind-up of a nociceptive spinal reflex, the hindlimb extensor reflex, was dose-dependently reduced by intravenous indomethacin (a NSAID) and a selective cox-2 antagonist, SC58125. Intrathecal indomethacin also concentration-dependently reduced the wind-up. An effect was therefore shown in hyperalgesic (and allied) but not non-hyperalgesic states.

A possible model for the role of spinal prostaglandins in the development of hyperalgesia is proposed.

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CONTENTS

ABBREVIATIONS

AA	arachidonic acid
ACh	acetyl choline
AP5	aminophosphonovaleric acid
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-proprionate
ATP	adenosine triphosphate
anova	analysis of variance
BK	bradykinin
CGRP	calcitonin gene-related peptide
7CK	7-chlorokynurenate
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
cox	cyclooxygenase
cox-1-ir	cyclooxygenase-1 immunoreactivity
cox-2-ir	cyclooxygenase-2 immunoreactivity
CSF	cerebrospinal fluid
D-AP5	5-amino-phosphonovaleric acid
D-CPP	D-3-((±)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid
EAA(s)	excitatory amino acid(s)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid
emg	electromyograph
EPSP	excitatory postsynaptic potential
FCA	Freund's complete adjuvant
GABA	gamma amino butyric acid
5-HT	serotonin
IL-1β	interleukin-1β
ir-PGE_2	prostaglandin 2 immunoreactivity
K/C	kaolin and carrageenan

kD	kilaDaltons
L-NAME	N(G)-nitro-L-arginine methyl ester
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d]-cyclohepten-5,10-amine maleate; dizocilpine
L-AP3	L-2-amino-3-phosphonopropionic acid
mRNA	messenger ribonucleic acid
n	number
NGF	nerve growth factor
NKA	neurokinin A
NMDA	*N*-methyl-D-aspartate
NORA	noradrenaline
NOS	nitric oxide synthase
NS	nociceptive specific
NSAID	non-steroidal anti-inflammatory drug
p	probability factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PG(s)	prostaglandin(s)
PKC	protein kinase C
PLA_2	phospholipase A_2
PMSF	phenylmethylsulfonyl fluoride
psi	pounds per square inch
rpm	revolutions per minute
RT-PCR	reverse transcriptase-polymerase chain reaction
SC58125	1-[(4-methylsulfonyl) phenyl]-3-trifluoromethyl-5-(4-fluorophenyl) pyrazole
SDS	sodium lauryl sulfate
SE	standard error of the mean
SP	substance P
TEMED	N,N,N′,N′-tetramethylethylenediamine
Tris/Trizma base	tris[hydroxymethyl]aminomethane
TTBS	tween/tris-buffered saline
Tween 20	polyoxyethylene-sorbitan monolaurate

UV	ultraviolet
V	volts
VIP	vasoactive intestinal polypeptide
WDR	wide dynamic range

Chapter 1

General Introduction

The sensation of pain is universal to sentient beings and is an almost universally unpleasant experience. Medically, pain relief is one of the most important areas of a physician's art. The national drugs bill for pain relief runs into billions of pounds a year and the pharmaceutical companies are keen to chase the shareholders' dream of a cheap, easily manufactured, safe panacea for this problem.

The dream has not yet been realised, but the importance of this area of medicine is such that vast resources are being poured into researching new drug therapies. A basic understanding of the physiological processes by which pain is perceived by an individual is of fundamental importance. Until we know how and why we feel pain under different circumstances, we cannot produce a rational and studied strategy to abolish or reduce the sensation.

Physiologists, anatomists, pharmacologists, pathologists, chemists, biochemists, physicians and surgeons can all contribute to the overall fund of knowledge. Each fragment of knowledge adds a drop to the shared pool from which a simple answer to the simple question 'why does it hurt?' may emerge.

1.1 Anatomy and Physiology of Somatic Pain Pathways

The sensation of pain serves a protective function to signal the danger of tissue damage. Somatic sensory information is transmitted from the periphery to the spinal cord by both large- and small-diameter nerve fibres. These fibres have been subdivided into groups based upon histological characteristics such as diameter and the presence or absence of myelination as shown in Table 1.1. Nociceptive information is transmitted by two such classes of nerve fibre, the Aδ- and C-fibres, although there is good evidence that Aβ-fibres may signal pain in pathological states (Neumann et al. 1996).

Low-intensity stimulation of somatic primary afferents activates large-diameter, myelinated Aα- and Aβ-fibres and produces the sensation of touch. Activation of

Table 1.1 Classification of afferent fibres (from Burgess & Perl 1973).

Fibre type	Function	Diameter (μm)	Myelinated/ unmyelinated	Conduction velocity (m/s)
Aα/Aβ	mechanoreceptors proprioceptors	6 - 20	myelinated	30 - 120
Aδ	mechanoreceptors	1 - 5	thinly myelinated	4 - 30
C	mechanoreceptors thermoreceptors polymodal nociceptors	0.3 - 1.5	unmyelinated	0.4 - 2

fast-conducting, myelinated Aδ-fibres produces a sensation of sharp, well localised pain. With increasing intensities of stimulation the small-diameter, slowly conducting, non-myelinated C-fibres are also recruited and this produces a prolonged perception of pain, usually subjectively described as 'dull' or 'aching'. The sensation of pain can be elicited by noxious thermal, chemical or mechanical insults to the tissues.

Aδ- and C-fibres arborise and terminate in free, non-corpuscular nerve endings. The ultrastructure of such nerve endings in the cat knee joint has been studied (Heppelmann et al. 1990) where they were shown to have a 'string of beads' appearance consisting of a series of spindle-shaped thick segments connected by waist-like thin segments, as well as end bulbs. Both the beads and end bulbs have characteristic features suggestive of receptor sites, being partly free of Schwann-cell covering and containing mitochondria, glycogen granules and vesicles.

'Pain receptors', or nociceptors, respond to potentially damaging mechanical stimuli (mechanical nociceptors), extremes of temperature (thermal nociceptors) or multiple types of harmful stimuli (polymodal nociceptors). Polymodal nociceptors have been described innervating skin, muscle and joints (Besson & Chaouch 1987).

Afferent fibres have their cell bodies in the dorsal root ganglia and enter the spinal cord mainly via the dorsal roots, although there is also evidence that some afferents may enter via the ventral roots (Besson & Chaouch 1987). The spinal cord is a segmented, bilaterally symmetrical structure with a somatotopic organisation such that afferents from the upper body enter more cranially and those from the hindlimbs enter in the lumbar region. It can be crudely subdivided into the grey matter, consisting of cell bodies and synaptic connections, and the surrounding white matter, consisting of nerve fibres that contribute to the ascending and descending tracts (Fig 1.1). On entry to the spinal cord, the afferent fibres split into ascending and descending bundles in Lissauer's tract, located dorsolateral in the white matter. The fibres run for one or two segments before terminating in the grey matter.

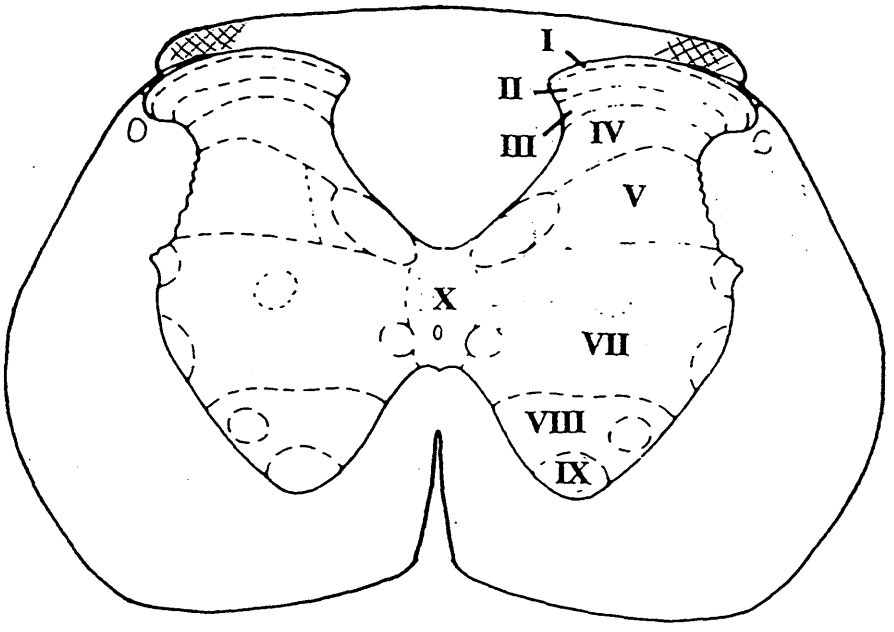


Figure 1.1. Cross-section of an idealised rat lumbar spinal cord. The position of Lissauer's tract is marked by crosshatching. Rexed's laminae are marked I – X. Adapted from Rexed (1952).

The grey matter of the spinal cord of the cat was divided into a number of dorsoventral layers or laminae on histological features by Rexed (1952) and this classification has been extended to the rat (Brichta 1985). These laminae are shown diagrammatically in Figure 1.1. Such divisions may also be functional. Fine fibres from the skin terminate mainly in the superficial layers of the dorsal horn, in Rexed's laminae I and II_0 (the substantia gelatinosa) while fine afferents from muscle terminate in laminae I and V. Most of the nociceptive afferents terminate in the superficial dorsal horn and nociceptive information is transmitted to the deep dorsal horn (laminae V - VII) by one or more interneurons.

Electrophysiological techniques have located cells responding to noxious stimulation in both the superficial (laminae I and II_0) and the deep dorsal horn (laminae IV-VII; Menétrey & Besson 1982; Schaible et al. 1986; Neugebauer & Schaible 1990; Grubb et al. 1993). Cells in the deep dorsal horn may receive both skin and deep tissue inputs due to convergence of information relayed by interneurons from more superficial layers. Distinct types of responsive neuron have been described: a nociceptive specific (NS) type which responds only to high intensity, noxious stimulation; and a wide dynamic range (WDR) type which responds in a graded fashion to a range of intensities of stimulation, with noxious stimulation provoking a greater response then innocuous stimuli (Grubb et al. 1993). Low-threshold neurons can also be found. NS neurons have been located in superficial layers and laminae VII and VIII and contribute to ascending tracts (Schaible et al 1987; Neugebauer & Schaible 1990); WDR neurons are most commonly found in the deep dorsal horn (laminae V and VI; Neugebauer & Schaible 1990).

The dorsal horn and the dorsal root ganglia, containing the cell bodies of the primary afferents, have been shown to contain many neuroactive agents and their receptors. Fast primary afferent transmitters, acting in milliseconds, may include excitatory amino acids (EAAs; glutamate, aspartate) and purines (ATP, adenosine) and slow primary afferent transmitters, acting over a period of seconds, may include neurokinins (substance P, neurokinin A), somatostatin, CGRP, nitric oxide (NO), galanin, bombesin and vasoactive intestinal peptide (VIP). Of these, the neurokinins, CGRP and EAAs are excitatory whereas

somatostatin is inhibitory. Primary afferents may contain two or more neuroactive substances, which may be coreleased or differentially released. A number of agents have been suggested to produce either local or descending inhibitory influences within the spinal cord, including endogenous opioids, noradrenaline, serotonin, GABA, neuropeptide Y, neurokinin B and adenosine. Cholecystokinin may be excitatory locally. There is thus a host of potential candidates that could play a role as fast or slow transmitters in nociceptive pathways within the spinal cord.

Nociceptive information is transmitted to the brain from the dorsal horn via decussating, ascending fibres in the anterolateral system, namely in the spinothalamic, spinoreticular, spinomesencephalic and other tracts (Besson & Chaouch 1987). Descending inhibitory influences appear to arise mainly from the periaqueductal grey and ventromedial medulla and may involve serotonin, endogenous opioids and noradrenaline (NORA) as inhibitory transmitters (Clarke et al. 1988; Harris & Clarke 1992). Both serotonin and NORA act at pharmacologically distinct receptor subtypes, which may have specialised roles within the central nervous system.

Tonic spinal inhibition has been described in the cat where neurons with knee input had larger receptive fields in spinalised as compared with intact cats (Neugebauer and Schaible 1990). Such inhibitory influences may be reduced in the anaesthetised animal. For example, Collins et al. (1990) recorded from dorsal horn neurons in the spinal cord of cats before and after giving intravenous pentobarbitone. The response properties of some spinal cord neurons were altered by the anaesthetic such that some cells classified in the awake animal as low-threshold cells were reclassified as WDR after pentobarbitone. These observations were interpreted as indicating a disruption of tonic inhibition by intravenous pentobarbitone. The possible mechanisms and significance of tonic descending inhibition in suppressing flexor reflexes to permit conscious motor activity are discussed by Duggan & Morton (1988). Most experiments involving spinal cord electrophysiological recordings, including those presented in this thesis, are performed on anaesthetised animals for ethical reasons. The effects of

anaesthetics in altering spinal responses must be borne in mind when interpreting the significance of such results.

The spinal cord is a site of integration for sensory inputs, with afferent, spinal and supraspinal components. Connections are not 'hard-wired' and can adapt to ongoing circumstances. Rather there is a network of connections within the cord such that a number of synaptic inputs may converge upon a single target cell, causing activation or inhibition depending upon the overall balance of individual inputs, the frequency of stimulation, the type and quantity of transmitters released and the duration of action of these transmitters. The spinal cord thus has a very flexible and sophisticated capacity for processing information. The spinal cord is said to exhibit plasticity, meaning that its response can be modified depending upon the prevailing circumstances (Woolf 1996).

Nociceptive information is ultimately transmitted to the brain but it may also trigger a spinal reflex invoking a motor reaction before the pain is consciously perceived. Spinal reflexes consist of the sensory receptor, the afferent pathway to the spinal cord, integration within the spinal cord, the motor nerve and an effector, such as a muscle or secretory cell. In its simplest form, the afferent fibre may synapse directly with the motor neuron: a monosynaptic reflex arc eg the knee-jerk reflex. Alternatively, one or more interneurons may be interposed between the afferent and efferent neurons producing a slower response via a polysynaptic reflex arc, eg nociceptive reflexes.

1.2 The Flexor Withdrawal Reflex

The flexor withdrawal reflex is a spinal reflex whereby muscles are rapidly activated to remove a part of the body from a real or perceived noxious stimulus in order to minimise tissue damage. Responding muscles have a highly organised cutaneous receptive field. An example of a flexor withdrawal reflex is the contraction of biceps femoris posterior, which flexes the knee joint and extends the hip joint in response to a noxious stimulus. The receptive field for this nociceptive reflex in the anaesthetised rat is the entire paw and the anterior side of

the lower hindlimb (Schouenborg & Kalliomäki 1990). Afferent information is transmitted through fibres in the sural nerve which project to spinal ganglia L3 - L6, projections to lamina II being in L3 - L4 (Molander & Grant 1986).

This sural nerve/biceps femoris reflex has been studied by many workers (Wall & Woolf 1984; Xu et al. 1991; Falinower et al. 1994; Herrero & Cervero 1996 a,b; Parsons et al. 1996). Low-intensity sural nerve stimulation elicits a rapid twitch in the muscle, which is considered to be due to an A-fibre reflex. At higher intensities and longer stimulus widths (of the order of 500 μs), an additional longer latency twitch can be observed evoked by recruitment of unmyelinated, slowly conducting C-fibres. These responses can be recorded as two distinct sets of action potentials from the motor efferent or from a single motor unit in the responding muscle. This dual component response was recorded from single fibre units in biceps femoris in chronic spinal animals (Falinower et al. 1994) indicating that the A- and C-fibre components are true spinal reflexes, without involvement of higher centres.

The latency of a response is composed of the time taken for electrical conduction along the afferent and the efferent neurons plus the time taken for synaptic transmission within the spinal cord. Electrical conduction is more rapid in large, myelinated fibres than in small, unmyelinated fibres (Table 1.1); transmission through polysynaptic reflex arcs takes longer than monosynaptic reflex arcs because a larger number of synapses must be negotiated. In the adult rat, the average latency for a single motor unit in biceps femoris to respond following low-intensity stimulation of the sural nerve was 17.5 $\pm$ 2.3 ms (Falinower et al. 1994). This was deemed to be the response to Aδ-fibre stimulation. At greater stimulus strengths and stimulus widths a second, additional well defined component was recorded 162.4 $\pm$ 5.1 ms after sural nerve stimulation, suggesting that both A-fibre and C-fibre reflexes were evoked at these stimulus parameters. The longer latency for the C-fibre reflex is due both to the relatively slow conduction velocity of the small, unmyelinated afferents and to the polysynaptic nature of the C-fibre reflex arc.

Willer (1977) stimulated the sural nerve transcutaneously in humans and showed that the threshold of stimulation required to elicit a nociceptive reflex response in biceps femoris was the same as the stimulation threshold to produce a sensation of pain. This suggests that the study of nociceptive reflexes and their modulation is of relevance to clinical pain (Arendt-Neilsen & Petersen-Felix 1995).

1.3 Models of Arthritis

A commonly used experimental model for a chronic pain state, which is considered to be of clinical relevance, is an experimental arthritis. This can be produced by the parenteral inoculation of arthritogenic material such as *Mycobacterium tuberculosis* or *M butyricum*. It is thought that an epitope on the mycobacterial 65-kD heat-shock protein is cross-reactive with an antigen in joint cartilage (van Eden et al. 1989), thereby producing immunologically mediated damage similar to that seen in human arthritic conditions. The clinical signs, together with neurochemical and neurophysical changes, have been reviewed by Pearson (1963) and Colpaert (1987). Briefly, behavioural evidence suggests that pain is severe for weeks 2 and 3 after inoculation and persists for 4 - 5 weeks. Pathological studies suggest that these models have similar, although not identical, features to human rheumatoid and other chronic inflammatory diseases (Pearson 1963).

Adjuvant-induced polyarthritis is a severe condition with widespread systemic disease, which develops from 1 - 4 weeks after inoculation. A less severe form of adjuvant arthritis, with fewer welfare problems or complicating factors, can be induced by the inoculation of a small quantity of *M tuberculosis* (Freund's Complete Adjuvant; FCA) subdermally around a single joint, such as the rat ankle joint. This produces a rapid initial inflammation reaching a peak at 4 days' postinjection followed by a monoarthritis at 14 days (Grubb et al. 1988; Donaldson et al. 1993). Neurophysiologically this model appears to induce a similar sensitisation to that seen with polyarthritis (Menétrey & Besson 1982; Guilbaud et al. 1985; Grubb et al. 1988, 1991, 1993). It could be argued that since many human arthritides are symmetrical, a monoarthritis is not a suitable model.

However larger doses of *M tuberculosis* will produce a bilateral disease (Donaldson et al. 1993) with identical electrophysiological changes in the primary afferents, suggesting that this is a valid model as well as being more ethically acceptable than the more severe polyarthritis.

Acute forms of arthritis, which develop within hours rather than days, are useful experimental tools. The injection of carrageenan or kaolin and carrageenan (K/C) into a joint cavity has been used in the cat (Coggeshall et al. 1983; Schaible & Schmidt 1985; Neugebauer & Schaible 1990; Schaible et al. 1991) and the rat (Neugebauer et al. 1995; Herrero & Cervero 1996 a,b; Yang et al. 1996). The injected joint becomes swollen, hot and tender and conscious animals will show a guarding of the affected limb with reduced weight-bearing and behavioural hyperalgesia which is maximal by 1 hour postinjection (Yang et al. 1996). Histologically there are macroscopic and microscopic signs of acute arthritis with marked oedema and cellular infiltration (Schaible & Schmidt 1985). Electrophysiologically, changes typical of hyperexcitability as discussed in the next section develop in parallel with the clinical signs of inflammation both peripherally (Coggeshall et al. 1983; Schaible & Schmidt 1985) and centrally (Neugebauer & Schaible 1990; Neugebauer et al. 1995).

These experimental models have been shown to produce neurotransmitter and neuromodulator changes in the spinal cord similar to those seen in hyperalgesia, which are discussed in detail in Section 1.4.2. Briefly, K/C arthritis induced an increased release of excitatory amino acids (Sluka & Westlund 1993; Yang et al. 1996), tachykinins (Hope et al. 1990; Schaible et al. 1990; Sluka & Westlund 1993), CGRP (Schaible et al. 1994) and PGE_2 (Yang et al. 1996; Ebersberger et al. 1999) from the spinal cord. FCA inoculation similarly induced an increase in substance P-immunoreactivity (Abbadie et al. 1996; Southall et al. 1998) and CGRP-immunoreactivity (Southall et al. 1998) in the superficial dorsal horn, along with an increase in the mRNA encoding for both preprotachykinin and CGRP in the dorsal root ganglia over a rapid time course (2 - 3 hours; Donaldson et al. 1992).

1.4 Hyperalgesia

Pain in the normal animal serves a useful protective function to prevent or minimise tissue damage. Even after injury, a degree of pain could be argued to be beneficial by limiting movement and thus speeding up healing processes. One of the methods by which the body can harness pain in this way is to develop a state of hyperalgesia.

Hyperalgesia is a condition of enhanced responsiveness to noxious stimulation when compared to the normal state. A related phenomenon, allodynia, can be defined as an alteration in responses such that normally innocuous stimuli are perceived to be painful. Hyperalgesia is commonly produced by inflammation; pain being one of the four classical signs of inflammation (Celsus 30 BC-38 AD). Hypersensitivity to pain can result both from an increase in the excitability of the primary afferent fibres (a peripheral sensitisation or primary hyperalgesia) and from an increase in the excitability of the spinal cord neurons (a central sensitisation or secondary hyperalgesia; Woolf 1983). Increased excitability both peripherally and centrally could result from a decrease in the stimulus thrshold, an increase in the rate of firing of sensory neurons, an increase in the duration of the response or a combination of these. Thus there may be both quantitative and qualitative differences in the encoding of sensory information when comparing the normal with the hypersensitive state.

1.4.1 Peripheral Hyperalgesia

Much work has been done on the sensitisation of primary afferents in a variety of chronic and acute models, such as a Freund's complete adjuvant (FCA) monoarthritis in rats (Grubb et al. 1988; Birrell et al. 1990), kaolin/carrageenan arthritis in cats (Coggeshall et al. 1983; Schaible & Schmidt 1985), Randall-Selitto-type tests (Nakamura-Craig & Gill 1991), neurogenic inflammation (Ferrell & Russell 1986; Levine et al. 1990) and the formalin test in rats (Malmberg & Yaksh 1992a,b; Chapman & Dickenson 1992). In inflammatory conditions there is evidence that a number of the inflammatory mediators produced by damaged or inflamed tissues contribute to this sensitisation, such as

prostanoids (Heppelmann et al. 1985; Schaible & Schmidt 1988, Birrell et al. 1991; Grubb et al. 1991), bradykinin (Kanaka et al. 1985; Grubb et al. 1991; Birrell et al. 1993), serotonin (Grubb et al. 1988; Birrell et al. 1990), leukotrienes (Levine et al. 1984a), histamine (Handwerker et al. 1991), substance P (Yaksh 1988; Nakamura-Craig & Gill 1991) and neurokinin A (Nakamura-Craig & Gill 1991).

Bradykinin Bradykinin (BK) is a nonapeptide that has been implicated as a mediator of pain, inflammation, vascular and gastrointestinal function. It is produced from inactive precursors, kininogens, in the plasma by the action of kallikreins and other proteolytic enzymes. BK and other kinins are potent vasodilators and increase the permeability of blood vessels, producing oedema. BK is present in inflammatory exudates and there is evidence that many of the effects of inflammation can be reproduced by the application of BK. BK has long been known to be a potent algogen.

The effects of BK are mediated by two main classes of receptor, B_1 and B_2. Both are G-protein-coupled receptors and both have been implicated in hyperalgesia brought about by persisting inflammation. The B_1 receptor has been described as a constitutively expressed receptor. BK application excites fine afferents (Kanaka et al. 1985; Birrell et al. 1993) and B_2 receptor activation is thought to modulate activity in sensory nerve terminals, producing pain. BK is thought to have synergistic effects with other algogens such as prostaglandins and serotonin (see below) in addition to stimulating the release of prostanoids and cytotoxins, including NO, activating immune cells and inducing the degranulation of mast cells to release histamine, serotonin and other inflammatory mediators. These processes were reviewed by Walker et al. (1995).

Serotonin Serotonin (or 5-HT) is a lipid-insoluble amine which binds to multiple receptors and functions both as a fast neurotransmitter and as an endocrine hormone. In inflammatory conditions, serotonin is released from platelets and mast cells and, like BK, is a potent algogen. Serotonin excites sensory neurons (Grubb et al. 1988) and the 5-HT_2, 5-HT_3 and 5-HT_4 receptors have been implicated (Todorovic & Anderson 1990; Cardenas et al. 1997).

Histamine In damaged tissue another amine, histamine, is released by degranulation of mast cells and is the main inducer of vasodilatation, acting together with BK and serotonin to produce oedema. Application of histamine to the skin is associated with an itching and burning sensation, along with the classic wheal and flare responses. Histamine has been shown directly to excite polymodal C-fibres (Handwerker et al. 1991). Like serotonin, histamine also functions as a neurotransmitter.

Neuropeptides The neuropeptides substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) are all present in the synaptic terminals of nociceptive neurons and synthesis of these substances increases in inflammatory conditions. SP is a tachykinin that acts mainly at Neurokinin-1 (NK_1) receptors. Electrical stimulation of the peripheral nerve produced a SP release that induced an extravasation of plasma proteins and erythrocytes in the knee joint of the cat (Ferrell & Russell 1986) and SP infusion increased the severity of an adjuvant arthritis (Levine et al. 1984b). An NK_1 antagonist reduced C-fibre stimulation-induced plasma extravasation (Wiesenfeld-Hallin & Xu 1993) and pretreatment of a rat knee with a SP antagonist reduced the swelling induced by carrageenan by 93% (Lam & Ferrell 1989), implying a major role for peripheral SP release in animal models of joint inflammation.

Eicosanoids Eicosanoids are fatty acid derivatives formed throughout the body that include the prostaglandins (PGs; described in more detail in Section 1.6) and leukotrienes. Leukotrienes act on white blood cells to induce the release of mediators such as PGs and cytokines at the site of inflammation (Levine et al. 1984a). A variety of PGs have been shown both to excite primary afferents directly (Schaible & Schmidt 1988; Birrell et al. 1991) and to sensitise joint afferents to movement (Heppelmann et al. 1985; Schaible & Schmidt 1988; Birrell et al. 1991; Grubb et al. 1991).

Prostaglandins, together with a variety of other inflammatory mediators, are found in increased concentrations at the site of inflammation in many different experimental models, eg rabbit monoarticular arthritis (Blackham et al. 1974), FCA adjuvant arthritis in rats (Parnham et al. 1978) and carrageenan-evoked paw

swelling in rats (Willis 1969; Arai & Aizawa 1978). Adjuvant polyarthritis induces a 5 - 10-fold increase in prostacyclin metabolite urinary excretion, reflecting a dramatic increase in total body PGI_2 synthesis (Stichtenoth et al. 1995). Non-steroidal anti-inflammatory drug (NSAID) treatment reduces both the inflammation and the increase in PG concentrations in synovial fluid (Blackham et al. 1974; Arai & Aizawa 1978). PGs are also present in increased concentrations in human clinical disease, for example in synovial fluid in rheumatoid synovitis (Trang et al. 1977; Egg 1984; Moilinen 1994).

PGs have more specifically been implicated in nociception. Intradermal injections of PGs in humans produced pain (Ferreira 1972) and injection of prostaglandins peripherally into joint cavities has been shown to cause hypersensitivity as measured by reflex rises in blood pressure in dogs (Moncada et al. 1975; Ferreira et al. 1978). Local application of PGI_2 to peripheral nerve terminals facilitated discharge both by a direct excitation (an activation of previously silent units and/or an increase in ongoing activity) and by the sensitisation of neurons (a lowering of threshold of excitation; Birrell et al. 1991).

PGs may interact with other inflammatory mediators such as bradykinin (BK). PGE_2 potentiates the sensitising effects of BK on mechanoreceptor responsiveness (Grubb et al. 1991; Birrell et al. 1993) and BK formation is thought to induce prostaglandin production (Schuligoi et al. 1994). There is evidence that PGE_2 and PGI_2 may act directly on sensory neurons to enhance the release of neuropeptides such as substance P and CGRP from sensory neurons and spinal cord slices *in vitro* (Hingtgen & Vasko 1994; Southall et al. 1998) and these effects, together with interactions with bradykinin or capsaicin, appear to be mediated by cAMP transduction (Hingtgen et al. 1995).

Figure 1.2 is a diagrammatic summary of the interactions between some of these mediators during inflammation. These processes were reviewed by Schaible & Grubb (1993).

Electrophysiologically, peripheral nerve discharges are altered in a majority of sensory units during the development of hypersensitivity. Acute joint

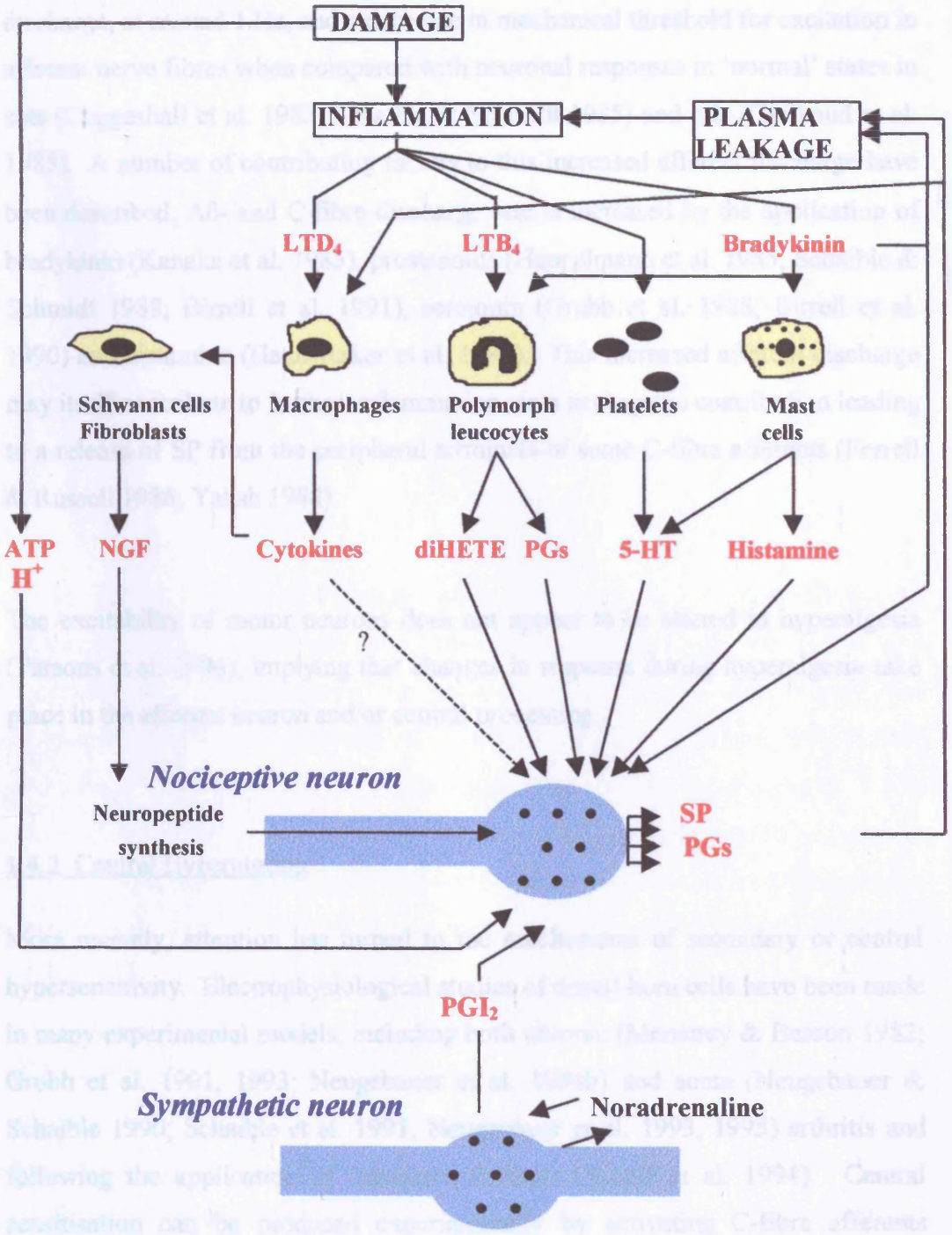


Figure 1.2. Diagrammatic representation of some of the inflammatory mediators released during inflammation.

inflammation induced increased spontaneous activity with a higher frequency of discharge, at around 1 Hz, and a decrease in mechanical threshold for excitation in afferent nerve fibres when compared with neuronal responses in 'normal' states in cats (Coggeshall et al. 1983; Schaible & Schmidt 1985) and rats (Guilbaud et al. 1985). A number of contributing factors to this increased afferent discharge have been described. Aδ- and C-fibre discharge rate is increased by the application of bradykinin (Kanaka et al. 1985), prostanoids (Heppelmann et al. 1985; Schaible & Schmidt 1988; Birrell et al. 1991), serotonin (Grubb et al. 1988; Birrell et al. 1990) and histamine (Handwerker et al. 1991). This increased afferent discharge may itself contribute to further inflammation via a neurogenic contribution leading to a release of SP from the peripheral terminals of some C-fibre afferents (Ferrell & Russell 1986; Yaksh 1988).

The excitability of motor neurons does not appear to be altered in hyperalgesia (Parsons et al. 1996), implying that changes in response during hyperalgesia take place in the afferent neuron and/or central processing.

1.4.2 Central Hyperalgesia

More recently, attention has turned to the mechanisms of secondary or central hypersensitivity. Electrophysiological studies of dorsal horn cells have been made in many experimental models, including both chronic (Menétrey & Besson 1982; Grubb et al. 1991, 1993; Neugebauer et al. 1994b) and acute (Neugebauer & Schaible 1990; Schaible et al. 1991; Neugebauer et al. 1993, 1995) arthritis and following the application of cutaneous irritants (Woolf et al. 1994). Central sensitisation can be produced experimentally by activating C-fibre afferents mechanically, electrically or chemically (Wall & Woolf 1984; Cook et al. 1987; Ma & Woolf 1995b, 1996). Dorsal horn cell characteristics have been observed to change in these hypersensitive states, with enlargement of receptive fields (Cook et al. 1987), fewer cells being categorised as nociceptive-specific and more as wide dynamic range, a decrease in firing thresholds, more spontaneous activity (eg Neugebauer & Schaible 1990; Stiller et al. 1993), a higher probability of an

afterdischarge and sometimes a bursting pattern of firing (Grubb et al. 1996). The time course of such changes correlates with the development of hyperalgesia as assessed behaviourally (Hylden et al. 1989).

Nociceptive reflex activity has been studied by comparing normal rats with those rendered hyperalgesic by FCA-induced paw inflammation (Parsons et al. 1996). A- and C-fibre thresholds were unchanged but 50% of hyperalgesic animals showed spontaneous activity in the muscles and the duration of the flexor reflex was enhanced. Changes in the wind-up of nociceptor reflexes were observed in arthritic animals (Herrero & Cervero 1996a); this is discussed in detail in Section 1.5.

A number of biochemical changes occur within the spinal cord during the development of central hyperalgesia. Microdialysis studies have shown that both glutamate and aspartate were released from the spinal cord during the development of hyperalgesia induced by arthritis (Sluka & Westlund 1993; Sorkin & Moore 1996) or following formalin injection into a paw (Malmberg & Yaksh 1995). Glutamate was also released from the spinal cord of the rat in response to C-fibre stimulation, imitating the increased afferent barrage due to an increase in spontaneous activity that occurs in peripheral inflammation (Sorkin & Moore 1996).

Glutamate and aspartate are naturally occurring excitatory amino acids (EAAs) that act at two structurally distinct groups of receptor site: ionotropic and metabotropic sites. Ionotropic receptors are associated with ion channels and have been designated as 'NMDA', 'AMPA' or 'kainate' after the main ligand found experimentally to activate them. AMPA and kainate receptors control non-selective cation channels. Activation of NMDA channels permits the entry of Ca^{2+} and Na^{+} into the cell. Some agonists and antagonists that act at these receptors are shown in Table 1.2. Metabotropic receptors, on the other hand, are members of the G-protein-coupled family of receptors. A number of metabotropic receptors have been cloned ($mGlu_1$–$mGlu_8$). Some of these have splice variants

Table 1.2 Classification of excitatory ionotropic amino acid receptors.

Receptor	Main agonist(s)	Main antagonist(s)
NMDA	NMDA	D-AP5 MK-801 Ketamine Arcaine
Kainate	Kainate Domoic acid (S)-5-Iodowillardine	CNQX NBQX
AMPA	AMPA Quisqualate (S)-5-Fluorowillardine	CNQX NBQX (RS)-AMOA CFM-2

($mGlu_1$, $mGlu_4$ and $mGlu_5$). The metabotropic receptors have been grouped according to sequence homology, agonist pharmacology and second messenger coupling. A fourth group of metabotropic receptors which couple to phospholipase D has been reported. The main agonists and antagonists that act at the metabotropic receptor sites are shown in Table 1.3. mGluR1,3,4,5 and 7 subtypes have been detected in the grey matter of the rat spinal cord (Boxall et al. 1998).

Both L-glutamate and L-aspartate given intrathecally enhanced the behavioural responses in the formalin test, as did intrathecal *N*-methyl-D-aspartate (NMDA; Coderre & Melzack 1992). Intrathecal NMDA application produced mechanical (Sher & Mitchell 1990) and thermal (Malmberg & Yaksh 1992b) hyperalgesia as well as an increase in the spontaneous firing rate of convergent neurons in both deep and superficial dorsal horn of the rat (Sher & Mitchell 1990), similar to the increase in spontaneous activity seen in hyperalgesia. Intrathecal NMDA administration provoked a large EAA and PG release from the spinal cord (Sorkin & Moore 1996). Behaviourally, intrathecal NMDA can provoke a 'scratching, biting and licking' response which was reduced by systemic NSAIDs (Björkman 1995). This evidence suggests that glutamate acting at NMDA receptors within the spinal cord plays a role in nociception.

Confirmation that this is so comes from behavioural experiments which have shown that EAA antagonists given intrathecally are antinociceptive (Coderre & Melzack 1992; Malmberg & Yaksh 1992b; Näsström et al. 1992; Ren et al. 1992). NMDA antagonists (ketamine, AP5) and a non-NMDA antagonist (CNQX) administered iontophoretically were shown to reduce the responses of dorsal horn cells, rendered hyperexcitable by both an acute (Neugebauer et al. 1993) and a chronic arthritis (Neugebauer et al. 1994b), to noxious and innocuous mechanical stimulation of the inflamed joint. A similar reduction in the electrophysiological changes associated with hyperalgesia following peripheral inflammation was seen with intrathecal MK-801, a non-competitive NMDA receptor antagonist (Ren et al. 1992). There is therefore evidence for the involvement of NMDA and AMPA receptor activity in hyperexcitability phenomena.

Table 1.3 Classification of metabotropic amino acid receptors.

Receptor	Agonist(s)	Antagonist(s)	Transduction mechanism
Group I ($mGlu_1$, $mGlu_5$)	Quisqualate (S)-3,5-DHPG	(S)-4-CPG CPCCOEt MPEP	↑ Phospholipase C
Group II ($mGlu_2$, $mGlu_3$)	L-CCG-1 (2R,4R)-APDC	LY341495 MCCG EGLU	↓ Adenylyl cyclase
Group III ($mGlu_4$, $mGlu_{6-8}$)	L-AP_4 L-SOP	CPPG MAP_4	↓ Adenylyl cyclase
Phospholipase D-coupled	L-CSA L-AP_3	(RS)-3,5-DHPG	↑ Phospholipase D

There is also evidence for the involvement of metabotropic receptors. Iontophoretic application of a metabotropic receptor antagonist (L-AP3) during the induction of an acute arthritis prevented the development of dorsal horn cell excitability (Neugebauer et al. 1994d) but had no effect upon responses to noxious or innocuous pressure applied to a normal knee joint. In situ hybridisation techniques have revealed a significant increase in the expression of the mGluR3 mRNA (but not of mGluR 1,2,4,5 nor 7 mRNA) in the lumbar spinal cord of rats rendered hyperalgesic by UV irradiation (Boxall et al. 1998).

Substance P is a neurotransmitter with a widespread distribution in sensory nerve fibres (Holzer et al. 1982). More recently substance P-like immunoreactivity has been shown, by antibody microprobe techniques, to be released into the superficial layers of the dorsal horn of the spinal cord in response to afferent electrical stimulation (Duggan et al. 1995), noxious heating of the skin (Duggan et al. 1987), noxious pressure applied to the knee joint of the rat (Neugebauer et al. 1994c) and innocuous stimulation of an acutely arthritic joint (Schaible et al. 1990). C-fibre stimulation and the application of capsaicin, considered selectively to excite small-diameter afferents, similarly evoked a release of SP-like immunoreactivity from the cat spinal cord (Go & Yaksh 1987). This release is believed to be from the primary afferent terminals in the superficial dorsal horn where SP colocalises with glutamate (De Biasi & Rustioni 1988). Certainly around 20% of the dorsal root ganglion cells in both rat and cat have been shown to contain SP (Lawson 1992). More recently, capsaicin was shown to evoke a release of both immunoreactive SP and immunoreactive CGRP from spinal cord slices which was doubled in slices from the side ipsilateral to a 5-day FCA inflammation of the paw of rats (Southall et al. 1998). This is evidence of an enhanced release of these substances in hyperalgesia.

Autoradiographic studies of human spinal cord showed NMDA-, quisqualate- (AMPA and metabotropic) and kainate-receptors and SP to be densely concentrated in lamina II of the dorsal horn, the site of termination of primary afferent fibres (Jansen et al. 1990). There is dense NK_1-receptor immunoreactivity in lamina I of the lumbar enlargement of the spinal cord of rats and the substantia gelatinosa contains dorsally directed dendrites of large NK_1-

receptor-immunoreactive neurons located in laminae III - V (Abbadie et al. 1996). Electron microscope immunocytochemistry has revealed that the majority of NK_1 receptors in the superficial dorsal horn of rats are located on the plasma membrane. Intrathecal NMDA treatment induces a reversible internalisation of NK_1 receptors to the cytoplasm on a time-scale corresponding with behavioural hyperalgesia (Liu et al. 1997). Structural changes, consisting of the appearance of dendritic varicosities packed with NK_1 receptors, were concentrated in regions targeted by SP-containing primary afferent fibres, including dendrites of lamina I neurons and the dorsally arborising dendrites of lamina III neurons. Ablation of NK_1-expressing primary afferent fibres markedly reduced behavioural responses to noxious stimulation and to mechanical and thermal hyperalgesia but had no effect upon responses to mild noxious stimuli (Mantyh et al. 1997), suggesting that the SP-containing primary afferent fibres are involved in the maintenance of hyperalgesia.

The expression of mRNA for preprotachykinin and CGRP in the dorsal root ganglia (DRG) innervating an inflamed joint increases during the development of adjuvant-induced monoarthritis although the proportion of neurons expressing these mRNAs does not change (Donaldson et al. 1992), demonstrating an increased synthesis within the DRGs in this situation. Both SP and glutamate immunoreactivity increase in the superficial spinal cord during the development of hyperalgesia (Sluka & Westlund 1993). This evidence is consistent with glutamate and SP playing a role in the transmission of nociceptive information.

Experimental data confirms this. SP applied intrathecally produced thermal hyperalgesia (Malmberg & Yaksh 1992b) and provoked a caudally directed biting/scratching behaviour in mice (Mjellem-Joly et al. 1991). Superfusion of the spinal cord with SP induced electrophysiological changes associated with hyperalgesia in superficial dorsal horn cells (Liu & Sandkühler 1995). Iontophoresis of SP close to NS or WDR neurons in the dorsal horn sensitised or excited a majority of cells (Neugebauer et al. 1994c), including cells in the deep dorsal horn. 'Knockout' mice, which are unable to produce SP or NKA because the preprotachykinin gene has been deleted, do not show significant pain behaviour in a formalin test (De Felipe et al. 1998) and have an increased pain

threshold as measured by the hotplate test (Zimmer et al. 1998). This suggests that SP (and/or NKA) is essential for some specific responses to pain. Interestingly the responses to the tail flick test and acetic acid-induced writhing tests, where a state of central hyperalgesia is not induced, are normal in these mice, implying further that tachykinins are involved specifically in central hyperalgesia (De Felipe et al. 1998; Zimmer et al. 1998)

An NK_1 antagonist given intrathecally reduced the hypersensitivity of a flexor motor reflex induced by C-fibre stimulation (Ma & Woolf 1995b) and CFA paw inflammation (Parsons et al. 1996). Both an NMDA-receptor antagonist and two NK_1 tachykinin receptor antagonists given intrathecally reduced thermal hyperalgesia in adjuvant-induced inflammation of a rat paw (Ren et al. 1996). The effects of the two classes of antagonist were additive, suggesting that both NMDA and NK_1 receptors contribute independently to hyperalgesia. In agreement with this, coapplication of NMDA and SP intrathecally produced a potentiation of the biting/scratching behaviour provoked (Mjellem-Joly et al. 1991). A contrary finding was that of Masuyama et al. (1996) who considered low doses of intrathecal SP to reduce NMDA-induced behaviour, although higher SP doses did potentiate the caudally directed licking and biting behaviour. The exact mechanism of interaction between SP-receptor activation and NMDA-receptor function has not yet been elucidated but the balance of evidence is that NMDA and SP both contribute to central hypersensitivity. Various possible mechanisms were discussed by Rusin et al. (1993) and Traub (1996).

Interestingly, intrathecally applied non-steroidal anti-inflammatory drugs reduced behavioural hyperalgesia induced by intrathecal NMDA, AMPA and SP (Malmberg & Yaksh 1992b), and inhibited the electrophysiological changes seen in dorsal horn cells associated with reperfusion hypersensitivity (Gelgor & Mitchell 1995), suggesting a role for prostanoids in the processing of nociceptive information in the spinal cord. More direct evidence that spinal PGs are involved in hyperalgesia is presented in Section 1.6.

Opioids have traditionally been used as analgesics. There are high levels of opioid receptors in the superficial dorsal horn: 70% μ receptors, 20% δ receptors and 10% κ receptors (Besse et al. 1991). Sixty to seventy per cent of these opioid receptors are on terminals of peripheral C-fibres (μ and δ subtypes) and the remainder are on dendrites of nociceptive cells or interneurons (μ, δ and κ subtypes). Spinally applied opioids reduced the responses of spinal cord neurons to C-fibre stimulation in the anaesthetised rat (Dickenson & Sullivan 1986). Activation of opioid receptors reduced the release of the primary afferent transmitters, ie excitatory amino acids and peptides, thereby reducing the excitatory processes discussed above (Besse et al. 1991).

The concentration of serotonin, a metabolite of tryptophan, has been shown to increase in both dorsal and ventral horns of the spinal cord of arthritic, as compared to normal, rats. All serotonin-containing axons in the spinal cord originate from supraspinal sites (Ruda 1990) and they are found in all laminae of the dorsal horn.

A mechanism that all these processes for producing central sensitisation have in common is an elevation of intracellular calcium: activation of NMDA-receptors allows direct calcium influx; activation of neurokinin receptors releases intracellular calcium stores (Heath et al. 1994); a summation of action potentials may allow sufficient calcium to enter the cell; and activation of neuropeptide or EAA metabotropic receptors also increases intracellular calcium. These possible mechanisms are summarised in Figure 1.3. Woolf (1996) suggested that any process which elevates intracellular calcium can produce central sensitisation; one such process is wind-up.

1.5 Wind-up

Wind-up is a phenomenon involved in the changes of central responses during noxious stimulation. It is characterised by an increasing response to afferent stimulation of a constant strength when given in a train at a sufficiently high

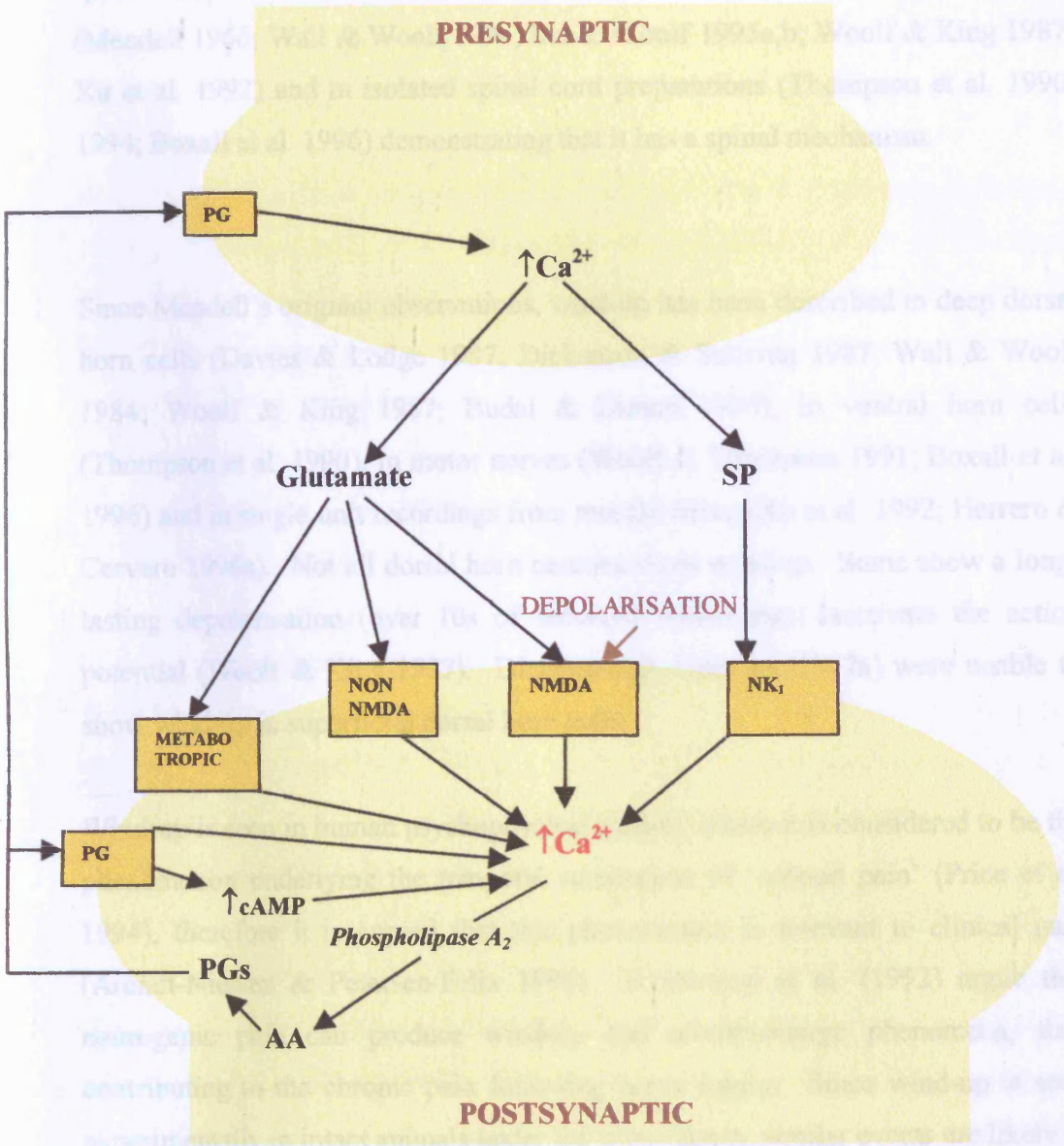


Figure 1.3. Diagram of the processes that may play a role in increasing intracellular calcium in the postsynaptic neuron.

frequency. A progressive increase in the discharge of neurons in the dorsolateral column on repeated stimulation was first described by Mendell (1966) in the spinalised, decerebrate cat. Wind-up has been observed in spinal animals (Mendell 1966; Wall & Woolf 1994; Ma & Woolf 1995a,b; Woolf & King 1987; Xu et al. 1992) and in isolated spinal cord preparations (Thompson et al. 1990, 1994; Boxall et al. 1996) demonstrating that it has a spinal mechanism.

Since Mendell's original observations, wind-up has been described in deep dorsal horn cells (Davies & Lodge 1987; Dickenson & Sullivan 1987; Wall & Woolf 1984; Woolf & King 1987; Budai & Larson 1996), in ventral horn cells (Thompson et al. 1990), in motor nerves (Woolf & Thompson 1991; Boxall et al. 1996) and in single unit recordings from muscle fibres (Xu et al. 1992; Herrero & Cervero 1996a). Not all dorsal horn neurons show wind-up. Some show a long-lasting depolarisation (over 10s of seconds) which may inactivate the action potential (Woolf & King 1987). Dickenson & Sullivan (1987a) were unable to show wind-up in superficial dorsal horn cells.

Wind-up is seen in human psychophysical studies, where it is considered to be the phenomenon underlying the temporal summation of 'second pain' (Price et al. 1994), therefore it is argued that this phenomenon is relevant to clinical pain (Arendt-Nielsen & Petersen-Felix 1995). Kristensen et al. (1992) argue that neurogenic pain can produce wind-up and afterdischarge phenomena, thus contributing to the chronic pain following nerve injury. Since wind-up is seen experimentally in intact animals under full anaesthesia, similar events are likely to occur in humans under general anaesthesia, which is of relevance to the management of post-operative pain (Dickenson 1991).

Nociceptors terminating in the superficial layers of the dorsal horn release glutamate, which may act at AMPA, kainate, NMDA and metabotropic receptors as discussed in Section 1.3.2. The tachykinins, SP and NKA, are also released and act at NK_1 and NK_2 receptors respectively to produce slow, excitatory synaptic potentials. These are by themselves unable fully to depolarise the

postsynaptic neuron to produce an action potential but a build up in local concentration during repetitive afferent input may produce a summating, subthreshold depolarisation. Intracellular calcium is one controlling factor in neuronal excitability and the interactions of receptor activation with intracellular calcium increases are illustrated diagrammatically in Figure 1.3.

Wind-up depends upon the activation of NMDA receptors (Dickenson & Sullivan 1987a; Thompson et al. 1990; Dickenson & Aydar 1991; Woolf & Thompson 1991). These are both ligand- and voltage-gated ionotropic receptors that are highly permeable to calcium ions. At resting membrane potentials the ion channel is blocked by a magnesium ion that prevents ion flow even if the ligand, glutamate, binds to the receptor. On membrane depolarisation, the magnesium ion is ejected from the channel allowing an inflow of sodium and calcium ions, thereby further depolarising the membrane.

Wind-up is inhibited by NMDA antagonists such as D-AP5 (Dickenson & Sullivan 1987a; Thompson et al. 1990; Boxall et al. 1996), dextromethorphan (Price et al. 1994) and D-CPP (Woolf & Thompson 1991), channel blockers such as dizocilpine (MK 801; Woolf & Thompson 1991; Wiesenfeld-Hallin & Xu 1993), phencyclidine, ketamine (Davies & Lodge 1987), and Mg^{2+} and glycine-site antagonists such as 7 chlorokynurenate (Dickenson & Aydar 1991). Indeed, the NMDA antagonist, CPP, has been successfully used clinically to relieve the wind-up and afterdischarge elements of a severe neurogenic pain syndrome in a human (Kristensen et al. 1992).

Davies & Lodge (1987) postulated that the long-lasting slow excitatory postsynaptic potentials (EPSPs) evoked by C-fibre stimulation summate to induce a gradual, cumulative depolarisation of the postsynaptic membrane in the dorsal horn which progressively relieves the voltage-dependent NMDA receptor block and allows increased transmission via the NMDA receptors, manifested as postsynaptic hypersensitivity. A summation of the slow synaptic potentials mediated by tachykinins may contribute to the postsynaptic depolarisation. If the stimulus frequency is sufficiently high, temporal summation, ie wind-up, can occur (Thompson et al. 1990; Sivilotti et al. 1993).

C-fibres generate EPSPs lasting seconds. Aβ-fibres, on the other hand, elicit fast excitatory postsynaptic potentials lasting milliseconds, a thousand-fold difference in magnitude. The stimulus frequency would therefore have to be a thousand times higher to generate a cumulative depolarisation due to temporal summation in Aβ-fibres, ie of the order of 100 Hz (Davies & Lodge 1987) which is much greater than frequencies measured in vivo.

The observations that NMDA antagonists reduce wind-up but do not affect the initial or baseline response (Davies & Lodge 1987; Dickenson & Sullivan 1987a; Price et al. 1994) together with the evidence that NMDA-receptor antagonists have no effect in nociceptive behavioural tests on non-hyperalgesic animals (Näsström et al. 1992) suggest that this initial acute response is mediated by non-NMDA receptors. As with central hyperalgesia, wind-up has been also been reduced by metabotropic-receptor antagonists (Boxall et al. 1996).

As would be expected for a hyperalgesia-related phenomenon, substance P has been demonstrated to have effects upon wind-up. Wind-up is reportedly absent in mice lacking the NK1 receptor (de Felipe et al. 1998). The increase in excitability of a nociceptive withdrawal reflex in the rat by C-fibre stimulation could be imitated by the intrathecal injection of both SP and CGRP (Woolf & Wiesenfeld-Hallin 1986). There was multiplicative synergy between C-fibre stimulation and neuropeptide administration, that is the effect of both stimuli applied together was greater than the sum of their separate effects. Similarly NK_1-receptor antagonists blocked wind-up of biceps femoris muscle units and dorsal horn neurons (Xu et al. 1992; Budai & Larson 1996) and low doses of NMDA- and NK_1-receptor antagonists acted synergistically to block wind-up and reflex facilitation (Wiesenfeld-Hallin & Xu 1993). SP administered iontophoretically close to WDR neurons in the dorsal horn gave an apparent decrease in wind-up, as defined by the authors (Budai & Larson 1996). In fact it produced a marked increase in the baseline response, ie made the neuron more excitable, which is not at variance with a role for SP in hyperalgesia. Intrathecal administration of the excitatory amino acids L-glutamate and L -aspartate, on the other hand, had no facilitatory

effects upon the reflex and produced no synergy with C-fibre stimulation (Woolf & Wiesenfeld-Hallin 1986), implying that an increase in the rate of release of EAAs into the synaptic cleft is not a modulating factor in nociceptive flexor withdrawal reflex facilitation.

Herrero & Cervero (1996a) looked at wind-up in normal rats and in rats rendered hyperalgesic by an acute carrageenan joint inflammation. They observed an enhanced C-fibre wind-up following joint inflammation and a novel (if small) A-fibre wind-up in hyperalgesic but not normal rats. These A-fibre effects were abolished by acute spinalisation, implying that they depend upon supraspinal influences (Herrero & Cervero 1996b). A similar enhancement of both A- and C-fibre evoked responses was reported in an *in vitro* preparation rendered hyperexcitable by UV irradiation, and was correlated with observed behavioural hyperalgesia (Thompson et al. 1994).

To conclude, C-fibre wind-up is a spinal mechanism dependent upon post-synaptic NMDA-receptor activation and SP release.

1.6 Prostaglandins

In many clinical situations pain is excessive, inappropriate and inconvenient. Some form of pain relief is required and one frequently used class of drug is the non-steroidal anti-inflammatory drugs (NSAIDs). These are a group of compounds, such as aspirin, ibuprofen and indomethacin, that share the common ability to prevent the synthesis of prostaglandins by inhibiting the action of cyclooxygenase enzymes (Vane 1971, 1983). The possible role of prostaglandins (PGs) in inflammation has been mentioned in Section 1.4.1 and it is reasonable to explain the anti-inflammatory actions of NSAIDs in terms of an inhibition of PG production. The fact that NSAIDs are also analgesic has led to the hypothesis that PGs are involved more directly in nociception.

Prostaglandins are a group of 20-carbon unsaturated fatty acid derivatives with a myriad of functions within the body. Different PGs have specific roles in

different tissues. For example in the kidney they regulate salt and water absorption, in the blood stream they control platelet aggregation, they affect smooth muscle tone and there is a large body of evidence to suggest that they play a key role in inflammatory processes. In the central nervous system they are implicated functionally in the sleep/wake cycle (Hayaishi 1988).

The synthesis pathway of PGs from arachidonic acid (AA) is shown in Figure 1.4. AA is produced by phospholipases, principally phospholipase A_2, acting upon phospholipids in the cell membrane. AA is converted to PGG_2 and PGH_2 by the action of cyclooxygenases (cox) and these two PGs are converted by specific enzymes to the active forms: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TxA_2 (thromboxane). PGs are thought not to be stored in or secreted from synaptic vesicles but are produced as required and, being lipophilic, diffuse easily out through the cell membrane.

The active prostanoids bind to specific G-protein-linked receptors which have been classified on the basis of their most potent endogenous agonist. PGD_2, PGI_2, $PGF_{2\alpha}$ and TxA_2 appear each to act at a single receptor site (termed DP, IP, FP and TP respectively) whereas PGE_2 acts at four pharmacologically distinct receptor classes: EP_1, EP_2, EP_3 and EP_4 (Coleman et al. 1987a,b, 1994). Some of the agonists and antagonists used pharmacologically are shown in Table 1.4. This classification has recently been confirmed by the cloning of many of these receptor types, as discussed by Pierce et al. (1995).

All the cloned prostanoid receptors are 7-transmembrane-domain, G-protein-coupled receptors. The G-protein coupling depends upon the C-terminal domain (Irie et al. 1994; Hasegawa et al. 1996) and it is possible that some prostaglandin receptors are coupled to more than one second messenger, as shown in Table 1.4.

The possible role of PGs in peripheral sensitisation has been discussed above in Section 1.4.1. PGs have also been implicated in central sensitisation. Results of behavioural experiments have been published which suggest that central application of PGs either intrathecally or intracisternally will cause hyperalgesia

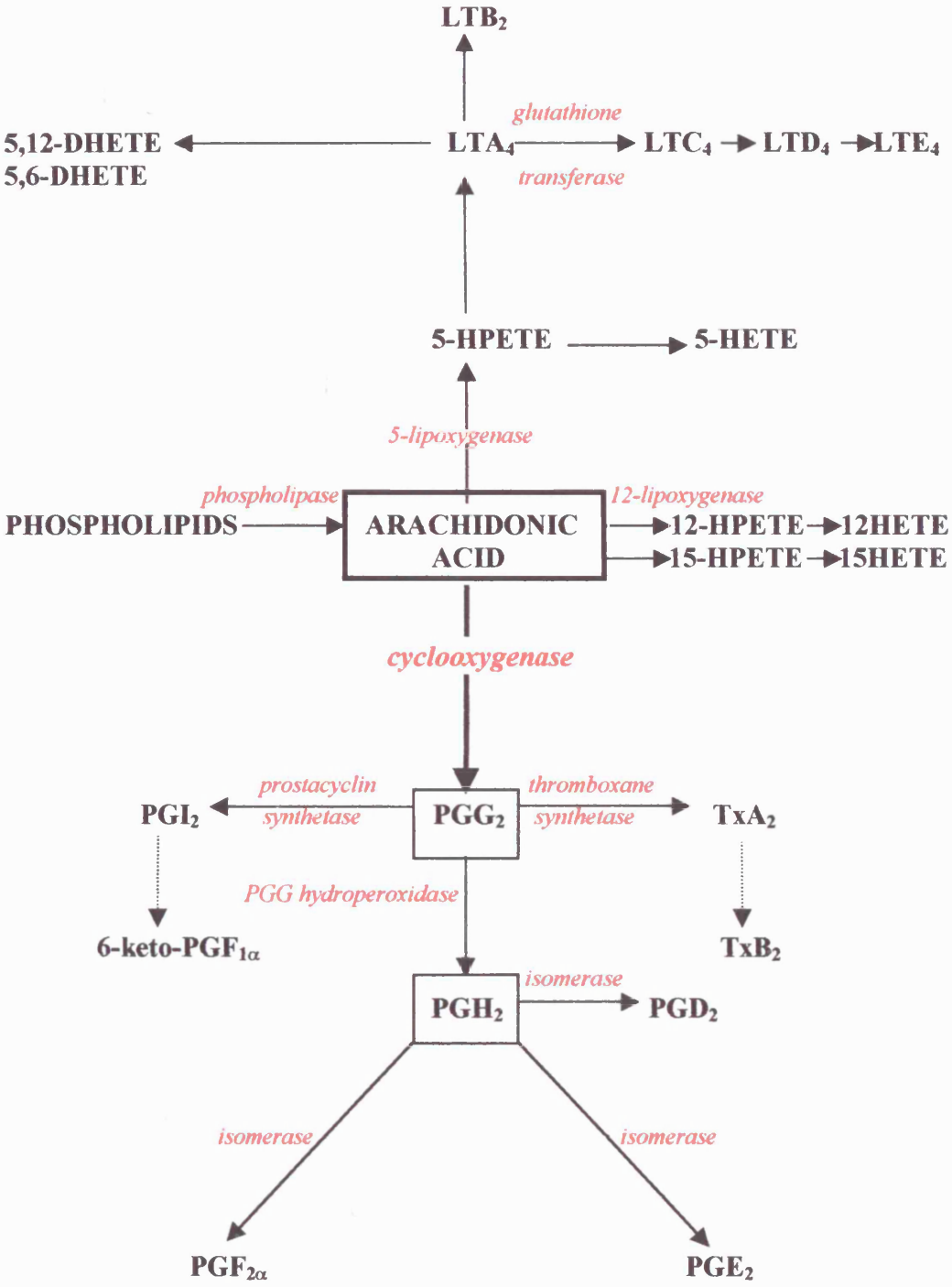


Figure 1.4. Diagram showing the formation of physiologically active prostaglandins and other eicosanoids from arachidonic acid.

Table 1.4 Table showing the prostaglandin receptor (sub)types, their agonists and antagonists and proposed second messengers.

Receptor	Natural agonist	Agonist(s)	Antagonist(s)	Proposed second messenger(s)
DP	PGD_2	BW245C	BWA868C	↑ Ca cAMP stimulation
EP_1	PGE_2	17 phenyl trinor PGE_2 sulprostone	AH6809 SC19220 SC51089 SC51234A meclofenamic acid	↑ Ca phospholipase C
EP_2	PGE_2	butaprost AH13205 AY23626 misoprostol	AH6809	cAMP stimulation
EP_3	PGE_2	enprostil GR63799 MB28767 sulprostone misoprostil SC-46275		cAMP inhibition cAMP stimulation ↑ Ca ↑ inositol phosphate turnover
EP_4	PGE_2	11-deoxy-PGE_1	AH22921X AH23848B AH13205	cAMP stimulation

Table 1.4 cont.

FP	$PGF_{2\alpha}$	fluprostenol cloprostenol ICI 81008	phloretin	phospholipase C ↑ Ca
IP	PGI_2	cicaprost ZK 96489 iloprost	FCE22176?	cAMP stimulation
TP	TxA_2	STA_4 U46619 IBOP SQ 26,655	GR 32191 SQ 29548 AH 19437 BAY u3405 AH 23848 CGS 22652 ICI 19605	↑ Ca ↑ inositol phosphate turnover phospholipase Cβ

although there is some disagreement as to which PGs are involved. Uda et al. (1990) and Horiguchi et al. (1986) suggested that PGD_2 and PGE_2 but not $PGF_{2\alpha}$ had an effect whereas Taiwo & Levine (1986) record an effect with PGE_2 and $PGF_{2\alpha}$. Horiguchi et al. (1986) described a biphasic effect of PGE_2 and PGD_2. Ferreira & Lorenzetti (1996) report a PGE_2-induced hyperalgesia that is NMDA dependent. Bhattacharya (1986), on the other hand, concluded that intra-cerebroventricular PGD_2 was antinociceptive in behavioural tests on rats.

Malmberg et al. (1995) showed that a high concentration (1mM) PGE_2 administered spinally in rats via a dialysis tube produced allodynia that correlated with a spinal release of glutamate and aspartate. Lower concentrations of PGE_2 enhanced the release of glutamate and aspartate by capsaicin, an irritant and C-fibre excitant, thought to release neurotransmitters such as SP. PGE_2 has been shown to stimulate a release of glutamate and aspartate from rat spinal cord synaptosomes (Nishihara et al. 1995).

Many of these experiments relied upon behavioural tests in 'normal' animals, ie a state of hypersensitivity had not been induced. Drugs were also delivered in a relatively concentrated form (often millimolar) via catheters, which may have caused CNS damage both by trauma and by chemical/pH/osmotic considerations. In addition, the site of application needs careful consideration. The relevance of such experiments to the physiology of nociception, unless appropriately controlled, must therefore be critically examined.

If we are to postulate that PGs are in some way involved in nociception centrally, and knowing that PGs are not stored but must be constantly produced, we would expect to be able to find PGs and their receptors within central nervous system (CNS) tissue.

Some prostaglandins and their receptors have been identified within the CNS. PGD_2 is actively produced and metabolised in the CNS (Vesin 1992) and binds to a specific protein (receptor) on synaptic membranes (Hayaishi 1983), especially in the pituitary, hypothalamus, olfactory bulb (Narumiya et al. 1982; Shimizu et al.

1982) and spinal cord (Vesin et al. 1995). Autoradiography shows dense PGD_2-binding protein in the substantia gelatinosa (Watanabe et al. 1985). The DP receptor has been found by RT-PCR in the rat brain (L. Donaldson, Leicester University, personal communication). DP receptor mRNA transcripts have been localised in the sensory neurons of the dorsal horn and the motor neurons of the ventral horn of the rat spinal cord (Wright et al. 1999).

PGE_2 binding sites in rat brain have been mapped by autoradiography (Matsumura et al. 1992) with binding in many specific locations throughout the CNS including laminae I and II of the dorsal spinal cord, regions associated with nociceptive processing. EP_1 mRNA has been found in nerve cells of the paraventricular and supraoptic nuclei of the hypothalamus (Batshake et al. 1995) and approximately 30% of mouse dorsal root ganglion cells express mRNA for EP_1 receptors (Oida et al. 1995). Iloprost-binding sites (which encompass IP and EP_1 receptors) have been found in the substantia gelatinosa. EP_3 mRNA has been detected in the brain and in about half of sensory ganglion neurons (Sugimoto et al. 1994; Kotani et al. 1995; Oida et al. 1995). More recent work has demonstrated the EP_3 receptor in the afferent terminal areas only in the superficial dorsal horn of the rat spinal cord (Beiche et al. 1998a). Mouse EP_4 mRNA has been found in 20% of mouse dorsal root ganglion cells (Oida et al. 1995). mRNA for all EP receptors has recently been found in both rat dorsal root ganglion and spinal cord by RT-PCR (Donaldson et al. 2001). Significant amounts of FP mRNA transcripts were detected in rat astrocytes and whole brain (Kitanaka et al. 1994).

No IP receptors were found in the brain or spinal cord by autoradiography but, interestingly, mouse IP mRNA was found in about 40% of neurons in dorsal root ganglia, with 70% of the dorsal root ganglion cells expressing preprotachykinin mRNA coexpressing IP mRNA (Oida et al. 1995).

There are also reports of the release of PGs from the spinal cord. Many years ago Ramwell et al. (1966) reported the presence of PG-like substances in frog spinal cord perfusates, together with other stimulating substances including serotonin and ACh. PG and ACh release were increased by electrical stimulation of the hindlimb at C-fibre strength. Recently the presence of PGD_2 and PGE_2, but not

$PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$ (the stable metabolite of PGI_2), in rat lumbar spinal cord perfusates has been reported (Gardiner et al. 1997; Willingale et al. 1997; Gardiner 1998). An increase in the release of PGE_2, but not $PGF_{2\alpha}$ or 6-keto-$PGF_{1\alpha}$, was evoked from rat spinal cord by a noxious thermal stimulus (Coderre et al. 1990). Similarly, injection of formalin into the paw of rats provoked a significant release of PGE_2-like immunoreactivity from the spinal cord, which was suppressed by an intrathecal NSAID (Malmberg & Yaksh 1995). PGE_2 release in a lumbar dialysate was increased both by C-fibre stimulation in normal and arthritic rats and by intrathecal NMDA (Sorkin & Moore 1996). A similar release has been demonstrated in the formalin test (Björkman et al. 1994; Muth-Selbach et al. 1999). Recent studies using antibody microprobes have shown that the enhanced release of PGE_2 in the dorsal horn occurs very rapidly, as early as 6 hours after the induction of a K/C arthritis (Ebersberger et al. 1997, 1999). There is thus evidence that PGs, specifically PGE_2 and PGD_2, are produced by the spinal cord and that PGE_2 release is increased by noxious stimulation and in hyperalgesia.

There is some experimental pharmacological evidence, using specific antagonist/agonists, that specific EP receptors may be involved in central nociceptive processing. Malmberg et al. (1994) suggest that EP_1 receptors are involved in nociceptive processing at the spinal level, perhaps by facilitating SP release, and EP_1 receptors may be involved in allodynia (Minami et al. 1994). The analgesic effect of a high dose of PGE_2 instilled into the cerebral ventricles appears to be mediated by EP_1 receptors (Oka et al. 1994). Hallinan et al. (1994) showed that SC-51322, an EP_1 antagonist, produced analgesia in the mouse writhing test. Oka et al. (1994) consider that the hyperalgesia seen when PGE_2 is applied to the lateral cerebral ventricle in the rat may be mediated by EP_3 receptors. EP receptors have also been implicated in allodynia (EP_1) and hyperalgesia (EP_2 and EP_3; Minami et al. 1994). However evidence produced using receptor agonists/antagonists must be viewed critically as with all procedures involving the application of high concentrations of a chemical, perhaps at unphysiological pH, to parts of the central nervous system. Additionally PG receptor agonists/antagonists are generally neither potent nor specific. Perhaps

more convincing evidence of EP receptor involvement in the spinal cord in hyperalgesia is the finding that, in spinal cord, mRNA for an EP_3 receptor subtype is significantly downregulated in the first days of a FCA monoarthritis (Donaldson et al. 2001).

Possible modes of action of PGs within the spinal cord include: i) action at PG receptors to alter intracellular concentrations of second messengers eg cAMP, Ca^{2+}, IP_3 (Table 1.3); ii) a presynaptic action to affect the release of neurotransmitters eg SP (Vasko et al. 1993); iii) an effect upon endogenous inhibition eg by inhibiting the release of noradrenaline from bulbospinal pathways (Taiwo & Levine 1988); and iv) an effect upon membrane excitability. Possible mechanisms were discussed by Buritova et al. (1996). Some of these are summarised diagrammatically in Figure 1.5.

1.7 Cyclooxygenases

The enzyme commonly termed cyclooxygenase (cox) is, more properly, also designated prostaglandin endoperoxide synthase. This enzyme encompasses both fatty acid bis oxygenase (cyclooxygenase) and prostaglandin hydroperoxidase activities. Cyclooxygenase activity is the first, essential and rate-limiting step in the pathway that converts arachidonic acid (AA) to the prostaglandins (PGs) and thromboxanes, as shown schematically in Figure. 1.4 and discussed in Section 1.6.

Two structurally distinct isoforms of cox have been identified: a 'constitutive' form (cox-1) and an 'inducible' form (cox-2). The isoforms have approximately 60% sequence homology and differ in their distribution, apparent function and inhibition by NSAIDs and other inhibitors (Mitchell et al. 1993; Engelhardt 1996). The three-dimensional structures for both isoforms have been published (Picot et al. 1994; Luong et al. 1996). Both enzymes have a molecular weight of 71 Kd and have similar active sites for the natural substrate and inhibitors.

Genes encoding for cox-1 have been cloned in the sheep (Merlie et al 1988; DeWitt & Smith 1988), human (Hla et al. 1986; Yokoyama & Tanabe 1989) and

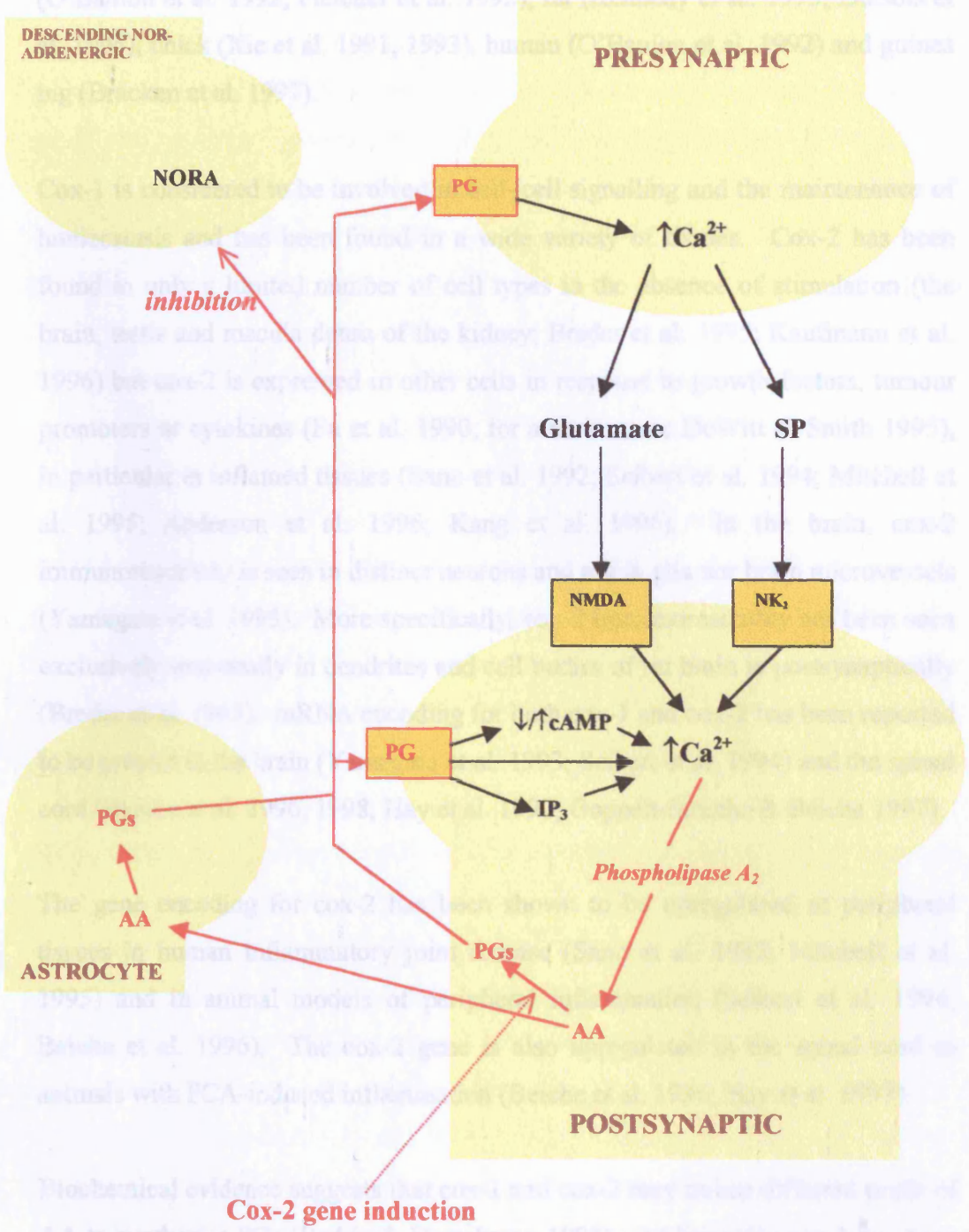


Figure 1.5. Possible modes of action of prostaglandins within the spinal cord.

mouse (DeWitt & Smith 1990) and those for cox-2 have been cloned in the mouse (O'Banion et al. 1992; Fletcher et al. 1992), rat (Kennedy et al. 1993; DuBois et al. 1994), chick (Xie et al. 1991, 1993), human (O'Banion et al. 1992) and guinea pig (Bracken et al. 1997).

Cox-1 is considered to be involved in cell–cell signalling and the maintenance of homeostasis and has been found in a wide variety of tissues. Cox-2 has been found in only a limited number of cell types in the absence of stimulation (the brain, testis and macula densa of the kidney; Breder et al. 1995; Kaufmann et al. 1996) but cox-2 is expressed in other cells in response to growth factors, tumour promoters or cytokines (Fu et al. 1990; for a review, see DeWitt & Smith 1995), in particular in inflamed tissues (Sano et al. 1992; Seibert et al. 1994; Mitchell et al. 1995; Anderson et al. 1996; Kang et al. 1996). In the brain, cox-2 immunoreactivity is seen in distinct neurons and not in glia nor brain microvessels (Yamagata et al. 1993). More specifically, cox-2 immunoreactivity has been seen exclusively neuronally in dendrites and cell bodies of rat brain ie postsynaptically (Breder et al. 1995). mRNA encoding for both cox-1 and cox-2 has been reported to be present in the brain (Yamagata et al. 1993; Seibert et al. 1994) and the spinal cord (Beiche et al. 1996, 1998; Hay et al. 1997; Goppelt-Struebe & Beiche 1997).

The gene encoding for cox-2 has been shown to be upregulated in peripheral tissues in human inflammatory joint disease (Sano et al. 1992; Mitchell et al. 1995) and in animal models of peripheral inflammation (Seibert et al. 1994; Beiche et al. 1996). The cox-2 gene is also upregulated in the spinal cord in animals with FCA-induced inflammation (Beiche et al. 1996; Hay et al. 1997).

Biochemical evidence suggests that cox-1 and cox-2 may utilise different pools of AA to synthesise PGs (Reddy & Herschman 1994). Additionally cox-1 has been shown to function mainly in the endoplasmic reticulum whereas cox-2 also produces PGs within or on the nuclear membrane (Morita et al. 1995). These observations suggest that the two isoforms are part of discrete PG synthetic pathways, with cox-2 having a possible role as an early inducible gene (Fletcher et al. 1992; Xie et al. 1991).

1.8 Non-steroidal Anti-inflammatory Drugs

The non-steroidal anti-inflammatory drugs (NSAIDs) are a structurally diverse group of drugs, such as aspirin, the fenemates, indomethacin and ibuprofen, which are anti-inflammatory and analgesic. They share the common ability to inhibit the action of cyclooxygenase to some extent (Vane 1971, 1983) and have therefore been considered to produce their clinical effects by preventing the production of prostaglandins.

As discussed above, there is a large body of evidence to implicate PGs as important modulators in peripheral inflammation. PGs are among the inflammatory mediators which are involved in experimental (Blackham et al. 1974; Arai & Aizawa 1978; Parnham et al. 1978; Heppelmann et al. 1985; Schaible & Schmidt 1988, Birrell et al. 1991; Grubb et al. 1991) and clinical situations (Trang et al. 1977; Egg 1984; Moilinen 1994) (Figure 1.2). NSAID treatment can be used to reduce inflammation and to reduce PG concentrations in joint disease (Blackham et al. 1974; Trang et al. 1977; Arai & Aizawa 1978; Tokunaga et al. 1981).

NSAIDs can be effective in reducing the electrophysiological changes seen in afferent fibres in hypersensitivity. For example, aspirin reduced the spontaneous and mechanically-induced activity recorded from mechanoreceptors from a FCA-induced arthritic joint in the rat (Guilbaud & Iggo 1985; McQueen et al. 1991) and intravenous aspirin and indomethacin were similarly effective in reducing the responses of primary afferents to movement in a kaolin/carrageenan joint inflammation (Heppelmann et al. 1986). NSAID administration has been reported to reduce or prevent the electrophysiological changes seen in dorsal horn cells rendered hyperexcitable by an acute arthritis (Neugebauer et al. 1995) or showing reperfusion hyperexcitability (Gelgor & Mitchell 1995).

NSAIDs have also been shown to be analgesic. Oral, intravenous or intraperitoneal NSAID pretreatment has been shown to produce analgesia in a variety of behavioural models, such as the second phase of the formalin test (Drower et al. 1987; Chapman & Dickenson 1992; Malmberg & Yaksh 1992a;

Muth-Selbach et al. 1999), the writhing test (Drower et al. 1987) and the tail-flick test in hyperalgesic animals (Bianchi & Panerai 1996).

It has traditionally been argued, in the light of the above evidence, that NSAIDs produce their anti-inflammatory and analgesic effects by inhibiting the production of PGs in the periphery, at the site of inflammation or tissue damage, and that analgesic effects are due to a dampening down of the increased electrical activity seen in afferent sensory fibres during the development of hyperalgesia. NSAIDs could inhibit local PG production thus a) reducing directly any excitatory effects of PGs on nociceptors, b) limiting the degree of inflammation thereby indirectly decreasing afferent discharges, and c) decreasing facilitatory effects on other inflammatory mediators therefore indirectly reducing the degree of nociceptor excitation.

Some workers became sceptical that this was the only mode of action of NSAIDs when it was suggested that some NSAIDs such as paracetamol (acetaminophen) had few anti-inflammatory effects but were clinically useful analgesics (McCormack & Brune 1991; McCormack & Urquart 1995; Bianchi & Panerai 1996). There have been suggestions that the mode of action of some NSAIDs is entirely unrelated to cox inhibition (Lorenzetti & Ferreira 1985; Björkman et al. 1994). There also appeared to be a dissociation between analgesic efficacy and cyclooxygenase-inhibiting ability in some cases (Brune et al. 1991). This led workers to look for evidence of central effects of NSAIDs.

Some evidence was supplied by behavioural tests where NSAIDs were given centrally, usually intrathecally. Antinociception by centrally applied NSAIDs was produced in the formalin test (Chapman & Dickenson 1992; Malmberg & Yaksh 1992a, 1993; Yamamoto & Nozaki-Taguchi 1996), tail-flicks following reperfusion hyperalgesia (Gelgor & Mitchell 1995) and writhing following intraperitoneal irritants (Björkman 1995; Akman et al. 1996). The relative potencies of NSAIDs in suppressing capsaicin-evoked release of PGE_2 from spinal cord slices was generally the same as their antinociceptive activities, providing evidence that central NSAID analgesia is due to the inhibition of PG production (Malmberg & Yaksh 1994a). Recent work showing that

acetaminophen both reduced flinching behaviour in the second phase of the formalin test and decreased spinal PGE_2 release (Muth-Selbach et al. 1999) has given weight to this hypothesis.

Electrophysiological effects of centrally applied NSAIDs on spinal cord were also demonstrated. Spinally applied NSAIDs were shown to reduce or inhibit the electrophysiological changes seen in dorsal horn cells during the development of hypersensitivity, as discussed in Section 1.4.2 (Gelgor & Mitchell 1995; Neugebauer et al. 1995). NSAIDs will also reduce nociceptive spinal reflexes, providing additional evidence for a central role for PGs.

Experimental evidence from both animals and humans was reviewed by Urquart (1993) who discussed the possible sites of action of NSAIDs, in particular the acidic NSAIDs. Bannwarth et al. (1995) similarly reviewed possible sites of action for NSAIDs, including supraspinal mechanisms.

Intrathecal NSAIDs do not reduce flinching behaviour in the first phase of the formalin test however (Malmberg & Yaksh 1992a; Chapman & Dickinson 1992). The first phase of flinching in the formalin test (1-10 min) is thought to be due to the direct chemical stimulation of the primary afferents, producing an initial barrage of nociceptor activity. The second phase of paw-flinching behaviour corresponds to an increase in the background activity in the primary afferent C-fibres that innervate the skin at the site of the injection (Dickenson & Sullivan 1987b) and to the development of hyperalgesia. Intrathecal NSAIDs have also been found to be ineffective in providing behavioural analgesia in conventional tail-flick or hot-plate tests where there is no hyperalgesia (Yaksh 1982; Björkman 1995; Akman et al. 1996; Bianchi & Panerai 1996; Yamamoto & Nozaki-Taguchi 1996). A hypothesis could be made that PGs are only involved in hyperalgesic states but not in acute nociception.

There is thus a body of evidence for a central component to NSAID actions, which could either be due to the inhibition of PG synthesis within the spinal cord or to other mechanisms not directly related to PG synthesis. Pharmacological evidence relating cox-inhibiting activity to antinociceptive activity must be re-

examined in the light of recent knowledge that there are two cox isoforms, with cox-2 being found in the brain (Yamagata et al. 1993; Breder et al. 1995) and cox-2 induction being linked to inflammation (Sano et al. 1992; Mitchell et al. 1995; Anderson et al. 1996; Kang et al. 1996). Early work on cox inhibition used preparations that measured almost exclusively anti-cox-1 activity. Also the activity in intact cells may not be the same as that against purified enzyme preparations (Mitchell et al. 1993) indicating that apparent anti-cox efficacies quoted in earlier works must be considered critically.

It is thought that the therapeutic activity of NSAIDs could be accounted for by their anti-cox-2 activity while inhibition of cox-1, the constitutively expressed isoform, produces unwanted side effects such as gastric (Seibert et al. 1994) and renal damage. Epidemiological data suggest that there is a parallel relationship between gastrointestinal side effects and cox1/cox2 ratios, such that NSAIDs that show more selectivity for cox-2 produce fewer clinical problems (data quoted in Vane & Botting 1998). This has led to a search for drugs that will selectively inhibit cox-2 with minimal effect against cox-1. Most 'traditional' NSAIDs such as aspirin and indomethacin inhibit cox-1 much more potently than cox-2 and are relatively ulcerogenic (Mitchell et al. 1993). Vane has divided NSAIDs into three broad groups based upon the these activities: high cox-2/cox-1 ratios for indomethacin, naproxen and piroxicam; equi-activity for diclofenac and ibuprofen; and selectivity for cox-2 for nimesulide, meloxicam and etodolac (Vane & Botting 1998). New drugs have been developed such as NS-398 (Masferrer et al. 1994; Ouellet & Percival 1995), meloxicam (Engelhardt 1996), SC58125 (Seibert et al. 1994) and SC58635 (celecoxib, rofexocib; quoted in Vane & Botting 1998) which claim to be more selective as cox-2 inhibitors than drugs currently used in the hope that these will prove to be therapeutically effective but with fewer unwanted renal and gastric side-effects.

1.9 Aims of this Investigation

Evidence has been presented above to suggest that PGs may be involved in nociception centrally in the spinal cord as well as peripherally. If this is so, then there are a number of expectations that could be tested:

- Since PGs are not stored and transported, the biosynthetic machinery to manufacture PGs locally must be present in the spinal cord. Cyclooxygenase is the first and rate-limiting step in the synthesis of active PGs from arachidonic acid. One or more isoforms of cyclooxygenase (cox) should therefore be present in the spinal cord.

- PG production by the spinal cord would be increased in hypersensitive states.

- Inhibition of PG production in the spinal cord should modify nociception, specifically by reducing or preventing the electrophysiological changes seen during the development of hypersensitivity.

Further, a hypothesis is proposed that PGs within the spinal cord are involved in nociception in hyperalgesic states but not in acute nociception in the normal anaesthetised animal.

To provide evidence to support the view that PGs are involved in spinal nociceptive processing in the hyperalgesic animal this study addressed the following aims:

- To look for cox isoforms in spinal cord tissue.

- To determine if cox concentrations in the spinal cord are increased in a model of hypersensitivity (K/C arthritis).

- To inhibit PG production in the spinal cord using topically applied non-steroidal anti-inflammatory drugs (NSAIDs) and:
    i) determine if there is an alteration in the dorsal horn neuronal response to noxious mechanical stimulation of normal joints
    ii) determine if there is an alteration in the dorsal horn neuronal response to noxious mechanical stimulation of hypersensitive joints (chronic FCA and acute K/C arthritis).

- To examine whether the wind-up (hyperexcitability) of a nociceptive withdrawal reflex can be modified by systemic and/or spinally applied cox inhibitors.

Chapter 2

Materials and Methods

2.1 Animals

Animals used in all experiments were adult Wistar rats. For electrophysiological experiments male (and one female) rats were used, weighing 220 - 320g. For tissue sampling rats generally weighed 200 - 300g; 14 male rats and 3 female rats were used.

All animals were housed in groups in a 12-h light:12-h dark regime with free access to food and water. The environmental temperature was controlled at 18 - 20°C.

All experiments were performed in compliance with the Animals (Scientific Procedures) Act 1986.

2.2 Surgery

2.2.1 Anaesthesia

For initial experiments, anaesthesia was induced with intraperitoneal thiopentone sodium ('Intraval'; Rhône Mérieux; dissolved in 0.9% sodium chloride solution) at an initial dose of 100 mg/kg. Further intraperitoneal doses were given as required to achieve surgical anaesthesia, such that the hindlimb withdrawal and corneal reflexes were abolished throughout the experiment. It was found that intraperitoneal thiobutabarbital sodium ('Inactin'; RBI; dissolved in 0.9% sodium chloride solution) gave a more stable level of anaesthesia during prolonged recordings. Thiobutabarbital sodium was given intraperitoneally at an initial dose of 120 mg/kg followed by further intraperitoneal injections of 12 - 24 mg as required in order to abolish both hindlimb withdrawal and corneal reflexes for the duration of the experiment.

2.2.2 Standard Surgical Preparation

A cannula was placed in the trachea to aid respiration. The carotid artery and external jugular vein were catheterised with Portex cannulae filled with heparinised saline at 12.5 units/ml and 1 unit/ml respectively, prewarmed to 37°C. Blood pressure was monitored continuously via the carotid arterial catheter using a World Precision Instrument blood pressure monitor (BP-1). A gentle jet of oxygen was aimed at the opening of the tracheal cannula to aid oxygenation. Rectal temperature was maintained at 36 - 38°C using a homeothermic blanket system (Harvard).

2.3 Western Blotting

2.3.1 Tissue Collection

Rats which had previously been prepared as described in section 2.2.2 were killed by an intraarterial or intravenous injection of 30 mg thiobutabarbital. Rats killed without prior surgery were given an intraperitoneal (ip) injection of 240 mg thiobutabarbital followed by further ip thiobutabarbital as required to inhibit cardiac and respiratory activity. Once respiration and cardiac activity had ceased, the chest cavity was opened and animals were perfused transcardially with 250 ml 0.9% saline at 37°C. A laminectomy was rapidly performed to expose the sacral, lumbar and lower thoracic spinal cord. By reference to the site of the lowest ribs, segments L3 - L5 were exposed, removed and placed in a plastic pot on dry ice. Samples were stored at -80°C before further processing.

2.3.2 Tissue Preparation

Homogenisation

Frozen spinal cord samples comprising the entire spinal cord from segment L3 to L5 were chopped into flakes on dry ice and 1 ml homogenisation buffer (see

Appendix 2) was added on wet ice. The mixture was vortexed and then homogenised on wet ice. Samples were spun at 20 000 rpm for 15 min at 4°C. The supernatant was removed carefully and stored at -20°C.

Protein Assay

A 50-µl sample of the supernatant was mixed with 450 µl 1 M NaOH and incubated at room temperature overnight. This was then diluted with 500 µl MilliQ water.

One hundred-µl duplicate aliquots were further diluted 1:2 with MilliQ water and vortexed. Protein standards were made up at 0, 5, 10, 20 and 40 µg/200 µl. One ml of a mixture of 2% Na_2CO_3, 0.4% NaOH, 0.01% $CuSO_4$ and 0.02% Na^+/K^+ tartrate was added to each aliquot and left to incubate for 10 minutes. One hundred µl of a 3 times dilution of folin and Cocalteu's phenol reagent was added and samples were vortexed and left for 20 minutes at room temperature. One ml MilliQ water was added, the mixtures vortexed and protein concentrations were read on a Beckman Counter at 750 nm.

Using the results, further dilutions of the samples were made and assayed such that each sample gave a consistent protein concentration of 1.5 µg/µl. These dilutions were used to make samples at constant protein concentrations for Western Blotting.

Protein Linearisation ('Cracking')

One hundred µl diluted supernatant was mixed with an equal volume of sample buffer (see Appendix 2) containing 0.05 mM dithiothreitol. Samples were heated to 100°C for 5 minutes and then stored at -20°C. Cox-1 (Cayman) and cox-2 (OBR) standards were treated in the same way.

2.3.3 Western Blotting

Minigels were set up between ethanol-washed plates using a Bio-Rad Protean system. A 5-cm depth of 10% acrylamide resolving gel was poured and overlayered with 1% sodium lauryl sulfate (SDS) solution (for all recipes see Appendix 2). Once the gel had set the gel surface was washed with MilliQ water and an imidazole stacking gel was layered over the resolving gel to the top of the plates and a 10-well comb inserted. After 30 min the combs were removed and the plates set up in tanks with the running buffer. The denatured samples were incubated at 40°C for 5 minutes, vortexed and then centrifuged for 5 minutes at 12 000 rpm. SDS-PAGE pre-stained markers and cracked cox-1 or cox-2 standards were incubated at 40°C for 5 minutes and vortexed.

Wells were loaded with 5 µl pre-stained markers (Bio-Rad), 12 µl sample (containing 18 µg protein) and 1 µl of a 1:2 dilution of purified cox-1 or cox-2 standard (containing 110 ng and 50 ng protein respectively). Gels were electrophoresed for 1 hour at 200 V.

The stacking gel portion was then removed and the gels were soaked in transfer buffer at room temperature on a plate shaker for 10 - 20 min. The blots were transferred to nitro-cellulose paper using a Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell at 15 V for 30 min following the manufacturer's instructions. The blots could be checked for bands to see if transfer had taken place by placing the blots in a 0.2% Ponceau Red staining solution.

Blots were soaked in blocking buffer (10% Marvel in TTBS) for 2 hours on a plate shaker, given three 5-min washes in TTBS and then incubated in primary antibody (in 10% Marvel in TTBS containing 0.01% sodium azide) at room temperature overnight. Blots were given three 5-min washes in TTBS and then incubated in secondary antibody for 1.5 hours.

After 5 further 5-minute washes in TTBS, blots were developed with ECL reagent (Amersham) following the manufacturer's instructions. Blots were visualised on blue sensitive X-ray film (Genetic Research Instrumentation Ltd) using an Amersham hyperprocessor.

2.3.4 Antibodies

Purified protein extracts were purchased from Cayman (cox-1) and OBR (cox-2). Primary antibody for cox-1 analysis was used at 1:1000 (Cayman) and primary antibody for cox-2 analysis was used at 1:750 (Cayman). Peroxidase-conjugated secondary antibody for cox-1 analysis was anti-mouse antibody (Amersham) used at 1:1000 and peroxidase-conjugated secondary antibody for cox-2 analysis was anti-rabbit antibody (Cayman) at 1:1000.

The manufacturers' literature provided with the primary antibodies indicated that the monoclonal murine antibody raised against ovine cox-1 bound sheep, rat, mouse and human cox-1 with relatively little or no crossreactivity with sheep, mouse or human cox-2. The polyclonal rabbit antibody raised against murine cox-2, bound sheep, rat, guinea-pig and human cox-2 with little or no crossreactivity to sheep, mouse or human cox-1.

2.3.5 Quantitative Densitometry

In order to compare the amount of cox protein in different samples, the cox bands on the radiographic films were analysed using a Bio-Rad scanning densitometer and Molecular Analyst software. The band in each sample running to the same distance as the purified cox protein on each blot was identified. The smallest block size which would just encompass the whole of each of these bands on a blot was chosen and the average optical density of each of these identically sized blocks containing each band, corrected for background exposure, was recorded.

2.4 Dorsal Horn Extracellular Recordings

2.4.1 Surgery

Animals were anaesthetised and prepared as described in Sections 2.2.1 and 2.2.2. The dorsal and lateral mammillary processes from T12 to L5 were exposed and animals were suspended in a stereotaxic frame using swan-necked clamps under the lateral processes.

A laminectomy was performed to expose the spinal cord from approximately vertebral segments T12 to L5. The incised skin over the back was tied up to make a pool over the spinal cord into which 3% agar in a 0.9% saline solution at 38°C was poured. Once the agar had set, a window was cut in it over the lumbar spinal cord and paraffin oil warmed to 37°C was poured into the well in the agar to prevent desiccation of the spinal cord. A durotomy was performed to expose the intact spinal cord. The frame was screwed to an air-table during recording sessions.

2.4.2 Electrophysiology

Electrodes made from acid-washed (with 0.5 M nitric acid) glass capillary tubing (1.5 mm external diameter, 1.2 mm internal diameter) were pulled, filled with 0.5 M sodium acetate and then the tips were broken against a glass rod to give a tip diameter of 1 - 2 μm and a resistance in the region of 2 - 10 MΩ.

The electrode was advanced into the spinal cord on the right side, i.e. ipsilateral to any arthritic lesion, using a stepping motor. During its descent the ipsilateral hindlimb of the animal was squeezed to try to identify cells which had ankle- or knee-joint deep tissue input. In the case of Freund's complete adjuvant lesions this was the ankle joint and for kaolin/carrageenan lesions this was the knee joint.

Extracellular activity was visualised on an oscilloscope (Gould DSO 1602) and stored on a Dell 450/L computer as a spike rate histogram using a Spike 2 or MRATE data capture program via a CED 1401 unit. The bin size was 1 s. The recordings were also stored on video tape via a VR-10B and a video recorder (JVC HR-J210) (Fig. 2.1) for back-up and further off-line analysis.

Graded mechanical stimulation to the appropriate joint was provided by a pneumatic pincher device with jaws capable of squeezing at up to 50 psi over an area of 60 mm^2. A 50-psi pinch applied to a human finger is very uncomfortable, a 20-psi pinch is felt as a firm but innocuous grip.

When a suitable unit had been identified its receptive field was mapped and the stimulation threshold was assessed subjectively as high, low or moderate. Its depth within the spinal cord was recorded from the stepping motor readout. Further characteristics such as the presence of spontaneous activity and any contralateral excitatory or inhibitory input were recorded. Methods of collection and analysis of data are detailed in Section 5.2.4.

At the end of the recording session the animal was killed by an intravenous overdose of anaesthetic.

2.5 Spinal Reflex Recordings

2.5.1 Surgery

Animals were anaesthetised and prepared as described in Sections 2.2.1 and 2.2.2. The dorsal and lateral mammillary processes from T12 to L5 were exposed and animals were suspended in a stereotaxic frame using swan-necked clamps under the lateral processes.

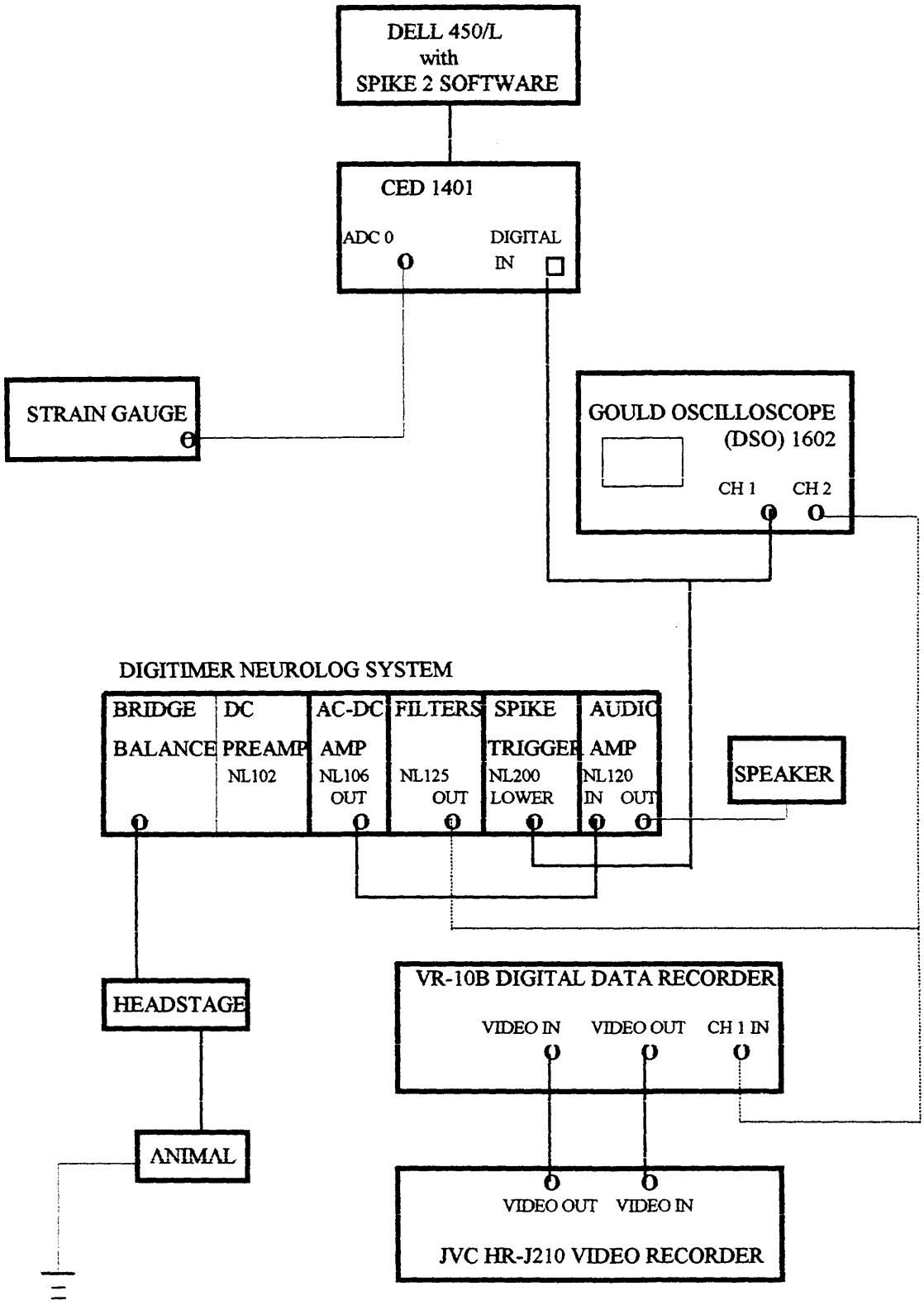


Figure 2.1. Diagrammatic representation of the electrical set-up for extracellular recording experiments.

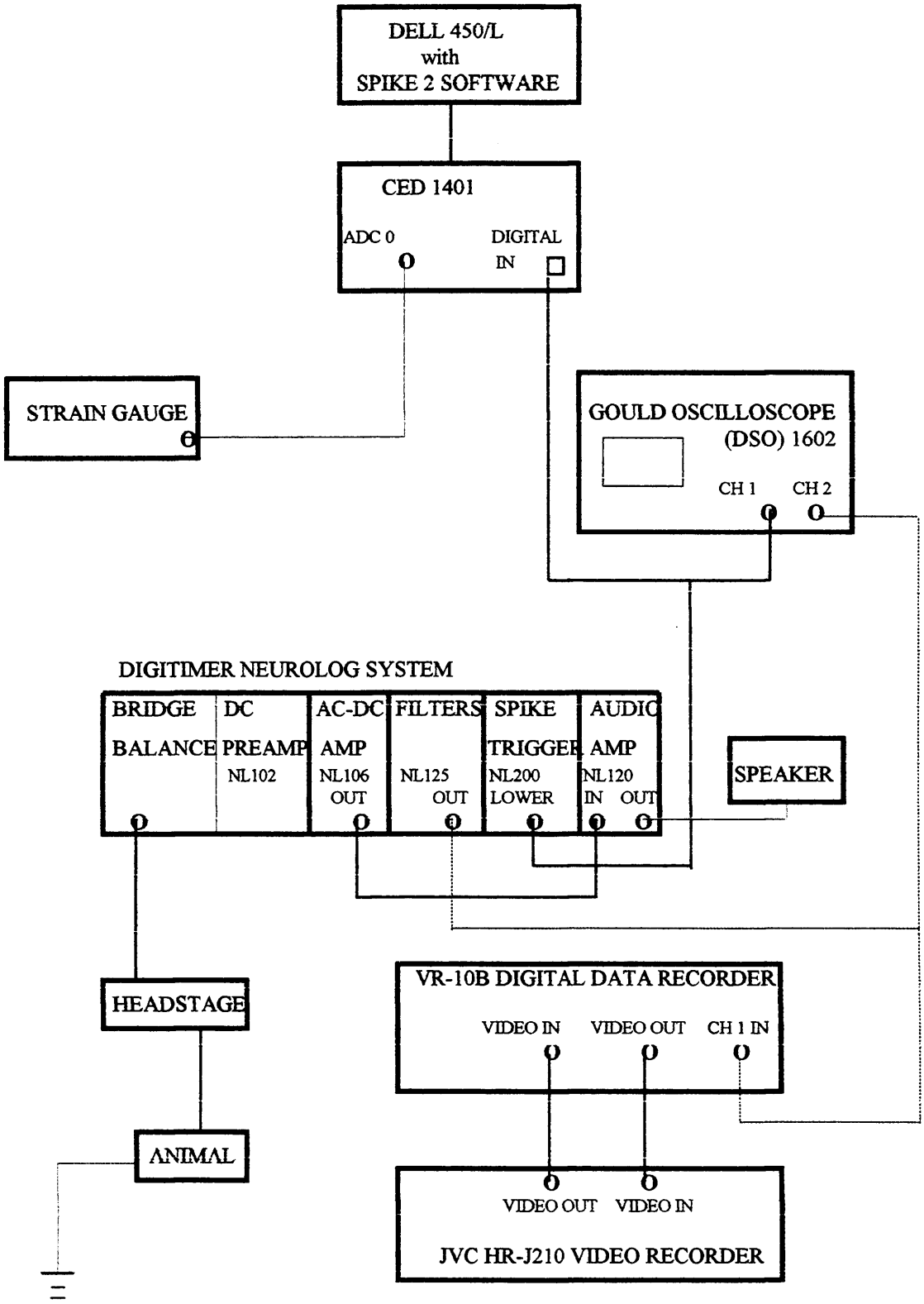


Figure 2.1. Diagrammatic representation of the electrical set-up for extracellular recording experiments.

The right hindlimb was immobilised by taping the foot to a horizontal bar behind the animal. An incision was made longitudinally on the posterior surface of the right hindlimb from the thigh to the ankle. The skin was reflected and tied with a series of sutures to a metal former clamped above it to make a pool into which warm paraffin oil was poured. The sural nerve was located under biceps femoris and dissected free from the ankle to the knee region. The nerve was tied distally and cut.

2.5.2. Electrophysiology

The cut sural nerve was laid over a pair of silver wire electrodes and stimulated with square-wave pulses of 500-μs duration. A single-unit emg was recorded via a pair of tungsten electrodes inserted into biceps femoris posterior.

The emg was visualised on an oscilloscope (Gould DSO 1602) and stored on a Dell 450/L computer as a spike rate histogram with a bin size of 20 ms using an MRATE data capture program via a CED 1401 unit. The recordings were also stored on videotape via a VR-10B and a video recorder (JVC HR-J210; Fig. 2.2) for further off-line analysis. The number of spikes in each response to each stimulus was counted using MRATE software. A-fibre and C-fibre spikes were counted separately using the criteria that A-fibre spikes occurred with a latency of 100 ms or less and C-fibre spikes occurred with a latency of greater than 100 ms. Further details of the counting methods used are given in Section 6.2.3.

At the end of the recording session, the animal was killed by an intravenous overdose of anaesthetic.

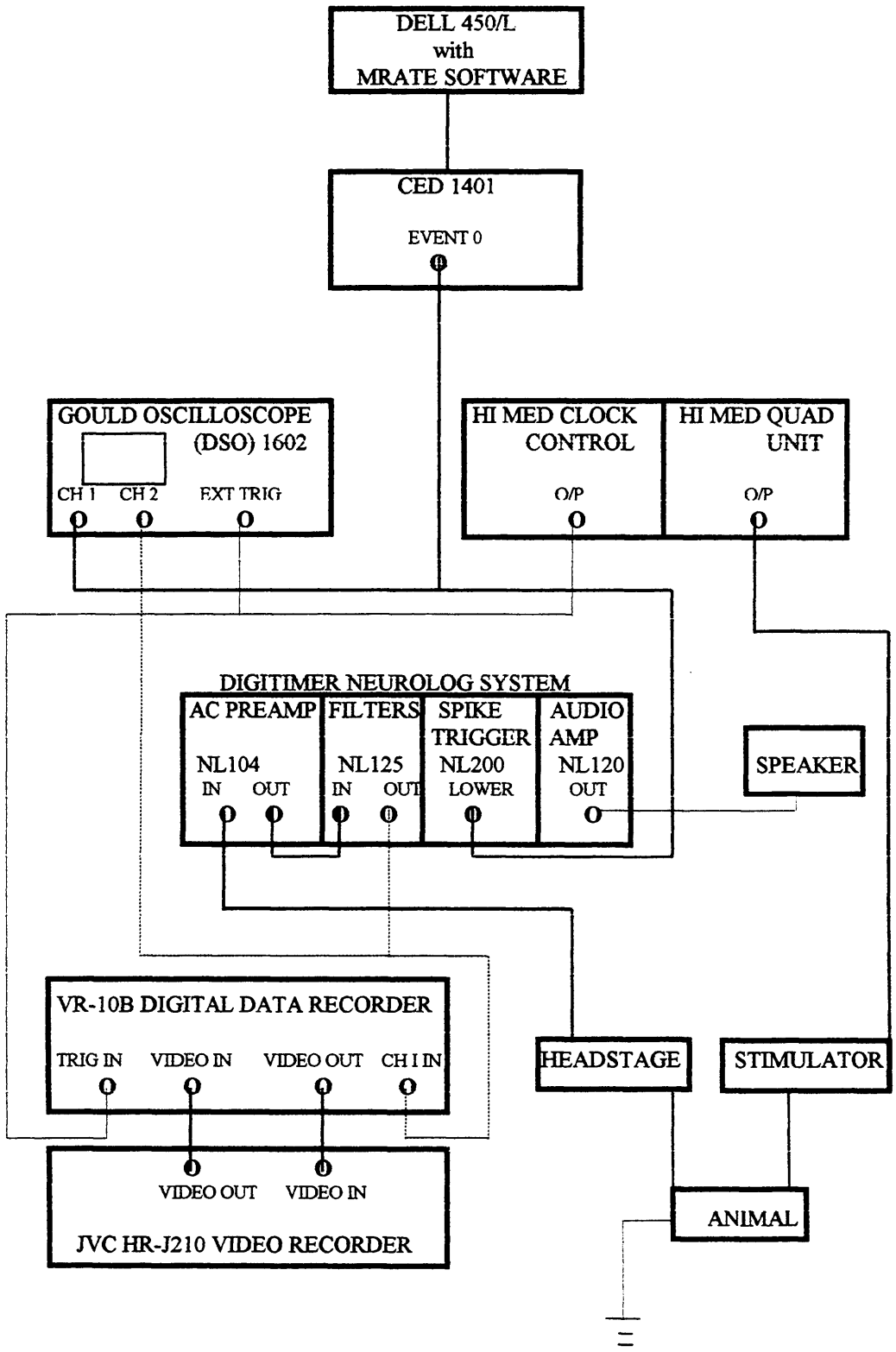


Figure 2.2. Diagrammatic representation of the electrical set-up for spinal reflex recordings.

2.6 Induction of Arthritis

2.6.1 Freund's Complete Adjuvant Monoarthritis

Rats were anaesthetised briefly with 5% halothane until they had lost their hindlimb withdrawal reflex. The skin of the ankle (usually the right limb) was swabbed with hibitane and a total of 0.15 ml Freund's Complete Adjuvant (FCA; heat-killed *Mycobacterium tuberculosis* (1 mg/ml) in paraffin oil and mannide monooleate; Sigma) was injected around the ankle at 3 - 4 sites. The circumference of each ankle was recorded. Rats were allowed to recover immediately in air.

The Animal House Technicians made daily measurements of the circumference of both ankles. Body weight was also recorded daily and scores of gait, motility, scratching behaviour, the appearance of the head and hindlimbs and stress on handling were made according to the protocol in Table 2.1. Rats were used for electrophysiological experiments within 1 - 3 days of injection. None needed to be culled due to reaching a 'three star' condition.

2.6.2 Kaolin/Carrageenan (K/C) Arthritis

An acute arthritis was induced by the injection of 0.07 ml of a 4% kaolin suspension (in MilliQ, warmed to 37°C) into the right knee joint of the surgically anaesthetised rat followed 15 - 25 minutes later by 0.07 ml of a 2% λ carrageenan solution (in MilliQ, heated to dissolve the carrageenan then maintained at 37°C). The knee joint was manipulated for 30 s at 10-min intervals following these injections for a period of 30 minutes after the carrageenan injection. Kaolin and λ carrageenan were purchased from Sigma.

Table 2.1 Behavioural scoring system for rats following periarticular injection with Freund's Complete Adjuvant.

Observations made daily by Animal House technicians:

1. Motility	*	Normal
	**	Responds when disturbed
	***	Very little activity
2. Scratching	*	Normal
	**	Increased scratching
	***	Scratching causing wounding
3. Inflammatory signs (hindlimbs and tail)	*	Normal
	**	Some redness/swelling
	***	Severe inflammation and wounds
4. Inflammatory signs (snout and ears)	*	Normal
	**	Some redness
	***	Severe inflammation and wounds
5. Stress on handling	*	Normal
	**	Increased heart rate/defecation/squeaking
	***	Agressive on handling
6. Gait	*	Normal
	**	Limping (reduced weight-bearing)
	***	No weight-bearing (leg held up)

Body weight and ankle circumferences were monitored daily.

If any condition reached *** the procedure was terminated immediately.

Chapter 3

Identification of Cox Proteins in Spinal Cord of Normal Rats

3.1 Introduction

If prostaglandins (PGs) are involved in spinal nociceptive processing, the biosynthetic enzymes required to produce them must be present within the spinal cord. PGs are produced locally when required and, being lipophilic, diffuse easily out through cell membranes. PGs are derived from arachidonic acid (AA) as shown diagrammatically in Figure 1.4. AA is converted to PGG_2 then to PGH_2 by the action of cyclooxygenase (cox) as discussed in Section 1.6. These two PGs are converted by specific enzymes to the biologically active metabolites: PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin) and TxA_2 (thromboxane). Phospholipase A_2 activity is the rate-limiting step in this synthesis.

As discussed in Section 1.7, two isoforms of cox have been identified in a variety of tissues: cox-1 and cox-2. The two known isoforms of cox have been shown to have different anatomical distributions. Cox-1 activity is constitutively expressed in a wide variety of tissues whereas cox-2 has been found in a limited number of tissues under normal conditions (brain, testis, kidney; Breder et al. 1995; Kaufmann et al. 1996). Cox-2 is, however, found in inflamed tissues (Sano et al. 1992; Seibert et al. 1994; Mitchell et al. 1995; Anderson et al. 1996; Kang et al. 1996) which has led to it being designated as an 'inducible' enzyme. Cox-2 production has been shown to be stimulated by growth factors, tumour promoters and cytokines (Fu et al. 1990; DeWitt & Smith 1995).

Cox activity is essential for the production of PGs, so if, as postulated, spinal PGs are involved in central nociceptive processing, cox-1 and/or cox-2 must be present in spinal cord tissue. Cox protein has previously been shown to be present in rat brain (Kawasaki et al. 1993; Yamagata et al. 1993; Breder et al. 1992, 1995; Kaufmann et al. 1996). More recently cox-2 mRNA and cox-1 mRNA have been identified in spinal cord tissue in the rat (Hay et al. 1997; Hay & De Belleroche 1998; Ichitani et al. 1997) using hybridisation techniques.

In order to determine whether cox protein is present in the spinal cord, and therefore by inference if PGs could be present, we looked for both cox-1 and cox-

2 in a lumbar spinal cord homogenate taken from a normal male rat using a Western blotting technique. Care was taken to provide rapid, stress-free euthanasia, rapid removal of tissues onto dry ice and careful subsequent processing of the tissues in order to prevent the induction or *de novo* synthesis of PGs. The results would therefore accurately reflect the presence or absence of cox activity in the spinal cord under normal circumstances.

Preliminary work by N Gardiner in this laboratory had established that the primary antibodies chosen for this work, manufactured by Cayman, gave reliable binding to purified cox protein extracts. Four other antibodies obtained from other manufacturers gave unsatisfactory or inconsistent binding in preliminary experiments.

3.2 Methods

Six adult male Wistar rats were killed by an intraperitoneal injection of 240 mg thiobutabarbital followed by further intraperitoneal doses until respiration and cardiac activity had ceased. Spinal cord tissue was removed from the freshly euthanased, perfused rats as described in Sections 2.3.1 and total protein was extracted, assayed and Western blotted as described in detail in Sections 2.3.2 - 2.3.3.

Following the protocols described in Section 2.3.3, wells were loaded with 22.8 μg spinal cord protein extract in 12 μl together with a control well loaded with either purified cox-1 or cox-2 protein (110 ng and 50 ng in 1 μl respectively). The molecular weight of the bands running in the same position as the purified cox-1 and cox-2 proteins was estimated by interpolation from a graph of molecular weight of the prestained SDS-PAGE markers plotted against distance travelled during electrophoresis. Blots were also performed omitting either the primary or the secondary antibody. Additionally a blot using ovine cox-1 purified protein was incubated in cox-2 primary antibody and a blot of ovine cox-2 purified

protein was incubated in cox-1 primary antibody to check for crossreactivity of the antibodies. Details of the antibodies used can be found in Section 2.3.4.

3.3 Results

Figure 3.1 shows blots of total spinal cord protein (sc) extracted from the lumbar spinal cord of a normal rat. Panels A and B show blots incubated in cox-1 antibody and cox-2 antibody respectively, exhibiting bands at approximately 75 kD; the same level as purified cox-1 and cox-2 protein standards. This shows that both cox-1 and cox-2 reactivity is present in the spinal cord of a normal rat. Note that a further band at approximately 68 kD is present on the cox-2 blots.

Similar cox-1 and cox-2 bands were seen in spinal cord samples taken from the 5 other rats sampled.

Panels C and D of Figure 3.1 show Western blots of ovine cox-1 and cox-2, incubated in cox-1 antibody and cox-2 antibody respectively. Cox-1 but not cox-2 purified protein blots incubated in cox-1 primary antibody produced a band and cox-2 but not cox-1 purified protein blots incubated in cox-2 primary antibody showed a band. There was no crossreactivity even at very long exposure times (data not shown). This suggests that there is little crossreactivity between the two rat isoforms using the primary and secondary antibodies employed in this study. Blots where primary or secondary antibody had been omitted showed no bands (data not shown).

3.4 Conclusions

The evidence from Western blotting shows that both cox-1 and cox-2 are present in the lumbar spinal cord of saline-perfused, normal rats with no surgical interventions beyond the administration of an intraperitoneal barbiturate anaesthetic. Transcardial perfusion with warmed isotonic saline removed blood contamination from the sample, and the spinal cord was removed rapidly to

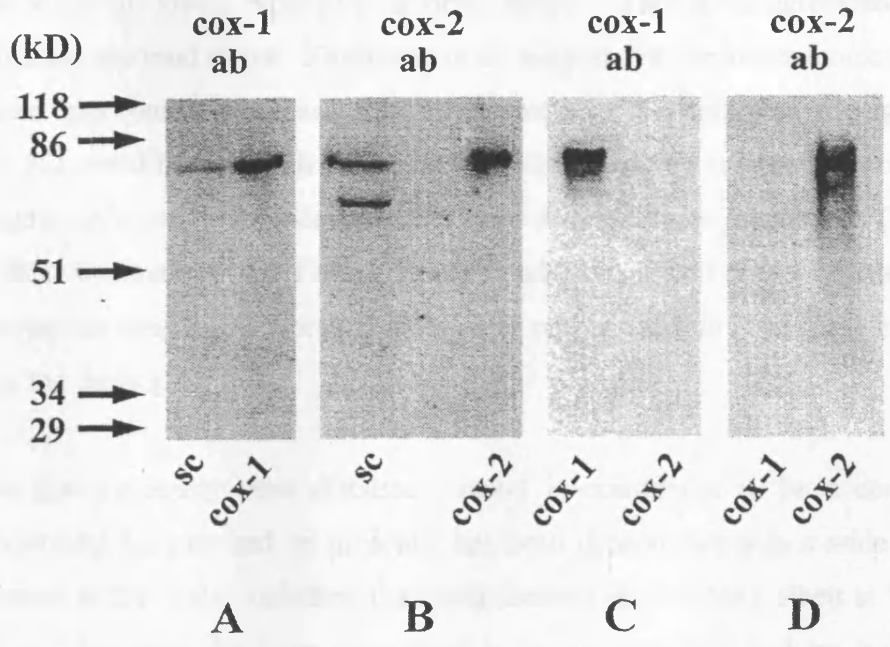


Figure 3.1. Scans of blots demonstrating the presence of cox protein in a spinal cord homogenate from normal rats. **A.** Blot incubated with anti-cox-1 antibody (cox-1 ab). Lane 1, spinal cord sample (sc); lane 2, cox-1 protein (cox-1). **B.** Blot incubated with anti-cox-2 antibody (cox-2 ab). Lane 1, spinal cord sample (sc); lane 2, cox-2 protein (cox-2). **C.** Ovine cox-1 and cox-1 incubated in cox-1 antibody. **D.** Ovine cox-1 and cox-2 incubated in cox-2 antibody. Both cox-1 and cox-2 are present in the spinal cord sample. There is no crossreactivity between the two isoforms.

minimise any *de novo* cox synthesis during the surgical removal of the spinal cord. The entire procedure took in the region of 3 - 5 minutes from the start of surgery to the placing of the excised spinal cord sample onto dry ice. It could therefore reasonably be concluded that both cox-1 and cox-2 activity is present in the spinal cord of a non-hyperalgesic rat. The presence of both cox-1 and cox-2 proteins was later reported by Goppelt-Struebe & Beiche 1997.

Kaufmann et al. (1996) reported seeing a double band at about 70 kD and a further single band at about 43 kD when using Western blotting techniques to identify the presence of cox-2, especially in brain tissue. This is in agreement with the findings reported above. Kaufmann et al. suggest that the lower-molecular weight band may consist of a cox-2 breakdown product. The multiple banding at 68 and 75 kD could be the result of partial degradation during sample preparation or to deglycosylation, which occurs naturally or during sample preparation (Feng et al. 1993; Kaufmann et al. 1996). The second band is certainly at the appropriate molecular weight to be the deglycosylation product and this is a likely explanation for the finding.

As has previously been discussed, cox-1 is considered to be a constitutively expressed enzyme and its presence has been demonstrated in a wide variety of tissues in the body, including the brain (Seibert et al. 1994), albeit at low levels. Cox-2 has generally been considered to be an inducible isoform but has been previously reported to be present in the normal brain (Kawasaki et al. 1993; Yamagata et al. 1993; Breder et al. 1995). mRNA encoding for both cox-1 and cox-2 has been isolated from both brain (Yamagata et al. 1993; Seibert et al. 1994) and spinal cord (Beiche et al. 1996; Goppelt-Struebe & Beiche 1997; Hay et al. 1997; Hay & De Belleroche 1998). The proteins synthesised from these two cox transcripts, cox-1 and cox-2, have now been demonstrated for the first time in the spinal cord.

Western blotting can do no more than identify the presence of these cox isoforms, saying nothing about their location. Cox-2 in brain has been located specifically in neurons by immunocytochemical techniques (Yamagata et al. 1993; Breder et al. 1995; Goppelt-Struebe & Beiche 1997). Subsequent immunocytochemical

studies were performed in this laboratory on lumbar spinal cord slices taken from normal rats killed rapidly in the same manner as for this Western blotting study. The presence of symmetrical cox-2 immunoreactivity (cox-2-ir) in superficial dorsal horn, diffusely in deep dorsal horn (laminae V-VI), around the central canal (lamina X) and some immunolabelling of neurons in the deep dorsal horn and in the motor nuclei of ventral horn was demonstrated, as shown in Figure 3.2 (Gardiner et al. 1997; Willingale et al. 1997). Similar patterns of cox-2 staining were reported by Beiche et al. (1998). Cox-2 immunoreactivity has therefore later been shown to be present in anatomical locations previously associated with nociception (Schaible et al. 1986; Neugebauer & Schaible 1990; Grubb et al. 1993).

Where the cellular location of cox-2-ir could be identified, staining was mainly neuronal and confined to the cell cytoplasm in rat lumbar spinal cord slices (Willingale et al. 1997). This is consistent with previous studies of brain tissue (Breder et al. 1995) and cultured cells (Kaufmann et al. 1996) although Goppelt-Struebe and Beiche (1997) report prominent staining in the nuclear envelope. Diffuse cox-2-ir seen throughout the grey matter of the spinal cord may be due to labelling of glial cells which, together with neurons, have been shown to synthesise eicosanoids (Bishai & Coceani 1992). Glial-cell labelling was reported by Goppelt-Struebe and Beiche (1997) and Beiche et al. (1998a).

Immunocytochemistry also showed that cox-1-ir is limited to a few axons in the very superficial layers of the dorsal horn of the rat spinal cord with more profuse staining in the dorsal roots (Willingale et al. 1997).

The biosynthetic machinery required to produce PGs in the spinal cord has thus been shown to be present and in an anatomical location associated with nociceptive afferent input. This is consistent with the hypothesis that PGs are involved in spinal nociceptive processing.

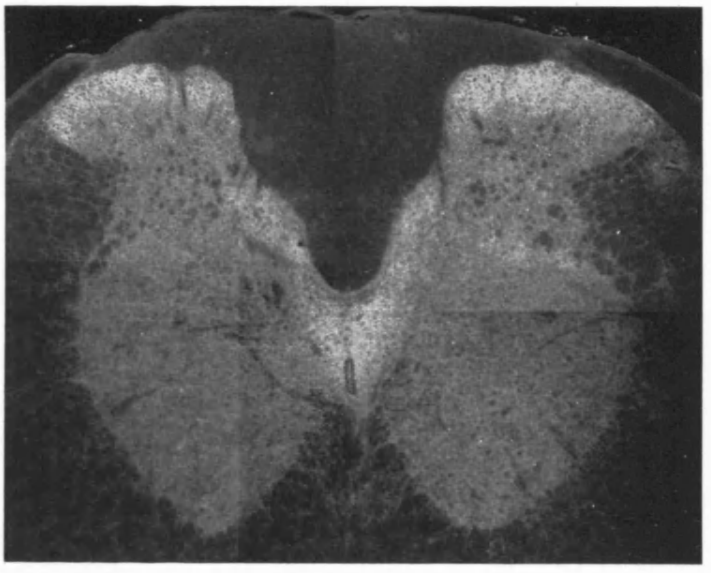


Figure 3.2. A composite confocal photomicrograph of a transverse section of rat lumbar spinal cord illustrating cox-2-like immunoreactivity. Cox-2-like immunoreactivity was present bilaterally in the superficial dorsal horn, around the central canal in lamina X and in the deep dorsal horn in laminae IV and V. Figure taken from Willingale et al. 1997.

Chapter 4

Cox Protein Synthesis in Spinal Cord of Arthritic Rats

4.1 Introduction

The presence of cox-2 and cox-1 in the lumbar spinal cord of normal adult rats has been established in the preceding chapter. Cox-2 is considered to be an inducible enzyme in peripheral tissues and has been shown to be upregulated in inflamed tissues (Sano et al. 1992; Seibert et al. 1994; Mitchell et al. 1995; Anderson et al. 1996; Kang et al. 1996) in response to growth factors, tumour promoters or cytokines (Fu et al. 1990; for a review, see DeWitt & Smith 1995). In the absence of stimulation, cox-2 has been found only in the brain, testis, macula densa of the kidney (Breder et al. 1995; Kaufmann et al. 1996) and now in the spinal cord. Cox-2 protein concentrations in the brain are increased by a number of processes such as epileptiform seizures and cold stress (Yamagata et al. 1993), demonstrating that cox-2 is actively and inducibly synthesised by central nervous system tissue in response to a stimulus.

A hypothesis has been proposed in the introduction to this thesis that PGs are involved in central nociceptive processing in states of hyperexcitability. A logical premise would therefore be that PG production would be altered in conditions of hypersensitivity as compared with the normal state. The enzyme essential for PG production (cox) has been shown to be present in the spinal cord under normal conditions in the rat. The cox-2 enzyme is autosuicidal so that an increase in PG synthesis will require a concomitant increase in cox production. A series of experiments was designed to determine if cox expression in the spinal cord increases in a model of hypersensitivity, where there is an increased nociceptive afferent input into the spinal cord producing central hyperalgesia.

Both chronic and acute models of arthritis have been shown to induce hyperexcitability of dorsal horn cells (Menétrey & Besson 1982; Guilbaud et al. 1985; Grubb et al. 1988, 1991, 1993; Neugebauer & Schaible 1990; Neugebauer et al. 1995) with an increased production of excitatory amino acids and neuropeptides (Hope et al. 1990; Schaible et al. 1990; Sluka & Westlund 1993; Schaible et al. 1994; Abbadie et al. 1996; Yang et al. 1996). It was therefore of

interest to see whether expression of cox-1 and/or cox-2 would vary during the development of an acute arthritis induced by the injection of kaolin and carrageenan (K/C) into the knee joint of rats, a model of peripheral inflammation described in Section 1.8.

Electrophysiologically, changes typical of hypersensitivity as discussed in Section 1.4 develop in parallel with the clinical signs of inflammation both peripherally (Coggeshall et al. 1983; Schaible & Schmidt 1985) and centrally (Neugebauer & Schaible 1990; Neugebauer et al. 1995). In acute models of arthritis, behavioural signs of hyperalgesia occur from 1 hour post-injection (Yang et al 1996) and electrophysiological changes have been observed up to 24 hours post-injection (Herrero & Cervero 1996a). Electrophysiological changes develop concomitantly with the clinical signs of inflammation, as discussed in Section 1.3. It was therefore considered reasonable to expect biochemical changes to be taking place in the spinal cord over a time course of 1 – 24 hours. The experiments reported below used sample times of 3, 6 and 12 hours post-injection. If there were an upregulation of the gene for cox-2 expression, then it would be expected to occur during this time period.

Relative concentrations of both cox-1 and cox-2 in lumbar spinal cord homogenates taken from animals at these time points during the development of kaolin/carrageenan were estimated by Western blotting followed by quantitative densitometry. Samples consisted of lumbar spinal segments L3 - L5 since this is the area of spinal cord receiving sensory input from the hindlimb (Molander & Grant 1986).

4.2 Methods

Four groups of four rats (193 - 400 g) were anaesthetised and prepared as described in Sections 2.2.1 and 2.2.2. Rats were maintained on a blanket heated to 37°C in an oxygen-enriched atmosphere. Blood pressure was monitored at approximately half-hourly intervals. Four rats were killed by massive intravenous thiobutabarbital overdose immediately after surgery was complete ('Con').

An acute kaolin/carrageenan arthritis was induced in 3 groups of 4 rats as soon as surgical anaesthesia had been achieved, as described in Section 2.6.2. These groups were killed by intravenous anaesthetic overdose 3, 6 and 12 hours after the induction of arthritis ('3K/C', '6K/C' '12K/C').

Tissue preparation and Western blotting were performed as described in Sections 2.3.1 - 2.3.3. Four blots were made comparing lumbar spinal cord total protein extracts from the 4 individuals in group Con with 3K/C, 6K/C and 12K/C and purified cox-1 protein using cox-1 primary antibody. The series of wells for a blot was loaded with 18 μg of spinal cord extract from an individual in each group with a final lane loaded with 110 ng purified protein. A repeat set of blots substituting 50 ng purified cox-2 protein in the final lane was incubated with cox-2 antibody. Details of the antibodies used are given in Section 2.3.4.

The relative amounts of cox-1 and cox-2 in the protein extracts were compared by quantitative densitometry as described in section 2.3.5. The band comprising cox protein was determined by comparison with the lane containing the purified protein extract. The average optical density of each cox band was measured and recorded. Results derived from the 4 bands corresponding to each treatment group were averaged. The average optical densities for the different treatment groups were compared by 1-way anova (significance < 0.05).

4.3 Results

Blots made from protein extracts from spinal cord homogenates taken from rats at different time points (3, 6, 12 h) following the induction of K/C arthritis of the knee and from surgically prepared, but non-arthritic, rats confirmed the presence of both cox-1 and cox-2 in the spinal cord of rats.

As can be seen from the example of a blot shown in the lower panel of Figure 4.1, the bands running level with the cox-2 standard were substantially denser for the tissue extracts from the 3, 6 and 12 h K/C arthritic samples when compared with the lumbar spinal cord tissue extract from a rat killed immediately after completion of neck surgery (Con). A similar pattern was observed with all 4 blots incubated with cox-2 antibody although one blot had a more erratic pattern of staining of cox-2. Densitometric analysis of the films showed that the cox-2 protein levels were increased 4-fold and 3 and 6 h and 6-fold at 12 h. The upper panel of Figure 4.1 shows an example of a blot incubated with cox-1 primary antibody, showing that the cox-1 bands did not noticeably vary in density between the different treatment groups.

Figure 4.2 is a histogram showing the averaged optical densities from the four tissue samples in each treatment group, demonstrating a significant increase in cox-2 protein concentrations ($p < 0.01$, one-way anova), but no significant change in cox-1 concentrations ($p > 0.05$, one-way anova), in spinal cord samples derived from rats at 3 - 12 hours postinduction of K/C arthritis.

4.4 Conclusions

These results show that cox-1 levels within the lumbar spinal cord were not significantly changed 3 - 12 h after the induction of an acute K/C arthritis of the knee as compared with the control animals. A similar series of experiments were performed by Gardiner (1997) using a chronic FCA monoarthritis as a model of

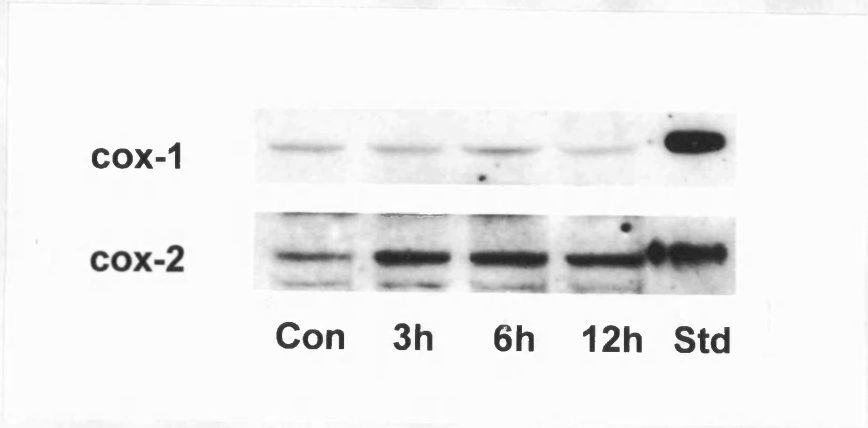


Figure 4.1. Individual western blots showing bands of protein samples taken from the lumbar spinal cord of control rats (Con) or from rats 3, 6 and 12 hours after the induction of a kaolin/carrageenan arthritis incubated in cox-1 antibody (upper panel) or cox-2 antibody (lower panel). Std: cox-1 and cox-2 purified protein standards.

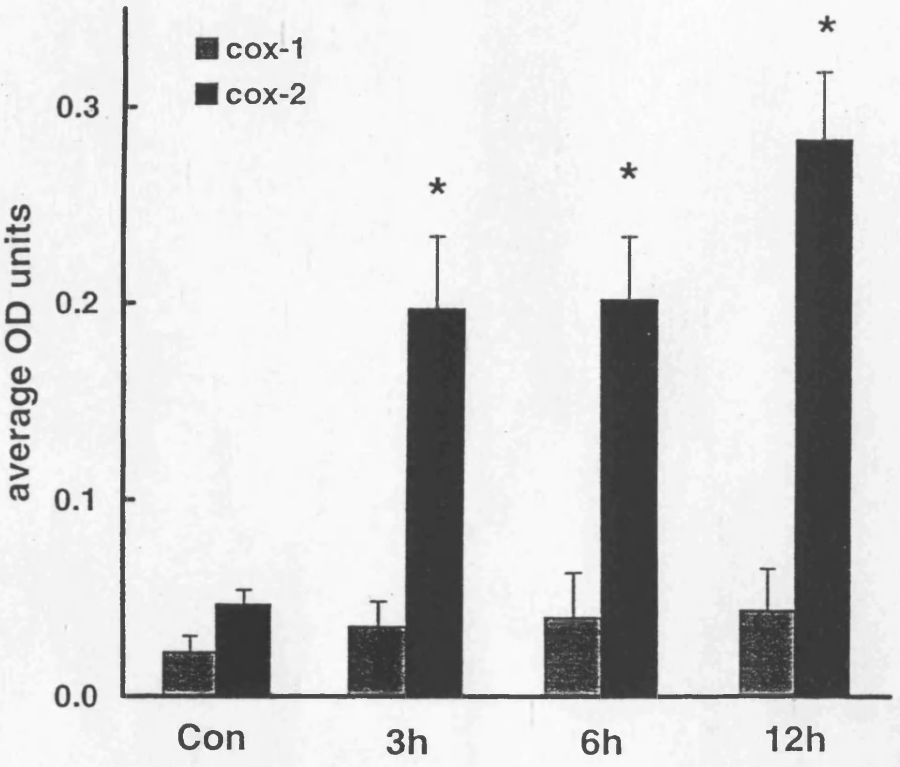


Figure 4.2. Histogram showing the relative concentrations of cox-1 and cox-2 in lumbar spinal cord homogenates 3 hours (3h), 6 hours (6h) and 12 hours (12h) following the induction of kaolin/carrageenan arthritis and in non-arthritic animals (Con), as assessed by semi-quantitative densitometry. The results are an average of 4 blots. Cox-2 levels increased rapidly and significantly following the induction of arthritis (* $p < 0.01$, one-way anova) but there is no significant change in cox-1 levels ($p > 0.05$, one-way anova).

chronic inflammation. Cox-1 concentrations in the lumbar spinal cord did not significantly vary over a time period of 0 - 14 days. This is in line with the idea that cox-1 is a housekeeping enzyme that does not readily respond to external stimuli. It also supports the histochemical findings that there is little cox-1 in spinal cord tissue (Willingale et al. 1997), as discussed in Section 3.4. The results suggest that cox-1 protein synthesis is not significantly induced in the spinal cord during the development of hyperalgesia.

These results indicate that cox-2 concentrations were significantly increased 3 - 12 h after the induction of an acute K/C arthritis when compared to concentrations in the spinal cord of rats subjected only to neck surgery. This supports the hypothesis that cox synthesis, and thereby PG production, is increased in the spinal cord during the development of central hyperalgesia, allowing the possibility that PGs within the spinal cord play a role in central nociceptive processing in hypersensitivity.

Gardiner (1998) showed, using similar methods, that cox-2 concentrations in the spinal cord of rats were significantly increased 3.5-fold at time points 12 h - 3 days post-induction of FCA monoarthritis in the ankle. As has been discussed in Section 1.3, FCA injection around the ankle joint produces a chronic monoarthritis. Gardiner (1998) further showed that injection of paraffin vehicle into the ankle caused neither a significant increase in the ankle circumference over this timespan nor induced a significant increase in cox-2 protein concentrations in the lumbar spinal cord. This chronic model of arthritis did not produce a significant alteration in cox-1 concentrations, demonstrating that cox-1 concentrations do not significantly increase in the spinal cord in either an acute or a chronic model of arthritis. mRNA for cox-2 was shown to be upregulated in the spinal cord in a similar model of peripheral inflammation over a similar time course (Beiche et al. 1996, 1998b; Goppelt-Struebe & Beiche 1997) which was reflected in a small increase in cox-2 protein from spinal membrane preparations by Western blot analysis.

In this acute model of arthritis, cox-2 protein expression was markedly increased by 3 h after the induction of inflammation. Workers used the same model of inflammation to study the time course for enhanced release of PGE_2 using an antibody microprobe technique (Ebersberger et al. 1999). The time courses for the increase in release of PGE_2-immunoreactivity (ir-PGE_2) and the increase in cox-2 expression were similar in that both developed within a few hours of the induction of a K/C arthritis. The enhanced ir-PGE_2 release was generally found later than 6 h post-induction, although insertion of microprobes started 175 min after kaolin injection at the earliest so a transient, early ir-PGE_2 release would have been missed. Antibody microprobe studies have also been used to demonstrate the release of ir-CGRP and ir-SP during the development of an acute inflammation (Schaible et al. 1990; Schaible et al. 1994). An increase in both ir-SP and ir-CGRP release was demonstrated in the first few hours after the induction of K/C inflammation in cats and rats. This therefore follows a similar time course to the induction of cox-2 and release of PGE_2. As discussed in Section 1.4.2, SP and CGRP are released centrally following stimulation of nociceptors in hyperalgesic animals.

Cox-2 mRNA has previously been shown to be rapidly and transiently induced in neurons of the cerebral cortex and hippocampus by electroconvulsive shock (Yamagata et al. 1993). A similar increase in cox-2 mRNA was also induced by NMDA-dependent synaptic activity. Adams et al. (1996) investigated the effects of chemically-induced seizures, using unilateral kainate injection into the basal forebrain and showed that cox-2 mRNA was ipsilaterally induced 4 - 8 hours after kainate injection and that MK-801 (an NMDA antagonist), lamotrigine (which blocks glutamate release) and dexamethasone (which indirectly inhibits PLA_2 via lipocortin) all attenuated this response. Taken together, these observations support the postulation that cox-2 induction in the brain is dependent upon glutamate release acting at NMDA receptors in the brain.

Cox-2 expression in the lumbar spinal cord has been shown to be increased over a rapid time course (by 3 hours post-injection) following the injection of kaolin and

carrageenan into a joint. By analogy with the induction of cox-2 mRNA in the cerebral cortex, it could be postulated that this is also an NMDA-dependent process. A series of experiments to investigate this was planned but not completed owing to time constraints. It was intended to investigate whether the increase in cox-2 concentrations could be attenuated by intravenous injections of ketamine, a non-competitive NMDA antagonist, at half-hourly intervals. This is an area for future research.

Chapter 5

Extracellular Recordings

5.1 Introduction

As discussed in Chapter 1, there is an accumulation of evidence to suggest that NSAIDs produce their analgesic effects by the inhibition of cox activity. Further, more recent evidence such as the behavioural studies of Malmberg and Yaksh (1992a, 1993) and Chapman and Dickenson (1992) together with electrophysiological studies (Neugebauer et al. 1994a, 1995) provide evidence that NSAIDs can act centrally in addition to any peripheral effects.

For example, intrathecal application of a variety of NSAIDs was shown to reduce the second phase of the flinching response following intraplantar formalin injection (Malmberg & Yaksh 1992a) at doses of 100 - 1000 times less than the intraperitoneal dose required to produce an equivalent effect. Antinociception was dose-dependent and both intrathecal and intraperitoneal routes of administration produced the same maximal degree of suppression of phase 2 flinching behaviour. Furthermore, the S(+) but not the R(-) isomer of ibuprofen given spinally produced suppression and the S(+) but not the R(-) isomer has been shown to have cox-inhibiting activity (Malmberg & Yaksh 1994b). The rank order of potency of intrathecal NSAIDs was roughly the same as their rank order for cox inhibition (Malmberg & Yaksh 1992a). Intrathecally applied specific EP_1 antagonists also suppressed flinching in the second phase of the formalin test (Malmberg et al. 1994) providing additional evidence for the involvement of PGs, in particular PGE_2, in pain-related behaviour.

Intrathecal NSAIDs have also been linked with central antinociception by electrophysiological studies. Dipyrone was shown to be effective in reducing the electrophysiological changes seen in the spinal cord during the development of hypersensitivity due to a kaolin and carrageenan (K/C) arthritis (Neugebauer et al. 1995) and indomethacin and diclofenac given intrathecally prevented the hypersensitivity of dorsal horn neurons during reperfusion following ischaemia of the rat tail (Gelgor & Mitchell 1995).

The observations that intrathecal NSAIDs were not antinociceptive in circumstances where there was no hyperalgesia, such as the first phase of the

formalin test (Malmberg & Yaksh 1992a; Chapman & Dickinson 1992) or tail-flick or hot-plate tests in non-hyperalgesic animals (Yaksh 1982; Björkman 1995; Akman et al. 1996; Bianchi & Panerai 1996) suggests that PGs are only involved in nociceptive processing in hyperalgesia, even though cox is present in the spinal cord in non-hyperalgesic rats.

In order to test this hypothesis two series of experiments were carried out. In the first series, the responses to noxious mechanical pressure of neurons in the deep dorsal horn with either ankle or knee joint input were recorded. The NSAIDs meclofenamic acid and indomethacin were applied topically to the spinal cord to determine if they would modify these responses in the normal, non-hyperalgesic rat. In the second series of experiments, a similar set of recordings were made from wide dynamic range (WDR) cells in the deep dorsal horn of rats that had a peripheral inflammation, either an acute kaolin/carrageenan (K/C) arthritis of the knee or a chronic Freund's complete adjuvant (FCA) arthritis of the ankle.

Neurons responsive to noxious joint stimulation have previously been found in the deep dorsal horn (laminae IV-VII) of the spinalised cat (Schaible et al. 1986; Neugebauer & Schaible 1990). This study concentrated on units responsive to joint stimulation in the deep dorsal horn, which was assumed to be 400 - 1200 μm from the dorsal surface of the cord of the 200 - 400 g rat. Such neurons are a focus for the convergent input of cells involved in nociception and play a pivotal role in the transmission of nociceptive information to motoneurons and the higher centres.

WDR cells have been defined as neurons, which respond to a low intensity stimulus (such as gentle compression of a joint or muscle, joint flexion or brushing the skin) and show a graded increase in response as the stimulus intensity increases into the noxious range (Grubb et al. 1993). Nociceptive-specific (NS) neurons are defined as those that respond only to noxious stimuli applied to skin or deep tissues. This study looked at WDR neurons which are of interest because their responses change during the hypersensitivity brought about

by, for example, peripheral inflammation (Cook et al. 1987; Grubb et al. 1993, 1996; Neugebauer et al. 1993, 1995).

Under conditions of peripheral inflammation the characteristics of cells within the deep dorsal horn of the spinal cord change, as previously discussed in Section 1.4. Fewer cells are categorised as nociceptive-specific and more as WDR, receptive fields increase, mechanical thresholds decrease and more cells are spontaneously active, frequently with bursting activity (Grubb et al. 1993, 1996). These changes have been described in both chronic (FCA polyarthritis in rats, Menétrey & Besson 1982; FCA monoarthritis in rats, Grubb et al. 1991, 1993) and acute (K/C in cats, Neugebauer & Schaible 1990; Schaible et al. 1991; and in rats, Neugebauer et al. 1995) models. The changes in dorsal horn activity, in particular the increase in receptive field size, are considered to be a correlate of central hyperexcitability.

Central sensitisation in hyperalgesic states could be due to the recruitment of WDR cells at lower thresholds than normal, producing patterns of firing at low stimulus intensities which would be similar to those normally associated with high-intensity stimulation. It could also be that the firing rate of individual neurons increases, or that the duration of the response increases in hyperalgesia. Indeed, it is possible that a combination of these factors is involved.

The acidic NSAIDs meclofenamic acid (N-[2,6-dichloro-*m*-tolyl]anthranilic acid) and indomethacin were used in this study. Both have been shown to inhibit cox-1 and cox-2 in a time-dependent, competitive manner (Laneuville et al. 1994; Ouellet & Percival 1995). Both could be dissolved at a physiological pH at the desired concentration and so were convenient to use for direct spinal application.

5.2 Methods

5.2.1 Animals

A total of 14 non-arthritic male Wistar rats weighing 212 - 315g were used in the first series of experiments involving extracellular recording in the spinal cord .

For the second series of experiments involving hyperalgesic animals, 21 male Wistar rats and one female Wistar rat, weighing 184 - 308g, were used. A chronic FCA monoarthritis of the ankle was induced in 8 animals 1 - 3 days prior to recording as described in Section 2.6.1 (2 animals used on Day 1 post-injection, 2 animals on Day 2 and 4 animals on Day 3). An acute K/C arthritis of the knee was induced in 14 anaesthetised rats after completion of the neck surgery, as described in Section 2.6.2.

5.2.2 Electrophysiology

Full details of the experimental preparation and electrophysiological apparatus and techniques are described Section 2.4. At least 1 h was allowed after preparative surgery was completed before a suitable cell was sought. The electrode was advanced into the spinal cord in the region of L2 - L4 in 2 - 4 μm steps while gently squeezing the ankle joint for meclofenamic acid experiments and the knee joint for indomethacin experiments. The second series of experiments was designed to look at two models of arthritis: a chronic arthritis of the ankle and an acute arthritis of the knee. For comparative purposes therefore, both joint inputs were investigated in normal animals.

To assess cell characteristics, the limb was subjected to both manual stimulation and probing with a pair of blunt forceps, avoiding any tissue damage. A cell was chosen for recording which had excitatory input from the deep tissue of the appropriate ipsilateral joint and was identified as a WDR cell using the criteria that such cells respond to low-intensity stimulation with a graded increase in the intensity of the response as the stimulus intensity is increased to noxious levels.

When a suitable unit had been identified its receptive field was mapped and the stimulation threshold was assessed subjectively as low, moderate or high. Its depth within the spinal cord and hence its anatomical location was recorded from the stepping motor read-out. Further characteristics such as the presence of spontaneous activity and any contralateral excitatory or inhibitory input were recorded.

In the second series of experiments, a similar protocol was followed. Cells were found at depths of 400 - 1200 μm which responded to mechanical stimulation of the inflamed joint. These were characterised subjectively as hyperexcitable cells (low threshold, bursting behaviour, with or without afterdischarge) or non-hyperexcitable cells (low to high threshold, no bursting behaviour, no afterdischarge). In practice, hyperexcitable cells were easily distinguished from non-hyperexcitable cells both by the sight of characteristic doublets or triplets of spikes with short inter-spike intervals on the oscilloscope screen and by the characteristic sound that these recordings produced. The depth within the spinal cord, threshold to mechanical stimulation, presence or absence of spontaneous activity, receptive field and presence or absence of contralateral input were assessed and recorded for each cell.

Mechanical stimulation was provided by pneumatic pinchers fixed immobile across the joint being used. For the first series of experiments using non-arthritic animals, the pneumatic pinchers were set up to squeeze the appropriate joint at 25 - 50 psi over a maximum area of 60 mm^2. Fifty psi was found to be uncomfortable when applied to the skin web between two human fingers and was generally used to produce a noxious pinch. However, sometimes a lower pressure was used either to prevent tissue damage if a high pressure was causing reddening of the skin or if the control response was reducing with time.

In the second series of experiments involving peripheral inflammation, pressures of 15 - 50 psi, usually 20 - 25 psi, were used. The exact pressure used in each individual experiment was determined by the pressure found to provide a consistent response of a sufficiently high level to record (ie with a high signal to

background noise ratio) balanced against the requirement not to cause tissue damage to the tissues contacted. In practice the lowest possible pressures which gave a good recording with no tissue damage were determined by trial and error over a preliminary period and recording was started only when the stimulation parameters were considered subjectively to be correct. The aim was to provide an equivalence of afferent input. In general the cells recorded from in the arthritic animals had a lower threshold, which was why lower stimulation pressures were used.

In the case of K/C arthritis, recordings were made from 4.5 - 12 h after the induction of arthritis. In all cases at least 1 hour was allowed to elapse after preparative surgery was complete before recording was started.

Control stimuli of 15-s duration were given every 3 - 4 min until the responses to 6 successive stimuli were observed to be stable. Drugs, pre-warmed to 37°C, were then applied directly onto the spinal cord in the region of the electrode in a volume of 40 - 50 μl via a thin polythene catheter (125 μm external diameter) attached to a 1-ml syringe taking care not to touch the spinal cord. Drugs were removed by suction using a 0.63 mm Portex tube.

For the first series of experiments, the usual protocols followed are shown in Figures 5.1 and 5.2. A total of 13 cells were studied in the meclofenamic acid series and a total of 6 cells in the indomethacin series. On occasion the protocol described in Figure 5.1 was performed on more than one cell in an individual animal for the meclofenamic acid series of experiments; one cell only was recorded from per animal in the indomethacin series.

For the second series of experiments, in animals with a peripheral inflammation, the usual protocols followed are shown in Figures 5.2 and 5.3. On occasion more than one cell was recorded from in an individual animal in the meclofenamic acid series; one cell from each animal was recorded in the indomethacin series. Only cells which maintained their spike amplitude throughout the course of the experiment are included in the results.

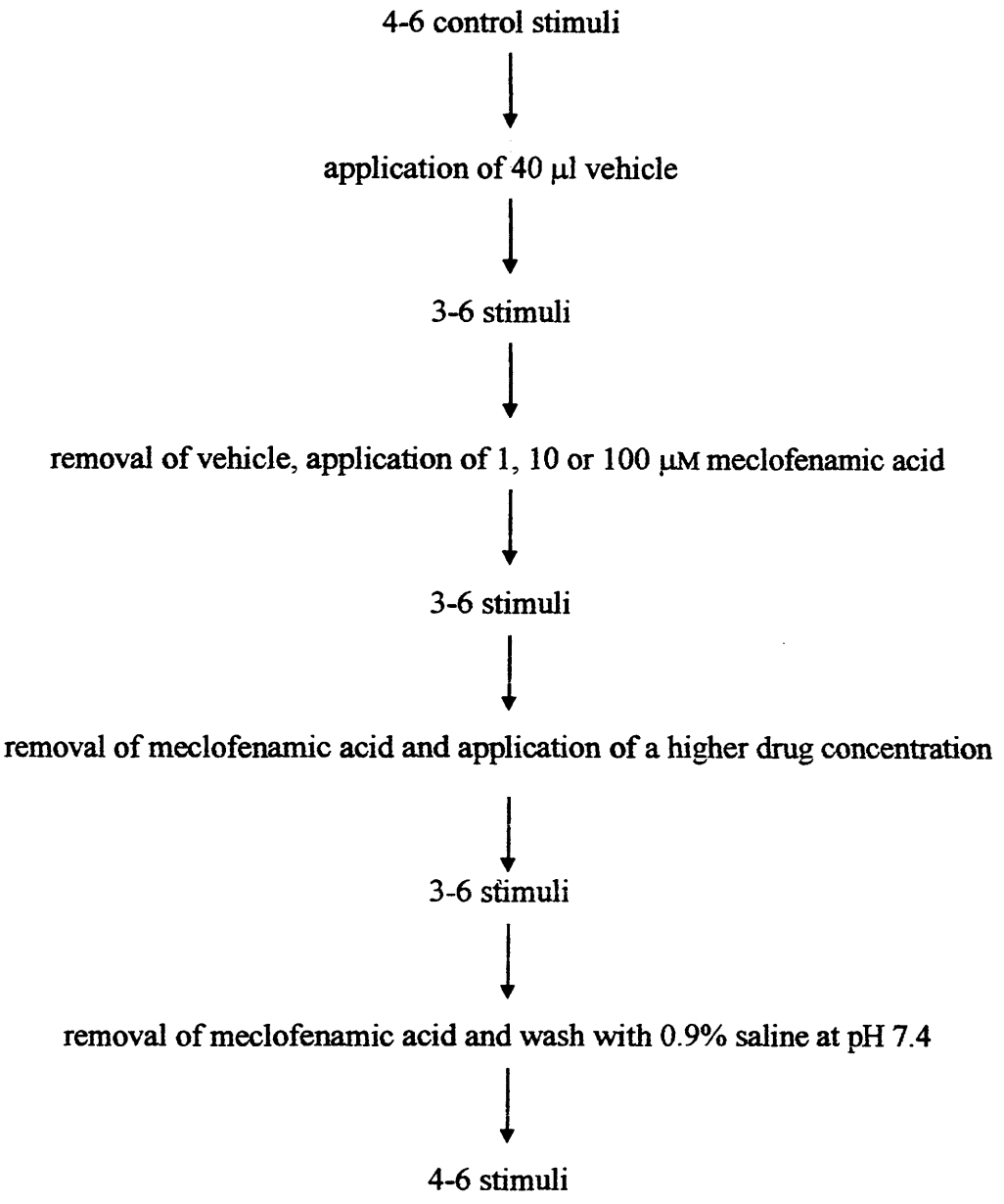


Figure 5.1. Protocol followed for the first series of experiments using spinally applied meclofenamic acid. A total of 13 cells were recorded before and after the application of vehicle (*n*=11), 1 (*n=4*), 10 (*n=4*) and/or 100 (*n=13*) µM meclofenamic acid.

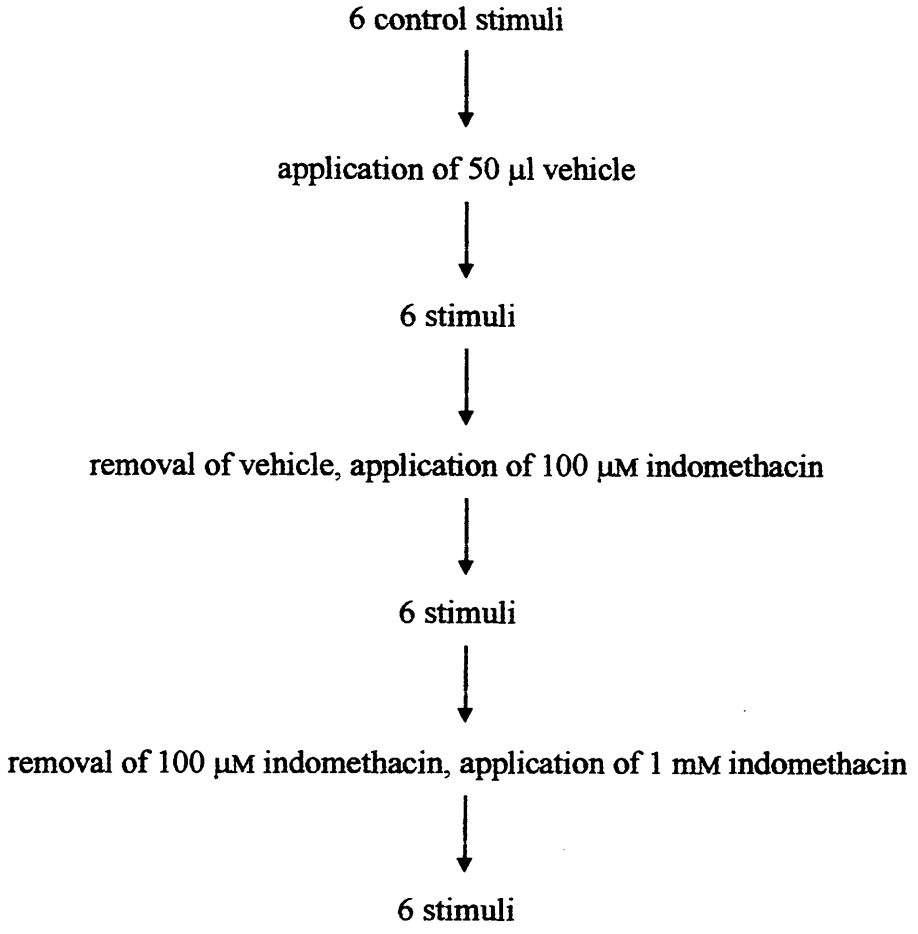


Figure 5.2. Protocol followed for the first series of experiments involving spinal application of indomethacin. A total of 6 cells were recorded before and after the application of vehicle, 100 µM and 1 mM indomethacin. This protocol was also followed for the indomethacin series of experiments in rats with a K/C arthritis of the ankle. A total of 14 cells were recorded before and after the application of vehicle, 100 µM and 1 mM indomethacin.

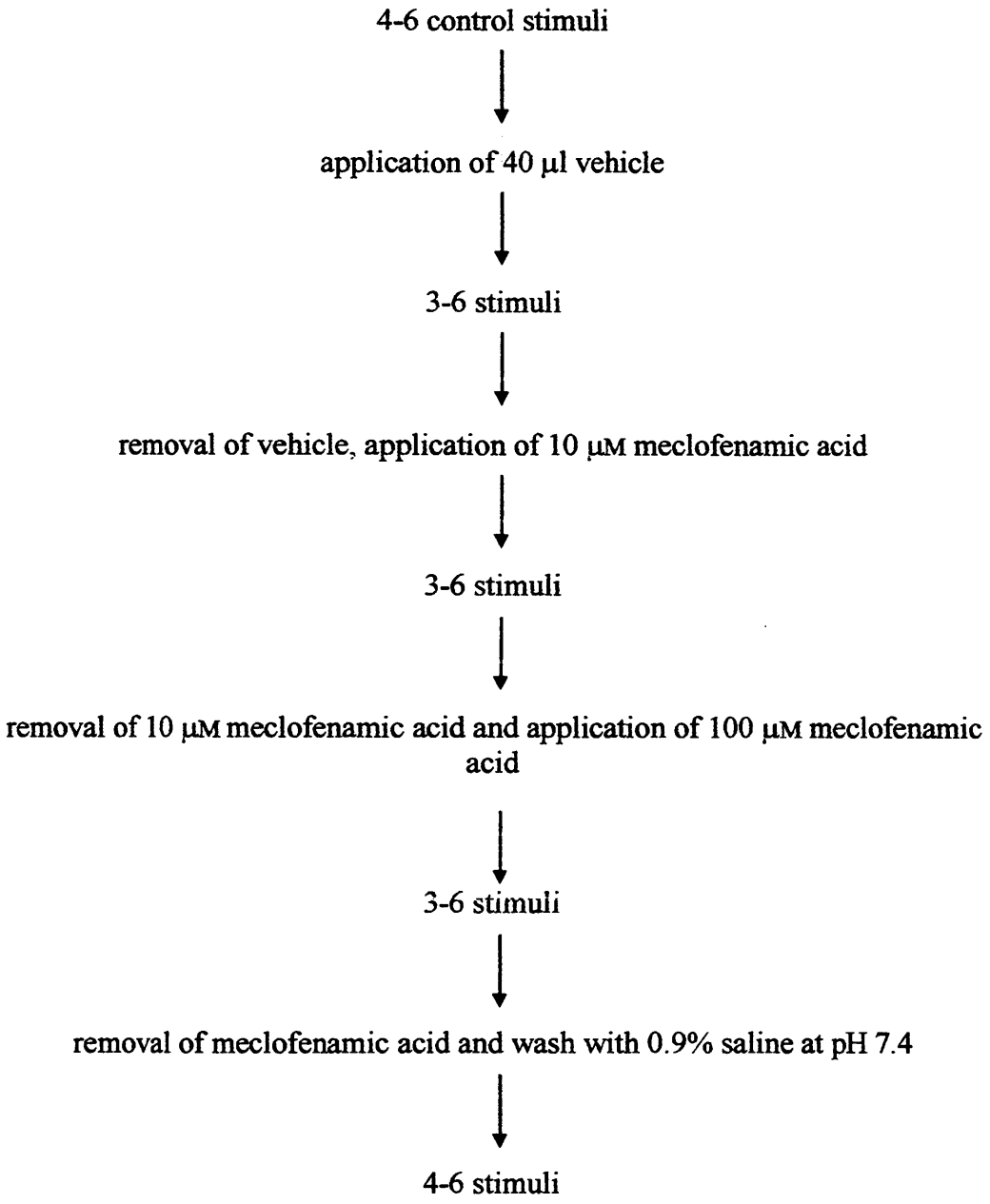


Figure 5.3. Protocol followed for the meclofenamic series of experiments in rats with a FCA arthritis of the ankle. A total of 15 cells were recorded before and after the application of vehicle (*n=15*), 10 (*n=5*) and 100 (*n=15*) µM meclofenamic acid.

5.2.3 Drugs

Meclofenamic acid was dissolved in 0.9% saline and adjusted to pH 7.4. Vehicle control was 0.9% saline at pH 7.4. These solutions were dispensed into 1-ml aliquots and stored at -20°C until use. Indomethacin was dissolved in warmed 10% $NaHCO_3$ and then diluted 1:10 with warmed PBS, the pH of which had been adjusted to give a final pH of 7.4. Vehicle control was 1 part 10% sodium bicarbonate and 9 parts PBS; pH 7.4. These solutions were made up freshly on the day of use. Meclofenamic acid and indomethacin were purchased from Sigma.

5.2.4 Data Analysis

Data for the first series of meclofenamic acid experiments (n=13) were recorded as a spike rate histogram using a Spike 2 data collection program. Results were counted automatically using Spike 2 DOS analysis scripts (see Appendix 1). The activity of a single neuron was discriminated from the background noise by setting a cursor by eye to a level between the amplitude of the background noise and the constant spike amplitude of the neuron being studied. Data from the first series of indomethacin experiments (n=6) and from all of the second series of experiments were recorded as a spike rate histogram using an MRATE data capture program and counted using MRATE software.

In all cases, the number of spikes in a 16-s period from the start of mechanical stimulation was counted and defined as S. The number of spikes in the 16-s period immediately preceding the stimulation was designated B (background). The response (R) was defined as:

$$R = S - B$$

The responses to stimulation during the control period were averaged, as were the responses following drug or vehicle application. The average responses post-treatment were expressed as a percentage of the average control value. The

maximum drug effect was calculated by taking the average of the lowest post-drug response in individual experiments. In the second series of experiments, the results from hyperexcitable cells (meclofenamic acid, *n=8*; indomethacin, *n=11*) and from non-hyperexcitable cells (meclofenamic acid, *n=7*; indomethacin, *n=3*) were averaged. Effects of vehicle or drug were tested for significance using 1-way anova (significance when $p < 0.05$).

For rats in which a FCA monoarthritis of the ankle joint was induced (*n*=19, not all these rats were used in the reported experiments), daily ankle joint circumference measurements were expressed as a percentage of the circumference of the non-injected ankle joint at Day 0 and averaged (Day 1, *n=19*; Day 2, *n=13*; Day 3, *n=9*; Day 4, *n=3*). Body weight was expressed as a percentage of the weight on Day 0 and averaged for all rats. Gait scores were averaged for all rats and compared to 'normal' scores ie 1. The significance of changes was assessed by 1-way anova and paired 2-tailed t-test (significance = $p < 0.05$).

5.3 Results

5.3.1 Freund's Complete Adjuvant (FCA) Arthritis

A total of 19 male Wistar rats were given periarticular injections of FCA as described in Section 2.6.1 and daily recordings of weight, gait, behaviour, appearance and the circumference of both ankle joints were made by the Animal House technicians as detailed in Table 2.1 over a period of 1 - 4 days post-injection.

The results depicted in Figure 5.4 show that injection of FCA into the periarticular tissues caused an immediate increase in ankle joint circumference to an average (± SE) of 109.0% (± 1.6%) of the non-injected ankle circumference. By Day 1 the swelling had increased to an average (± SE) of 149.7% ± 4.5% of the circumference of the non-injected ankle on day 0 ($p < 0.001$, t-test) and this swelling was maintained for the 4 days over which recordings were made ($p < 0.001$, 1-way anova). Body weight showed a non-significant reduction at Day 1

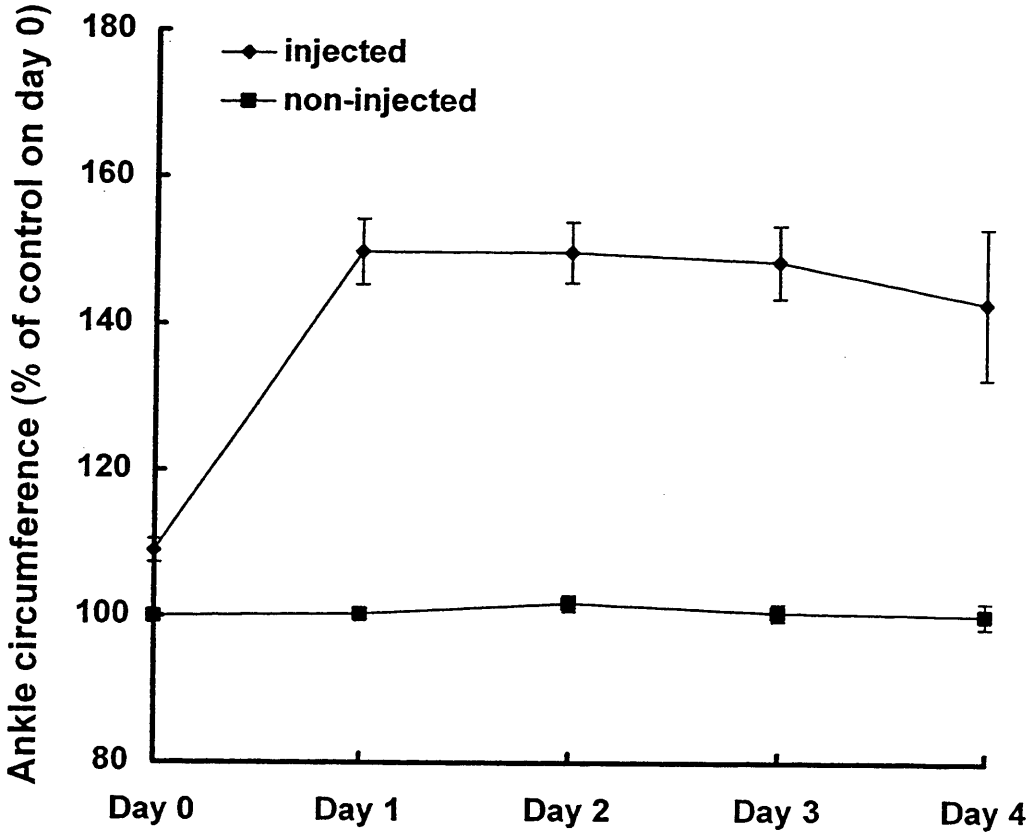


Figure 5.4. Graph illustrating the ankle joint swelling caused by periarticular inoculation of FCA. The injected ankle shows a significant increase in ankle joint circumference ($p < 0.001$, 1-way anova) compared to the circumference of the non-injected ankle on Day 0, whereas the non-injected ankle shows no significant change in circumference. Error bars = SE. (Day 0,1, *n=19*; Day 2, *n=13*; Day 3, *n=9*; Day 4, *n=3*.)

but thereafter increased at a steady rate (Fig. 5.5) implying that the rats were not suffering chronic pain. Figure 5.6 shows the averaged gait scores increased to an average (± SE) of 1.58 ± 0.12 by Day 1, paralleling the increase in injected joint circumference ($p < 0.001$, one-way anova) and showing that the arthritis had functional consequences.

5.3.2 Non-arthritic Rats

Cell Characteristics – Ankle Input

Twelve cells were recorded from in this series, lying at depths of 420 - 1180 μm from the spinal cord surface (in laminae III - VI; Table 5.1). 1/12 cells had a low threshold of response, 2/12 were classified as low/moderate threshold and 9/12 as moderate threshold. 4/12 cells were spontaneously active. Three cells had an inhibitory input and no cells showed an afterdischarge, ie an increased firing rate for at least 2 s after the mechanical stimulus had ceased. The average firing rate during the 16-s period of noxious stimulation was 40 ± 5.5 spikes/s (range 17 - 93 spikes/s).

Cell Characteristics – Knee Input

Five cells were recorded from in this series, lying at depths of 400 - 1048 μm from the spinal cord surface (in laminae III - VI; Table 5.1). One cell had a low threshold of response, one was classified as low/moderate threshold, 2 as moderate threshold and 1 as a moderate/high threshold cell. Three cells were spontaneously active. One cell showed an afterdischarge. The average firing rate during the 16-s period of noxious stimulation for 6 cells with knee input was 27 ± 4.2 spikes/s (range 15 - 44 spikes/s).

Tables 5.1 and 5.2 summarise the stimulation parameters and cell characteristics of these cells.

Meclofenamic Acid

WDR cells were recorded at depths of 420 - 1180 μm (in laminae III - VI; Table 5.1). Figure 5.7 shows that meclofenamic acid at 1, 10 and 100 μM and saline (pH

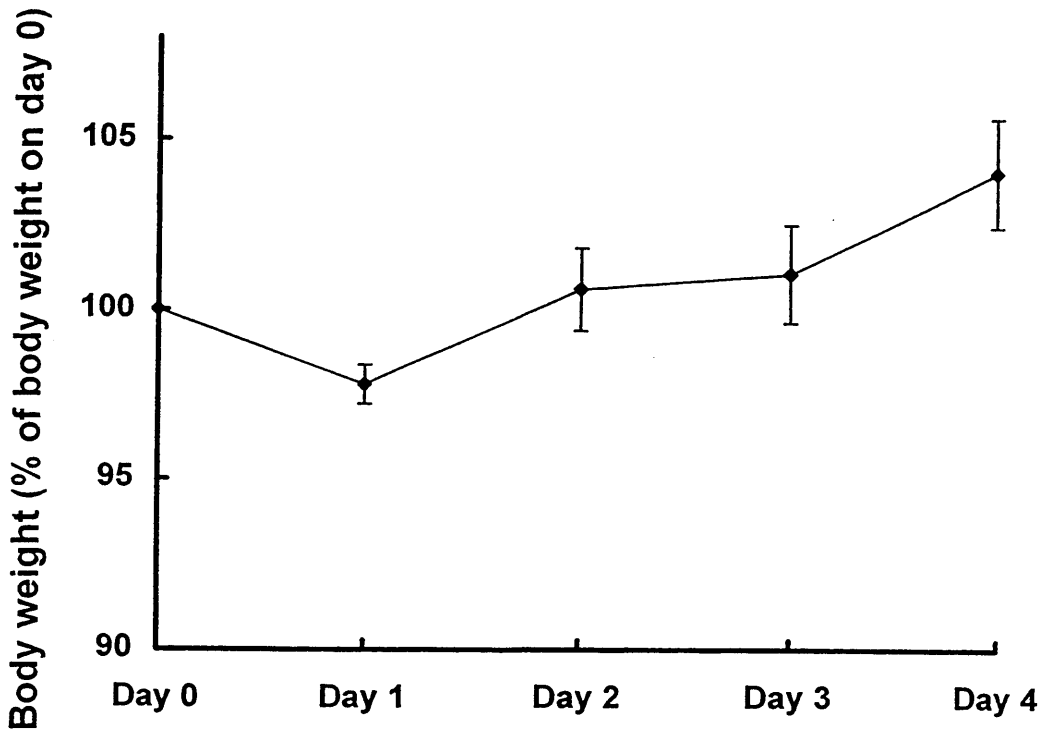


Figure 5.5. Graph showing the changes in average body weight (± SE) following the induction of a FCA monoarthritis on Day 0. There is a non-significant ($p > 0.05$, 2-tailed t-test) reduction in body weight on Day 1 followed thereafter by a steady weight gain. (*n* as in Fig. 5.4.)

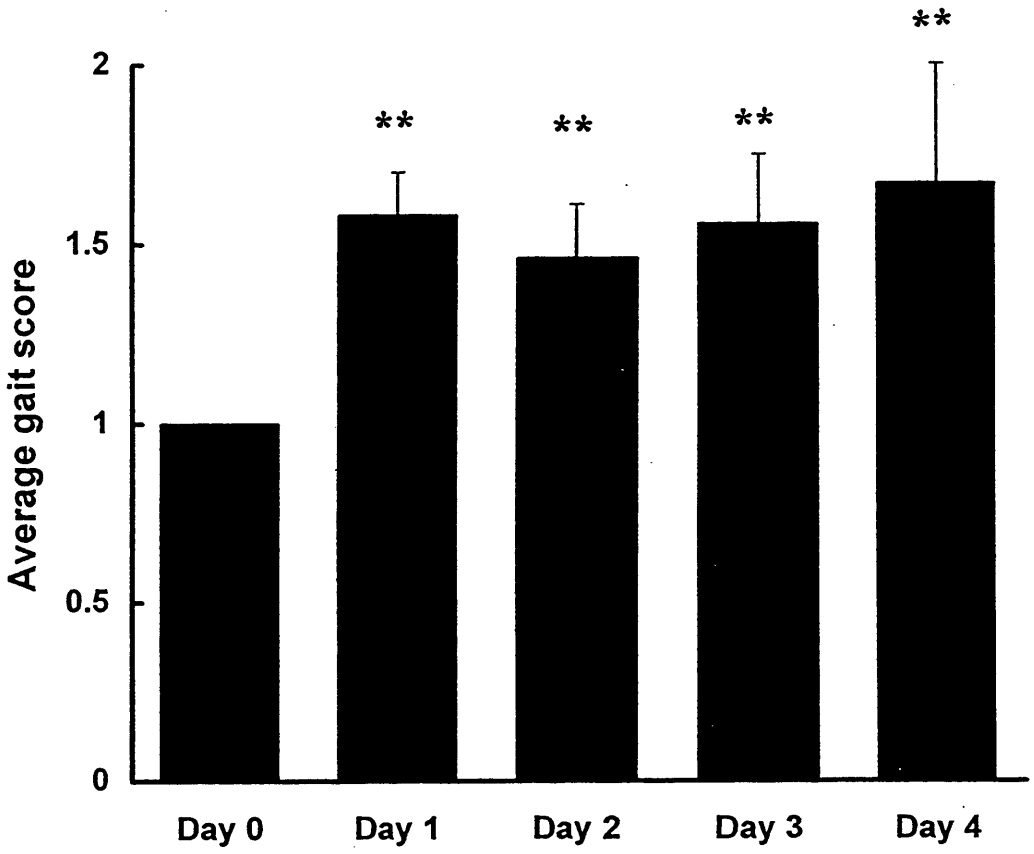


Figure 5.6. Histogram showing the average gait scores of animals following the induction of a FCA monoarthritis on Day 0 (error bars = SE). There is a significant increase in gait score from Day 1 (** $p < 0.001$, 1-way anova). (*n* as for Fig. 5.4.)

Table 5.1 Stimulation parameters and characteristics of cells recorded in the deep dorsal horn of the lumbar spinal cord.

Cell Number	Depth (μm)	Stimulation Pressure (psi)	Response Threshold	Average Evoked Firing Rate (spikes/s)
Ankle input – non-arthritic				
1	801	20	low/mod	36
2	922	30	mod	42
3	586	25	mod	46
4	863	25	mod	45
5	914	25	mod	36
6	1078	25	mod	47
7	1180	25	mod	93
8	878	40	low/mod	45
9	655	45	mod	17
10	420	45	mod	50
11	478	25	low	34
12	637	45	mod	27
Ankle input – arthritic, hyperexcitable				
1	845	20	low	20
2	491	15	low	43
3	1011	30	mod	22
4	667	25	low	25
5	462	40	mod	22
6	471	25	low	52
7	612	25	mod	30
8	485	20	low	45
Ankle input – arthritic, non-hyperexcitable				
1	487	50	mod	7
2	807	25	low/mod	33
3	552	25	low	28
4	737	30	mod	94
5	1151	25	mod/high	81
6	720	22	low	73
7	690	20	low	20

Table 5.1 cont.

Cell Number	Depth (μm)	Stimulation Pressure (psi)	Response Threshold	Average Evoked Firing Rate (spikes/s)
Knee input – non-arthritic				
1	965	50	mod	44
2	400	40	mod/high	24
3	694	50	low/mod	25
4	743	40	mod	15
5	1048	50	low	29
Knee input – arthritic, hyperexcitable				
1	679	25	low	9
2	550	25	low	19
3	733	30	low	11
4	468	20	low	23
5	842	15	low	16
6	598	20	low	53
7	544	20	low	22
8	588	20	low	18
9	391	20	low	61
10	1003	20	low	69
11	567	20	low	4
Knee input – arthritic, non-hyperexcitable				
1	703	25	mod	18
2	721	20	mod	22
3	826	20	mod	10

Table 5.2 Table showing the receptive fields of the cells recorded in the deep dorsal lumbar spinal cord.

Cell Number	Receptive Field	Skin Input (Yes/No)	Spontaneous Activity (Yes/No)
Ankle input – non-arthritic			
1	Ankle to knee	Yes	Yes
2	Proximal third of foot to lower leg	No	No
3	Foot to lower leg		Yes
4	Foot to lower leg		No
5	Ankle to lower leg	No	No
6	Foot to thigh	No	Yes
7	Toes to thigh	No	No
8	Ankle to knee	Yes	No
9	Ankle to knee		No
10	Upper foot to knee		No
11	Foot, whole of leg and some abdomen	Yes	No
12	Toes to thigh	Yes	Yes
Ankle input – arthritic, hyperexcitable			
1	Toes to thigh		Yes
2	Toes to thigh		Yes
3	Ankle to thigh	No	No
4	Foot to lower leg	Yes	Yes
5	Foot to knee		Yes
6	Upper foot to thigh		Yes
7	Upper foot to knee	Yes	No
8	Foot and ankle		Yes
Ankle input – arthritic, non-hyperexcitable			
1	Foot to lower leg		No
2	Foot to thigh	No	Yes
3	Toes to knee	Yes	No
4	Ankle to knee	No	No
5	Ankle to thigh	Yes	No
6	Ankle to lower leg	Yes	No
7	Toes to ankle	Yes	No

Table 5.2 cont.

Cell Number	Receptive Field	Skin Input (Yes/No)	Spontaneous Activity (Yes/No)
Knee input – non-arthritic			
1	Toes to thigh	No	No
2	Foot and ankle		No
3	Toes to lower leg		Yes
4	Toes to ankle	Yes	Yes
5	Toes to knee		Yes
Knee input – arthritic, hyperexcitable			
1	Knee to thigh	Yes	No
2	Knee	Yes	No
3	Knee and lower thigh		No
4	Knee	Yes	Yes
5	Knee and thigh	No	No
6	Ankle to hip	Yes	Yes
7	Knee	Yes	No
8	Knee to hip	Yes	Yes
9	Lower leg to thigh	Yes	Yes
10	Knee to hip	Yes (little)	No
11	Ankle to hip and some abdomen	Yes	Yes
Knee input – arthritic, non-hyperexcitable			
1	Foot to thigh	Yes	No
2	Knee	No	Yes
3	Lower leg and knee	No	Yes

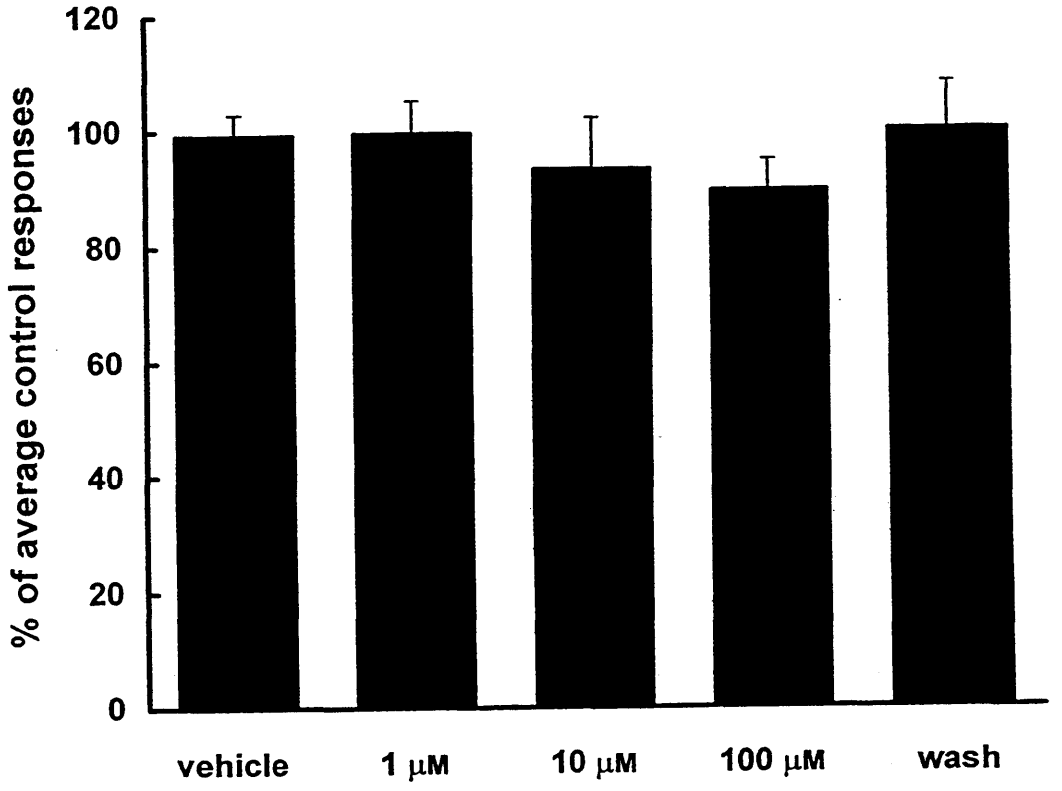


Figure 5.7. Histograms of the effect of meclofenamic acid on the average responses of WDR cells (± SE; *n=13*) in the deep dorsal horn of anaesthetised rats, with no peripheral joint inflammation, to nociceptive mechanical ankle joint stimulation. Application of vehicle (*n=11*), 1 (*n=4*), 10 (*n=4*) and 100 μM (*n*=13) meclofenamic acid onto the surface of the spinal cord produced no significant change in response ($p > 0.05$, 1-way anova).

7.4) had no significant effect upon the number of spikes recorded from deep dorsal horn WDR cells elicited by noxious mechanical stimulation of the ankle ($p > 0.05$).

Indomethacin

In this series, WDR cells were recorded at depths of 400 - 1048 μm, ie in laminae III - VI. The results displayed in Figure 5.8 show that neither indomethacin at 100 μM and 1 mM nor vehicle significantly affected the number of spikes in the response of WDR cells in the deep dorsal horn to noxious mechanical stimulation in the non-arthritic animal ($p > 0.05$).

In those cells which exhibited spontaneous activity, no significant effect of intrathecal NSAID or vehicle upon this spontaneous firing could be seen ($p > 0.05$).

None of the cells recorded from exhibited hyperexcitability, as defined subjectively by the criteria of low threshold, bursting behaviour, with or without afterdischarge. This implies that, despite preparative surgery in the neck and lumbar regions, cells in the deep dorsal horn of lumbar spinal cord in L2 - L4 were not rendered hyperexcitable on this definition.

5.3.3 Arthritic Rats

A total of 15 cells with ankle input in rats with a FCA arthritis of the ankle were recorded from at depths of 462 - 1151 μm from the spinal cord surface (ie in laminae III - VI) and 14 cells with knee input in rats with K/C arthritis at depths of 391 - 1003 μm were recorded. These cells were therefore in the same anatomical area of the spinal cord as the cells recorded from in non-arthritic animals (Fig. 5.9). Both hyperexcitable cells, as defined by the criteria of low/moderate threshold, bursting characteristics, with or without an afterdischarge, and non-hyperexcitable cells were recorded.

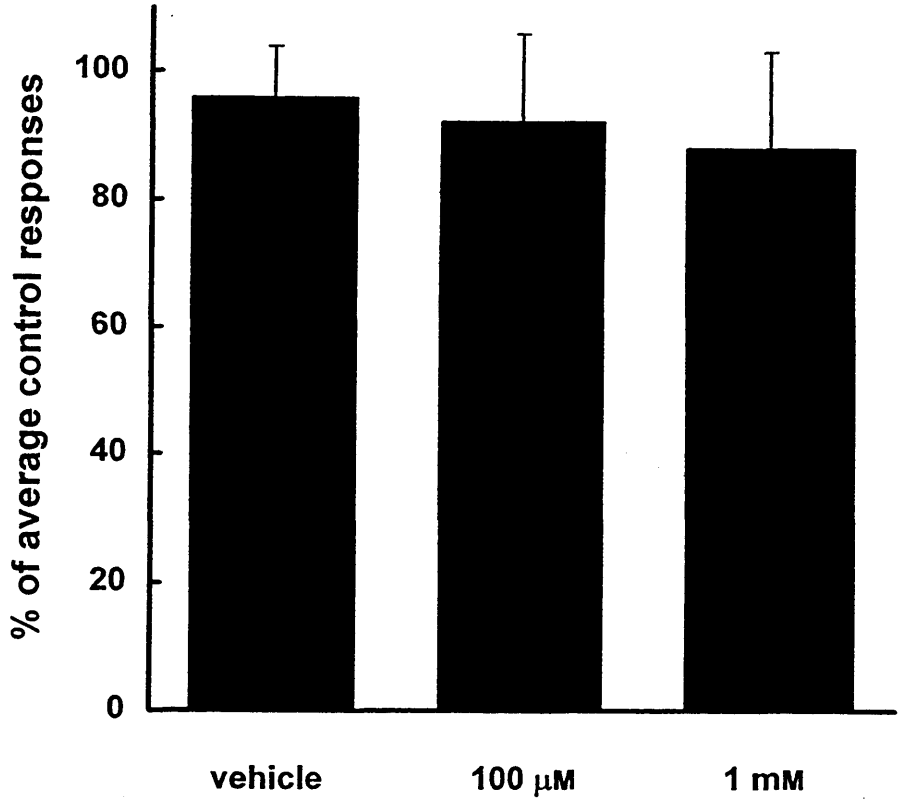


Figure 5.8. Histograms of the effect of indomethacin on the average responses of WDR cells (± SE; *n=6*) in the deep dorsal horn of anaesthetised rats with no peripheral joint inflammation to nociceptive mechanical knee joint stimulation. Application of vehicle, 100 μM or 1 mM indomethacin onto the surface of the spinal cord produced no significant change in response ($p > 0.05$, 1-way anova).

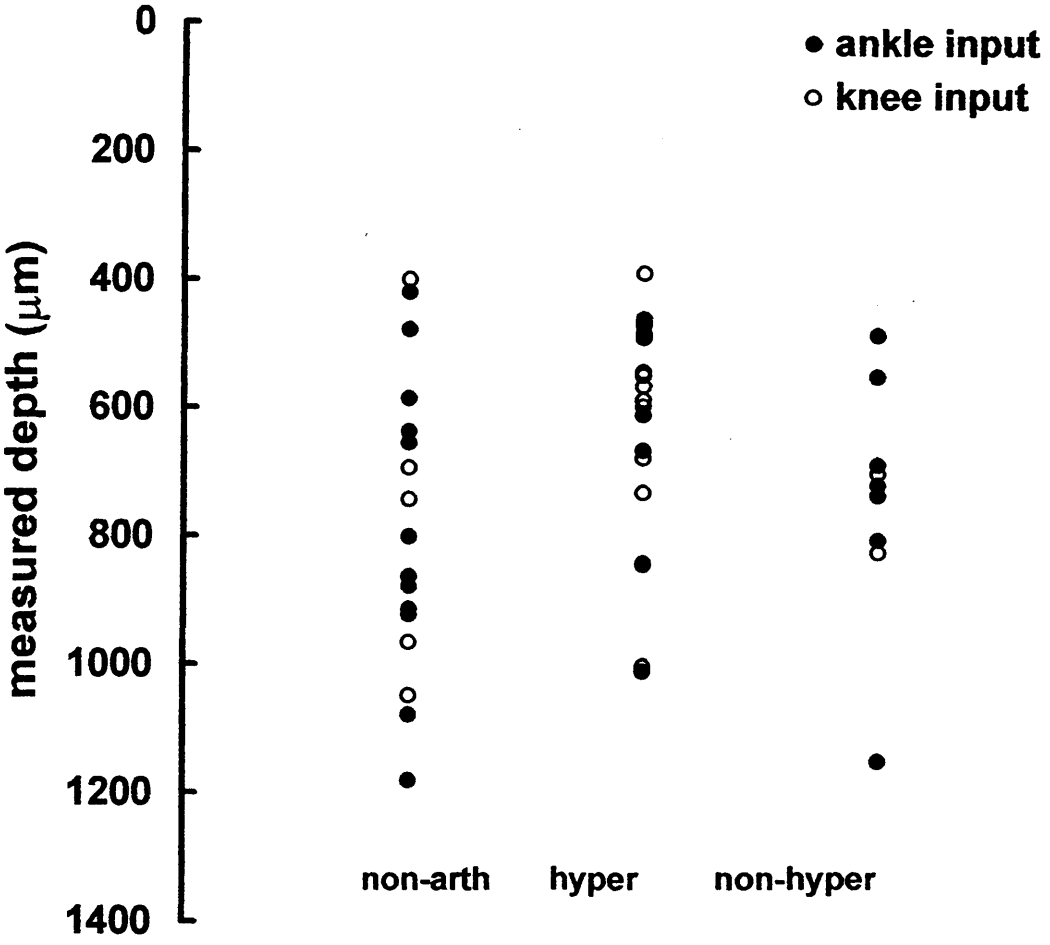


Figure 5.9. Diagram showing the depths at which cells with ankle or knee input were recorded. Zero is equivalent to the surface of the spinal cord. The distributions of cells in non-arthritic rats (non-arth), hyperexcitable cells in arthritic rats (hyper) and non-hyperexcitable cells in arthritic rats (non-hyper) are shown.

Cell Characteristics – FCA Arthritis

In animals with a FCA arthritis, both hyperexcitable and non-hyperexcitable cells were found in the deep dorsal horn of an individual animal. Both hyperexcitable and non-hyperexcitable cells were found at 1, 2 and 3 days postinoculation (hyperexcitable: 1 at Day 1, 2 at Day 2, 5 at Day 3; non-hyperexcitable: 2 at Day 1, 2 at Day 2, 3 at Day 3).

Five of 8 cells classified as hyperexcitable were considered to have a low threshold of response and 3/8 had a moderate threshold. Of the 7 cells classified as non-hyperexcitable, 3 had a low threshold of response, 1 had a low/moderate threshold, 1 had a moderate threshold and 1 had a moderate/high threshold of response. Hyperexcitable cells were thus generally classified as having lower thresholds than the non-hyperexcitable cells or the cells with ankle input recorded from normal animals.

Of the hyperexcitable cells, 6/8 showed some spontaneous activity compared to 1/7 non-hyperexcitable cells; 4/7 hyperexcitable cells had an inhibitory contralateral input and 1/5 showed an afterdischarge. All showed bursting characteristics, with frequent doublets or triplets of spikes.

Cell Characteristics – K/C Arthritis

All 11 cells classified as hyperexcitable were considered to have a low threshold of response. Of the 3 cells classified as non-hyperexcitable, all had a moderate threshold of response.

Of the hyperexcitable cells, 4/11 showed some spontaneous activity compared to 2/3 non-hyperexcitable cells. Two hyperexcitable cells showed an afterdischarge and all exhibited marked bursting characteristics. One of 3 non-hyperexcitable cells had an excitatory contralateral input and this same cell showed an afterdischarge. The average firing rate during the mechanical stimulus was 29 ± 7.8 spikes/s (range 4 - 73 spikes/s) for hyperexcitable cells and 16 ± 3.5 spikes/s (range 22 - 10 spikes/s) for non-hyperexcitable cells.

Table 5.1 summarises the stimulation parameters and cell characteristics for these cells, as for the cells recorded from non-arthritic animals. Cells at a similar range of depths within the spinal cord are included in each category, as shown in Figure 5.9. The response threshold of cells from arthritic animals was, generally, lower and concomitantly generally lower stimulation pressures were used. As can be seen from Figure 5.10, the distribution of response threshold, as assessed subjectively, of non-hyperexcitable cells in arthritic rats was similar to that of the WDR cells recorded from in non-arthritic rats. The hyperexcitable cells generally had a lower response threshold.

The average firing rate was very variable between cells. A stimulation pressure was chosen for each cell that gave a satisfactory, reproducible recording without causing visible tissue damage or swelling to the leg. Also the cells varied in the relative degree of dynamic and static phase responses. No conclusions should therefore be drawn from the variations in firing rate; the data are given for completeness.

Table 5.2 briefly describes the receptive field for each cell studied. Again, these vary widely between cells. In general it can be seen that cells recorded from arthritic rats were more likely to have skin input in additional to input from deep tissues: 55% of cells had skin input in arthritic rats compared with 29% of cells in non-arthritic rats.

FCA Arthritis and Meclofenamic Acid

Direct spinal application of vehicle had no significant effect upon the evoked responses to mechanical stimulation (15 - 40 psi) of the arthritic ankle joint of 8 hyperexcitable but there was a significant reduction to an average (± SE) of 89.2% ± 3.9% and 81%.2% ± 7.3% of control response following the direct spinal application of 10 and 100 μM meclofenamic acid respectively ($p < 0.05$, 1-way anova). After saline wash the responses returned towards control levels. The maximum average effect of 100 μM meclofenamic acid was a reduction to 72.8% ± 8.3% of the average control response. The averaged responses of 7 non-hyperexcitable cells to mechanical stimulation (20 - 50 psi) of the arthritic ankle

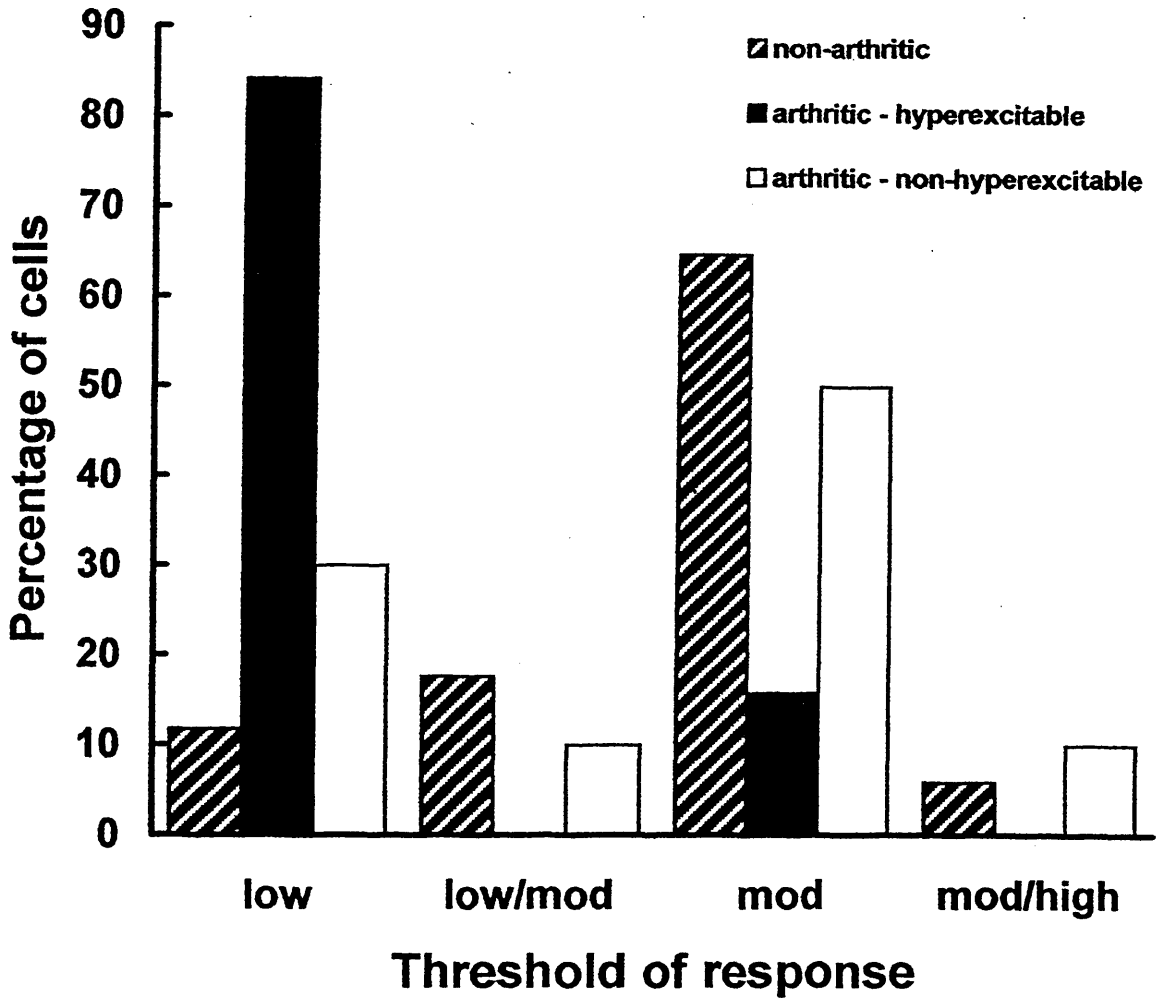


Figure 5.10. Histogram showing the response thresholds of cells recorded from non-arthritic rats and hyperexcitable and non-hyperexcitable cells recorded from arthritic rats. Response thresholds were assessed subjectively.

joint were not significantly affected by direct spinal application of vehicle, 10 μM or 100 μM meclofenamic acid or saline wash ($p > 0.05$). These results are shown in Figure 5.11.

K/C Arthritis and Indomethacin

A total of 14 cells was recorded from in this series of experiments: 11 hyperexcitable cells at depths of 391 - 1083 μm and 3 non-hyperexcitable cells at depths of 703 - 826 μm.

Indomethacin caused a dose-dependent reduction in response to mechanical stimulation in hyperexcitable cells. As shown in Figure 5.12, the average responses of 11 hyperexcitable cells were significantly reduced to 83.3% ± 4.5% (± SE) by 100 μM indomethacin and to 66.2 ± 6.5% (± SE;) by 1 mM indomethacin (drug effects, $p < 0.0001$, one-way anova). Application of the vehicle alone had no significant effect. The maximum effect of 100 μM and 1 mM indomethacin acid was a reduction to an average (± SE) of 67.2% ± 4.9% and 48.8% ± 6.9% of the average control response respectively. The averaged responses of 3 non-hyperexcitable cells to mechanical stimulation of the knee joint were not significantly affected by direct spinal application of 100 μM, 1 mM indomethacin or its vehicle ($p > 0.05$).

5.4 Conclusions

The results show that the responses of deep dorsal horn cells with input from deep joint tissues to noxious mechanical stimulation in rats with no peripheral joint inflammation were not significantly affected by the application of two NSAIDs, meclofenamic acid and indomethacin, directly onto the spinal cord.

These results were not unexpected in the light of previous evidence that NSAIDs may only be effective in producing antinociception following some insult such as inflammation or ischaemia (Yaksh 1982; Björkman 1995; Akman et al. 1996; Bianchi & Panerai 1996). They provide support for the hypothesis proposed that

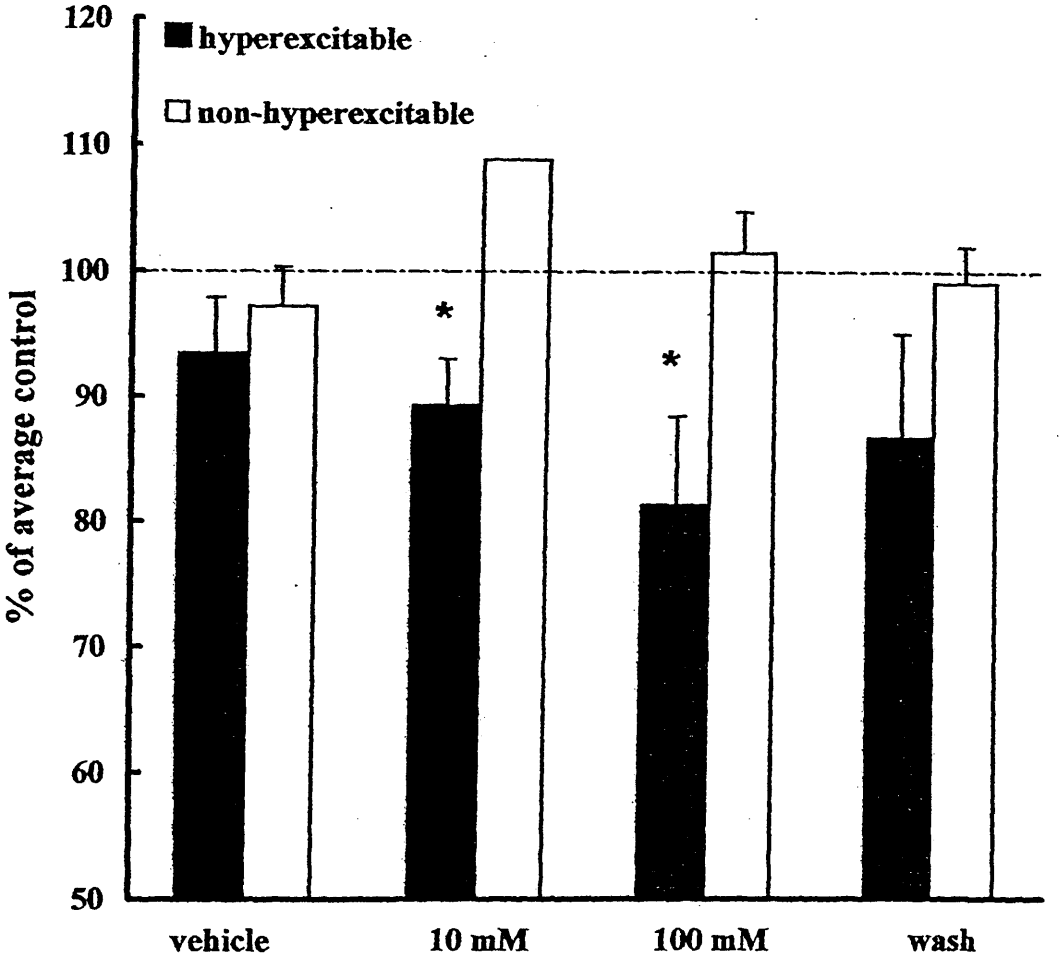


Figure 5.11. Histogram showing the effect of direct spinal application of meclofenamic acid on the average response (± SE) of hyperexcitable and non-hyperexcitable cells expressed as a percentage of the average control response. There is no significant variation from control for the non-hyperexcitable cells with any of the treatments (vehicle, n=7; 10 μm, n=1; 100 μm, n=7; wash, n=6; $p > 0.05$, 1-way anova) but 10 μM and 100 μM meclofenamic acid significantly and dose-dependently decreased the average response of the hyperexcitable cells (vehicle, n=8; 10 μM, n=3; 100 μM, n=8; wash, n=8; * $p < 0.05$, 1-way anova).

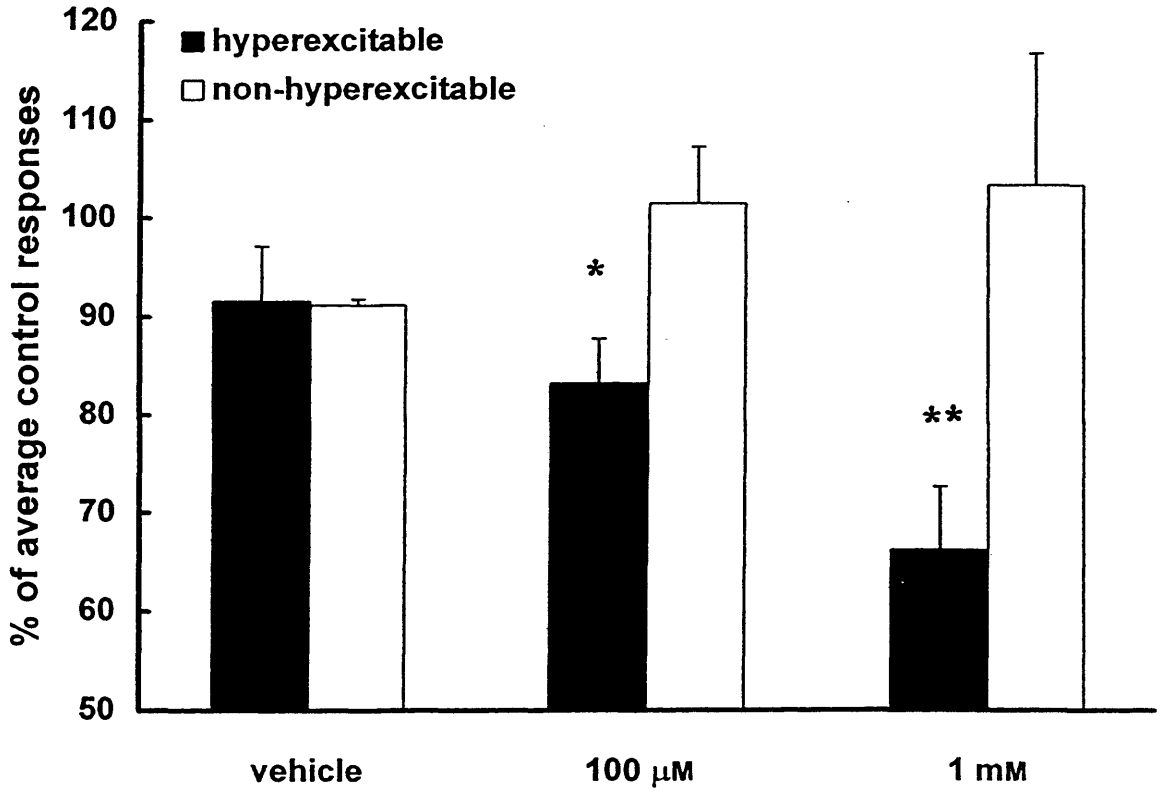


Figure 5.12. Histogram showing the effects of direct spinal application of indomethacin at 100 μM and 1 mM and its vehicle on the average control response (± SE) of hyperexcitable (*n=11*) and non-hyperexcitable (*n=3*) cells. Indomethacin dose-dependently reduced the average responses of hyperexcitable (** $p < 0.001$, 1-way anova) but not non-hyperexcitable cells ($p > 0.05$, 1-way anova).

prostaglandins are not involved in the processing of acute nociceptive information in the normal animal.

The second series of experiments used both an acute arthritis of the knee, induced by the intra-articular injection of kaolin and carrageenan, and a chronic model of arthritis, induced by the periarticular injection of FCA around the ankle joint. The time course of the development of inflammation and behavioural changes following the FCA arthritis are in agreement with those described by other authors (Grubb et al. 1991, 1993). Rats were used 1 - 3 days following the induction of arthritis. Gardiner et al. (1997) have shown that the amount of cox-2 protein within the spinal cord is significantly increased 3.5-fold at 12 and 24 hours following the induction of FCA monoarthritis, with levels no longer significantly different by 3 days postinoculation. Rats in the experiments reported above would therefore be expected to have increased cox-2 activity in the spinal cord as compared with non-arthritic rats, in particular on days 1 and 2. Both hyperexcitable and non-hyperexcitable cells were found at days 1, 2 and 3 postinoculation, suggesting that hyperalgesia is maintained even when cox-2 levels within the spinal cord are returning to control concentrations.

Comparing cell characteristics, it was observed that cells classified as hyperexcitable in rats with both chronic FCA arthritis and acute K/C arthritis had, generally, a lower threshold of response than cells recorded from at similar depths within the spinal cord in non-arthritic rats or non-hyperexcitable cells in arthritic rats. Some spontaneously active cells, cells with afterdischarges and cells with contralateral input were observed in cells in all classes. Only hyperexcitable cells in arthritic rats showed bursting characteristics. A larger proportion of cells studied in arthritic animals had skin as well as deep tissue inputs which is likely to reflect the enlarged receptive fields which have previously been described in arthritic animals (Cook et al. 1987).

The results from rats with both acute and chronic arthritis show that the non-selective cyclooxygenase inhibitors meclofenamic acid (10 and 100 μM) and indomethacin (100 μM and 1 mM) reduced the average response of spinal cord

neurons to mechanical stimulation in cells considered to be hyperexcitable but not those considered to be non-hyperexcitable in both models.

A simple reduction in response due to toxicity of the applied drugs or a general decline in responsiveness in the preparation can be ruled out because: i) non-hyperexcitable cells in these models showed no variation in response with mechanical stimulation with identical parameters and identically applied drugs; ii) the concentrations of drugs used did not reduce the spontaneous or elicited firing rate or spike amplitude; iii) the larger series of cells recorded from in normal animals and reported above also showed no significant variation in response with identically applied drugs and iv) observation of the spinal cord surface through a microscope revealed that the cord remained macroscopically healthy in appearance throughout the course of the experiment, which could often last for several hours.

Cells designated hyperexcitable showed similar characteristics to those described in earlier reports (Menétrey & Besson 1982; Neugebauer & Schaible 1990; Grubb et al. 1991, 1993, 1996; Schaible et al. 1991), ie they had a low response threshold to mechanical stimulation with a bursting pattern of firing, usually with a large receptive field including both deep and cutaneous tissues. Similar firing patterns were seen in the two models of arthritis.

A reduction in response to mechanical stimulation in lumbar dorsal horn cells rendered hyperexcitable by K/C arthritis of the knee in rats was seen following the intravenous application of both S- and R-flurbiprofen, another NSAID (Neugebauer et al. 1995). If we assume that intravenously applied drugs may also act within the spinal cord itself, then these results are in agreement with those reported above. Our results would also support the observations of Malmberg & Yaksh (1992a,b) and Chapman & Dickenson (1992) that intrathecally applied NSAIDs will reduce the flinching response in the second phase of the formalin test. The first phase of flinching in the formalin test (1 - 10 min) is thought to be due to the direct chemical stimulation of the primary afferents, producing an initial barrage of nociceptor activity. The second phase of paw-flinching behaviour corresponds to an increase in the background activity in the primary

afferent C-fibres that innervate the skin at the site of the injection (Dickenson & Sullivan 1987b). Thus the second phase of the formalin test can be considered to correlate with behavioural hyperalgesia. The results presented in this chapter demonstrate that intrathecal NSAIDs (indomethacin and meclofenamic acid) reduce the response of hyperexcitable deep dorsal horn cells, but not non-hyperexcitable cells, to a noxious stimulus.

Later work by Sotgiu et al. (1998) has confirmed an effect of NSAIDs on hyperexcitable WDR neurons. They showed that ketorolac applied iontophoretically close to spinal WDR neurons reduced the excitation produced by NMDA application. This is in agreement with the results reported above.

As was discussed in Section 1.4.2, neuropeptide release has been shown to occur within the spinal cord in central hyperalgesia. Southall et al. (1998) showed that capsaicin application would provoke a unilateral release of SP- and CGRP-immunoreactivity in rats rendered hyperalgesic by FCA injection. This release was attenuated by the intrathecal administration of the NSAIDs ketorolac and (S)-ibuprofen. This is interesting on two counts: first enhanced neuropeptide release was unilateral and so was an indicator of hyperalgesia; secondly it implies a positive role for prostaglandins in the enhanced neuropeptide release. This idea will be explored further in Section 7.2.

It is interesting to note that not all cells in the deep dorsal horn with input from the inflamed joint were hyperexcitable, as defined by the stated subjective criteria. It could be that these cells had input from areas of the joint which had not become inflamed, or that inhibitory inputs onto these cells outweighed the increased stimulation from peripheral inputs. No hyperexcitable cells, as defined by the criteria in Section 5.1, were found in non-arthritic animals.

A theory as to a possible mechanism by which prostaglandins within the spinal cord could contribute to hypersensitivity is discussed in Section 7.2.

Chapter 6

Spinal Reflex Recordings

6.1 Introduction

The study so far has established that cox isoforms are present in the spinal cord. Further, spinally applied NSAIDs (meclofenamic acid and indomethacin) had no effect upon the transmission of acute mechanical nociceptive information in the deep dorsal horn. The responses of deep dorsal horn cells rendered hyperexcitable by both an acute K/C arthritis and a chronic FCA arthritis to noxious mechanical joint stimulation were significantly reduced by NSAIDs applied locally to the spinal cord. It is presumed that this is due to the inhibition of prostaglandin production within the dorsal horn. This evidence supports the hypothesis that PGs are involved in the processing of nociceptive information in the hyperalgesic but not the normal rat.

Wind-up can be considered as a phenomenon with similarities to the development of hyperalgesia, as explained in detail in Section 1.5. It is possible to record a reproducible wind-up of a nociceptive withdrawal reflex by stimulating the cut sural nerve, at a strength sufficient to stimulate C fibres, and recording from a single muscle unit in biceps femoris posterior, hereafter referred to as biceps femoris (Xu et al. 1992; Herrero & Cervero 1996a). Preliminary experiments aimed to characterise this model by looking at the response to variations in frequency of stimulation.

Other workers (Davies & Lodge 1987; Dickenson & Sullivan 1987a; Dickenson & Aydar 1991; Woolf & Thompson 1991) have shown that wind-up is dependent upon NMDA receptor activation such that both competitive (D-CPP, AP5) and non-competitive (MK-801) NMDA antagonists both prevent wind-up and decrease established wind-up *in vivo* (Dickenson & Sullivan 1987a; Woolf & Thompson 1991) and *in vitro* (Thompson et al. 1990). The effects of the non-competitive NMDA antagonist, ketamine, on this wind-up of the spinal reflex were therefore measured to assess whether our model was NMDA-receptor dependent in line with other reports.

In order to establish the best possible experimental protocols for measuring the effects of drugs upon wind-up, we also looked at the duration of the effects of a

conditioning stimulus upon the size of the baseline reflex (defined as the response to the first stimulus in a train of stimuli).

Once the model had been characterised, the effects of both intravenous (indomethacin and SC58125) and intrathecal (indomethacin) cyclooxygenase inhibitors on wind-up were assessed. Indomethacin inhibits both cox-1 and cox-2 (Ouellet & Percival 1995) and so can be considered to be a non-selective cyclooxygenase inhibitor. SC58125 inhibits cox-2 at far lower concentrations than cox-1 (IC_{50} for cox-1/IC_{50} for cox-2 >2,500; Gierse et al. 1995) and so is considered to be a selective cox-2 inhibitor.

6.2 Methods

Male Wistar rats, weighing 230 – 320 g, were anaesthetised and surgically prepared as described in Section 2.5.

6.2.1 Intravenous Injections

Intravenous injections were given by slow pressure injection through the catheter implanted into the jugular vein, in a volume of 1 ml/kg for ketamine and indomethacin and 0.6 ml/kg for SC58125, followed by 0.2 ml 0.9% saline to flush the catheter. All solutions for injection were pre-warmed to 37°C.

6.2.2 Intrathecal Injections

A partial laminectomy was performed to expose the lumbar portion of the spinal cord. A slit was cut in the dura mater cranially. A catheter filled with warm phosphate-buffered saline (PBS; pH 7.4) with an external tip diameter of approximately 150 - 175 μm was carefully inserted through this slit until the tip was overlying the caudal part of the lumbar spinal cord. This was done under visual guidance to minimise damage to the cord or its associated blood vessels.

Solutions were prewarmed to 37°C and were administered slowly by pressure injection in a volume of 50 µl.

6.2.3 Electrophysiology

C-fibre Threshold

To determine the C-fibre threshold, stimuli at 0.1 Hz were applied to the sural nerve. The stimulus strength was gradually increased from 0 V until a consistent response with a latency of more than 130 ms was observed. This stimulus strength was defined as the C-fibre threshold. Experiments were performed at 1.5 times C-fibre threshold strength unless the threshold was unusually high (in the region of 15 V) in which case the nerve was stimulated at 1.2 - 1.3 times C-fibre threshold.

Responses were defined as the number of spikes counted above a threshold level, set to discriminate between background noise and the electrical response in the electromyograph (emg). This threshold was easily discriminated visually, there being a large signal to noise ratio in all cases (see typical recordings in Figures 6.1 and 6.2). The total magnitude of a response was defined as the sum of all the responses to a train of stimuli. As in all experiments reported here, the A-fibre response was taken to encompass the spikes occurring with a latency of less than 100 ms and the C-fibre response was defined as the number of spikes occurring with a latency of greater than 100 ms.

Frequency Response

A train of 20 stimuli was applied every 1.5 - 3 min at 0.2, 0.4, 0.6 and 0.8 Hz. The order in which the different frequencies of stimulation were applied was varied between animals. Four responses at each frequency were recorded, counted and then averaged for each animal. Frequency effects were calculated for each successive response in the stimulus train, as a percentage of the maximum response to stimulation at 0.8 Hz.

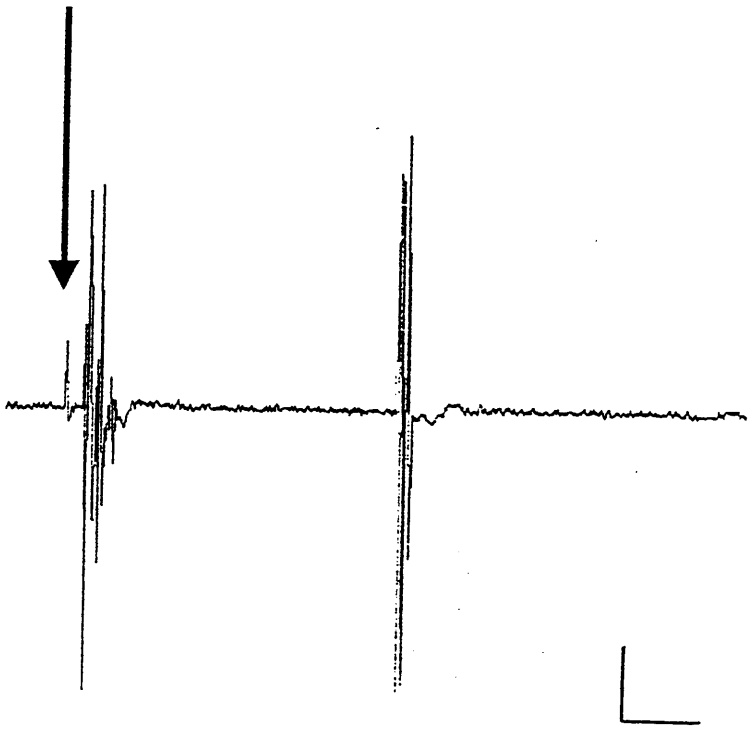


Figure 6.1. The A- and C-fibre elements of a single-unit emg response. Illustrated above is an oscilloscope trace of a single-unit emg response in biceps femoris following a single stimulus to the sural nerve (strength, 13.5 V: stimulus width 500 μs) showing the A-fibre response occurring, together with the stimulus artifact (indicated by the arrow), at a latency of less than 100 ms and the C-fibre response occurring with a latency of greater than 130 ms. Note there is a 10% delay on the oscilloscope trace. The A- and C-fibre elements can easily be distinguished. Scale bars: horizontal, 50 ms; vertical, 200 mV.

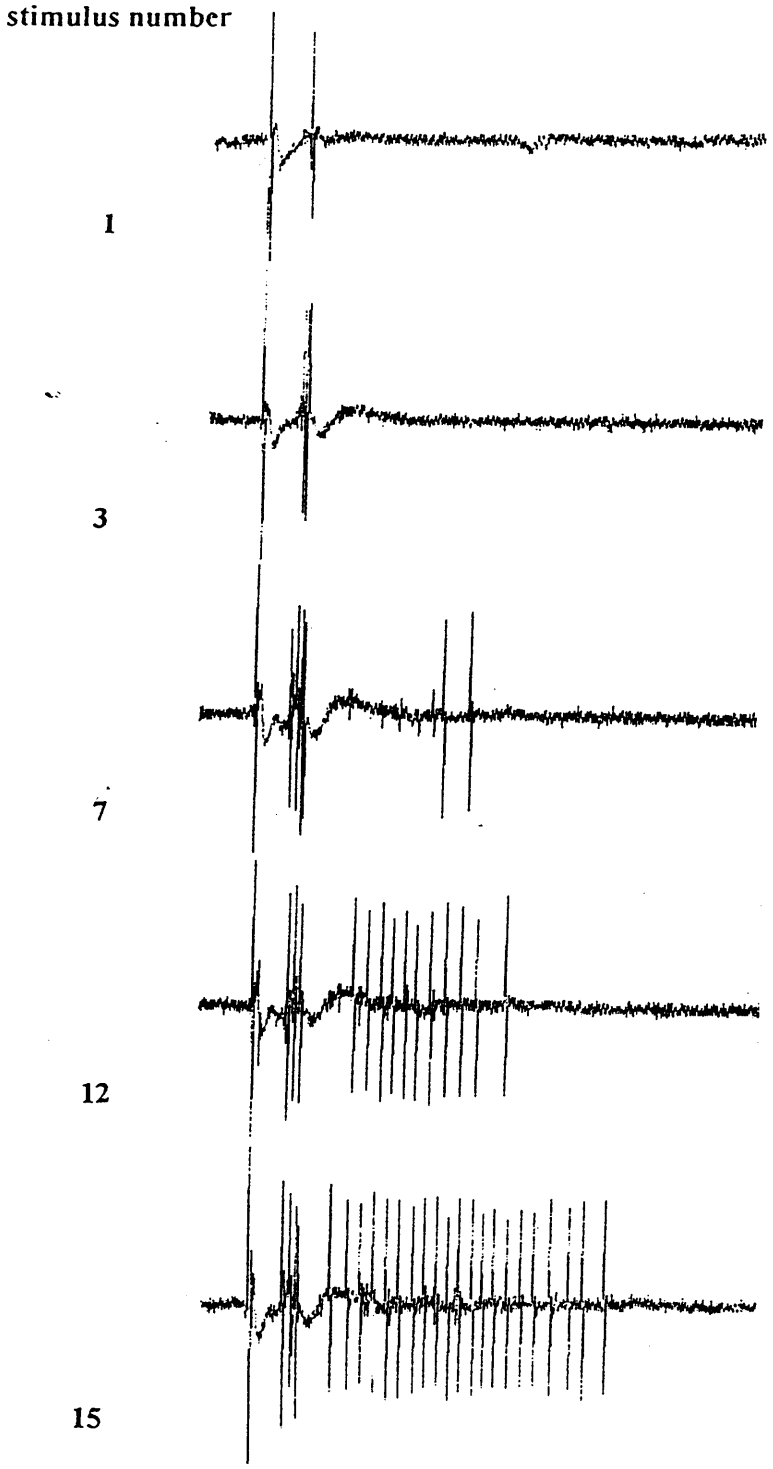


Figure 6.2. An example of C-fibre wind-up. A series of oscilloscope traces is illustrated showing the single-unit emg response in biceps femoris to stimulation of the sural nerve (frequency, 0.5 Hz; strength, 3.5 V; stimulus width, 0.5 ms) at a number of points in the stimulus train (indicated by the number to the left of the trace). The number of spikes in the C-fibre element increases. Each trace lasts for approximately 3 s.

Intravenous Ketamine

A train of 20 stimuli was applied every 2.0 - 4.5 min. A frequency was chosen which gave moderate, reproducible wind-up. After 6 control trains, a slow intravenous injection of 0.9% saline or ketamine (0.5, 1, 2 mg/kg) was given. Incremental doses of ketamine were given 15 - 20 min later after responses had returned to control levels. Four to six control trains before administration of ketamine were averaged for each animal and the post-drug effect was taken to be the response to the first stimulus train after the administration of ketamine. Post-drug wind-up was calculated by setting the number of spikes in the maximum control response to a stimulus to be 100%. Each response in the train was calculated as a percentage of this figure. The baseline response, ie the response to the first stimulus in the train, was calculated for each dose of ketamine as a percentage of the average control baseline response.

Inter-train Intervals

To assess the effect of differing intervals between trains, 5 - 10 stimuli were given at 0.1 - 0.2 Hz followed by a conditioning stimulus consisting of 20 stimuli at 1 Hz. After delays of 1 - 5 min, a further set of 5 - 10 stimuli at 0.1 - 0.2 Hz was given. Four trials using delays of 1, 2, 3, 4 and 5 min were performed on each animal. The responses to the trains before and after the conditioning stimulus were counted and responses to the four trials were averaged.

Indomethacin and SC58125

Trains of 20 stimuli were given every 5 min at a frequency which gave moderate, reproducible wind-up. After 6 control trains, vehicle or drug solutions (indomethacin: 0.1, 0.5, 1.0 and 5.0 mg/kg; SC58125: 1, 5 and 10 mg/kg) were injected slowly via the intravenous catheter or intrathecally (indomethacin: 10 μM, 100 μM and 1 mM; SC58125: 1 μM). Doses of drug were given incrementally at 30-min intervals.

Drug effects were assessed by comparing the average of the 6 responses after the drug to the average of the responses in the 6 control trains. Significance was assessed by 2-way anova. An assessment of the effect on wind-up could be made

by comparing the responses to the final, ie the 20th, stimulus in the train. The total magnitude of wind-up was defined as the total number of spikes summed over all 20 stimuli for the average responses. The average response to the first stimulus in the 6 control trains was defined as the baseline response and this was compared to the average baseline response of the 6 trains after each dose of drug. Drug effects on the total magnitude of wind-up and on the baseline response were determined by a 1-way anova or students' t-test (significance: $p < 0.05$).

6.2.4 Drugs

Ketamine was dissolved in 0.9% saline. SC58125 was dissolved in DMSO and then shaken in PEG 400 just before use to give a final concentration of 5% DMSO, 95% PEG 400. Indomethacin for intravenous administration was dissolved in 10% sodium bicarbonate and diluted 1 in 10 with 0.9% saline. For intrathecal use, indomethacin was dissolved in 10% sodium bicarbonate and diluted 1 in 10 with PBS adjusted with HCl to give a final pH of 7.4. Ketamine, indomethacin and SC58125 solutions were made up freshly for each experiment. Ketamine, DMSO, PEG 400 and indomethacin were purchased from Sigma and sodium bicarbonate was purchased from Fisher. SC58125 was a kind gift from Dr M Trevethick, Glaxo.

6.3 Results

6.3.1 C- and A-fibre Elements

When the sural nerve was stimulated at 1.5 times C-fibre strength, an emg was recorded which had two distinct elements: a short-latency element seen immediately following the stimulus artifact, which occurred with a latency of less than 100 ms, and a longer latency component which occurred with a latency of greater than 130 ms. The first element, which appeared at low stimulus intensities, consisted of the stimulus artifact and A-fibre response and the second, longer latency element was considered to be the C-fibre response. Figure 6.1 shows an example of these two components, which were usually easily

distinguished. In the experiments reported below, the C-fibre threshold varied from 2.8 - 16 V (average ± SE, 6.65 V ± 0.72; median, 5.80 V).

6.3.2 Frequency Response

When the sural nerve was stimulated at low frequencies, ie at or below 0.2 Hz, the number of spikes in the A- and C-fibre responses were unchanged throughout the train of 20 stimuli. At higher frequencies however, ie at 0.4 – 1.0 Hz, the number of spikes in the C-fibre element increased markedly as the stimulus train progressed, reaching a plateau level at around stimulus 16 - 20 as illustrated by the series of oscilloscope traces in Figure 6.2. The increase in response during the stimulus train, corresponding to wind-up, was frequency dependent as is shown in the graph in Figure 6.3. The number of spikes in the A-fibre element increased to a lesser extent, the numbers of spikes involved being markedly fewer than in the C-fibre response, and a small frequency-variation was observed ($p < 0.01$, 2-way anova; Fig. 6.4). The A- and C-fibre baseline responses were unaffected by the frequency of stimulation ($p > 0.05$, 1-way anova). Provided that stimuli were given at an interval of greater than 3 - 4 min, the wind-up was reproducible.

In subsequent experiments involving drug applications, a frequency was chosen in each case, which gave a moderate degree of wind-up so that possible increases or decreases in C-fibre wind-up could be observed. This varied from 0.2 - 0.9 Hz (average ± SE, 0.53 ± 0.04; median, 0.50).

6.3.3 Inter-train Interval

The inter-train interval had a profound effect upon subsequent responses. When a low-frequency train was given only 1 minute after the start of a conditioning train of 20 stimuli at 1 Hz (a treatment producing greater wind-up than used in any of the subsequent experimental protocols), the baseline C-fibre response was extremely variable and usually much larger than the baseline response measured before the conditioning train. At inter-stimulus intervals of 4 min and longer, the baseline response after a conditioning stimulus was reproducible at pre-conditioning levels, as shown in Figure 6.5. Subsequent experimental protocols

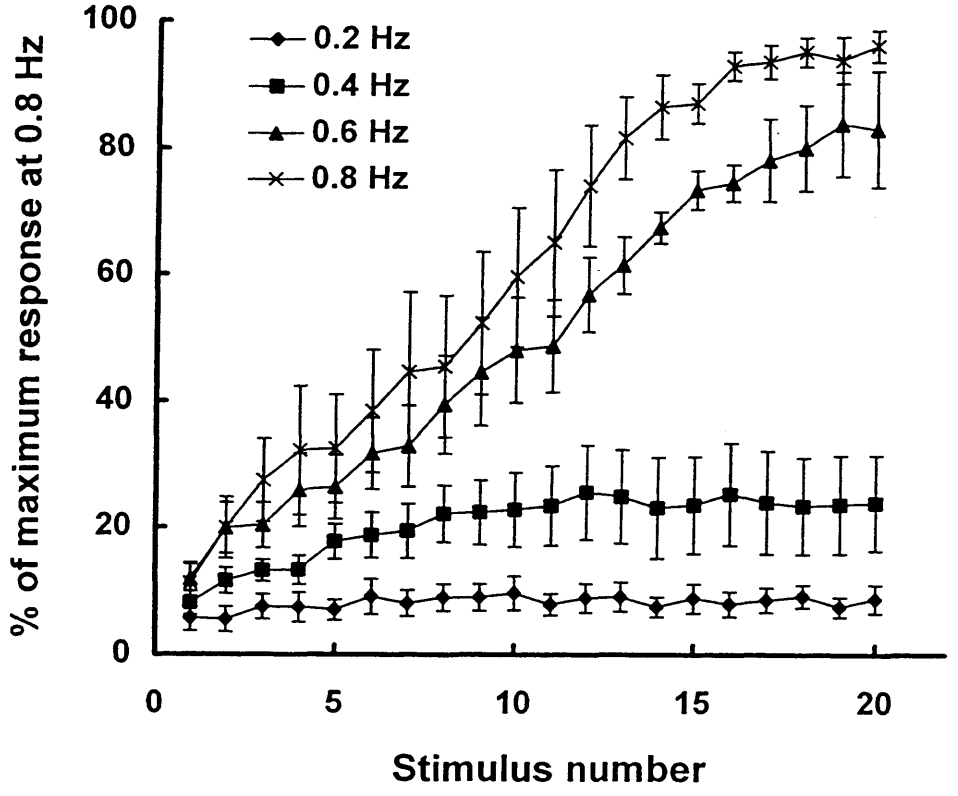


Figure 6.3. Frequency dependence of the C-fibre response. Results (n=5) are expressed as a percentage of the maximum response at 0.8 Hz (error bars = SE). At 0.2 Hz there is little change in the C-fibre response during the train whereas at higher frequencies, wind-up of the response is seen.

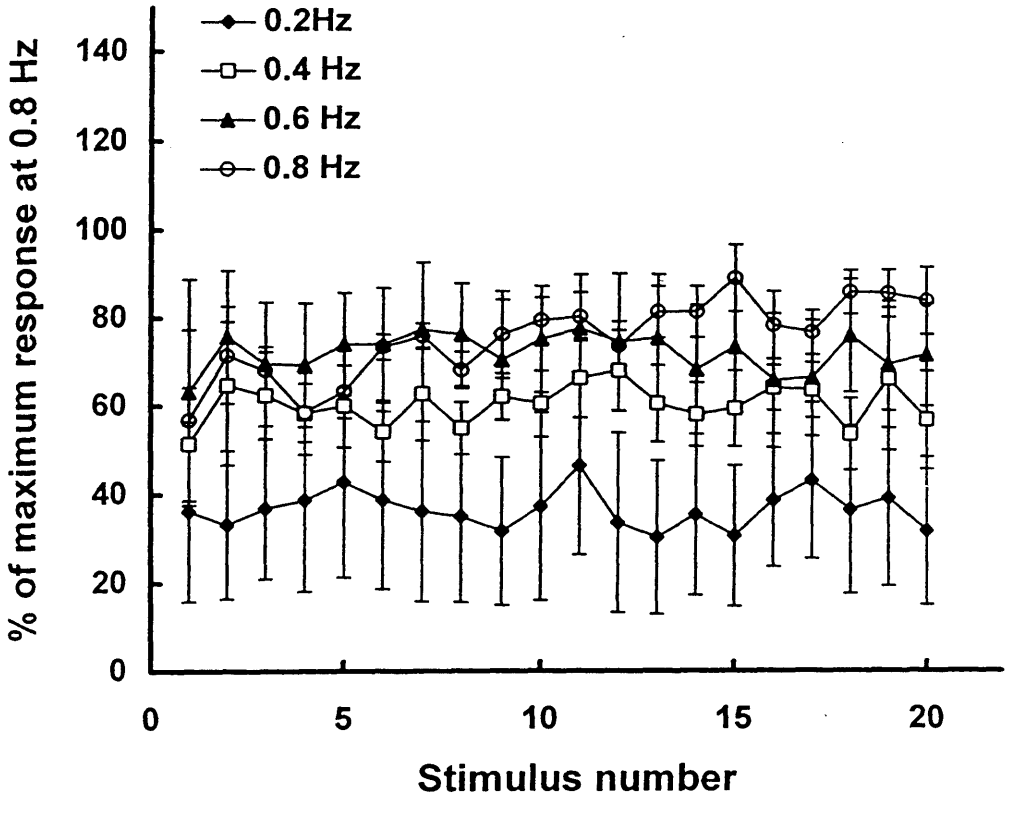


Figure 6.4. The effects of frequency on the A-fibre response. Results (*n*=4) are expressed as a percentage of the maximum response at 0.8 Hz (error bars = SE). At higher frequencies the response is increased ($p < 0.05$, 2-way anova).

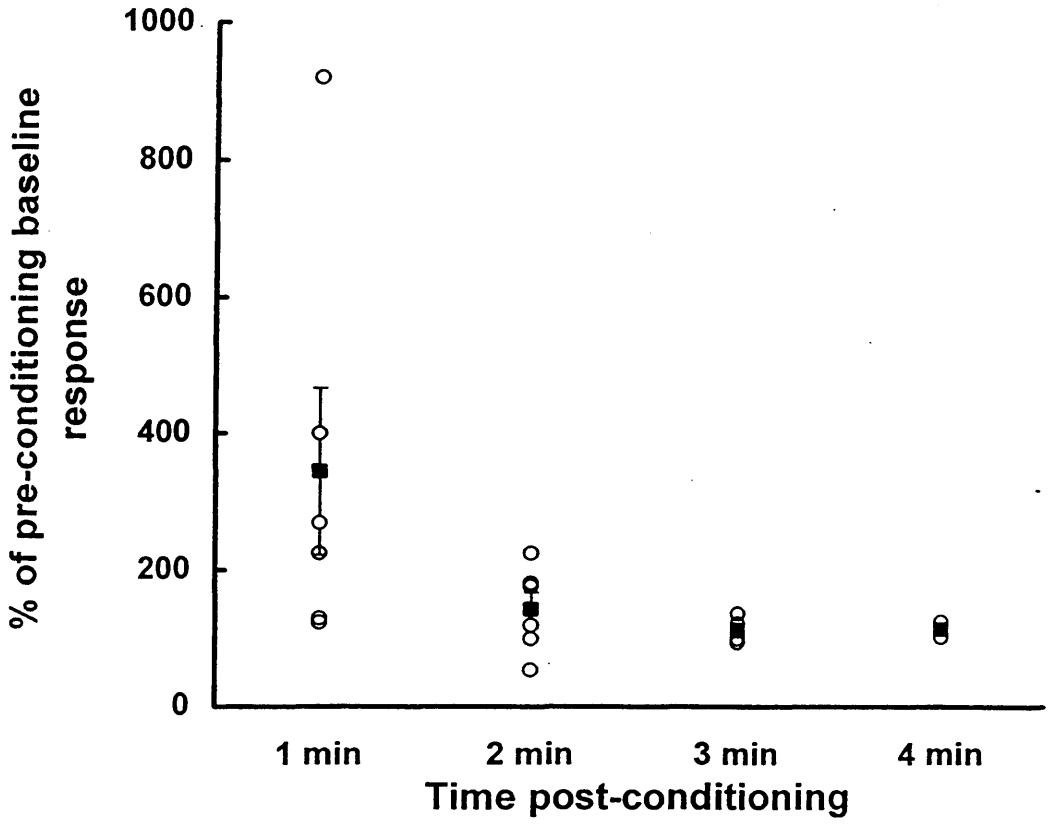


Figure 6.5. Effect of varying the inter-stimulus interval on the baseline response to C-fibre stimulation (*n=6*). O, individual animals; ■, average ± SE. At short intervals the post-conditioning response is altered in a highly variable manner. At inter-stimulus intervals of 3-4 min the post-conditioning response is at pre-conditioning levels and is more consistent.

therefore used an inter-stimulus interval of 5 min so that the baseline response did not vary during the experiment due to too frequent stimulation.

6.3.4 Intravenous Ketamine

Slow intravenous injections of 0.5 - 2.0 mg/kg ketamine gave an immediate reduction (within 2 min) in the C-fibre wind-up without an effect on blood pressure. This shows that the reduction in wind-up was not secondary to systemic effects of ketamine. The wind-up increased progressively back up to control values within 10 min as shown by the example in Figure 6.6. Figure 6.7 shows that the response to the 20th stimulus was reduced to 88% , 78% and 41% of maximum control levels by 0.5, 1.0 and 2.0 mg/kg ketamine respectively ($p < 0.001$, 1-way anova). The total magnitude of the response was significantly reduced to 48.1% of control levels by 2.0 mg/kg ketamine (Table 6.1; $p < 0.001$, t-test). Figure 6.8 shows that the baseline response was not significantly affected by doses of ketamine of 0.5 - 2.0 mg/kg ($p > 0.05$, 1-way anova). Intravenous vehicle alone had no significant effects upon any of these measures of wind-up.

Higher doses of 4 and 8 mg/kg were tested in a few animals; a rapid reduction in blood pressure by 20 - 30 mmHg was observed which returned to normal within 15 min. Wind-up and baseline responses were markedly reduced but in these animals ketamine was considered to be having severe systemic effects and data from these animals is not, therefore, shown.

The effect of ketamine on the A-fibre response was also investigated. Figure 6.9 shows that ketamine given intravenously at 2 mg/kg (*n=4)* produced a reduction in the total magnitude of wind-up to 74 % of control levels (Table 6.2; $p < 0.05$, t-test). There were no significant reductions in baseline response (Fig. 6.10).

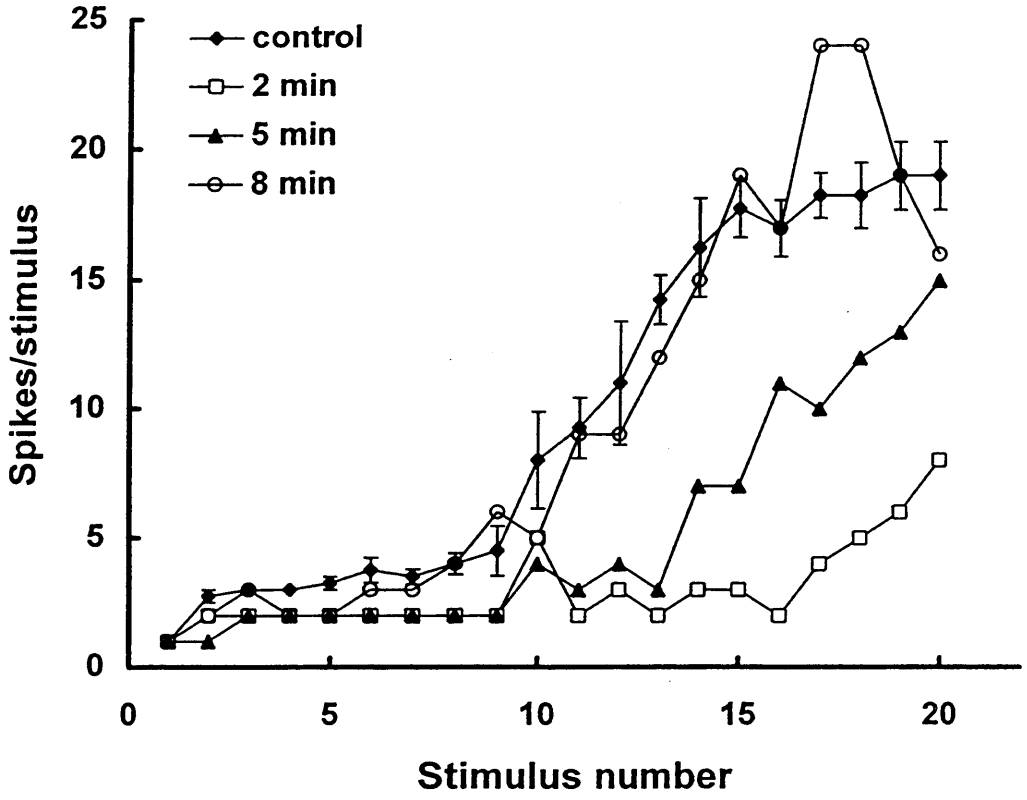


Figure 6.6. The transient effect of 2 mg/kg ketamine given intravenously on C-fibre wind-up at 2-8 minutes post-injection. There is a reduction in wind-up at 2 min but the responses rapidly return to control levels, in this case by 8 min. (Frequency, 0.5 Hz; strength, 5.4 V; stimulus width, 0.5 ms. Error bars = SE.)

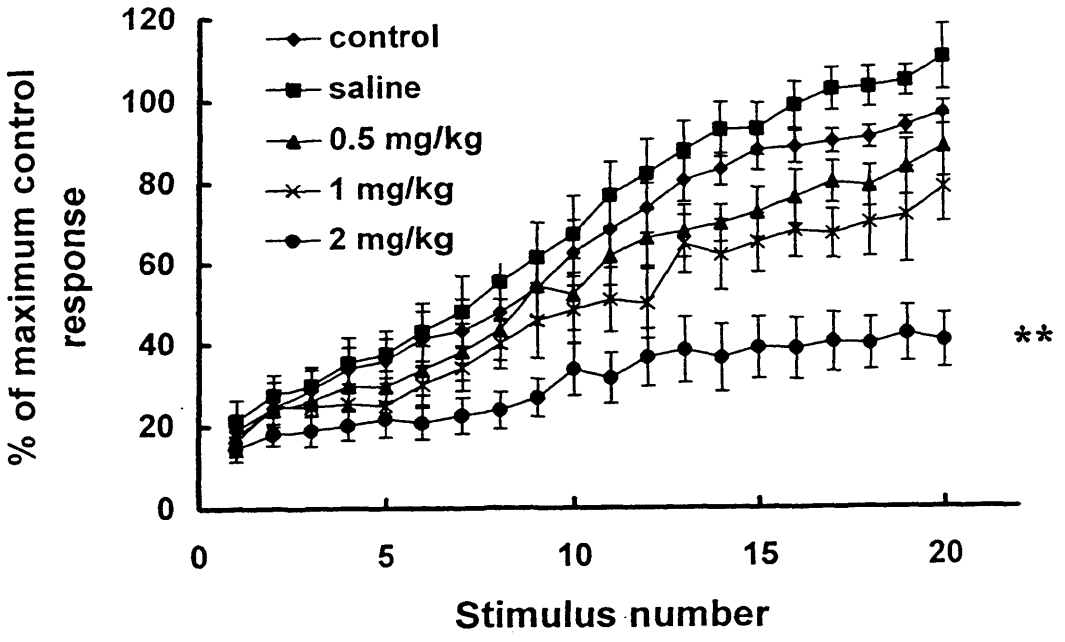


Figure 6.7. Effect of intravenous ketamine on C-fibre wind-up. Results (n=7) are expressed as a percentage of the maximum control value (error bars = SE). Ketamine dose-dependently reduced both the rate (** $p < 0.001$, 1-way anova) and total magnitude ($p < 0.001$, t-test) of the wind-up.

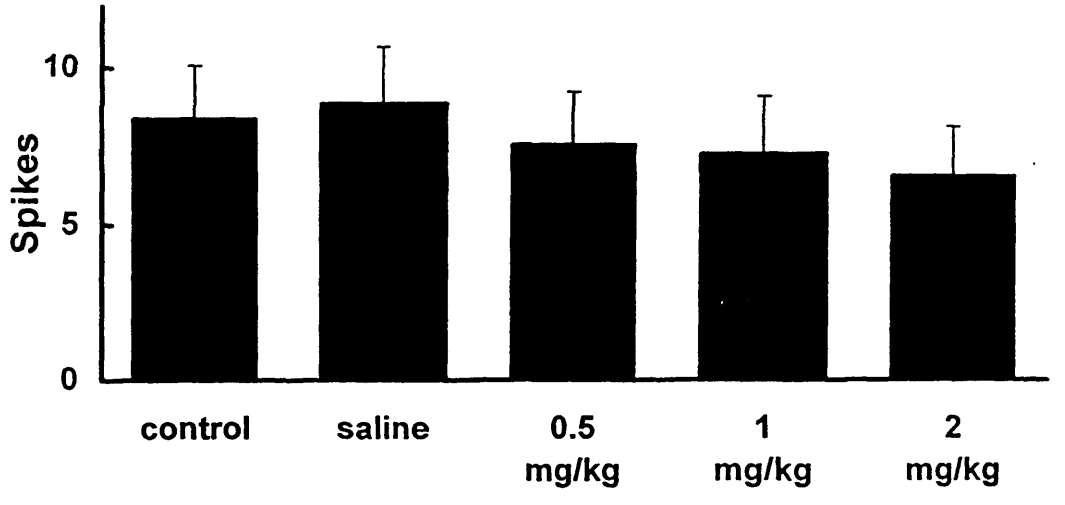


Figure 6.8. Effect of intravenous ketamine on the baseline response to C-fibre stimulation. Ketamine (n=7) and its vehicle control, 0.9% saline, had no significant effect upon the baseline response at doses of up to 2 mg/kg ($p > 0.05$, 1-way anova). Error bars = SE.

Table 6.1 Effects of drug treatments on the total magnitude of C-fibre wind-up.

Treatment	Effect (% of Control Response) ± Standard Error
Intravenous ketamine (n=7)	
vehicle	107.8 ± 4.8
0.5 mg/kg	88.9 ± 6.1
1.0 mg/kg	77.7 ± 7.3 *
2.0 mg/kg	48.1 ± 6.6 ***
Intravenous indomethacin (n=5)	
vehicle	120.9 ± 4.4 *
0.1 mg/kg	107.6 ± 6.2
0.5 mg/kg	100.5 ± 5.5
1.0 mg/kg	77.1 ± 7.4 *
5.0 mg/kg	52.2 ± 7.2 ***
Intrathecal indomethacin (n=5)	
vehicle	104.2 ± 3.1
10 μM	108.8 ±12.9
100 μM	67.5 ± 9.8 *
Intravenous SC58125 (n=5)	
vehicle	105.7 ±10.1
1 mg/kg	98.0 ± 4.3
5 mg/kg	73.1 ± 9.8
10 mg/kg	29.4 ± 8.4 ***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$ (t-test)

Table 6.2 Effects of drug treatments on the total magnitude of A-fibre wind-up.

Treatment	Effect (% of Control Response) ± Standard Error
Intravenous ketamine (n=4)	
2.0 mg/kg	73.9 ± 6.5 *
Intravenous indomethacin (n=5)	
vehicle	108.1 ± 3.7
5.0 mg/kg	87.0 ± 11.9
Intrathecal indomethacin (n=5)	
vehicle	118.7 ± 4.0 **
100 μM	111.8 ± 14.7
Intravenous SC58125 (n=5)	
vehicle	101.8 ± 4.6
10 mg/kg	65.3 ± 6.4 **

* $p < 0.05$; ** $p < 0.01$ (t-test)

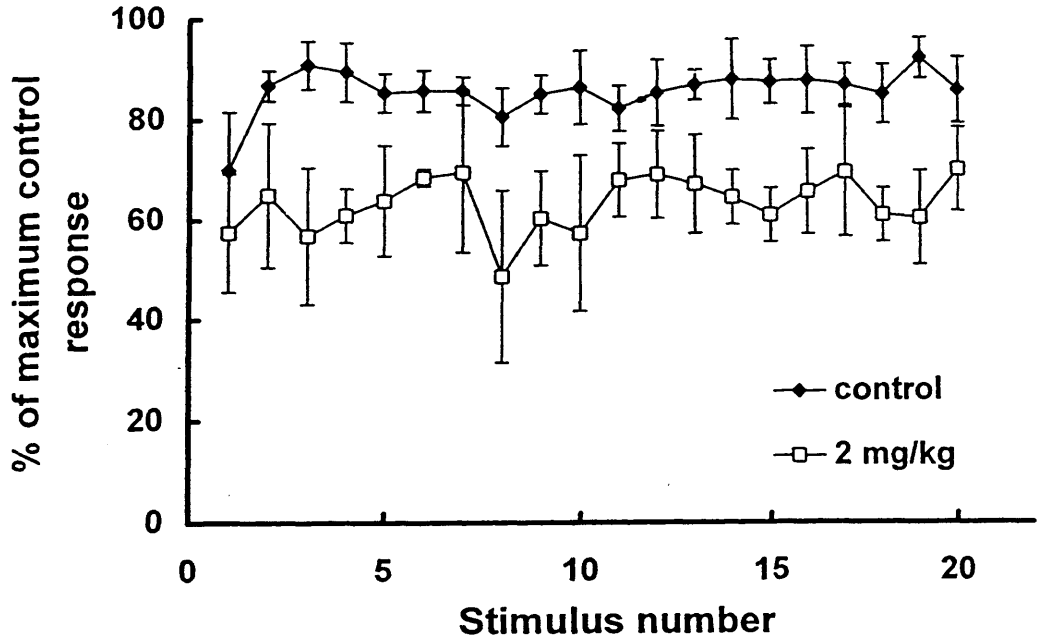


Figure 6.9. Effect of intravenous ketamine (2 mg/kg) on the A-fibre element of the spinal reflex response to C-fibre stimulation (*n=4*). Error bars = SE. A slight reduction in the total magnitude of the wind-up is seen ($p < 0.05$, t-test).

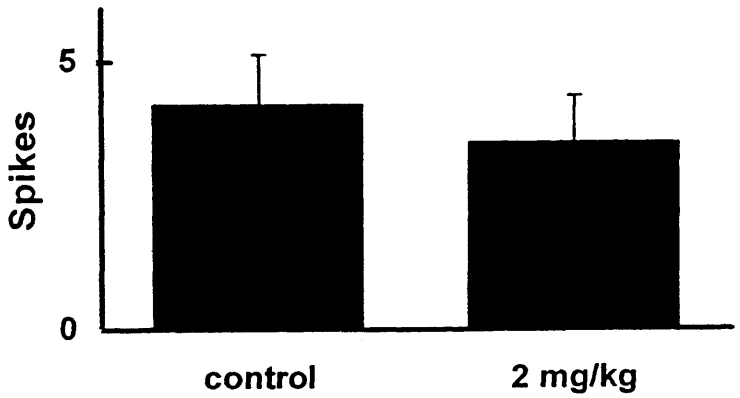


Figure 6.10. Effect of intravenous ketamine (2 mg/kg) on the A-fibre baseline response to C-fibre stimulation (*n=4*). There is no significant difference between control and treatment responses ($p > 0.05$, 1-way anova).

6.3.5 A-fibre Responses

Intravenous Indomethacin

Figure 6.11a shows that intravenous indomethacin at 5 mg/kg (n=5) reduced the total magnitude of wind-up to 87% of control but this effect was not statistically significant ($p > 0.05$). Vehicle alone had no statistically significant effects (Fig. 6.11b).

Intrathecal Indomethacin

Figure 6.12a demonstrates that intrathecal 100 μM indomethacin (n=5) had no statistically significant effects upon the A-fibre response. Its vehicle alone given intrathecally significantly increased the total magnitude of the response ($p < 0.01$) with no significant effect upon the baseline response (Fig. 6.12b).

Intravenous SC58125

Intravenous SC58125 given at 10 mg/kg (n=5) did produce a significant reduction in A-fibre response, as shown in Figure 6.13a. The total magnitude was significantly reduced to 65 % of control levels ($p < 0.01$, t-test). The baseline response was not significantly affected ($p > 0.05$, one-way anova) but the response to the 20th stimulus was significantly reduced to 72% of control levels by 10 mg/kg SC58125 ($p < 0.05$, t-test) but not vehicle alone (Fig 6.13b).

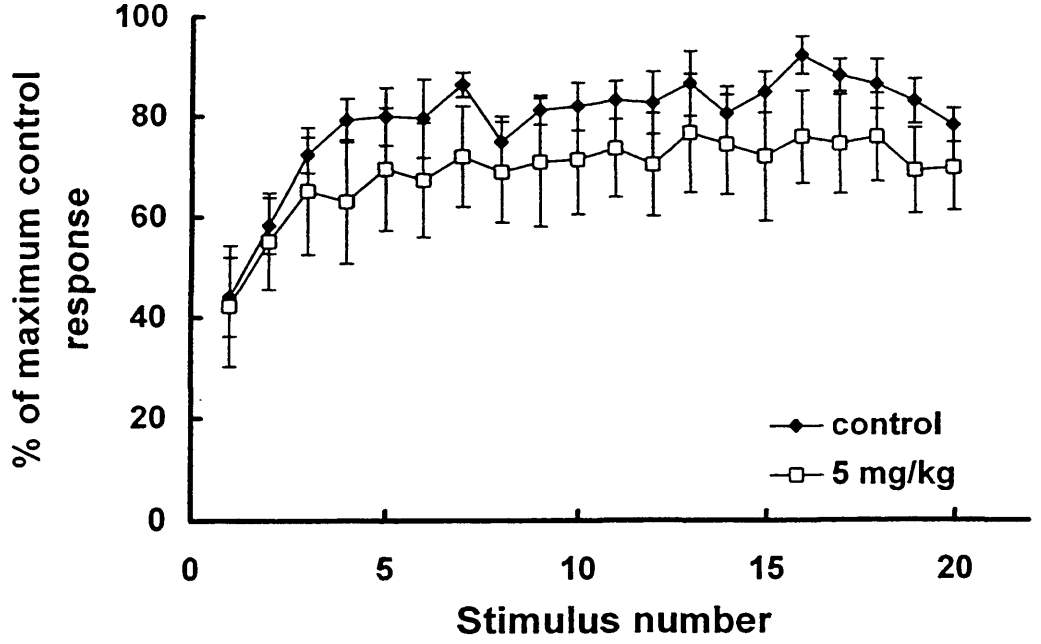
The effects of these drug treatments on the total magnitude of the response are summarised in Table 6.2.

6.3.6 C-fibre Responses

Intravenous Indomethacin

Intravenous indomethacin dose-dependently reduced C-fibre wind-up. Figure 6.14 shows that the response to the 20th stimulus was reduced to 46 % of maximum control levels by 5 mg/kg ($p < 0.005$, t-test) and the total magnitude of wind-up was reduced to 52 % of control (Table 6.1; $p < 0.005$, t-test). Intravenous

a



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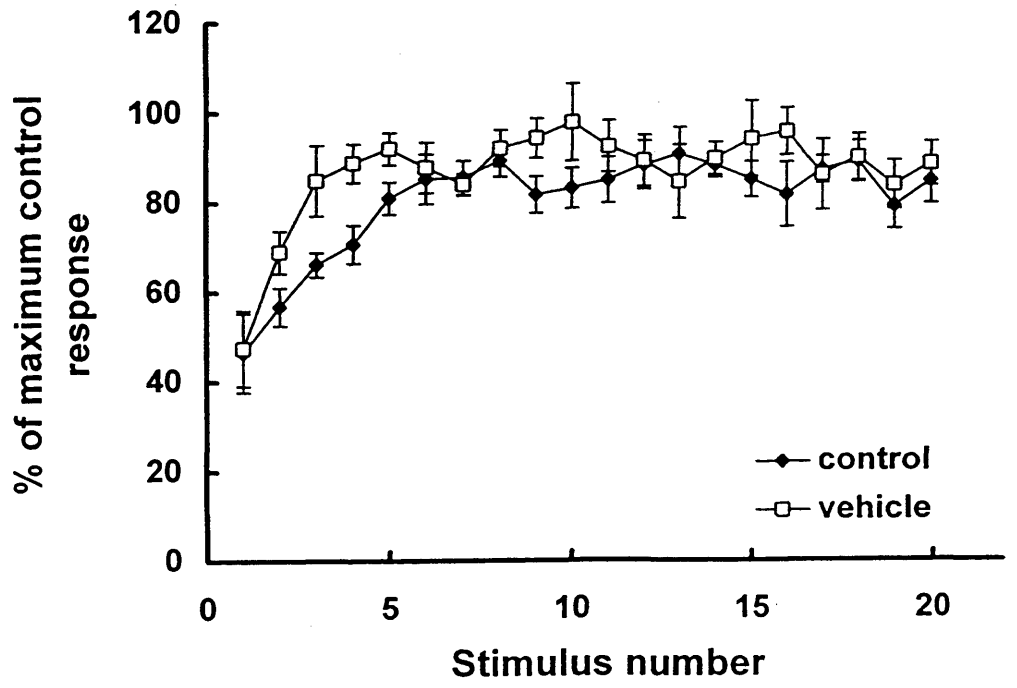
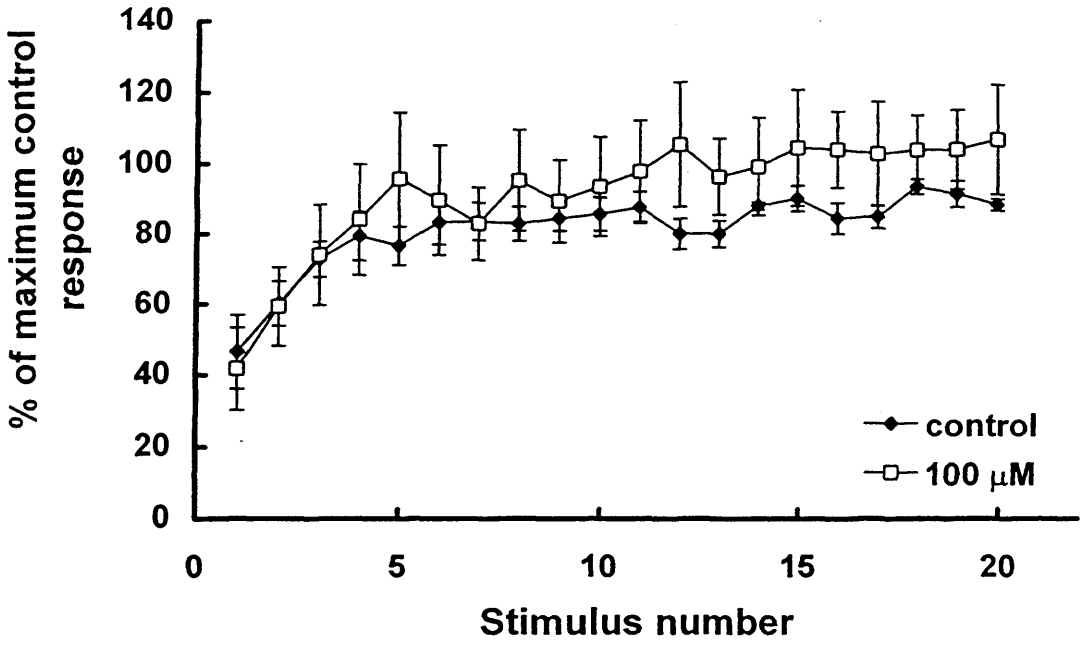


Figure 6.11. **a.** The effects of 5 mg/kg intravenous indomethacin on the A-fibre element of an emg response to C-fibre stimulation (*n=5*). There is a non-significant reduction in the response ($p > 0.05$, 1-way anova). **b.** Intravenous vehicle alone had no effect upon this A-fibre element (*n=5*; $p > 0.05$, 1-way anova). Error bars = SE.

a



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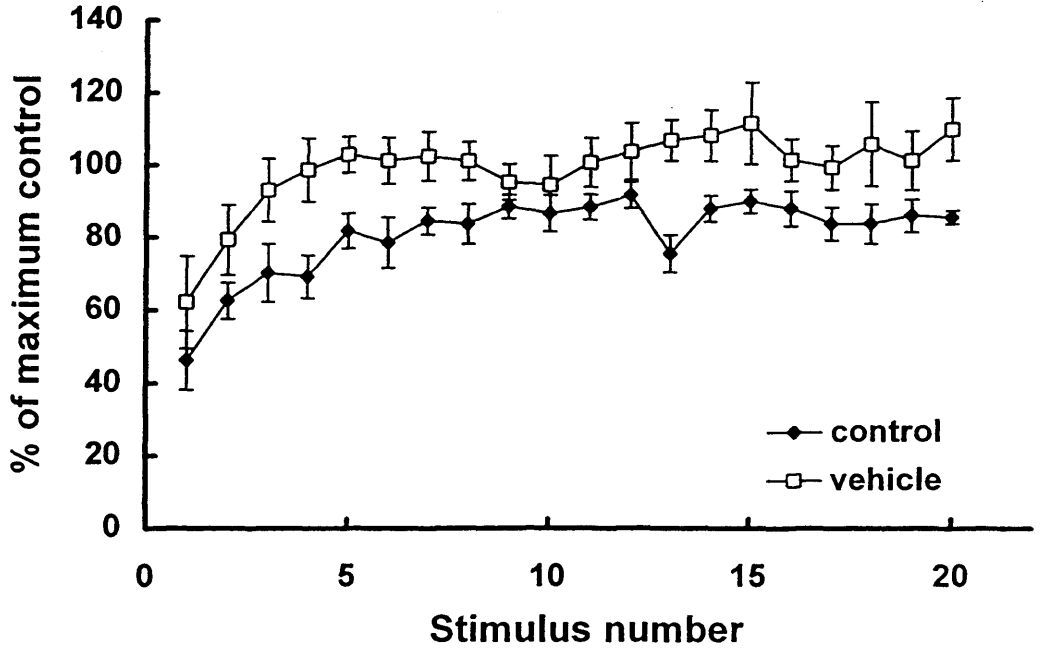
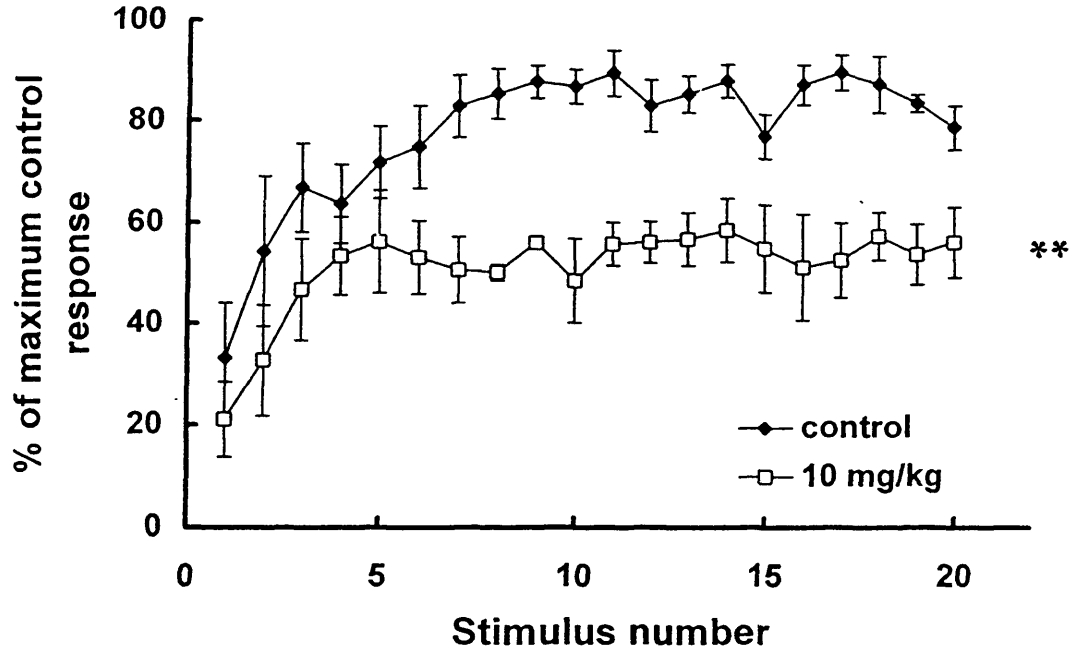


Figure 6.12. a. The effects of intrathecal indomethacin (100 μM; *n*=5) on the A-fibre element of an emg response to C-fibre stimulation. There were no significant effects on the response ($p > 0.05$, 1-way anova). **b.** Intravenous vehicle (*n*=5) significantly increased the total magnitude of the A-fibre element ($p < 0.01$, 1-way anova). Error bars = SE.

a



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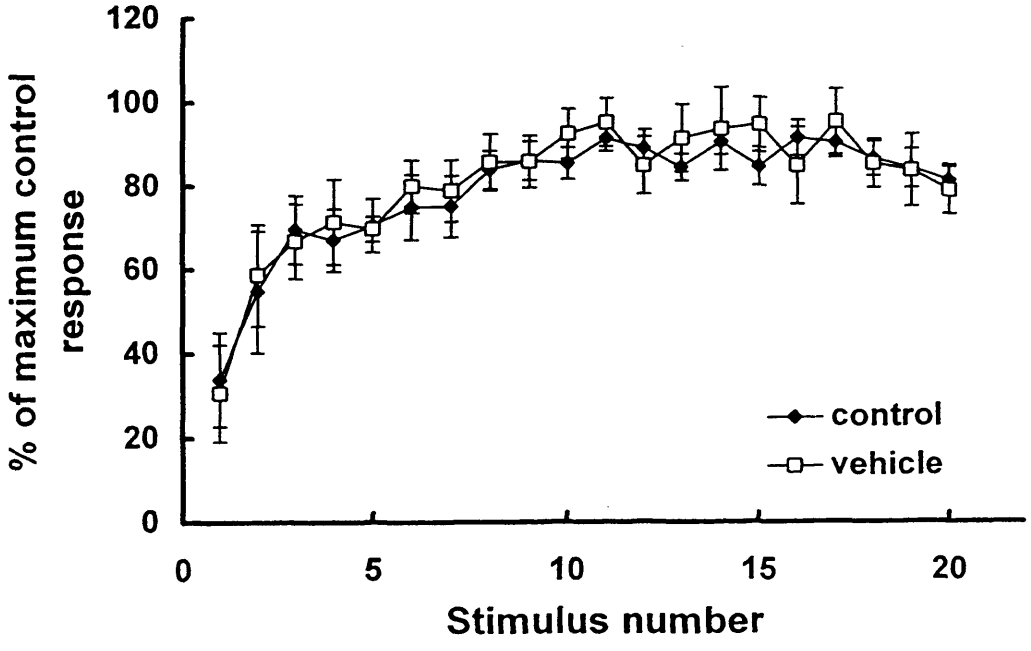

Figure 6.13. a. The effects of intravenous SC58125 at 10 mg/kg (*n=5*) on the A-fibre element of an emg response to C-fibre stimulation. There is a significant reduction in the total magnitude of the response (** $p < 0.01$, t-test). **b.** Intravenous vehicle alone (*n=5*) had no effect upon this A-fibre element ($p > 0.05$, t-test). Error bars = SE.

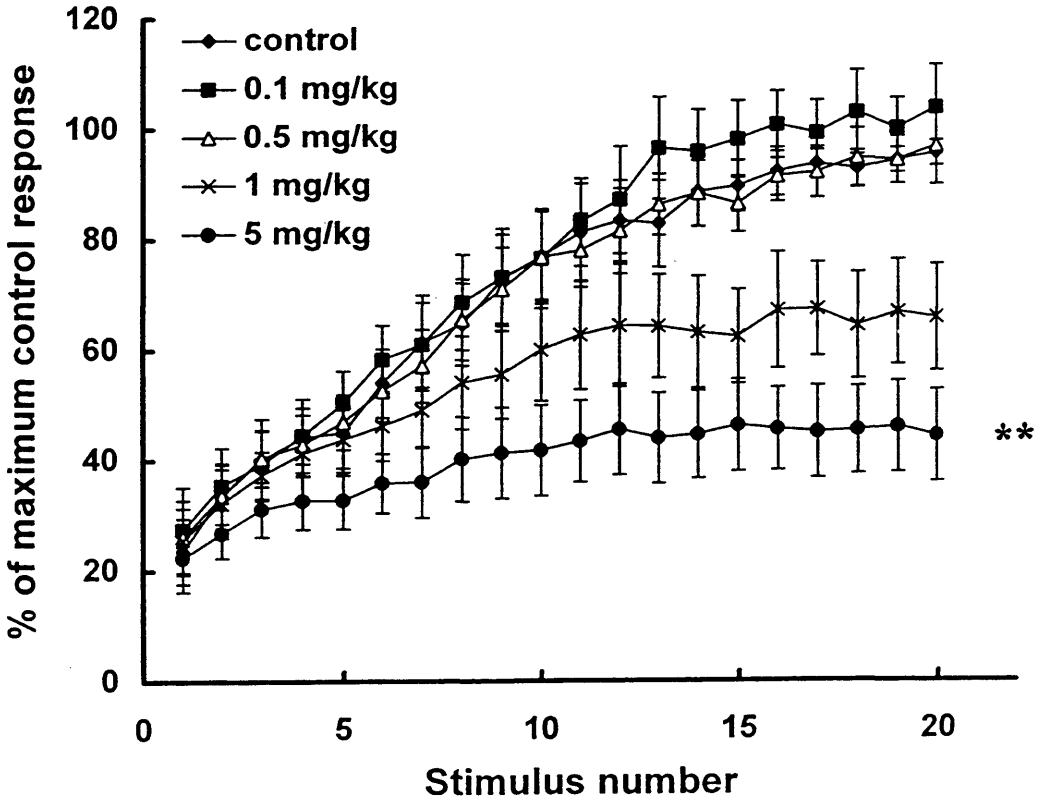

Figure 6.14. The effects of intravenous indomethacin on C-fibre wind-up. Both the rate and total magnitude of wind-up are significantly and dose-dependently reduced (n=5; ** $p < 0.005$, t-test). Error bars = SE.

vehicle alone produced a significant ($p < 0.05$) increase in the total magnitude of wind-up and the response to the 20th stimulus as shown in Figure 6.15. The baseline response was not significantly altered (Fig. 6.16; $p > 0.05$ 1-way anova). Two-way anova analysis of the drug effect showed a highly significant reduction in wind-up at the $p < 0.0001$ level.

Intravenous indomethacin was clearly having an effect on C-fibre wind-up. In order to determine whether it was acting solely peripherally or at other sites within the central nervous system, indomethacin was also applied directly onto the spinal cord via an intrathecal catheter.

Intrathecal Indomethacin

Intrathecal indomethacin at 100 μM - 1 mM concentration-dependently reduced both the rate of wind-up and its maximum level. Figure 6.17 shows that a dose of 100 μM indomethacin reduced the response to the 20th stimulus to 63 % of maximum control levels and the total magnitude of C-fibre wind-up was reduced to 67 % (Table 6.1; $p < 0.05$). This drug effect was significant at the $p < 0.001$ using a 2-way anova analysis. The lower dose of indomethacin (10 μM) and intrathecal vehicle alone had no significant effect upon the rate or total magnitude of C-fibre wind-up (Figs. 6.17, 6.18). Figure 6.19 shows that the baseline response was not significantly affected by intrathecal vehicle or indomethacin.

Intravenous SC58125

Intravenous SC58125 dose-dependently reduced both the rate and total magnitude of C-fibre wind-up, as shown in Figure 6.20. Intravenous vehicle alone had no significant effect (Fig. 6.21). Intravenous SC58125 at 10 mg/kg reduced the response to the 20th stimulus to 28 % of maximum control levels and the total magnitude of C-fibre wind-up was reduced to 29 % ($p < 0.005$, t-test). Drug effects were highly significant ($p < 0.001$ level, 2-way anova). Figure 6.22 shows that the baseline response was not significantly altered by intravenous vehicle or SC58125 at 1 or 5 mg/kg but was reduced by 10 mg/kg SC58125 ($p < 0.05$). It was found that intravenous injections of SC58125 could cause a rapid and

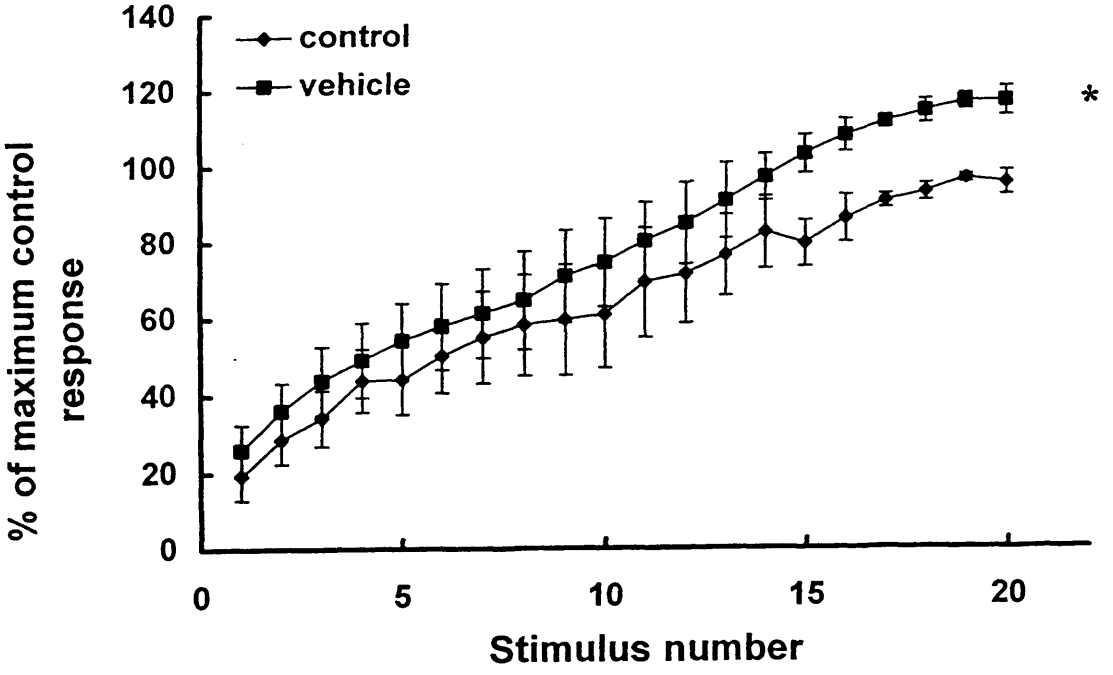

Figure 6.15. The effect of intravenous vehicle (1 part 10% sodium bicarbonate and 9 parts 0.9% saline; *n*=5) on C-fibre wind-up. Wind-up is significantly increased (* $p < 0.05$, 1-way anova). Error bars = SE.

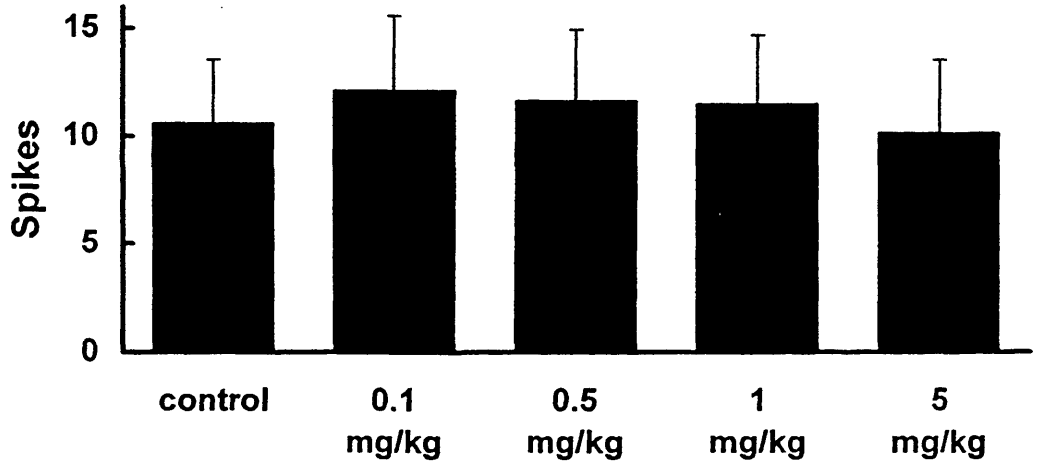

Figure 6.16. The effects of intravenous indomethacin on the baseline response to C-fibre stimulation (*n*=5). Error bars = SE. There is no significant change ($p > 0.05$, 1-way anova).

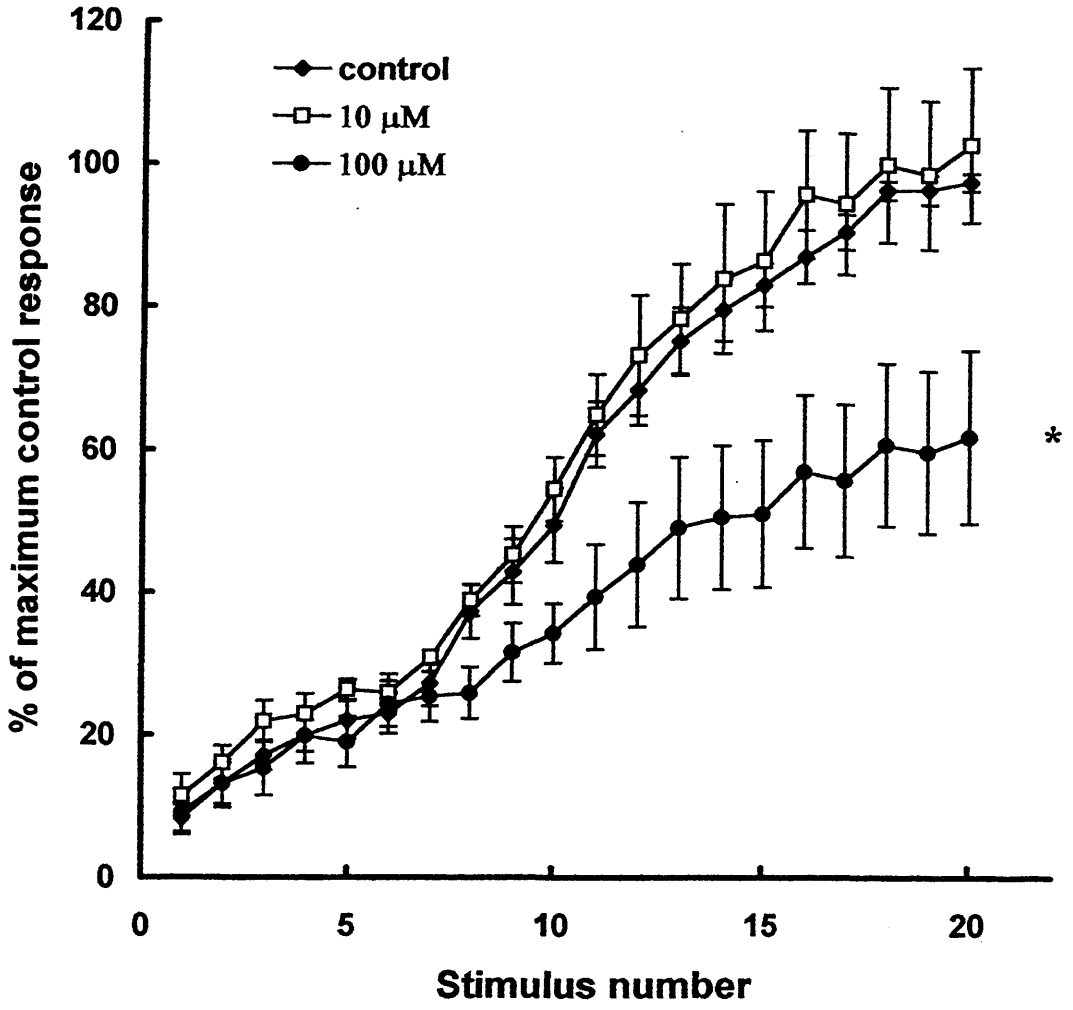

Figure 6.17. Effect of intrathecal indomethacin on C-fibre wind-up (*n=5*). Indomethacin at 10 μM had no significant effect upon wind-up ($p > 0.05$, 1-way anova) whereas indomethacin at 100 μM and 1 mM (*n=3*; data not shown) concentration-dependently reduced both the rate and total magnitude of wind-up (* $p < 0.05$, 1-way anova). Error bars = SE.

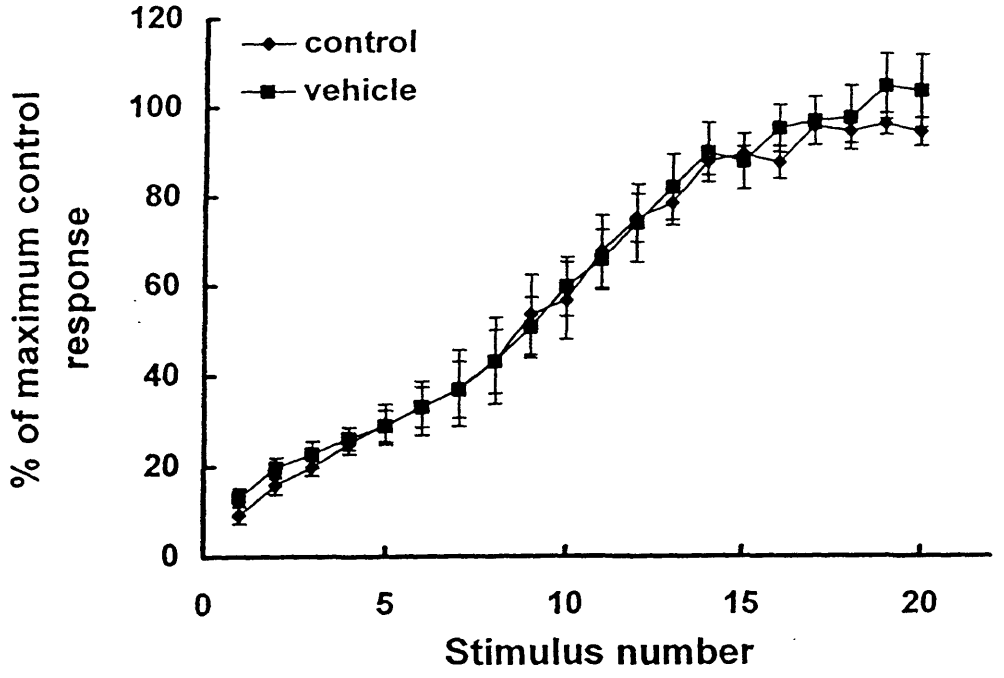

Figure 6.18. Effect of intrathecal vehicle on C-fibre wind-up (*n*=5). Error bars = SE. There is no significant difference from control ($p > 0.05$, 1-way anova).

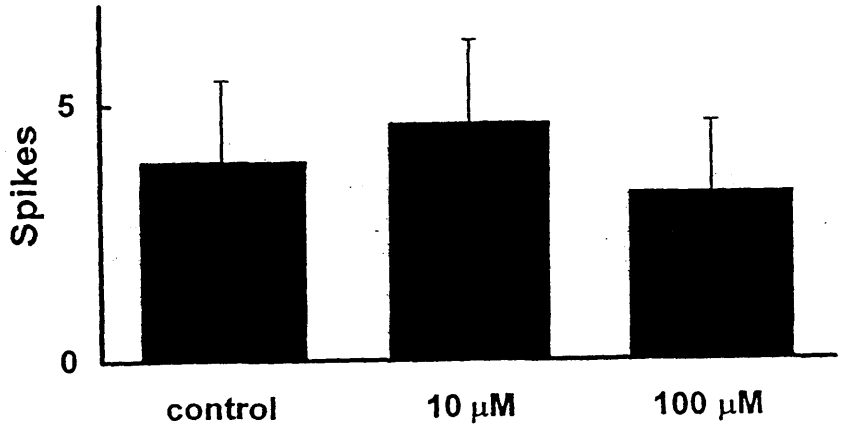

Figure 6.19. Effect of intrathecal indomethacin on the baseline response to C-fibre stimulation (*n*=5). There is no significant variation in the baseline response (in spikes; $p > 0.05$, 1-way anova). Error bars = SE.

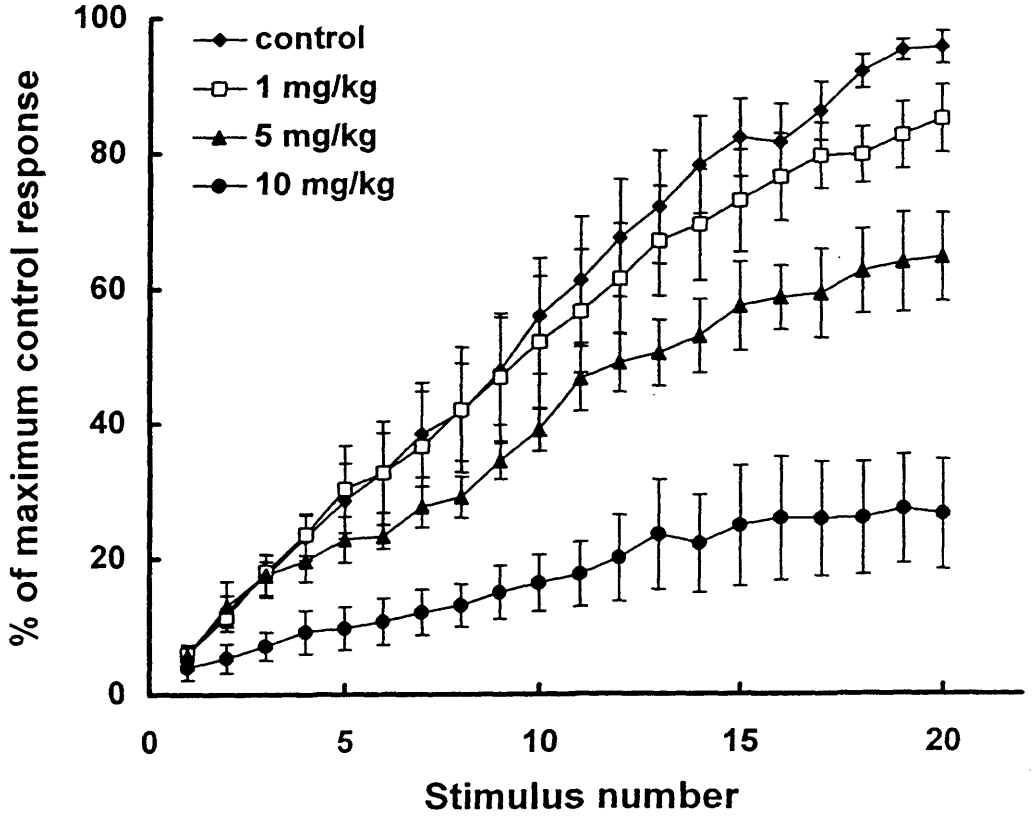

Figure 6.20. Effects of intravenous SC58125 on C-fibre wind-up (*n*=5). SC58125 significantly and dose-dependently reduced both the rate ($p < 001$, 2-way anova) and the total magnitude ($p < 0.005$, t-test) of the reflex wind-up. Error bars = SE.

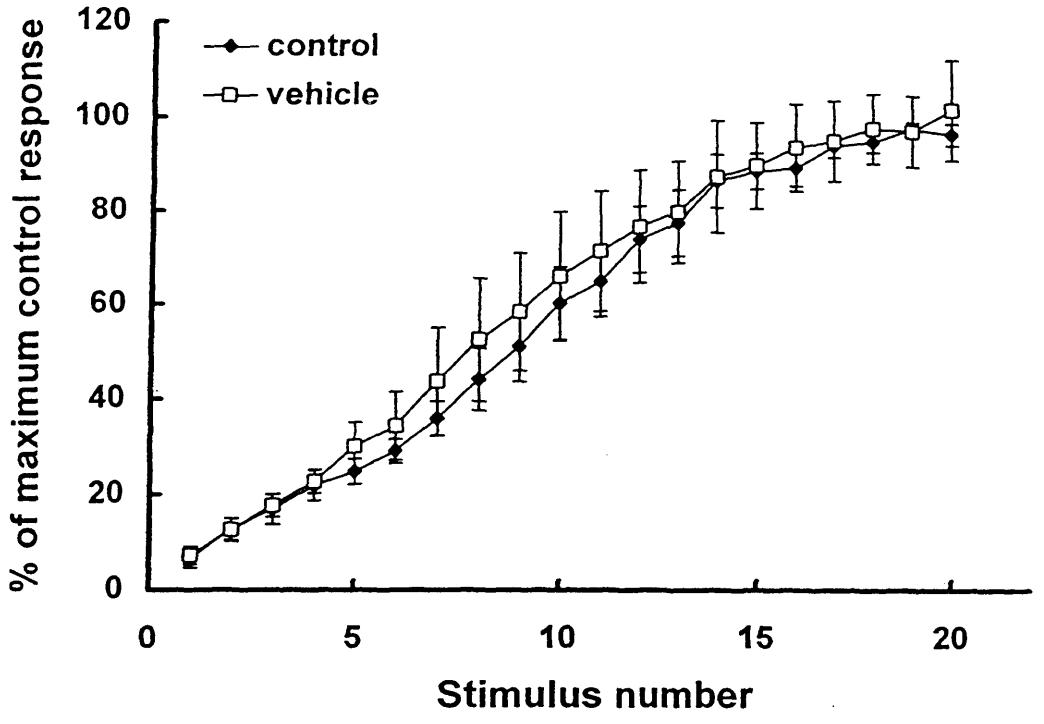

Figure 6.21. The effects of intravenous vehicle (5% DMSO, 95% PEG 400) on wind-up in response to C-fibre stimulation (*n*=6). Error bars = SE. There is no significant effect ($p > 0.05$, 1-way anova).

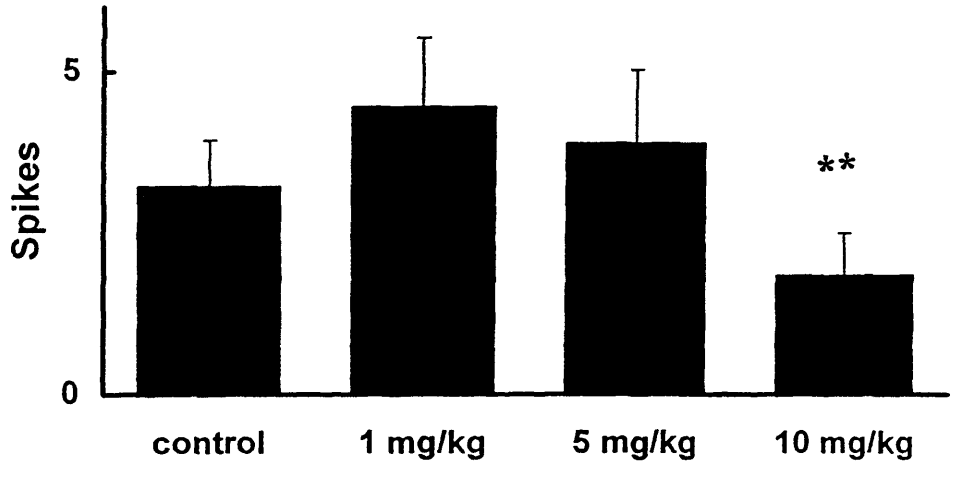

Figure 6.22. The effects of intravenous SC58125 on the baseline response (*n*=5). The total magnitude of the response is significantly reduced (** $p < 0.005$, t-test) by 10 mg/kg SC58125. Error bars = SE.

transient fall in blood pressure, which was avoided by administering the drug extremely slowly while constantly monitoring blood pressure.

The effects of intravenous and intrathecal drug treatments on the total magnitude of the C-fibre wind-up are summarised in Table 6.1.

Intrathecal SC58125

An attempt was made to apply SC58125 intrathecally in 2 rats. One experiment showed an apparent significant reduction in wind-up when 1 μM SC58125 but not when vehicle was applied onto the spinal cord, as shown in Figure 6.23. Problems were encountered when applying the drug due to blocking of the intrathecal catheter. A second experiment was set up and similarly showed no effect with vehicle alone but a progressive decline in response to stimulation following the application of 1 μM SC58125. Microscopic examination of the spinal cord showed a precipitation of drug out of solution onto the surface of the spinal cord. Attempts to modify the vehicle for this drug so that the precipitation did not occur were unsuccessful, therefore it was concluded that the reduction in response seen could be due to a toxic effect rather than to a pharmacological effect.

6.4 Conclusions

A- and C-fibre withdrawal reflexes were recorded from single muscle unit emgs at similar frequency and stimulus width criteria as those previously reported for dorsal horn cells (Mendell 1966; Davies & Lodge 1987; Dickenson & Sullivan 1987a), ventral roots (Thompson et al. 1990), motor nerves (Wall & Woolf 1984; Woolf & Wall 1986a) and muscle fibres (Falinower et al. 1994).

As can be seen from the results above, the preparation described was used to study the wind-up of a nociceptive withdrawal reflex in an anaesthetised animal using a novel stimulus protocol. When stimulating at C-fibre strength, a response with two distinct components could be seen; these were considered to be an A-fibre response appearing with a short latency of less than 100 ms and a C-fibre response

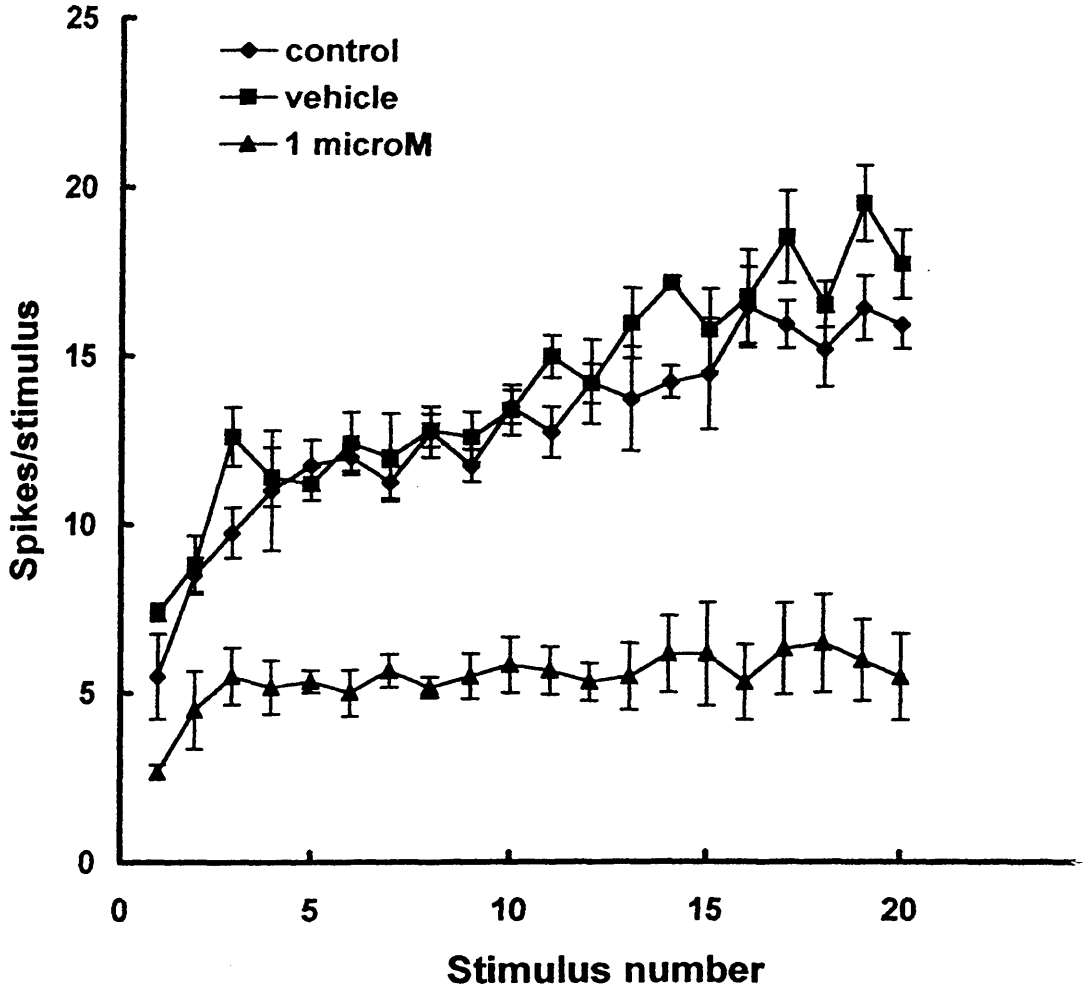

Figure 6.23. The effects of spinally applied SC58125 on wind-up (*n=1*). There is an apparent reduction in wind-up following the application of 1μM SC58125 but no reduction following the application of vehicle alone.

appearing with a latency of greater than 130 ms. These latencies are in agreement with those found by other workers. Falinower et al. (1994) found a latency of 17.5 ± 2.3 ms for the response to Aδ fibre stimulation and 162.4 ± 5.1 ms for the C-fibre response in a similar model using rats of the same size as ours. Herrero & Cervero (1996a,b) considered the A-fibre component of a single motor unit in the hindlimb flexor muscles of an adult rat to be that occurring within 150 ms of stimulation of the toe and the C-fibre component to be occurring at 150 - 500 ms after stimulation; figures in agreement with those of Wall & Woolf (1984) recording from biceps femoris/semitendinosus motoneurons following stimulation of the sural nerve. When recording wind-up from neurones in the deep dorsal horn, latencies of less than 20 ms for A-fibres and 100 - 300 ms for C-fibres were found (Dickenson & Sullivan 1987a). Latencies for responses in deep dorsal horn neurones would be expected to be shorter since recordings are made at an anatomically earlier point in the reflex arc.

The voltages used to evoke the responses were in the low range compared to those used by some other workers. That convincing C-fibre recordings were made at these voltages is likely to be due in part to the experimental technique used. Care was taken to cover the exposed sural nerve with a cotton-wool wick soaked in liquid paraffin both to prevent desiccation of the nerve and to ensure excellent electrical contact. To confirm that the responses recorded were truly C-fibre responses, recordings could have been made from the sural nerve during stimulation to demonstrate C-fibre firing at the voltages used in this series of experiments.

C-fibre wind-up was frequency dependent, with a sigmoid form that levelled out at approximately 1 Hz. It is interesting that this cut-off level, above which wind-up does not increase, is of the same order of magnitude as the frequency of spontaneous firing of primary afferents in arthritic conditions. This provides further weight to the hypothesis that the mechanisms underlying wind-up are allied to those seen in hyperalgesia.

The C-fibre wind-up involved NMDA-receptor function, as shown by the reduction in wind-up following intravenous ketamine. The reduction in wind-up with no effect upon the initial or baseline response is in agreement with the study by Davies & Lodge (1987) on WDR lumbar dorsal horn cells. That NMDA receptor function is required for wind-up supports observations by Davies & Lodge (1987), Dickenson & Sullivan (1987a), Thompson et al. (1990), Dickenson & Aydar (1991) and Woolf & Thompson (1991).

The results reported above indicate that an inter-train interval of 5 min was long enough to produce a consistent post-conditioning response but that there was a facilitation of the flexor response at intervals of less than 3 min. This is in agreement with the reports by Woolf & Wall (1986a) and Xu et al. (1991) that an identical conditioning stimulus to that used in this study transiently increased the flexor reflex for less than 3 - 5 min. A prolonged volley to the sural nerve of 2 Hz for 10 min elevated the flexion reflex for more than 3 h, but this was far in excess of any stimulus given in the reported experiments when testing drug effects, which never exceeded a C-fibre input of 0.9 Hz for 18 s. Wall & Woolf (1984) found that sectioning the sural nerve produced an increase in flexor reflex excitability lasting for 15 min. The recordings reported above were performed at least 1 h after sural nerve section.

C-fibre wind-up was significantly and dose-dependently reduced by intravenous indomethacin, a non-selective cox inhibitor. In these experiments it is not possible to say whether the indomethacin was acting peripherally, spinally or centrally, or at a combination of these sites. Indomethacin given intrathecally also produced a dose-dependent and significant reduction in wind-up and in these cases the small amounts given would eliminate the possibility of a peripheral effect. It is unlikely that a significant dose could travel to higher centres since the volume in which the indomethacin was administered was very small and excess cerebrospinal fluid tended to leak out through the incision in the dura. It is possible that some indomethacin entered local blood vessels and so could have travelled to other locations, however intrathecal indomethacin was most likely to be causing a reduction in wind-up through a direct effect upon the local tissues in the spinal cord. It is presumed that indomethacin applied to the surface of the

cord was able to diffuse through the spinal cord tissue or through local blood vessels to exert an effect on deep structures.

In each case of indomethacin administration the baseline response was unaffected. This indicates that the overall sensitivity of the spinal cord was stable throughout the experiments and that the reduction in wind-up was not due to variations in the level of anaesthesia or to variations in spinal excitability with time due to the surgery involved in preparing the animal.

Intravenous SC58125, a specific cox-2 inhibitor, also dose-dependently and significantly reduced C-fibre wind-up indicating that this wind-up is dependent upon prostaglandin synthesis at least in part via action of cox-2. Frustratingly, it was not possible to apply the drug spinally and be confident that there were no toxic effects due to precipitation of the drug.

Both SC58125 and ibuprofen, dissolved in 100% DMSO, were applied intrathecally using chronically implanted catheters by Yaksh's group (Dirig et al. 1998). They studied carrageenan-induced thermal hyperalgesia in rats and found that the thermal escape latency of the inflamed paw was not affected by these drugs given intrathecally after the establishment of hyperalgesia, whereas both intraperitoneal or systemic administration given 10 minutes before the carrageenan would dose-dependently reduce the thermal hyperalgesia. They concluded that spinal cox-2 is necessary for the initiation of thermal hyperalgesia but not for its maintenance. This would support my findings, in that spinally applied SC58125 was shown to prevent the establishment of hyperalgesia, possibly due to inhibition of wind-up. It would be interesting to repeat the experiments described above using SC58125 dissolved in 100% DMSO as it would be possible to observe any visible effects upon the spinal cord surface.

Chapman & Dickenson (1992) reported that direct spinal application of indomethacin had no effect upon C-fibre wind-up of deep dorsal horn spinal cord neurons. This contrary evidence may be explained by the different anaesthetic used: there is a suggestion that volatile anaesthetics, as used by these workers, reduce spinal sensitisation (O'Connor & Abrams 1995). More recent work by

Mazario et al. (1999), on rats anaesthetised with alpha-chloralose, demonstrated a significant inhibition of wind-up by the active enantiomer of ketoprofen.

The effects of the various treatments on the A-fibre element of the response were more equivocal, mainly due to the small size of the baseline response with little wind-up. This is in agreement with findings by Herrero & Cervero (1996a), who found that there was no wind-up of the A-fibre response in non-arthritic animals, and by Dickenson & Sullivan (1987a), who found that there was no wind-up in deep dorsal horn neurons when A fibres alone were stimulated at 0.5 Hz. Dickenson & Sullivan (1987a) further found that AP5, a selective NMDA-receptor antagonist, had little effect upon A-fibre discharges. The results presented above show a reduction in A-fibre response in the single-unit emg following intravenous ketamine (2 mg/kg) which is significant at the $p < 0.05$ level.

Intrathecal indomethacin had no effect upon the A-fibre response in these experiments. Jurna (1993) found similarly that intrathecal acetylsalicylic acid would reduce the response of ascending axons within the spinal cord to C-fibre stimulation of the sural nerve but had no effect upon the Aβ-fibre-evoked response. The specific cox-2 inhibitor SC58125 did, however, significantly reduce the A-fibre response without affecting the baseline response.

It should be remembered that since A-fibre and C-fibre discharges were defined on the basis of their latencies, any A-fibre afterdischarge occurring after 130 ms would have been counted together with the C-fibre response. Some caution needs to be exercised, therefore, when interpreting these results.

One final word of caution should be applied to the interpretation of these findings and their application to clinical situations. Houghton et al. (1995) observed an A-fibre enhancement of the sural nerve/gastrocnemius medialis reflex in the rabbit on repetitive stimulation that they showed was due to tachykinergic tone brought about by the nociceptive input during the surgical preparation of the leg. The preparation described above involved a major surgical insult that would have

resulted in an afferent bombardment of the spinal cord. The fact that the baseline responses remained constant over a prolonged period of time means that we can assume that the preparation was at a stable level of overall excitement during recordings. This is, of course, paralleling the situation during human surgery where the human spinal cord would be subject to similar stimulation. The results of these experiments are therefore directly applicable to an important practical situation. It can be concluded that NSAID administration before or during surgery reduces wind-up, thus contributing to the relief of post-operative pain.

Chapter 7

Discussion

7.1 Summary of Results

The purpose of this study was to test the hypothesis that prostaglandins (PGs) within the spinal cord have a role in nociception, specifically in hyperalgesic states. A number of aims were addressed: firstly, to ascertain if cyclooxygenases (cox), the enzymes responsible for the synthesis of PGs, are present in the spinal cord; secondly, to determine if cox levels can be altered by the induction of a hyperalgesic state; thirdly, to determine if NSAIDs, which inhibit cox activity, affect the responses of individual dorsal horn neurons to noxious mechanical joint stimulation in hyperalgesic and non-hyperalgesic animals; and fourthly, to determine if cox inhibitors affect the wind-up of a withdrawal reflex, wind-up being a phenomenon linked with central hyperalgesia.

A clear answer was found to the first question. Both cox-1 and cox-2 were found in lumbar spinal cord homogenates by Western blotting. Later studies by other workers have confirmed the presence of cox-1 and cox-2 protein in the spinal cord (Goppelt-Struebe & Beiche 1997).

Subsequent immunocytochemical studies located cox-1-ir in the cytoplasm and axonal processes of small- and medium-sized cell bodies (area < 1000 mm^2) in lumbar dorsal root ganglia (Willingale et al. 1997; Chopra et al. 2000). Cox-1 has more recently been shown to be co-localised with CGRP and isolectin B4, markers for peptidergic- and non-peptidergic-containing nociceptors respectively (Grubb et al. 1997; Chopra et al. 2000), suggesting that cox-1 is expressed in nociceptive neurons. In contrast, cox-2 immunolabelling was not detected in dorsal root ganglia.

Cox-2 was, however, found within the cytoplasm and perinuclear region of neurons in both superficial and deep dorsal horn of the spinal cord, as well as in lamina X and sparsely in motoneurons in the ventral horn (Figure 3.2; Willingale et al. 1997). Cox-2 is thus located in anatomical areas that have previously been associated with

nociception using electrophysiological techniques (Schaible et al. 1986; Neugebauer & Schaible 1990; Grubb et al. 1993).

That the PG biosynthesis pathway is active is demonstrated by the production of PGs by the spinal cord (Malmberg & Yaksh 1995; Sorkin & Moore 1996; Gardiner et al. 1977; Muth-Selbach et al. 1999). Also cox-2 protein levels (Chapter 4; Goppelt-Struebe & Beiche 1997; Gardiner 1998; Beiche et al. 1998b) and cox-2 mRNA expression (Beiche et al. 1998b; Hay & De Belleroche 1998) are increased by peripheral inflammation.

The second point to be addressed was whether spinal cord cox levels varied during the induction of hyperalgesia. Preliminary results indicated that cox-2, but not cox-1, protein levels increased following the induction of a K/C. A significant increase in cox-2 but not cox-1 levels has been shown by Gardiner (Gardiner et al. 1997) using a chronic model of arthritis, induced by FCA inoculation of the ankle joint, and Beiche et al. (1998b) following injection of CFA into the rat hindpaw.

The third aim of this study was to determine if spinally applied NSAIDs, which are inhibitors of cox activity, would affect the electrophysiological response of dorsal horn neurons to a noxious mechanical stimulus, and more specifically to see if an effect was apparent in hyperalgesic but not non-hyperalgesic states. The results showed that in the non-hyperalgesic rat, spinally applied meclofenamic acid or indomethacin had no effect upon the neuronal response. Following the induction of both an acute K/C arthritis and a chronic FCA arthritis some, but not all, deep dorsal horn neurons were classified as hyperexcitable using the criteria of Grubb et al. (1993, 1996). The responses of hyperexcitable but not non-hyperexcitable deep dorsal horn neurons were significantly reduced by the spinal application of both indomethacin and meclofenamic acid. The concentrations of indomethacin used were in the same range as those shown to significantly reduce the wind-up of a nociceptive spinal reflex in the experiments reported in Chapter 6. The results from these experiments, using both acute and chronic models of arthritis, support the theory that

spinal PGs are not involved in acute nociceptive responses but that PG production is part of the mechanism by which hyperexcitable neurons respond to noxious mechanical joint pressure in hyperalgesic animals.

The final aim of this study was to determine whether spinally administered cox inhibitors would affect the wind-up of a nociceptive spinal reflex. Preliminary experiments showed that it was possible, using controlled parameters, repeatedly to induce a reproducible wind-up of the hindlimb flexor withdrawal reflex of the rat recorded as a single-unit emg over a period of hours. This novel model was used to study the effects of a non-selective cox inhibitor (indomethacin) and a selective cox-2 inhibitor (SC58125) on C-fibre wind-up.

Briefly, it was shown that intravenous administration of both indomethacin and SC58125 dose-dependently reduced C-fibre wind-up. Further, intrathecally applied indomethacin at the same concentrations that reduced the responses of hyperexcitable dorsal horn cells to noxious mechanical pressure also reduced C-fibre wind-up. Solubility and toxicity problems prevented the intrathecal administration of SC58125. However, it is tempting to infer by analogy that selective cox-2 inhibitors administered intrathecally would also reduce C-fibre wind-up. Since it has been demonstrated that cox-2 is the main isoform found within the spinal cord, it is probable that the effects of intrathecal indomethacin were mainly, if not entirely, due to cox-2 inhibition. A component of the indomethacin effect could also be due to inhibition of cox-1 activity in the DRGs.

As wind-up is a phenomenon associated with hyperalgesia, these results support the hypothesis that PG production is involved in hyperalgesic responses.

7.2 Hypothetical Model of Spinal Cord Changes in Hyperalgesia

Evidence has been presented that cox-2 protein is present in the spinal cord in locations previously associated with nociception (Gardiner et al. 1997; Willingale et al. 1997). Levels are increased during the development of both acute and chronic arthritis (Gardiner et al. 1997; Gardiner 1998; Beiche et al. 1998b; Ebersberger et al. 1999), models that have previously been shown to be associated with the development of central hyperalgesia (Menétrey & Besson 1982; Neugebauer & Schaible 1990; Grubb et al. 1991, 1993; Schaible et al. 1991; Neugebauer et al. 1993, 1994b, 1995). There is a body of evidence to suggest that PGE_2 is released during central hyperalgesia (Willingale et al. 1997; Gardiner 1998; Muth-Selbach et al. 1999). A recent study using antibody microprobes demonstrated PGE_2 release from the spinal cord within hours of the induction of a K/C arthritis, in parallel with an increase in cox-2 levels (Ebersberger et al. 1999).

Inhibiting spinal PG release by intrathecal NSAIDs has been shown to:

- attenuate the response of hyperexcitable dorsal horn cells to mechanical stimulation (Chapter 5)
- reduce wind-up of a nociceptive reflex (Chapter 6)
- attenuate the enhanced neuropeptide release in the hyperalgesic spinal cord (Southall et al. 1998)
- reduce electrophysiological changes in spinal cord neurons during the development of hyperalgesia (Neugebauer et al. 1995)
- reduce flinching behaviour in the second phase of the formalin test (Muth-Selbach et al. 1999)

A simple theory is proposed to explain a role for PGs in the spinal cord in nociception associated with hyperalgesia. The development of central hyperalgesia is triggered by an increased afferent input from C-fibres, which may be caused by a variety of peripheral chemical, thermal or mechanical insults. Arachidonic acid is split from the cell membrane by the action of phospholipase A_2. Cox-2 activity converts the AA to

PGE_2, which diffuses out of the postsynaptic cell. PGE_2 acts at both pre- and post-synaptic EP_3 receptors (shown to be located in afferent terminal areas by Beiche et al. 1998a) to further increase intracellular calcium ion concentrations. Pre-synaptically, this enhances the release of SP and glutamate. Glutamate acts at AMPA receptors to increase the inflow of cations into the postsynaptic cell. SP acts at NK_1 receptors to activate protein kinase C (PKC), via inositol triphosphate and diacyl glycerol pathways. Phosphorylation of the NMDA receptor by PKC increases the cation influx. Rapid EPSPs due to the AMPA receptor activation and sustained depolarisation due to NK_1 receptor activation raise the membrane potential of the postsynaptic cell, overcoming the voltage-dependent block on the NMDA receptors and making the cell increasingly more likely to depolarise. A number of processes thus act together to produce positive feedback, leading to hyperexcitability of the cell. This is shown diagrammatically in Figure 7.1.

Wind-up has similarities with this model in that a C-fibre barrage causes temporal summation of the EPSPs, as discussed in Section 1.5, allowing depolarisation of the post-synaptic neuron with a similar train of consequences.

It is proposed that all the pathways shown in Figure 7.1 act in concert, and that interference with any stage can affect the development of hyperexcitability. Thus NSAIDs and other cox-2 inhibitors inhibit cox-2 activity, reducing the production of PGs and limiting the activation of pre- and post-synaptic PG receptors. It has been demonstrated in the work presented above that NSAIDs and a specific cox-2 inhibitor reduce the electrophysiological responses of hyperexcitable neurons and wind-up. This positive feedback pathway involving PG release would only come into play when there is an increase in the C-fibre firing rate, ie in the development of hyperalgesia (or wind-up). The nociceptive response in the non-hyperalgesic animal would not require this loop, which explains the lack of a spinal effect of NSAIDs in non-hyperalgesic states. There is a large body of work in the literature to show that NMDA receptor inhibitors and NK1 receptor inhibitors, as discussed in Section 1.4.2 reduce hyperexcitability and hyperalgesia. This model explains the additive effects of

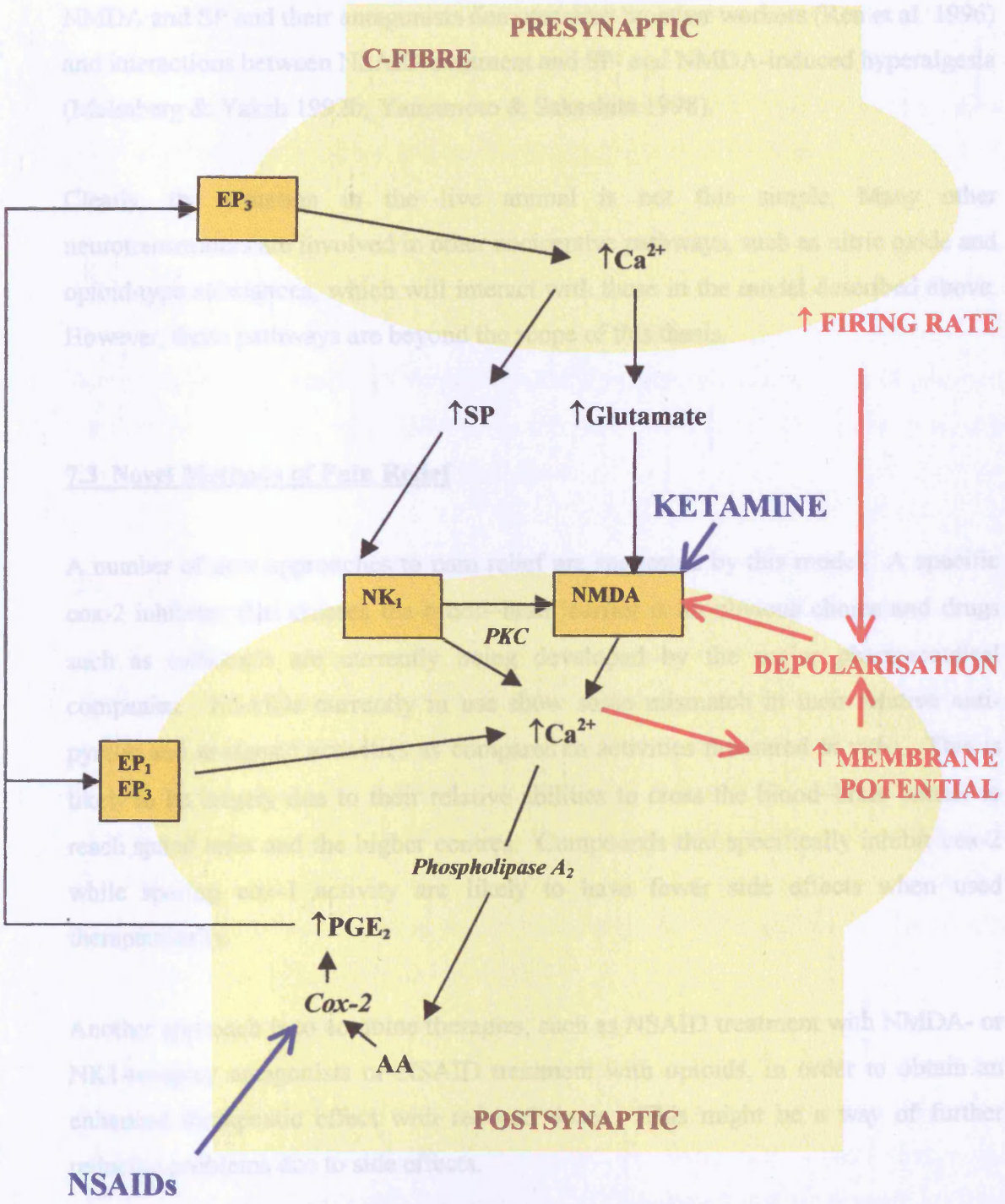

Figure 7.1. A hypothetical diagram of the processes that may occur during the induction of central hypersensitivity. Inhibitory actions are shown in blue.

NMDA and SP and their antagonists demonstrated by other workers (Ren et al. 1996) and interactions between NSAID treatment and SP- and NMDA-induced hyperalgesia (Malmberg & Yaksh 1992b; Yamamoto & Sakashita 1998).

Clearly, the situation in the live animal is not this simple. Many other neurotransmitters are involved in other nociceptive pathways, such as nitric oxide and opioid-type substances, which will interact with those in the model described above. However, these pathways are beyond the scope of this thesis.

7.3 Novel Methods of Pain Relief

A number of new approaches to pain relief are suggested by this model. A specific cox-2 inhibitor that crosses the blood–brain barrier is an obvious choice and drugs such as rofecoxib are currently being developed by the major pharmaceutical companies. NSAIDs currently in use show some mismatch in their relative anti-pyretic and analgesic activities as compared to activities measured *in vitro*. This is likely to be largely due to their relative abilities to cross the blood–brain barrier to reach spinal sites and the higher centres. Compounds that specifically inhibit cox-2 while sparing cox-1 activity are likely to have fewer side effects when used therapeutically.

Another approach is to combine therapies, such as NSAID treatment with NMDA- or NK1-receptor antagonists or NSAID treatment with opioids, in order to obtain an enhanced therapeutic effect with reduced doses. This might be a way of further reducing problems due to side effects.

NMDA receptor antagonists such as MK-801 and APV are toxic therefore cannot be used clinically. A recently suggested approach is to use antisense oligonucleotides targeted to the NMDA-RI receptor subunit. This has shown encouraging results in

rats (Garry et al. 2000) and suggests that gene therapy might be a novel method to manage chronic pain. EP receptors and the NK_1 receptor could similarly be targeted.

Whatever the future developments, the work described in this thesis has shown a clear role for prostanoids in spinal nociceptive processing in hyperalgesia.

7.4. Subsequent Findings

Since this experimental work was undertaken, a number of studies have extended our knowledge of the role of prostanoids in the spinal cord some of these have already been mentioned in the body of the work above.

The demonstration of an increase in the concentration of cox-2 in spinal cord during the development of an acute kaolin and carrageenan-evoked arthritis was complemented by a parallel study by Gardiner (Gardiner 1998) who demonstrated a similar increase in cox-2 concentration during the development of a chronic FCA arthritis. Subsequently mRNA for cox-2 was shown to be upregulated in similar models of peripheral inflammation over a similar time course (Goppelt-Struebe & Beiche 1997; Beiche et al. 1998b). Recently unilateral paw inflammation evoked by FCA injection into the rat hindpaw evoked a similar increase in cox-2 mRNA expression in the lumbar spinal cord that was initially unilateral but was bilaterally symmetrical by 12 hours (Samad et al. 2001).

Confirmation that the cox-2 concentration increases were of functional significance was the demonstration be Ebersberger et al. (1999) that there is an enhanced release of PGE_2 from the lumbar spinal cord over the same time course during the development of K/C arthritis. Samad et al. (2001) demonstrated an increase in cox-2 protein in the spinal cord following peripheral inflammation, associated with a substantial increase in PGE_2 concentrations in the cerebrospinal fluid (CSF). These

findings support the pattern of biochemical changes in the spinal cord described in this work.

Samad et al. (2001) further showed that phospholipase A_2 (PLA_2) activity in the spinal cord remained unchanged during inflammation, implying that the basal level of PLA_2 activity is sufficient to support an increase in central PG production following peripheral inflammation. Complete sensory and motor blockade of the sciatic nerve reduced but did not eliminate the induction of spinal cox-2 mRNA and PGE_2 CSF release. Samad et al. also showed that not only did peripheral inflammation cause an increase in type-1 interleukin-1β (IL-1β) in the CSF that preceded the peak up-regulation of cox-2 mRNA but also that intrathecal administration of type-1 IL-1β induced cox-2 mRNA upregulation and PGE_2 release. This work supports the hypothesis of spinal cord changes during hyperalgesia postulated above and proposes the possibility that inhibiting central IL-1β upregulation or IL-1β receptor activity may be another practical means of pain relief after local inflammation.

Semi-quantitative RT-PCR techniques were recently used to identify all the EP receptor subtype mRNAs and splice variants in the rat spinal cord (Donaldson et al. 2001). EP_4 receptor mRNA was upregulated and $EP_{3A/\alpha}$ receptor mRNA was downregulated during the 3 days following the induction of a FCA ankle inflammation. The significance of these findings has yet to be elucidated

Interestingly, a different pattern of EP receptor subtype regulation was seen in the dorsal root ganglia (Donaldson et al. 2001). It has been suggested that cox-1 rather than cox-2 is an important marker of putative nociceptive neurons in the dorsal root ganglia (Chopra et al. 2000), implying that the two cox isoforms play independent roles in nociception. Work to elucidate the role of cox in the dorsal root ganglia is ongoing.

Strains of cox-1 and cox-2 deficient mice have been developed which, although infertile, have normal longevity and no severe renal pathology (Ballou et al. 2000).

The mean reaction times of cox-1-null and cox-2-null mice to the hot-plate test were the same as for normal mice although cox-1-defiecient mice showed increased reaction times. Both cox-1-null and cox-1-deficient mice showed a decrease in nociception as measured by the number of writhes following intraperitoneal acetic acid. Cox-2-deficient and cox-2-null mice generally showed the same responses to this test as wild-type mice although cox-deficient female mice had reduced responses to this test. The reason for a sex-difference is unknown at this stage. Interestingly, cox-1 mRNA levels in spinal cord from cox-2 deficient and cox-2-null mice were increased compared with wild-type mice, suggesting that cox-1 mRNA levels may be raised to compensate for a lack of cox-2 mRNA. No reciprocal increase in cox-2 mRNA to compensate for a lack of cox-1 was seen in the spinal cord of cox-1-deficient mice. It would be interesting to use 'knockout' animals to investigate any differences in electrophysiological responses to mechanical stimulation in hyperalgesia and to study any alterations in C-fibre wind-up.

Subsequent studies have therefore supported the hypothesis proposed in this thesis that cox-2 in the spinal cord is of functional significance in increasing PG activity during peripheral inflammation and that PGs play a role in nociception in hyperalgesia. Such work is of practical significance since it has provided direction for the development of drugs not only against cox-2 but has also suggested other targets amongst the pathways involved in the development and maintainence of hyperalgesia.

Appendix 1

DOS script for analysing extracellular recording experiments using Spike2.

A1ANALYS.TXT

Analysis uses a cursor to discriminate the spikes from the background.

```
fkey 0                                  'sets function keys
fkey 1 2 mask"mask"                     'associate key 2 with mask
fkey 1 3 sum"sum"                       'associate key 3 with sum
repeat
fkey 4                                  'wait for function key, do proc
until escape
end


proc mask
view 1;rate 2 1                         'rate histogram on channel 2
minmax 2 0 maxtime a b;yrange 2 0 b     'set yrange of channel 2
draw 0 maxtime
hcursor 2                               'add horizontal cursor
cursors 0 1                             'display position of hcursor
interact "set horizontal cursor to discriminate stimuli"
clear
x:=10
y:=5
move to x y
printto 1
print "cell number %s" fname[3]+fname[4]  'prints cell number from filename at
                                          position (10,5) on screen
return
```

```
proc sum
a:=0
x:=10                                   'sets x position of display on screen
y:=10                                   'sets y position of display on screen
for i:=0 maxtime-1
count 2 i i+1 a                         'count channel 2 from i to i+1 and
                                        call it a
if a>hc;count 2 i i+16 s                'if value . HC, count next 16 s, call it
                                        s
        count 2 i-16 i b                'count previous 16 s, call it b
moveto x y                              'sets print position on screen
printto 1                               'print to screen
print "time:   %6.0d     evoked response:  (%4.0d-%4.0d) = %4.0d"i s b s-b
i:=i+50                                 'sets the search interval - can adjust
                                        this
y:=y+4                                  'set to 3 if there are a lot of results
endif
next i
return
```

DOS script for analysing the dynamic and static responses of extracellular recording experiments

DYSTAT

```
fkey 0                                  'sets function keys
fkey 1 2 mask"mask"                     'associate key 2 with mask
fkey 1 3 sum"sum"                       'associate key 3 with sum
repeat
fkey 4                                  'wait for function key, do proc
until escape
end


proc mask
view 1;rate 2 1                         'rate histogram on channel 2
minmax 2 0 maxtime a b;yrange 2 0 b     'set yrange of channel 2
draw 0 maxtime
hcursor 2                               'add horizontal cursor
cursors 0 1                             'display position of hcursor
interact "set horizontal cursor to discriminate stimuli"
clear
x:=10
y:=5
move to x y
printto 1
print "cell number %s" fname[3]+fname[4]  'prints cell number from filename at
                                          position (10,5) on screen
return
```

```
proc sum
a:=0
x:=1                                    'sets x position of display on screen
y:=10                                   'sets y position of display on screen
for i:=0 maxtime-1
count 2 i i+1 a                         'count channel 2 from i to i+1 and
                                        call it a
if a>hc;count 2 i i+3 d                 'd = dynamic phase: first 3 s
        count 2 i+3 i+16 s              's = static phase: 4-16 s
        count 2 i+13 i+16 o             'o = off phase: 13-16 s
        count 2 i-16 i-13 j             'j = background for d
        count 2 i-13 i k                'k = background for s
        count 2 i-3 i l                 'l = background for o
moveto x y                              'sets print position on screen
printto 1                               'print to screen
print "time: %6.0d  dynamic: (%4.0d-%3.0d) = %4.0d" i d j d-j
moveto x+42 y
print "static: (%4.0d-%3.0d) = %4.0d" s k s-k
moveto x+72 y
print "off: (%4.0d-%3.0d) = %4.0d" o l o-l
i:=i+50                                 'sets the search interval - can adjust
                                        this
y:=y+4                                  'set to 3 if there are a lot of results
endif
next i
return
```

Appendix 2

Western Blotting Recipes.

Homogenisation Buffer

150 mM NaCl

10 mM Tris (pH 7.4)

1 mM EDTA

1 mM EGTA

1% Triton X-100

Just before use add:

0.2mM PMSF

2 μg/ml Aprotinin

3X Running Buffer

For 1 litre:

10 g Tris Base

3.33 g SDS

48 g Glycine

Sample Buffer

For 100 ml:

1.7 ml Glycerol

2.5 ml 3X Running Buffer

0.29 ml 100 mM EDTA

0.171 g SDS

0.27 g Sucrose

0.007 g Bromophenol Blue

0.01 g Tris Base

0.048 g Imidazole

Resolving Gel

For 4 Minigels:

10 ml 30% Acryl

10 ml Water

10 ml 3X Running Buffer

Then add 40 µl TEMED

and 160 µl 10% Ammonium Persulphate Solution.

Swirl gently to mix and use immediately.

Stacking Gel

For 4 Minigels:

2.4 ml Acryl

6.0 ml Imidazole Stacking Buffer

9.6 ml Water

Then add 24 µl TEMED

and 150 µl 10% Ammonium Persulphate Solution

Swirl gently to mix and use immediately

Imidazole Stacking Buffer

For 100 ml:

1.29 g Tris Hydrochloride

4.75 g Imidazole

0.3 g SDS

pH to 6.8 with HCl then bring up to 100 ml with water.

SDS-PAGE Transfer Buffer

For 1 litre:

5.82g Tris Base

3.0 g Glycine

0.39 g SDS

200 ml Methanol

TTBS

For 1 litre:

6.05 g Tris Base

8.75 g NaCl

1 ml Tween 20

pH solution to 8 with HCl.

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