Investigations into central mechanisms of pain transmission

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Declaration

I, Jill Price, do hereby declare that the work carried out in this thesis is original, was carried out by myself or with due acknowledgement, and has not been presented for the award of a degree at any other University.

And a woman spoke, saying, Tell us of pain.

And he said:

Your pain is the breaking of the shell that encloses your understanding.

Even as the stone of the fruit must break, that its heart may stand in the sun, so must you know pain.

And could you keep your heart in wonder at the daily miracles of your life, your pain would not seem less wondrous than your joy……

And you would watch with serenity through the winters of your grief.

For much of your pain is self chosen.

It is the bitter potion by which the physician within you heals your sick self.

Therefore trust the physician, and drink his remedy in silence and tranquillity;

For his hand, though heavy and hard, is guided by the tender hand of the Unseen,

and the cup he brings, though it burn your lips, has been fashioned of the clay which the Potter has moistened with his own tears.

From: *The Prophet,* Kahlil Gibran

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Drugs and Chemicals

AMPA	α-amino-3-hydroxy-5-4-isoxazolproprionate
AR	adrenoreceptor
Ati	atipamezole
CaMKII	calcium calmodulin kinase II
CGRP	calcitonin gene-releasing peptide
CFA	complete Freund's adjuvant
COX	cyclo-oxygenase
Cx	carrageenan
CGRP	calcitonin gene-releasing peptide
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
FCS	foetal calf serum
GAD	glutamic acid decarboxylase
GABA	γ-amino butyric acid
HBSS	Hank's balanced salt solution
IEG	immediate early gene
ITF	inducible transcription factor
KA	kainate
MEM	memantine
MTT	3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H tetrazolium bromide
NADPHd	nicotinamide adenine dinucleotide phosphate diaphorase
NGS	normal goat serum
NK	neurokinin
NMDA	N-methyl D-aspartate
NTX	naltrexone
PFA	paraformaldehyde
SP	substance P
TPA	tissue plasminogen activator

Terminology and constituents of the central nervous system

EPSP	excitatory post-synaptic potential
IPSP	inhibitory post-synaptic potential
IR	immunoreactivity
ISH	*in-situ* hybridisation
LC	locus coeruleus
LSC	locus subcoeruleus
LTD	long term depression
LTP	long term potentiation
MWT	mechanical withdrawal threshold
NRM	nucleus raphe magnus
PAG	periaqueductal grey matter
PC	paw circumference
RVM	rostroventral medulla
SEM	standard error of the mean
TWL	thermal withdrawal latency
WDR	wide dynamic range

ABSTRACT

The pain transmission system is inherently plastic in nature; plasticity of nociceptive processing in the dorsal horn of the spinal cord is believed to contribute to clinical states of post-injury pain hypersensitivity. Both enhancement and tachyphylaxis of nociceptive processing have been reported previously following repeated carrageenan-induced inflammation. The present study aimed to investigate central mechanisms involved in the transformation of pain transmission from 'physiological' to 'pathophysiological' in adult rats, using a model of mild intraplantar inflammation induced by intraplantar administration of carrageenan at doses markedly lower than those standardly used in research into central mechanisms of inflammatory pain transmission. Changes in plantar inflammation, thermal and mechanical sensitivity were assessed following intraplantar injection of repeated doses of carrageenan (0.5%, corresponding to a dose of 0.25 mg and 0.1%, corresponding to a dose of 0.05 mg), administered at weekly (0.5% and 0.1%) and daily (0.1%) intervals. Expression of mRNA of key genes implicated in plasticity of central spinal pain transmission in laminae I, II and V of the dorsal horn of the lumbar spinal cord (laminae involved in central nociceptive transmission) was investigated using *in-situ* hybridisation techniques. The genes investigated were calcium calmodulin kinase IIα (CaMKIIα), a key intracellular molecule instantaneously activated by neuronal stimulation; alterations in CaMKIIα expression can rapidly induce nociceptive plasticity through modulation of many excitatory and inhibitory nociceptive mediators; the cyclo-oxygenase enzymes COX-1 and COX-2, which catalyse prostaglandin synthesis and are implicated in the modulation of the central nociceptive response to inflammatory injury; the immediate early genes zif//268, junD and tissue plasminogen activator (tPA), which have been implicated in the induction and maintenance of neuronal plasticity in higher centres, and the precursors for the inhibitory neurotransmitter molecules γ-amino butyric acid (GABA), enkephalin and dynorphin. A method for organotypic culture of neonatal spinal cord was developed and characterised with the aim of providing a useful technique for more detailed study of the molecular basis of nociceptive plasticity.

Mild inflammatory injury induced by 0.5% and 0.1% carrageenan treatment induced consistent hyperalgesic behaviour, which did not change following weekly repeated

injection. Temporary attenuation of hyperalgesia developed following daily repetitive administration of 0.1% carrageenan, but hyperalgesia returned when this repetitive inflammatory stimulation was maintained. Preliminary studies on the role of NMDA receptors, opioid receptors and α_{2A} adrenoreceptors in the mediation of this tachyphylaxis suggest that these receptor systems did not play a major role in the observed tachyphylaxis

In-situ hybridization studies did not identify changes in gene expression induced by repetitive carrageenan treatment in lamina V. In laminae I/II, changes were observed in expression of certain genes (notably CaMKIIα, COX-2 and proenkephalin), but not of immediate early genes, GAD 67 or prodynorphin. Hyperalgesia associated with weekly carrageenan treatment correlated closely with significantly enhanced transcription of CaMKIIα mRNA in laminae I/II; moreover, tachyphylaxis of hyperalgesic behaviour correlated with attenuation of CaMKIIα upregulation. Since increased expression of CaMKIIα, leading to regulation of expression of a range of kinase-dependent receptors and intracellular mediators, is a hallmark of neuronal plasticity in higher centers, this suggests that central plasticity of nociceptive transmission in the dorsal horn could have contributed to the development of hyperalgesia following carrageenan treatment. Weekly administration of carrageenan also consistently induced significant upregulation of COX-2 and proenkephalin mRNA expression in laminae I/II, suggesting that ultimate modulation of pain sensation following inflammatory injury is determined by the interaction of excitatory and inhibitory transmitter pathways. COX-1, prodynorphin and GAD 67 mRNA expression were not significantly changed in relation to the intensity of inflammatory injury or in relation to changes in nociceptive responses. This would suggest that these mediators did not play a key role in the modulation of spinal nociceptive transmission associated with mild inflammatory injury. With the possible exception of CaMKIIα, changes in gene expression did not correlate closely with plasticity of nociceptive behaviour induced by daily repeated carrageenan treatment.

200 μm transverse slices of postnatal spinal cord were cultured successfully for up to 5 days using a simple interface culture system. Histochemical and immunocytochemical assays indicated that the architecture of organotypically cultured spinal cord closely resembled that observed *in-vivo*.

This study presents a new approach to the investigation of neuronal plasticity associated with tissue injury and inflammation. Different mechanisms underlying plasticity of

nociceptive responses may be induced by induced by high intensity as opposed to low-intensity injury. The observation of tachyphylaxis of hyperalgesia following daily repeated carrageenan treatment may represent engagement of endogenous 'anti-hyperalgesic' mechanisms. Further investigation of the molecular basis of endogenous 'anti-hyperalgesia', facilitated by organotypic slice culture techniques, may identify new targets for the treatment and prevention of persistent pathological nociceptive transmission following inflammatory injury.

CHAPTER 1: INTRODUCTION

The truth is out there

(Carter, 1999)

1.1 'PHYSIOLOGICAL' PAIN TRANSMISSION: THE NOCICEPTIVE PATHWAY

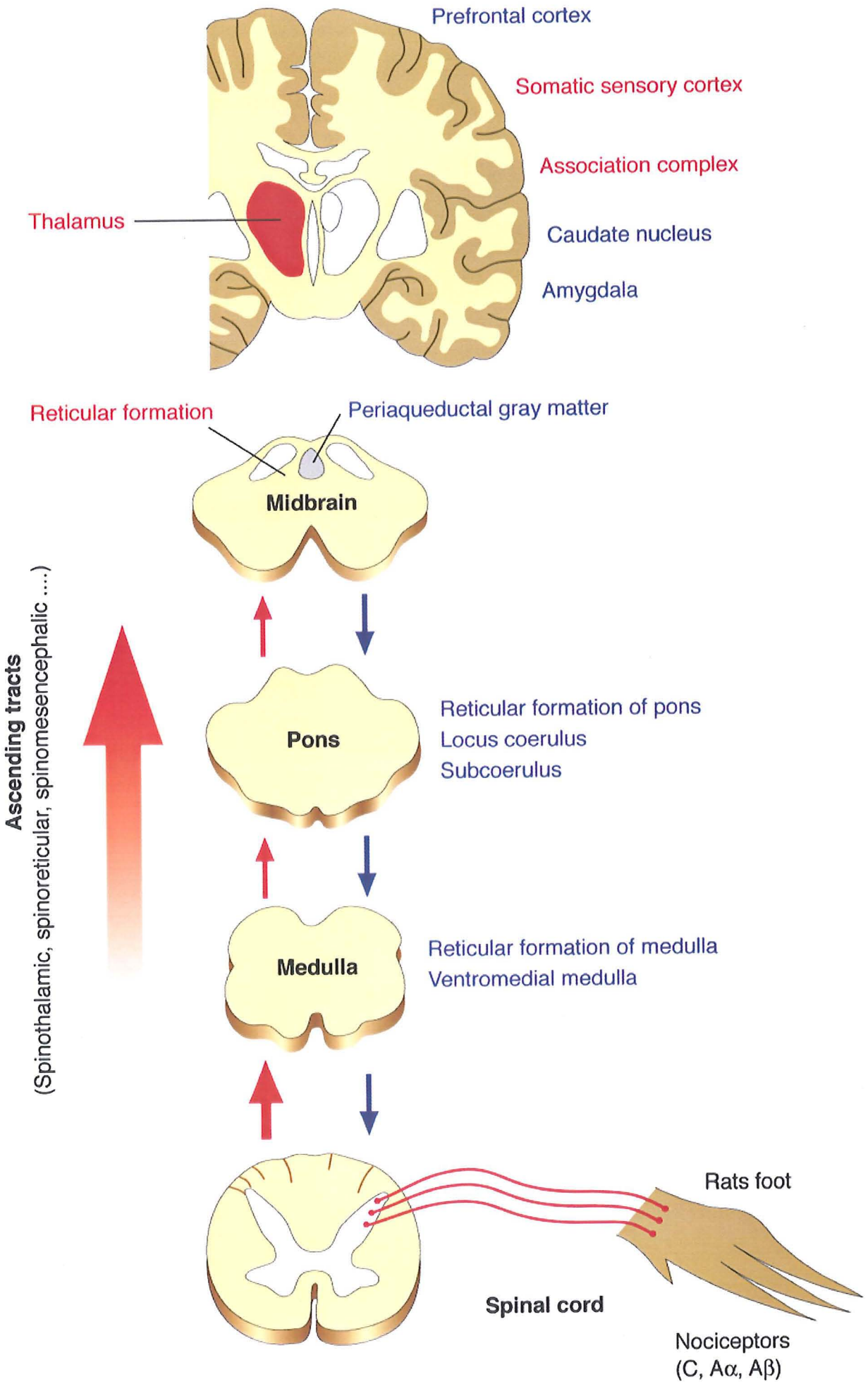
Mankind has struggled to understand the nature of pain since time immemorial. The conscious experience of 'pain' defies precise anatomical, physiological and or pharmacological definition; unlike any other sensory experience, it is a subjective emotion which can be experienced even in the absence of obvious external stimulation, and which can be enhanced or abolished by a wide range of behavioural experiences. It cannot be seen or directly measured, but its patterns can be recognised. While elusive and ill defined, 'pain' nonetheless has substance and specific characteristics. We now recognise that conscious perception of pain is not the result of a simple transfer of information via ascending nociceptive pathways from the periphery to the brain, but rather the result of interplay of facilitatory and inhibitory pathways, which use an orchestra of interacting neurotransmitter systems. The highly subjective nature of pain perception is one of the factors which make it difficult to define and treat clinically.

The neuroanatomy of the pain system and of the pathways that modulate pain perception are illustrated in figure 1.1.

Pain pathways

Following tissue injury, nociceptive information is relayed from the periphery to the CNS by nociceptors, the peripheral endings of primary sensory neurons whose cell bodies are located in the dorsal root and trigeminal ganglia. The structure and functional characteristics of the peripheral nociceptor have been reviewed by Casey (1992), Handwerker & Reeh (1992) and Willis *et al.* (1995a). Nociceptors, first described by Sherrington in 1906, are the least differentiated of the cutaneous sensory receptors, existing as free nerve endings lacking peripheral structures to filter or transduce external stimuli. Three types of afferent fibre conduct nociceptive information: C-fibres, Aδ fibres and Aβ fibres. Their properties are reviewed by Jessell & Kelly (1997) and Millan (1999).

Figure 1: The pain transmission pathway



Unmyelinated polymodal C fibres are activated by intense mechanical, chemical and thermal stimuli and conduct impulses relatively slowly at approximately 0.5m/s. Thermal and mechanical stimulation thresholds of C fibres are substantially higher than those of other types of afferent sensory fibre, requiring, for example, noxious thermal stimulation at temperatures above 45°C to elicit a response. Physiologically, the C-nociceptor responds directly to increasing intensity of noxious stimulation- the greater the stimulus strength, the more vigorous the response (reviewed by Besson & Chaouch, 1987). Repeated stimulation, however, enhances C-nociceptor responsivity to a given stimulus strength (LaMotte, 1984, reviewed by Campbell *et al.,* 1989). In contrast to other sensory afferent fibres, C-nociceptors have no background discharge pattern under physiological conditions but can develop a tonic discharge pattern following a single vigorous stimulation (Perl 1992). Pain in the absence of ongoing external stimulation has been associated with the development of background activity in sensitised C-nociceptors (Jessell & Kelly, 1997).

Thermal and mechanical nociceptors may also be supplied by finely myelinated Aδ fibres, which conduct impulses at 5-30m/s. Larger diameter low-threshold Aβ fibres (which normally relay sensory information from mechanoreceptors) are also capable of transmitting nociceptive signals to higher centres and are believed to contribute to the development of pathological pain sensation (reviewed by Ren, 1996) , although they are not normally involved in nociceptive transmission (Price & Dubner, 1977, Wiesenfeld-Hallin *et al.,* 1984).

Axons of Aδ and C nociceptive fibres bifurcate on entering the spinal cord as they pass through the dorsal root ganglia and ascend and descend for some segments as constituents of the dorsolateral fasciculus of Lissauer, while collaterals synapse with projection neurons in the dorsal horn of the spinal cord [reviewed by Grant (1995); Jessell and Kelly, 1997].

The division of the grey matter of the spinal cord into distinct laminae, according to neuronal type and function, was described by Rexed (1952; 1954) and reviewed by Molander & Grant (1995). Laminae I-V comprise the dorsal horn. Nociceptive fibres terminate primarily in the superficial dorsal horn (laminae I/II) although some fine nociceptor axons terminate in deeper laminae (notably laminae V-VI) and close to the central canal (lamina X). Nociceptive afferent neurons form direct or indirect connections with three major classes of neuron in the dorsal horn: projection neurons which relay incoming sensory information to higher centres in the brain; excitatory interneurons; and inhibitory interneurons which regulate and modulate the flow of nociceptive information to higher centres.

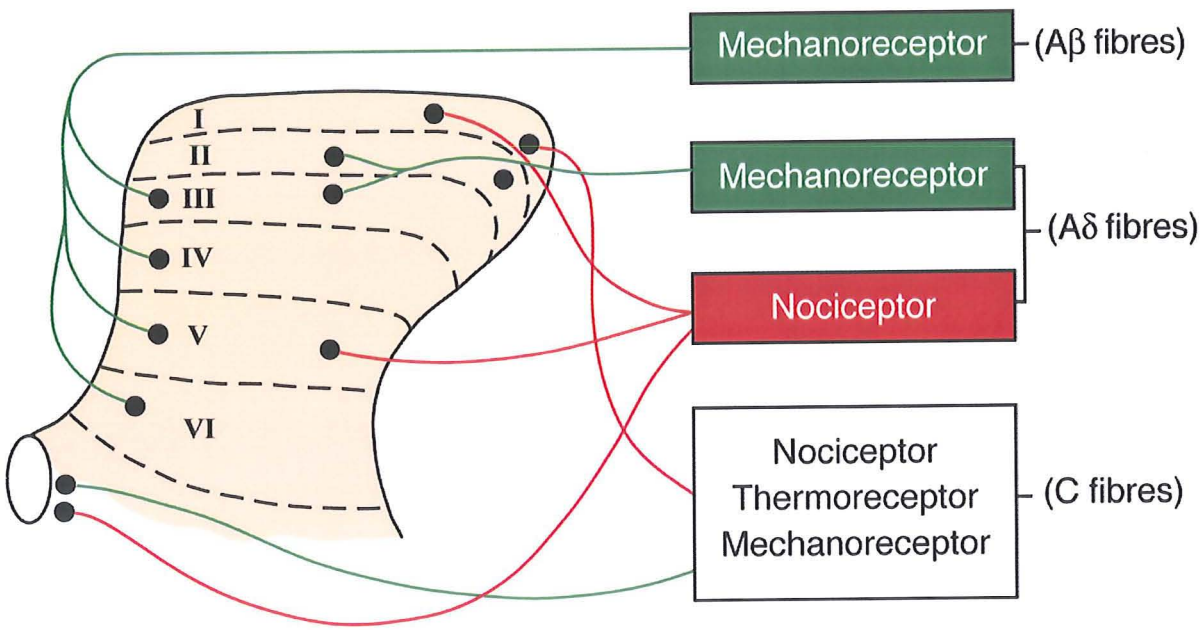
The neurons of the dorsal horn have been classified into three types according to the nature of their response to nociceptive input (reviewed by Millan, 1999). Non-nociceptive neurons occur predominantly in laminae II, III and IV, although a small number are present in lamina I. Nociceptive-specific (NS) neurons are activated exclusively by high-intensity noxious stimuli mediated by C and Aδ fibres and are concentrated in laminae I/II (although also present in lamina V). These neurons have very limited capacity to encode stimulus intensity. Wide-dynamic range (WDR) neurons display considerable convergence from cutaneous, muscular and visceral input (Mense, 1986; 1995). WDR neurons are found predominantly in lamina V but also in laminae I, II, III,VI and X and also occur in the ventral horn. WDR neurons encode stimulus intensity (Dubner *et al.,* 1989) and are excited by thermal, mechanical and thermal stimuli mediated by C, Aδ and Aβ fibres, producing a response which is directly related to the intensity of stimulation.

Figure 1.2 illustrates the laminar arrangement of the rat spinal dorsal horn.

Lamina I, classically named 'the marginal zone', contains a high density of projection neuronswhich process nociceptive information. It is composed principally of large horizontal neurons (the marginal cells of Waldeyer) and a plexus of horizontally arranged fine axons. Lamina I neurons have been divided into two populations on a functional basis (Jessell & Kelly, 1997): one 'nociceptor-specific' population is excited solely by C and A-δ nociceptor fibres, the second 'wide dynamic range' population receives input from low-threshold mechanoreceptors in addition to nociceptors. Axons of lamina I neurons project to other regions of the spinal cord and also to the thalamus, midbrain, reticular formation in the brain stem, and cerebellum. Lamina I receives input from both fine unmyelinated axons (C fibres) and larger myelinated A-δ axons)(Gobel *et al.,* 1981)

Lamina II, termed ' the substantia gelatinosa' due to its distinctive gelatinous appearance, is composed predominantly of small neurons and their unmyelinated processes. Lamina II constitutes the main projection area for cutaneous C-fibres while also receiving sparse primary afferent input from Aδ fibres (LaMotte, 1977; Sugiura *et al.,* 1986). Lamina II also receives C-fibre input from visceral areas. Most lamina II neurons display extensive local axonal arborisation, such that even 'projection neurons', which relay information to

Figure 1.2
The termination of afferent fibres in the dorsal horn of the spinal cord



distant centres, also integrate locally. Lamina II neurons have multiple projection targets. Some 'true' projection neurons project out of the spinal cord; some 'propriospinal' neurons project to distant spinal segments; some project to other laminae in the same or adjacent spinal segments; and some neurons communicate with other neurons in lamina II(Millan, 1999).

Lamina III forms a broad band across the dorsal horn, distinguished from lamina II by the presence of slightly larger neurons with myelinated axons. The predominant primary afferent input into lamina III is from fibres transmitting sensory information from hair follicles; other primary afferent fibres entering lamina III arise from pacinian corpuscles and Aβ fibres; many cells in lamina III respond only to mild mechanical stimulation (Cervero *et al.,* 1988)

Laminae IV and V are similar in terms of their composition and the axonal destinations of their projections. Primary afferent input to lamina IV comes from large primary afferent (Aβ) fibres organised into a prominent longitudinal plexus; as in lamina III, many cells in lamina IV respond only to light mechanical stimulation, but nociceptive specific and wide dynamic range fibres (C and Aδ) are also present (Cervero *et al.,* 1988). Some of the cells of lamina IV are post-synaptic dorsal column and spino-cervical tract cells which communicate with neurons located in superficial laminae. The axons of cells in lamina IV pass to the thalamus, the lateral cervical nucleus, the dorsal column nuclei, and other regions of the spinal cord. Lamina V forms a thick band across the narrowest part of the dorsal horn and is composed of a heterogeneous population of neuronswhich project principally to the thalamus, the lateral cervical nucleus, dorsal column nuclei and local destinations in the spinal cord.

Lamina VI, described only in the cervical and lumbosacral enlargements of the spinal cord, is a transition between the primary afferent dominated dorsal horn, and the ventral horn in which motor activity is co-ordinated and ultimately initiated (Willis & Coggeshall, 1991b). Lamina VII corresponds to the intermediate grey matter, while laminae VIII and IX comprise the medial and lateral ventral horn respectively. These laminae have not been accorded an important role in the transmission of nociceptive information. Lamina X is the area surrounding the central canal. The cells are generally small and more densely packed than those in the adjacent lamina VII. Many lamina X cells respond to noxious stimuli (Nahin *et al.,* 1982). Cells from lamina X project to the brainstem (Nahin *et al.,* 1983), the hypothalamus (Burstein *et al.,* 1987) and the thalamus (Burstein *et al.,* 1990).

Nociceptive input into the dorsal horn originating from sensory stimulation of the body is relayed to higher centres by projection neurons, which are organised into so-called 'ascending pathways'. Ascending pathways are species-specific, bilaterally symmetrical and for the most part cross to the contralateral cortex for perception and control. There are numerous differences between species in anatomical location and relative importance of ascending nociceptive pathways. The following description refers to the rat, where the major ascending nociceptive pathways in the spinal cord are the spinothalamic tract, the spinoreticular tract and the spinomesencephalic tract (reviewed by Willis (1985), Willis & Coggeshall (1991), Tracey (1995) and Willis *et al.* (1995).

The spinothalamic tract, the most prominent ascending pathway in the primate (Jessell and Kelly 1997), carries the axons of nociceptive-specific and wide-dynamic range neurons originating in laminae I, VI and VII to terminate in the thalamus. The spinoreticular tract in the rat originates from neurons concentrated in laminae V,VII, VIII and X (Chaoch *et al.,* 1983) to terminate in the thalamus and the reticular formation (Willis 1985; Willis & Coggeshall, 1991). The spinomesencephalic tract, another major ascending tract in the primate (Jessell & Kelly, 1997) carries axons originating in laminae I and V up to the midbrain, notably the mesencephalic reticulum and the lateral periaqueductal grey matter (PAG, which makes reciprocal connections with the limbic system through the hypothalamus). In the rat the spinomesencephalic tract is believed to process motivational-affective aspects of pain perception and stimulate activity in descending control systems rather than processing sensory-discriminative aspects of pain (Willis *et al.,* 1995).

Additional ascending tracts have been extensively studied in other species, notably primates and cats, but are less well documented in the rat (Willis *et al.*, 1995). The spinocervical tract, identified in the primate and the rat, carries axons from neurons originating in laminae III and IV (predominantly neurons which respond specifically to mechanical stimuli but also a small population of nociceptive neurons (Giesler *et al.,* 1979) to the lateral cervical nucleus, a small cluster of neurons lateral to the dorsal horn in the upper cervical segments of the spinal cord. Axons from this nucleus ascend in the contralateral medial lemniscus of the brainstem to midbrain nuclei and the thalamus. Spinal hypothalamic and spinotelencephalic tracts have also been described and ascribed a role in nociceptive processing in the rat (Burstein *et al.,* 1987, Burstein & Giesler, 1989). In the primate, a small number of nociceptive neurons in laminae III and IV project their axons, accompanied by the axon collaterals of large-diameter myelinated primary afferent

fibres, to the cuneate and gracile nuclei of the medulla (Jessell & Kelly, 1997) but this ascending pathway has not been reported in the rat.

Nociceptive input into the central nervous system originating from sensory stimulation of the head is transmitted via the trigeminal nerve (reviewed by Ranson & Clark, 1959).

The thalamus is the principal synaptic relay for information reaching the cerebral cortex and contains distinct sensory nuclei, divided into medial and lateral groups, which process input about different sensory modalities (reviewed by Lund & Webster, 1967). The medial nuclear group, whose neurons project widely to basal ganglia and many different cortical areas, is not concerned exclusively with processing nociceptive information, but is part of a non-specific arousal system, although many neurons in the medial thalamus do respond optimally to noxious stimuli. The lateral nuclear group, which includes the ventrobasal nucleus and the posterior thalamic nuclei - receives input primarily from nociceptive-specific and WDR neurons in laminae I and V. Certain neurons in the lateral thalamus respond exclusively to noxious stimuli while others respond to multiple sensory stimuli. In the rat, the most extensively studied thalamic nuclei associated with processing of nociceptive information are the ventral posteriolateral (VPL) nucleus, the posterior thalamic nuclear group, and the reticular thalamic nucleus (Guilbaud *et al.,* 1980; Peschanski *et al.,* 1981).

In higher centres, conscious perception of pain results from activation of a complex network of structures, each of which participates differently in the multiple aspects of the pain experience. Brain imaging studies performed in humans using positron emission tomography (PET) and functional magnetic resonance (fMRI) have identified many cortical and subcortical sites which demonstrate a multifocal and predictable pattern of activation in response to noxious stimulation, and it has been suggested that there is at least partial functional segregation of regions involved in the sensory and affective dimensions of pain (reviewed by Treede *et al.,* 1999; Bushnell *et al.,* 1999; Davis *et al.,* 1999; Tolle *et al.,* 1999; Saab *et al.,* 1999; Rainville *et al.,* 2000). Cortical activation has been identified predominantly in the contralateral hemisphere, in primary and secondary somatosensory areas (S1 and S2); parietal operculum; insula; the anterior cingulate cortex and the prefrontal cortex. It is probable that these areas process different aspects of pain sensation in parallel. Subcortical foci of activation have been identified in brain stem, thalamus, hypothalamus, lenticular nuclei, and cerebellum.

Descending modulation of nociceptive transmission

Ascending nociceptive transmission is subject to descending modulatory influences- both inhibitory and facilitatory- from supraspinal sites. These endogenous pain modulatory systems can be activated by numerous environmental stimuli, including stress, illness, cognitive behaviour and pain (reviewed by Urban & Gebhart, 1999). The functional anatomy of descending pain modulation was initially investigated using traumatic lesions, electrical stimulation or reversible local anaesthetic blocks of selected regions of the brainstem or fibre tracts (reviewed by Basbaum & Fields, 1984; Besson and Chaouch, 1987; Beitz, 1992). More recently, stimulation-produced neuronal activity associated with antinociception has been mapped at a cellular level (reviewed by Sandkuhler, 1996) and using non-invasive imaging techniques (reviewed by Bushnell *et al.,* 1999; Tolle *et al.,* 1999).

Descending inhibition

Many regions of the brain contribute to the modulation of excitatory nociceptive transmission (reviewed by Willis & Westlund, 1997). Early studies using electrical stimulation in rats identified numerous 'antinociceptive' regions in the forebrain, including the prefrontal cortex (Hardy, 1985; Hardy *et al.,*1985); the septal area of the ventral forebrain (Mayer & Liebeskind, 1974), the amygdala (Rogers, 1977) and the caudate nucleus (Schmidek *et al., 1971,* Lineberry & Vierck, 1975). In the brainstem and midbrain, the periaqueductal grey matter (PAG) and the rostral ventromedial medulla (RVM, which incorporates the nuclei raphe magnus (NRM), reticularis gigantocellularis and gigantocellularis pars alpha) have been accorded predominant roles in the endogenous modulation of excitatory nociceptive transmission, although many other regions also possess this capacity, including the midbrain reticular formation, locus coeruleus (LC) and locus subcoeruleus (LSC) of the pons (reviewed by Behbehani, 1995; Willis & Westlund, 1997). Of these supraspinal sites, the PAG was originally found to be the most effective site for stimulation-produced analgesia. The majority of PAG efferent fibres project to the RVM, which in turn sends bilateral descending projections that terminate in the spinal dorsal horn. Stimulation of the PAG inhibits both simple nociceptive spinal reflexes and behavioural responses to noxious stimulation in both rats (Fardin *et al* 1984 a,b) and humans (Hosobuchi, 1986). Stimulation of the dorsal raphe nucleus, adjacent to the PAG, also induces analgesia although it has been postulated that this antinociception is mediated through both ascending and descending pathways (reviewed by Wang & Nakai, 1994; Watkins *et al.,* 1998). Wei *et al.,* (1999) recently showed that chemical ablation of the

NRM or LC/LSC enhanced hyperalgesic behaviour associated with Freund's adjuvant induced intraplantar inflammation.

Millan (1999) reviewed the spinal targets of descending 'antinociceptive' pathways. The terminals of descending projections occur in laminae I-III and V-VII of the dorsal horn, where they synapse on central terminals of primary afferent nociceptors and cell bodies of ascending projection neurons. Descending inhibitory pathways originating in higher centres modulate (generally by reducing) release of neurotransmitters from the terminals of nociceptor-responsive primary afferent fibres. They also inhibit projection neurons located in the spinal cord, both directly and indirectly, through inhibition of excitatory interneurons and excitation of inhibitory interneurons. Substantial evidence suggests that this effect of descending modulatory pathways- essentially post-synaptic relative to primary afferent fibres- is the predominant mechanism through which descending pathways modulate nociceptive transmission (Willis & Coggeshall, 1991; Fields & Basbaum, 1994; Sandkuhler, 1996; Millan, 1997). Some studies indicate that descending pathways preferentially inhibit excitation of WDR neurons by noxious as compared to innocuous stimuli (reviewed by Fields & Basbaum, 1994; Willis, 1994; Millan, 1997). However, electrophysiological studies have indicated that under certain conditions, the inhibitory influence of descending systems on WDR neurons in deeper laminae can be non-selectively expressed against both noxious and innocuous (Aβ) input, suggesting direct monosynaptic inhibition of projection neuron excitability (Willis & Coggeshall, 1991; Fields & Basbaum, 1994; Willis, 1994).

Although descending pain modulatory systems were originally accorded an exclusively inhibitory role, it is now recognised that stimulation of supraspinal sites- including those implicated in descending inhibition- can also facilitate spinal nociceptive transmission. Chemical or electrical stimulation of the RVM, typically at lesser intensities than those used to produce descending inhibition, has been shown to enhance spinal behavioural responses to acute noxious stimuli, spontaneous responses and evoked responses of dorsal horn nociceptive neurons (Gebhart, 1993). Facilitatory and inhibitory pathways originating in the RVM are mediated by independent descending systems that are anatomically, pharmacologically and physiologically distinct (reviewed by Urban & Gebhart, 1999).

The stimuli which activate descending pain modulation have been reviewed by Beitz (1992) and Urban & Gebhart (1999). Descending inhibitory pathways can be activated naturally in response to various environmental factors, including noxious stimulation, and

indirectly through the limbic system; it has been proposed that descending inhibition represents a beneficial adaptive modulation of nociceptor sensitivity in response to aversive or potentially aversive environmental stimuli. Pain inhibition by noxious somatic input has formed the basis for analgesia produced by counter-irritant techniques and may contribute to the analgesic effect produced by certain forms of acupuncture and transcutaneous nerve stimulation techniques. It is well recognised that nociceptive transmission can be profoundly modulated by the emotional and attentional state of the individual, presumably through connections with the limbic system. The most extensively investigated example of descending inhibition is stress-induced analgesia (or hypoalgesia), which represents an adaptive response to prepare and protect the body in potentially injurious situations (reviewed by Yamada & Nabeshima, 1995; Menendez *et al.,* 1996). Antinociceptive mechanisms are also engaged in experimental models of conditioned fear in anticipation of noxious stimulation, or by other stressful situations, produced by both opioid and non-opioid mediated mechanisms (Millan, 1986; Wiertelak *et al.,* 1992; Watkins *et al.,* 1994; 1997). Opioid mediated stress-associated analgesia demonstrates tolerance with repeated stress, and cross-tolerance with morphine, while non-opioid mediated stress-induced analgesia demonstrates neither of these characteristics (Grau *et al.,* 1981; Watkins *et al.,* 1982; Terman *et al.,*1984 a,b). Descending facilitation can also be activated by environmental factors, which are opposite to those which trigger descending inhibition, suggesting that descending facilitation similarly represents a beneficial adaptive response in which enhanced pain perception encourages reduced mobility during illness, to conserve energy and promote healing (Maier *et al.,* 1992).

Extensive modulation of excitatory nociceptive transmission also occurs segmentally within the dorsal horn of the spinal cord through interaction of multiple excitatory and inhibitory transmitter systems, which shall be introduced and discussed in subsequent sections.

It is important to recognise that the ascending excitatory nociceptive transmission, and descending modulatory pathways are intimately interconnected and interdependent. Thus, nociceptive input is subject to modulation through spinal cord neuroplasticity and descending influences, both facilitatory and inhibitory, from supraspinal sites. The activation of these pathways is critically dependent on the nature of acute or persistent injury or noxious input, but also upon a melee of behavioural and environmental stimuli. , which may combine to produce dominant facilitation or inhibition of ascending nociceptive transmission (reviewed by Millan, 1999). An improved understanding of tonic 'antinociceptive' regulation of the induction of physiological and pathophysiological pain

may help to identify new strategies for the prevention and treatment of pathological hyperalgesia and persistent pain.

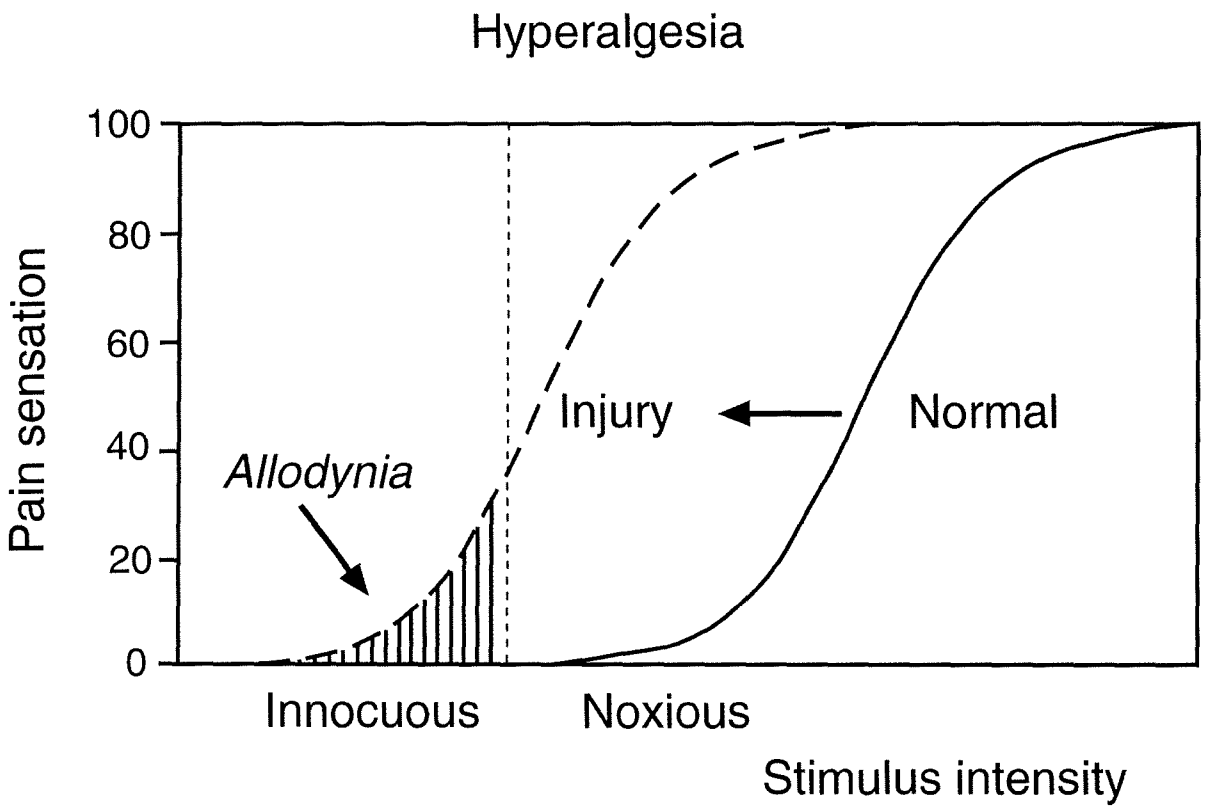
1.2 PATHOPHYSIOLOGICAL NOCICEPTIVE TRANSMISSION: PAIN HYPERSENSITIVITY

Peripheral inflammatory injury can enhance subsequent sensation of pain. This phenomenon is defined as hyperalgesia, an enhanced response to a stimulus which is normally painful, and allodynia, pain associated with an innocuous stimulus which does not normally provoke pain (Lindblom *et al* 1986). These are behavioural phenomena which develop subsequent to cutaneous (thermal, mechanical or chemical/inflammatory) injury. Cutaneous hyperalgesia can also develop in undamaged skin in neuropathic pain conditions, or as a consequence of somatic referral from visceral pain and injury (for example, from the heart) (Ruch 1961, reviewed by Ness & Gebhardt, 1990). Hyperalgesia and allodynia are illustrated diagrammatically in figure 1.3.

Hyperalgesia and allodynia may provide 'self-protection' mechanisms whereby the peripheral and central nervous systems work in concert to discourage further stimulation, or exacerbation of injury, to an already damaged area, thus enhancing the potential for recovery and healing. However hyperalgesia and allodynia are also hallmarks of persistent pain syndromes. Recognition of hyperalgesia and allodynia, which reflect hypersensitivity of peripheral or central nociceptive transmission, has changed our approach to clinical pain management.

The region of hyperalgesia which develops subsequent to cutaneous injury is commonly divided into primary and secondary components (reviewed by Woolf, 1995). Within the region of primary hyperalgesia, incorporating the actual injury site, 'peripheral sensitisation' develops, (reviewed by Treede *et al.*, 1992). The region of secondary hyperalgesia extends far beyond the site of injury into undamaged skin, mediated by peripheral and central mechanisms distinct to those which mediate primary hyperalgesia (Cervero & Laird 1996 [a, b]; Ren, 1996). The relative contribution of peripheral and central mechanisms to post-injury hypersensitivity remains a contentious topic.

Figure 1.3
Alterations in pain sensitivity induced by injury



Peripheral sensitisation

Peripheral sensitisation is defined as enhanced sensitivity of nociceptive sensory afferent neurons associated with modulation of transduction at peripheral terminals (Levine & Taiwo 1994). Inflammatory injury enhances peripheral afferent nociceptive transmission through a host of chemical and physical mechanisms (reviewed by Dickenson 1996;Yaksh 1996; Woolf & Salter, 2000). A multitude of chemical mediators are released into the immediate vicinity of the injured cell, including hydrogen ions, potassium ions, histamine, purines, neuropeptides, bradykinin, serotonin, arachidonic acid metabolites, cytokines and growth factors (reviewed by Dray & Perkins, 1993; Dickenson, 1997), which are illustrated in figure 1.4. These mediators alter nociceptor transduction sensitivity both directly and indirectly (through altering ionic conductance across the peripheral terminal membrane) so that less intense stimuli are sufficient to activate nociceptive fibres, more nociceptors are activated by any given suprathreshold stimulus, and sensory fibres which were previously resistant to excitation can be activated by natural stimuli. Peripheral sensitisation can also result from phenotypic modification of peripheral nociceptors in response to inflammatory injury, through up-regulation of expression of target-derived growth factors which results in altered expression of transmitters, synaptic neuromodulators, ion channels, G-protein-coupled receptors and structural proteins (reviewed by Woolf & Salter, 2000).

Recent studies have focused on the molecular mechanisms which enhance the excitability of peripheral nociceptors following injury. The predominant mechanism appears to be phosphorylation of ion/receptor channels or associated regulatory proteins within the primary nociceptor, which alter its intrinsic functional properties or cell-surface expression of ion channels such as the tetrodotoxin-resistant sensory neuron-specific sodium ion channel (SNS) (Khasar *et al.,* 1998) and the vanilloid receptor VR_1 (Fitzgerald *et al.,* 1999). Inflammatory injury results in up-regulation of expression of VR_1 receptors and SNS channels at peripheral nerve terminals, resulting in increased sensitivity to inflammatory mediators and enhanced peripheral sensitisation (reviewed by Wood *et al.,* 1999). Peripheral sensitisation may also result from enhanced expression of receptors in dorsal horn neurons for transmitters such as substance P which are then transported to the terminals of primary nociceptive neurons, priming the system to respond more strongly to any given stimulus (Woolf & Costigan, 1999).

Central sensitisation

Inflammatory injury can also induce changes in central nociceptive transmission. Ample evidence suggests that central plasticity of nociceptive transmission is mediated through

correlated alterations in the discharge rates in individual nociceptive neurons- in other words, by changing the strength and the duration of synchronisation of central neuronal responses-rather than by recruiting additional neuronal connections (Aertsen *et al.,* 1989). A similar pattern of neural transmission operates in other sensory systems, including the visual cortex (Engel *et al.,* 1991; Konig & Schillen, 1991) and the olfactory system (Johnson *et al.,* 1991).

Central sensitisation is classically defined as an increase in excitability of neurons within the spinal cord, triggered exclusively by C-fibres, and characterised by a decrease in response threshold, increased response to suprathreshold stimuli, and an increase in the receptive field area which may include recruitment of previously ineffective inputs (Woolf, 1983; 1984; Wall & Woolf 1984; Woolf and Wall 1986; Cook *et al.,* 1987; Woolf & King, 1990). However, central sensitisation has also often been associated with an increased response to Aβ fibre activity, which has been speculated to contribute to mechanical allodynia (Simone *et al.,* 1991; Woolf & Doubell, 1994). Enhanced sensitivity of central nociceptive neurons to stimulation from peripheral nociceptive afferents can persist long after the original stimulus has disappeared. It has been postulated that central plasticity of nociceptive processing is the 'common denominator' in a number of pathological persistent pain syndromes (Dickenson, 1991; Coderre *et al.,* 1993; Pockett, 1995; Randic, 1996).

A key advance in appreciation of the inherent plasticity of central pain processing came with the observation by Mendell & Wall (1965) that constant frequency stimulation of peripheral nerves induced a progressive augmentation in central neuronal excitability (Mendell & Wall 1965, Mendell 1966), which they named 'wind-up'. Windup is an electrophysiological phenomenon induced in response to mild low-frequency electrical stimulation, which synchronously activates many C-fibres (Woolf 1996) and is mediated by an N-methyl-D-aspartate (NMDA) receptor mechanism (Thompson *et al.,* 1990). Windup, which can amplify dorsal horn neuronal responses to a given peripheral input by a factor of 20, has been demonstrated in dorsal horn neurons (Mendell 1966) and in large motor neurons of the ventral horn (Thompson *et al.,* 1990).

Woolf (1983) demonstrated that conditioning stimulation of C-fibres could induce prolonged enhancement of the flexion withdrawal reflex in spinal-decerebrate rats, thus demonstrating central sensitisation of dorsal horn nociceptive neurons. Hallmarks of central sensitisation of dorsal horn nociceptive transmission (reviewed by Coderre *et al.,* 1993) include: increased responsiveness to supra-threshold inputs (Kenshalo *et al.,* 1979),

expansion of the receptive field size (Cook *et al.,* 1987; Hylden *et al.*, 1989), reduced response threshold (Simone *et al.,* 1989); and prolonged after-discharge (Mendell 1966; Schoeunberg & Dickenson (1985). Central sensitisation may also reflect depression of spinal inhibitory mechanisms. Electrophysiological evidence suggests that tonic descending inhibitory systems contribute to maintenance of 'functional synchronicity' of 'physiological' central nociceptive transmission in the dorsal horn by prolonging the synchronisation time between central nociceptive neurons (Eblen-Zajjur & Sandkuhler, 1997). The demonstration of central sensitisation marked a milestone in appreciation of the potential plasticity of central neuronal responses to peripheral stimulation, and led to the hypothesis that pathophysiological pain syndromes, including persistent hyperalgesia and allodynia, could represent synaptic plasticity of central spinal nociceptive processing (Woolf, 1984). This advance led in turn to the application of existing knowledge about neuronal plasticity- developed most extensively through studies in the hippocampus and neocortex- to central nociceptive transmission, in an attempt to relate central neuronal plasticity to persistent pain syndromes.

Persistent alteration of neuronal excitability is defined electrophysiologically as long-term potentiation (LTP) and long-term depression (LTD). LTP was first observed in the hippocampus following high frequency (100Hz) electrical stimulation of a synaptic pathway (Bliss & Lomo, 1973; Bliss & Collingridge, 1993) and the phenomenon has been accorded a key role in establishing memory and 'learning' pathways (Bliss & Collingridge, 1993). 'LTP' is a generic phrase used to describe a prolonged increase in the efficacy of synaptic plasticity, lasting for more than one hour. Many different types of LTP have been described in different regions of the central nervous system (reviewed by Holscher, 1999). Forms of prolonged synaptic plasticity which persist for a shorter duration (20 -60 minutes) are termed short-term potentiation.

LTP is generated through complex intraneuronal molecular pathways (reviewed by Bliss & Collingridge, 1993). Briefly, LTP induction critically depends upon the precise characteristics of the stimulation of presynaptic fibres, with synchronous high-frequency bursts being most effective in its induction at many central synapses. While NMDA receptor activation, leading to dramatic intracellular Ca^{2+} ion influx, plays a key role in the induction of LTP in the hippocampus (Herron *et al.,* 1986), associated activation of AMPA and metabotropic glutamate receptors also occurs (Bliss & Collingridge, 1993; Bashir *et al.,* 1993). The capability of dramatic Ca^{2+} ion influx alone to generate stable LTP remains a contentious issue. NMDA receptor-independent LTP has been documented in the hippocampus (Johnston *et al.,*1992), induced by multiple mechanisms, certain of which

involve metabotropic receptor activation or activation of non-NMDA-associated calcium channels by strong post-synaptic stimulation (reviewed by Johnston *et al.,* 1992). It has been postulated that afferent synaptic activity in addition to Ca^{2+} ion influx may also be a prerequisite for the induction of stable LTP, through a mechanism which involves metabotropic glutamate receptors (Kullman, 1994).

LTP has been observed in various regions of the spinal cord grey matter, including the dorsal horn (Liu & Sandkuhler, 1995; 1996; 1997; Svendson *et al.*, 1997), the intermediate grey matter (Pockett 1995) and the ventral horn (Pockett & Figurov, 1993). Sandkuhler & Liu (1998) demonstrated that afferent discharge patterns which occur during natural noxious stimulations (heat, mechano- or chemosensitive cutaneous nociceptor stimulation) or acute nerve injury produce robust LTP of C-fibre evoked field potentials in superficial spinal cord. Moreover, maintenance of LTP was not affected when afferent nerves were cut 1 hour or 5 minutes after noxious stimulation, indicating that ongoing afferent barrage was not required to maintain LTP. Notably, natural noxious stimuli induced LTP in animals which were spinalised but were ineffective in intact animals, suggesting that induction of LTP was tonically suppressed by supraspinal descending inhibition. Previous studies by Liu & Sandkuhler (1995; 1996) support this hypothesis. LTP was induced in spinalised rats through inflammatory injury [intense radiant skin heating, intraplantar formalin injection, and noxious mechanical stimulation using an artery clamp], sural nerve injury or superfusion of the spinal cord with substance P, but could not be induced using the same conditioning stimuli in rats with intact central circuitry. Consistent with findings in the hippocampus, the NMDA receptor is postulated to play a major role in the induction of many forms of LTP in the dorsal horn (Randic *et al.,* 1993).

NMDA-receptor independent LTP has been described in the spinal cord dorsal horn (Cerne *et al.,* 1991; Meller *et al.,* 1993; Kojic & Randic, 1993). Meller *et al.* (1993) reported that chemical irritant- induced mechanical hyperalgesia required co-activation of AMPA and metabotropic receptors while remaining independent of NMDA receptor antagonism. Kojic & Randic (1993) demonstrated that metabotropic receptor agonists could induce LTP in the spinal dorsal horn in the absence of tetanic stimulation, speculating that metabotropic receptors played an essential role in the induction of NMDA-independent LTP in the spinal cord.

Long term depression of primary afferent neurotransmission, the central complement of LTP, has been identified in the hippocampus (Dudek & Bear, 1992; Mulkey & Malenka, 1992). Both LTP and LTD are induced by highly specific changes in the post-synaptic

membrane potential; indeed, the same conditioning stimuli (intracellular Ca^{2+} influx, activation of glutamate receptors and tetanic stimulation of dorsal roots) can give rise to LTP or LTD depending on factors that have not yet been fully elucidated. LTD is broadly classified as homosynaptic or heterosynaptic depending on whether the decrease of synaptic efficiency occurs only at the stimulated synapses (homosynaptic) or also at inactive synapses adjacent to the activated synaptic region (heterosynaptic). LTD was first observed in the cerebellum (Ito *et al.,* 1982) and has since been demonstrated in many regions of the CNS, including the hippocampus (Mulkey & Malenka, 1992), visual cortex (Artola & Singer, 1987) and spinal cord (Randic *et al.,* 1993; Pockett & Figurov, 1993). LTD has been elicited by a wide variety of both high frequency (HFS-LTD) and low frequency (LFS-LTD) conditioning stimuli (reviewed by Bindman *et al.,* 1991; Artola & Singer, 1993; Malenka & Nicoll, 1993; Christie *et al.,* 1994).

In the spinal cord, LTD has been observed in both the dorsal and ventral horns following primary afferent stimulation (Cerne *et al.,* 1991; Randic *et al.,* 1993; Pockett & Figurov, 1993; Pockett, 1995). Both HFS-LTD and LFS-LTD have been induced in lamina II neurons *in vitro* through stimulation of primary afferent Aδ fibre input (Sandkuhler & Randic, 1996; Sandkuhler *et al.,* 1997); in these studies it was postulated that LTD of synaptic transmission in the dorsal horn may provide an alternative mechanism for suppression of excitatory central nociceptive transmission.

Distinctions between LTP/LTD and central sensitisation

LTP and LTD occur in many regions of the brain and spinal cord. Since cerebral LTP and LTD are typically associated with the processes of learning and memory (Bliss & Collingridge, 1993), a popular dogma has emerged that their principal roles in the superficial spinal dorsal horn may be related to plasticity of spinal nociception (Randic, 1993). However, as electrophysiological phenomena that cannot be studied (in the spinal cord) in free-moving animals, direct comparisons cannot be made between LTP/LTD and plasticity of nociceptive behaviour observed *in-vivo,* and the association between observations of LTP and LTD in the spinal cord, and plasticity of nociceptive transmission associated with inflammatory injury, remains ambiguous and inconclusive.

Willis (1997) and Millan (1999) discussed characteristic differences between LTP/LTD, as they are classically defined in higher centres, and central sensitisation of dorsal horn neuronal responses. LTP and LTD describe activity-dependent modulation of neuronal excitability which persists for at least 1 hour, but is of indefinite duration and may be permanent. It is worthy of note that permanent alterations in gene expression and

phenotypic changes are not prerequisites for LTP and LTD. Central sensitisation is similarly activity-dependent and of indefinite duration, but may theoretically persist for less than 1 hour following specific injury, although it is typically used to describe long-lasting alterations in central neuronal excitability.

LTP and LTD of central nociceptive transmission may represent transient and reversible alterations in central nociceptive transmission, associated with upregulation of expression of key nociceptive mediators but not associated with permanent alterations in gene expression or phenotypic alterations in dorsal horn neurons; or they may represent permanent alterations in gene expression which will produce a persistent state of hyperexcitability in the dorsal horn (Pockett, 1995).

It is important to recognise that while LTP and LTD in the spinal dorsal horn may be related to persistent states of enhanced excitability in the dorsal horn involving phenotypic alterations in central nociceptive neurons (a convincing explanation for certain pathological persistent pain states), they may also represent central nociceptive plasticity of shorter duration, mediated by stimulus-induced alterations in gene transcription which alter nociceptive transmission for a certain period in the absence of persisting stimulation, but subsequently subside, without the induction of permanent phenotypic alterations in dorsal horn neuronal excitability

1.3 PHARMACOLOGY OF PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL NOCICEPTIVE TRANSMISSION

Information about noxious peripheral stimulation is communicated to the spinal cord by a barrage of excitatory neurotransmitters released into the spinal cord from peripheral afferent neurons. Simultaneously, ascending excitatory nociceptive transmission is subject to modulation by descending inhibitory transmission originating within higher centres or within the spinal cord itself. The interaction of key excitatory and inhibitory neurotransmitter systems in nociceptive transmission is illustrated in Figure 1.4. The pharmacology of pain transmission and of central pain states has been reviewed by Yaksh (1996), Dickenson (1997) and Millan (1999).

Excitatory transmission

Neuropeptides

The tachykinins (TKs) are a family of small peptides which share the common C-terminal sequence Phe-X-Gly-Leu-MetNH_2. Three peptides of this family, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), have an established role as

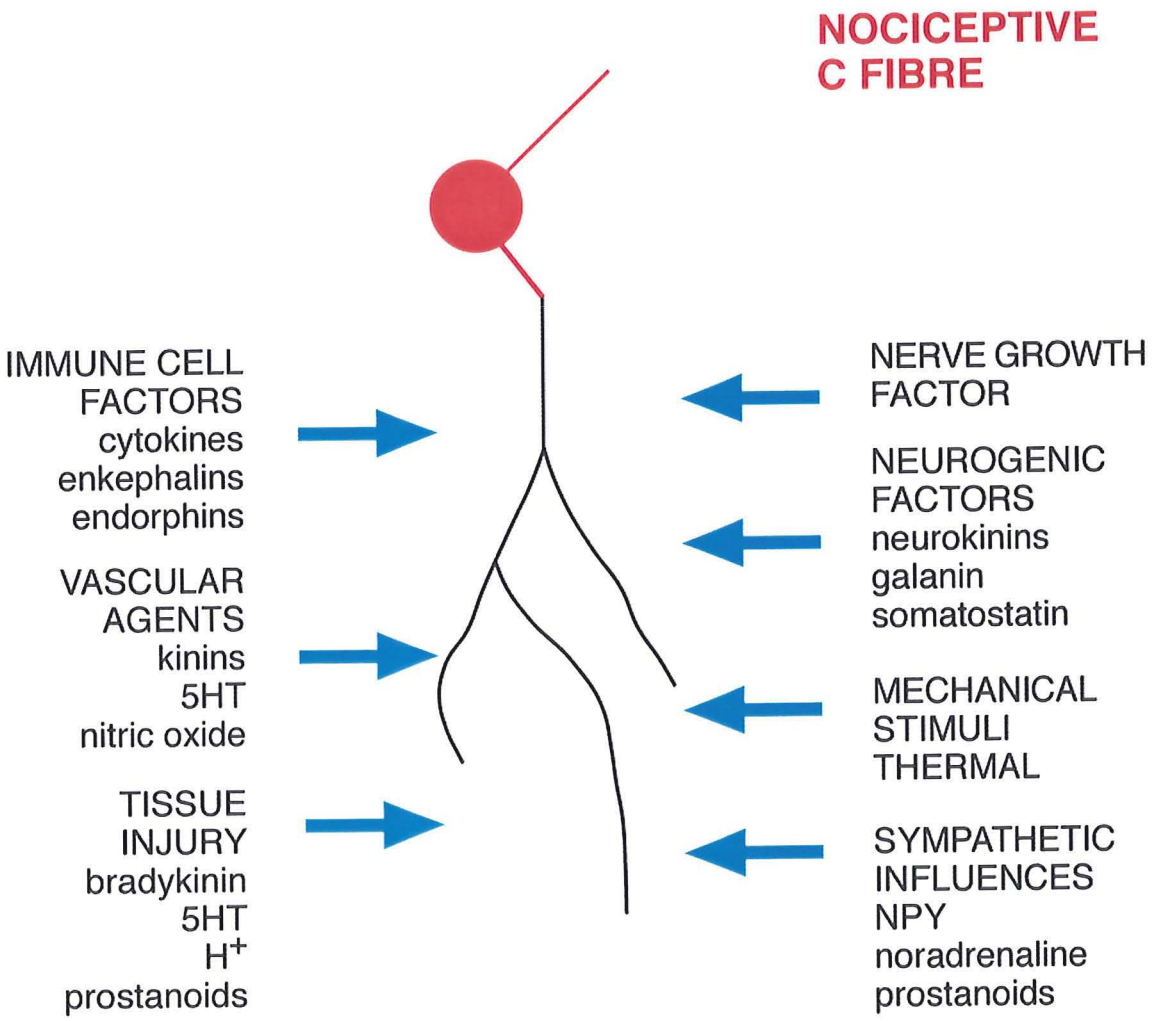
neurotransmitters in mammals; SP and NKA have been extensively studied as pronociceptive neurotransmitters. Three receptors for TKs have been cloned: NK_1 (SP-preferring), NK_2 (NKA -preferring) and NK_3 (NKB-preferring) although NKA may also exert actions at NK_1 sites and SP at NK_2 sites (Maggi, 1995). NK_1 receptors are located throughout the spinal cord grey matter although they are concentrated in laminae I and II, while NK_3 binding sites are limited to laminae I and II (Yashpal *et al.,* 1990).
The distribution of NK_2 receptors remains controversial. Tsuchida *et al.,* (1990) reported that NK_2 receptor expression was limited to peripheral tissues; however, numerous studies have demonstrated that NK_2 receptors are involved in the spinal processing of nociceptive information (Fleetwood-Walker *et al.,* 1990; Thompson *et al.,* 1994; Neugebauer *et al.,* 1996).

It has been speculated that tachykinin receptors play a minimal role in physiological nociceptive transmission, but are important mediators involved in enhanced nociceptive transmission following inflammatory injury and in the induction of central sensitisation (Dickenson, 1996).

It has been recently demonstrated, however, that tachykinin release from nociceptive primary afferent fibres is required to produce moderate to intense pain sensation (Cao *et al.,* 1998). Substance P (SP) and NKA are rapidly released following stimulation of C fibres and local intrinsic neurons, and this release is enhanced following inflammatory injury (Moochala & Sawnok 1984; Go & Yaksh, 1987; Okhubo *et al.,* 1990). Ample evidence supports the involvement of neuropeptides in the induction and maintenance of central sensitisation. Intrathecal administration of SP and NKA reduces behavioural nociceptive thresholds (Cridland & Henry, 1986) and enhances spinal flexor motor neuronal responses to noxious stimuli (Wiesenfeld-Hallin, 1986), while neurokinin receptor antagonists can block the development of central sensitisation following injury (Xu *et al.,* 1992; Ma & Woolf, 1995).

A further pro-nociceptive peptide, calcitonin gene-releasing peptide (CGRP) has been extensively studied in association with both physiological and pathophysiological nociceptive transmission. Two CGRP receptors ($CGRP_1$ and $CGRP_2$) have been characterised to date. CGRP is released following noxious stimulation of C-fibres and has an excitatory effect on dorsal horn neurons; intrathecal injection of CGRP increases the excitability of spinal flexor motor neurons (Ryu *et al.,* 1988). Substance P and CGRP are degraded by the same breakdown enzyme (CGRP preferentially) and thus in the presence of CGRP, SP diffuses more extensively in the spinal cord.

Figure 1.4
Transmitters and chemical mediators, released following tissue injury, implicated in the induction of peripheral sensitisation



Numerous other neuropeptides have been accorded potential roles in physiological and pathophysiological nociceptive transmission, notably neuropeptide Y (Hua *et al.,* 1991; Calza *et al.,* 1998; Yaksh *et al.,* 1999; Honore *et al.,* 2000;), nerve growth factor (NGF) (Lewin & Mendell, 1993; Bennet *et al.,* 1998; Theodosiou *et al.,* 1999); somatostatin and galanin (Calza *et al.,* 1998; Hokfeldt *et al.,* 1999; Honore *et al.,* 2000).

Excitatory amino acids (EAA's)

Glutamate is the major excitatory neurotransmitter in the central nervous system. EAA's produce their effects through two broad categories of receptors, ionotropic and metabotropic (reviewed by Schoepp & Conn 1992; Rang *et al.,* 1999a). Ionotropic receptors are classified into 3 subtypes based on selectivity of synthetic agonists, namely N-methyl-D-aspartate (NMDA); α-amino-3-hydroxy-5-0-methyl-4-isoxazolepropionic acid (AMPA); and kainate (KA) receptors. Activation of AMPA or KA receptors is essential for fast excitatory transmission in the CNS. Pathophysiologically, regulation of the voltage-dependent Mg^{2+} ion block of NMDA receptor calcium channels plays a major role in central sensitisation (Davies & Lodge, 1987; Woolf & Thompson, 1991; Chen & Huang, 1992).

The mechanism of NMDA receptor activation and the cascade of intracellular events which ensue have been reviewed by Pockett (1995). In the quiescent state, the Ca^{2+} channel of the NMDA receptor is blocked by physiological levels of Mg^{2+} ions. This channel block can only be removed by repeated depolarisation of the cell membrane. In the 'normal' situation, C-fibre stimulation following brief acute mechanical or thermal stimulation causes activation of AMPA receptors to give excitations of short duration. If this stimulation is maintained, or if the frequency or intensity of stimulation is increased, then the NMDA receptor will be activated to produce dramatic enhancement of neuronal responses. This is the mechanism by which the NMDA receptor is believed to induce central spinal hypersensitivity. The effects of NMDA receptor activation are the results of transient massive Ca^{2+} ion influx into the post-synaptic cell.

The role of the NMDA receptor in physiological and pathophysiological central nociceptive transmission has been reviewed by Dickenson (1996) and Millan (1999). NMDA antagonists typically exert minimal effects on acute nociceptive transmission but attenuate 'facilitated' states of processing (Dickenson & Sullivan, 1987; Simone *et al.,* 1989; Woolf & Thompson, 1990; Coderre & Melzack, 1992; Coderre *et al,* 1993). Ample evidence supports the involvement of the NMDA receptor in central sensitisation. Neuronal responses implicated in the induction of central sensitisation can be attenuated

by both intrathecal (Haley *et al.,* 1990; Nasstrom *et al.,* 1992) and systemically delivered (Hudspith *et al.,* 1999) NMDA antagonists. Hyperalgesic behaviour following inflammatory injury can be attenuated through intrathecal (Haley *et al.,* 1990; Ren *et al.,* 1992) and systemic (Hudspith *et al.,* 1999) administration of NMDA receptor antagonists. Intrathecal delivery of NMDA agonists induces spontaneous agitation, thermal hyperalgesia and tactile allodynia (Aanonsen & Wilcox 1986; Coderre & Melzack, 1992; Malmberg & Yaksh, 1992).

Controversy persists over the precise role of the NMDA receptor in different manifestations of pathophysiological pain transmission. There is evidence to suggest that the NMDA receptor mediates thermal hyperalgesia but not mechanical allodynia (Meller *et al.,* 1993; Tal & Bennet, 1994; Leem *et al.,* 1996). However there are several reports of NMDA mediation of mechanical hyperalgesia (Aanonsen *et al.,* 1990; Schaible *et al.,* 1991; Ma & Woolf, 1994; Munglani *et al.,* 1999; Chaplan *et al.,* 1997). It has also been proposed (Song and Zhao 1993) that while cutaneous hyperalgesia is mediated by NMDA receptor activation, muscular hyperalgesia is mediated by non-NMDA mediated mechanisms.

In addition to their central effects, NMDA receptors localised peripherally on primary afferent terminals in the skin may play a role in inflammatory pain states (Carlton *et al.,* 1995; Zhou *et al.,* 1996; Davidson *et al.,* 1997; Davidson & Carlton, 1998), since local application of the NMDA antagonist MK-801 attenuates hyperalgesia induced by intraplantar injection of complete Freunds adjuvant (Jackson *et al.,* 1995) or formalin (Davidson *et al.,* 1997).

Metabotropic receptors

Eight subtypes of metabotropic glutamate receptors have been identified, grouped into three distinct groups (mGluR's 1, 2 and 3) according to their transduction mechanisms (reviewed by Pin & Duvoisin, 1995). Metabotropic receptors are coupled to GTP-binding proteins in the cell membrane, and act in conjunction with initial transient calcium influx to activate various second messenger systems. Group 1 mGluR's are coupled to phopsholipase C, while group 2 and group 3 mGluR's are negatively coupled to adenyl cyclase. Metabotropic receptors induce numerous effects on central neurons, including depression of excitatory (and inhibitory) post-synaptic potentials (Baskys & Malenka, 1991; Hayashi *et al.,* 1993) and enhancement (or attenuation) of glutamate release (Glaum & Miller, 1992). There is evidence to suggest that metabotropic receptors play an important role in the induction and/or maintenance of synaptic plasticity in the central

nervous system (Knopfel *et al.,* 1995; reviewed by Conn & Pin, 1997) but this role is less well defined for metabotropic receptors than for NMDA receptors.

Prostaglandins

Prostaglandins are metabolites of arachidonic acid, produced by many different types of cell, which produce a multitude of effects throughout the body. PG receptors are present in peripheral tissues and in many organ systems (reviewed by Rang & Dale, 1999) and throughout the central nervous system (Watanebe *et al.,* 1989; Matsumura *et al.,* 1992). Four major prostaglandin receptors have been described (DP, EP, FP and IP), derived from predicted amino acid sequences and operational pharmacology (reviewed by Bley *et al.,* 1998). Further subtypes of each prostaglandin receptor have been subsequently identified; for example, the PGE receptor has been further subtyped into EP_1 EP_2, EP_3 and EP_4. In the spinal cord, the majority of binding sites for PGE_2 are located in laminae I and II (Mastumura *et al.,* 1992).

Prostaglandins play a key role in peripheral transmission of nociceptive responses to injury and in the development of peripheral sensitisation (Ferreira, 1978a; Geisslinger & Yaksh, 1999). Prostaglandin synthesis is a key component of the acute inflammatory response. Following acute injury PGE_2 and PGI_2 are generated by local tissues and blood vessels, while mast cells generate PGD_2. In chronic inflammatory conditions monocyte-macrophage cells also synthesise prostaglandins (reviewed by Vasko, 1995). Peripheral PG administration reduces the activation threshold of peripheral nociceptive neurons (Martin *et al.,* 1987; Schaible *et al.,* 1988) and induces hyperalgesic behaviour (Ferreira, 1978b; Taiwo & Levine, 1986).

PG's also fulfill an important role as neurotransmitters within the central nervous system, playing a key role in central excitatory transmission of nociceptive responses to injury and in the development of central sensitisation (reviewed by Yamagata *et al.,* 1993; Geisslinger & Yaksh, 1999). Prostaglandins are synthesised in the spinal cord following stimulation of fine-diameter (nociceptive) afferent fibres (Ramwell, 1966) and following noxious stimulation (Malmberg & Yaksh, 1995a,b; Scheuren *et al.,* 1997). Intrathecal injection of PGE_1, PGE_2, PGF_2 and PGD_2 induces dose-dependent hyperalgesia (Coceani & Viti, 1975; Ferreira, 1983; Taiwo & Levine 1986). Intrathecal delivery of non-steroidal anti-inflammatory drugs (NSAID's) which inhibit prostaglandin synthesis) induces dose-dependent attenuation of hyperalgesia and attenuation of spinal nociceptive neuronal responses associated with inflammatory injury, although these agents do not alter acute

nociceptive thresholds (Malmberg & Yaksh, 1992a,b; Chapman & Dickenson 1992; Dirig *et al.,* 1998).

The two isoforms of COX (COX-1 and COX-2) catalyse a key stage in PG synthesis, namely the oxygenation of free arachidonic acid to PGH_2, which is then further converted into alternative prostaglandins or thromboxane A_2 by specific synthase enzymes (Smith & Marnett, 1991; Xie *et al.,* 1991). COX-1, classically referred to as the 'constitutive' isoform, is expressed in many cell types including gastro-intestinal and renal cells, where it is involved in physiological prostanoid-associated homeostatic mechanisms. COX-2 is cytokine-inducible and present in inflammatory cells (Crofford *et al.,* 1994; Ristimaki *et al.,* 1994) but is also constitutively expressed in numerous tissues including the macula densa of the kidney (Harris *et al.,* 1994), developing ovarian follicles (Sirois & Richards, 1992), the testes (Sirois & Richards, 1992), and the central nervous system (Yamagata *et al.,* 1993; Herrting & Seregei, 1989) . In the rat brain COX-2 represents the predominant constitutive isoform (Breder *et al.,* 1995; Beiche *et al.*, 1996; Kaufmann *et al.,* 1996). In the spinal cord, constitutive expression of mRNA and protein for both COX isoenzymes has been identified (Beiche *et al.,* 1996; Hay & de Belleroche, 1997). COX-2 protein expression has previously been identified in neurons in laminae I-IV of the dorsal horn and lamina IX motorneurons (Struebe & Beiche, 1998).

The factors which regulate COX-2 expression in the central nervous system remain undetermined and somewhat contentious. In the brain, COX-2 expression is dynamically regulated by specific forms of activity associated with long-term neuronal plasticity, and can be rapidly and transiently induced in neurons by seizures or NMDA-receptor activation Yamagata *et al* (1993). Immunocytochemical studies have identified high levels of COX-2 expression in dendrites and neuronal cell bodies, typically post-synaptic structures, in the cerebral cortex (Breder *et al* 1994).

The precise role of each COX isoform in inflammatory pain is also a controversial topic. Studies in cyclo-oxygenase gene-knockout mice suggest a role for both isoenzymes in the generation of peripheral inflammation (Langenbach *et al.,* 1995). Selective COX-2 knockout only partially reduces prostaglandin concentration at sites of acute and chronic inflammation (Langenbach *et al.,* 1995) while non-selective cyclo-oxygenase blockade using non-steroidal anti-inflammatory drugs prevents peripheral prostaglandin induction (Seibert *et al.,* 1994; Portanova *et al.,* 1996), suggesting that both isoenzymes are involved in the peripheral inflammatory response. Centrally, there is mounting evidence that COX-2 is the dominant isoform associated with nociceptive transmission and the development

and maintenance of hyperalgesia associated with inflammatory injury (Beiche *et al.,* 1996; Hay *et al.,* 1997; Hay & de Belleroche, 1997; Ichitani *et al.,* 1997).

Inhibitory transmission

Gamma-amino-butyric acid

Gamma-amino-butyric acid (GABA) is the most ubiquitous inhibitory neurotransmitter in the central nervous system. The pharmacology of the GABAergic system has been reviewed by Rang *et al.,* (1999b). GABA inhibits neuronal excitability and neurotransmitter release through two main classes of receptor, both of which have been cloned and subsequently subdivided into receptor subtypes. $GABA_A$ receptors are ligand-gated ion-channels selectively permeable to Cl^- ions (Stephenson 1995), which are located post-synaptically and mediate fast post-synaptic inhibition. $GABA_B$ receptors are coupled through G proteins to K^+ and Ca^{2+} channels (Bowery, 1993) and are located both pre- and post-synaptically. Both receptor types are present in the dorsal horn, co-existing on Aδ and C primary afferent fibres (Desarmenien *et al.,* 1984; Price *et al.,* 1986; 1987). Both $GABA_A$ and $GABA_B$ receptors are concentrated in lamina II of the superficial dorsal horn of the spinal cord. In the spinal cord, GABA protein expression has been identified predominantly in interneurons in the superficial dorsal horn which receive afferent input from small-diameter nociceptive fibres (Carlton & Hayes, 1990; Hayes & Carlton, 1992; Magoul *et al.,* 1987; Todd & McKenzie, 1989).

These inhibitory interneurons are themselves targeted by C and Aδ nociceptive fibres, as well as by Aβ fibres (Coggeshall & Carlton, 1992; Yoshimura & Nishi, 1992; 1995; Todd & Spike, 1993; Todd *et al.,* 1994; Bernardi *et al.,* 1995). This targeting suggests that C and A δ nociceptive fibres may exert 'counter-regulatory' inhibitory feedback control upon their parallel excitation of WDR and nociceptive specific projection neurons in the dorsal horn, so that the pain elicited by projection neuron stimulation by nociceptors, may be limited by simultaneous activation of GABAergic inhibitory interneurons, at least for short duration sub-maximal stimulation (Millan, 1999). Numerous behavioural studies support this hypothesis. Antagonism of spinal $GABA_A$ receptors using specific antagonists (Reeves *et al.,* 1998; Roberts *et al.,* 1986), and selective $GABA_A$ gene- knockout (Ugarte *et al.,* 1999) induce mechanical allodynia accompanied by modest thermal hyperalgesia, suggesting that GABA acting at $GABA_A$ receptors tonically suppresses afferent transmission of noxious information in the spinal cord. Antagonism of spinal $GABA_A$ (but not spinal $GABA_B$) receptors enhances allodynic responses in spinalised rats, suggesting that $GABA_A$ receptors mediate tonic GABAergic inhibition through 'local' segmental

spinal inhibitory circuitry (Sivilotti & Woolf, 1994). Buritova *et al.*, (1996) demonstrated that systemic (but not intraplantar) administration of baclofen, a $GABA_B$ agonist, reduced cfos expression in the spinal dorsal horn and also attenuated paw and ankle oedema following high-dose intraplantar carrageenan treatment, suggesting that $GABA_B$ receptors may also mediate tonic inhibition of excitatory nociceptive transmission. Activation of spinal GABAergic neurons through stimulation of peripheral nociceptive fibres (Aβ, Aδ and C) has also been shown to inhibit spinothalamic tract neurotransmission (Carlton *et al.*, 1992).

Hammond (1999) has reviewed the role of GABA in pathological pain. Changes in GABAergic transmission are highly dependent on the type of injury and the duration of the post-injury period; while nerve injury attenuates GABAergic responses (Castro-Lopez *et al.*, 1995), intense inflammatory injury enhances GABAergic transmission (Castro-Lopes *et al.*, 1992; 1994; Kaneko & Hammond, 1997). Intraplantar injection of CFA (Castro-Lopes *et al.*, 1992) or carrageenan (Castro-Lopez *et al.*, 1994) enhances spinal GABA protein expression and increases the number of GABA-immunoreactive cells in the superficial dorsal horn. Intraplantar injection of CFA induces prolonged ipsilateral enhancement of glutamate decarboxylase (GAD) mRNA expression and GABA immunoreactivity, from 4 days following CFA injection (Nahin & Hylden, 1991; Castro-Lopes *et al.*, 1994).

GABA and $GABA_A$ receptors are involved in the development and induction of synaptic plasticity in higher centres including the amygdala, (Watanebe *et al.*, 1995), cortex (Kanter & Haberly, 1993), dentate gyrus (Tomasulu *et al.*, 1993), and hippocampus (Yasui *et al.*, 1993). Thus modulation of GABAergic transmission may also be implicated in the induction and/or maintenance of LTP or LTD of spinal nociceptive processing, associated with central sensitisation.

Endogenous opioids

While the pain-relieving properties of *papaver somniferum* were familiar to Sumerians and pre-dynastic Egyptians some 5 millenia ago, the active analgesic and euphoric component of the humble poppy was not identified until the 19th century, when the German chemist Friedrich Sertuner isolated the principal active compound of opium and named it morphine in honour of Morpheus, God of dreams. Opioids remain the 'gold standard' against which all other analgesic agents are compared. Our understanding of the mechanisms through which opioids induce analgesia has been obscured by the intrinsically complex nature of

'pain' sensation as a mixture of sensory transmission and subjective emotional experience, since opioids are able to alter both of these components of pain sensation.

The pharmacology of the endogenous opioid system has been reviewed by Loughlan *et al.*(1995) and Feldman *et al.* (1996). Endogenous opioid ligands were first identified in 1975 and differentiated into two 'families' of closely related peptides, enkephalins (Hughes *et al.,* 1975) and dynorphins (Goldstein *et al.,* 1979). Three distinct genes coding for endogenous opioid peptides have since been identified, each of which encodes a collection of peptides. These are prepro-opioimelanocortin (POMC), which encodes endorphin peptides, preproenkephalin, which encodes enkephalin peptides, and preprodynorphin, which encodes dynorphins. Expression of the precursor peptides varies markedly between different tissue types and regions of the CNS. POMC is concentrated in the pituitary gland and the hypothalamus, while proenkephalin and dynorphin peptides are distributed widely throughout the CNS.

Three major opioid receptor types have been cloned, classified as μ (MOR-1) (Chen *et al.,* 1993; Wang *et al.,* 1993); δ (DOR-1) (Evans *et al.,* 1992, Kiefer *et al.,* 1992) and κ_1 (KOR-1) (Chen *et al.,* 1993; Li *et al.,* 1993). It is now becoming evident that each of these main receptor types can be further subdivided into multiple splice variants (reviewed by Pasternak, 2000). Receptor distribution in the central nervous system and elsewhere varies significantly between the different receptor types and is species specific. The distribution of endogenous opioid receptors in the rat spinal cord has been characterized by Morris & Herz, 1987, and Besse *et al.,* 1990). While opioid receptors are present in all laminae of the spinal grey matter they are more abundant within the dorsal horn; the precise localisation of each receptor type is believed to reflect their particular mode of action. All three major receptor types occur within the dorsal horn, predominantly within lamina II. Besse *et al.* (1990) reported that 70% of opioid receptors in lamina II are μ; 24% are δ, and 6% are κ. Although enkephalin, dynorphin and β-endorphin do show selectivity for μ, κ and δ receptors respectively, there is no clear correspondence between the anatomical distribution of any opioid peptide and a particular receptor type. Hollt (1986) proposed that this mismatch may reflect the processing of propeptide precursors into a number of different peptides with differing receptor selectivities. Opioid receptors are also located in higher centres including the peri-aqueductal grey matter (Budai & Fields, 1998) and the forebrain (Grau 1987; Meagher *et al.,* 1989; 1990); thus, endogenous opioids also regulate central nociceptive transmission through modulation of descending control systems located in the midbrain and brainstem (reviewed by Gogas *et al.,*1991; Duggan, 1992). While most

exogenously administered opioids interact with several different opioid receptors, morphine and the majority of opioid agents used in clinical analgesia act through μ-opioid receptors (Pasternak, 2000). Selective κ opioid receptor activation produces numerous adverse behavioural side effects including psychomimetic behaviour and dysphoria in rats and humans and thus κ selective agents have not been developed as clinical analgesic agents. μ-selective receptor activation produces the most potent and effective opioid-mediated inhibition of excitatory nociceptive transmission (reviewed by Pasternak, 2000)

All opioid receptors belong to the G-protein-coupled receptor superfamily and induce their intracellular effects through interaction with guanine nucleotide-binding proteins (reviewed by Childers, 1993 and Cox, 1993). The mechanisms through which opioids modulate spinal nociceptive transmission- principally by means of modulating synaptic transmission and neurotransmitter release- have been reviewed by Yaksh (1993)and Dickenson (1997). Opioids exert both pre- and post-synaptic influence on primary afferent nociceptive neurons and the spinal neurons on which they converge, and alter activity in ascending excitatory pathways and descending inhibitory pathways. It has been proposed that numerous central nociceptive circuits may exist under tonic opioid inhibitory control (reviewed by Dickenson, 1997). Opioids selectively inhibit synaptic transmission in Aδ and C nociceptive afferent fibres within the dorsal horn rather than generally inhibiting all sensory transmission, and modulate release of numerous key neurotransmitters involved in pain transmission pathways, including substance P, CGRP, noradrenaline, acetylcholine, dopamine and serotonin (5-HT).

It is well established that endogenous opioids exert tonic inhibitory control on central nociceptive processing , and that this system can be modulated by injury (Kayser & Guilbaud, 1987; 1991; Kayser *et al.,* 1986; 1988; Lombard & Besson, 1989). The inherent plasticity of endogenous opioid systems is well documented; the spinal effects of opioids can be enhanced or reduced depending on pathology and activity in other segmental and non-segmental pathways (Dubner & Ruda 1992; Dickenson 1994; Cesselin 1995; Mao *et al.,* 1995; Stanfa & Dickenson 1995; Stanfa *et al.,* 1994; Dickenson 1997).

It is worthy of note that plasticity of opioidergic inhibition subsequent to injury may reflect not only alterations in pro-opioid ligand mRNA expression and translation into active peptide within the spinal cord, but also recruitment of different levels of the neuroaxis; alterations in opioid receptor sensitivity; and modulation of opioid activity by complementary transmitter systems, including adrenergic, and glutamate/NMDA

neurotransmitter systems (Dickenson, 1997; Mao, 1999) and anti-opioid peptides (Faris *et al.,* 1983).

Stanfa & Dickenson (1995), and Dickenson (1997) have reviewed the contribution of the opioid system to induction and maintenance of plasticity of central nociceptive transmission associated with inflammatory injury. Inflammation alters the dynamics of endogenous opioid transmission, both through modulating synthesis of endogenous transmitters and through altering the activity of both peripheral and central opioid receptors. In inflammatory states there is dramatic upregulation of dynorphin mRNA expression, and moderate upregulation of enkephalin mRNA (Nahin *et al.,* 1989; Dubner & Ruda, 1992). While upregulation of enkephalin activity results in enhanced μ-opioid receptor mediated inhibition of excitatory nociceptive transmission, the functional consequences of enhanced dynorphin expression are considerably more complex; while dynorphin can emulate certain increases in central excitability which occur following inflammation (e.g. enhanced responses of nociceptive neurons), it inhibits others (Knox and Dickenson, 1987). Dynorphin and its 2-17 fragment may exert central analgesic action partly through non-opioid receptors (Hooke *et al.,* 1995). Dynorphin (1-17) has been reported to act as an antagonist at the glutamate binding site of the NMDA receptor, while other dynorphin peptides have been reported to inhibit NMDA receptor channels of isolated trigeminal neurons (Dyn 2-17) (Chen *et al.,* 1995) and AMPA and kainate responses in dorsal horn neurons (Dyn 1-17) (Kolaj *et al.,* 1995). κ-opioids also appear to antagonise the μ receptor in the spinal cord (Stanfa *et al.,* 1992). However, spinal dynorphin levels are also enhanced in neuropathic pain models, where opioid actions tend to be reduced, suggesting that upregulation of dynorphin in isolation does not play a key role in the induction or maintenance of plasticity of central nociceptive transmission.

There is some evidence that inflammatory injury also alters the expression and/or activity of central opioid receptors; Goff *et al.* (1998) reported significant upregulation of μ-receptor labelling in the lumbar dorsal horn associated with persistent intraplantar inflammation induced by CFA treatment. However, the majority of studies have failed to identify conclusive changes in the number or ligand affinity of any of the three opioid receptor types in response to inflammatory stimulation (Iadarola *et al.,* 1988; Millan *et al.,* 1988; Delay-Goyet *et al.,* 1989).

Opioids have been shown to play a role in the mediation of certain types of long-term potentiation in the brain, notably in the lateral perforant and mossy fibre pathways of the

hippocampus, although the precise role of each receptor group remains undetermined. There is ample evidence that μ-and δ receptors regulate LTP induction (Satoh *et al.,* 1989; Derrick *et al.,* 1991; Xie *et al.,* 1991; Bramham *et al.,*1991; 1992; Xie & Lewis, 1995a; Williams & Johnston, 1996), partially through interaction with the GABAergic inhibitory system (Bramham & Sarvey 1996). However, Xie & Lewis (1995b) reported that LTP of inhibitory neurotransmission in the dentate gyrus of the hippocampus required δ-receptor antagonism. κ-receptor activation through dynorphin administration attenuates LTP in the hippocampus in a naloxone-reversible manner (Wagner *et al.,* 1993; Drake *et al.,* 1994). Opioids have also been implicated in the induction of LTD in the hippocampus (Francesconi *et al.,* 1997; Ikeda *et al.,* 1999) and in the spinal cord (Pockett, 1995) where LTD induction has been both attenuated and inhibited by naloxone-mediated opioid receptor antagonism.

Catecholamines

Noradrenergic and adrenergic pathways, originating in the medulla and the pons (locus coerulus and subcoerulus) and projecting to the spinal dorsal horn, constitute a third key transmitter system which inhibits excitatory nociceptive transmission in the central nervous system. Adrenergic mediation of antinociception in the central nervous system has been thoroughly reviewed by Tasker *et al.,* (1992), Kingery *et al.,* (1997) and Millan, 1998).

Multiple types of α_1, α_2 and β_1 ,β_2 and β_3 adrenergic receptors (AR's) have been described, the physiological significance of which is still under investigation (Millan, 1998). α_1 AR's are present at only low concentrations in the dorsal horn and have not been accorded a significant role in central nociceptive processing (Millan 1998). β AR's have been identified in the spinal cord (Nicholas *et al.,* 1993) but there is presently no evidence to support a significant role for these receptors in nociceptive transmission. α_2 AR's are abundant in the superficial laminae of the DH and in the intermediate grey matter and are present at a somewhat lower density in deeper laminae of the dorsal horn as well as in the ventral horn (Dashwood *et al.*, 1985; reviewed by Millan, 1998). Investigation of central adrenergic mediation of antinociception has demonstrated a dominant role for the α_2AR (Howe *et al.,* 1983; Osipov *et al.,* 1984; Barbaro *et al.,* 1985; Jones & Gebhart, 1986). α_2AR-activated pathways inhibit central nociceptive transmission through various mechanisms (reviewed by Kingery *et al.,* 1997), including pre-synaptic inhibition of glutamate release from nociceptive fibres (Belcher *et al.,* 1978; Fleetwood-Walker 1985) and post-synaptic inhibition of wide dynamic range spinal neurons (Belcher *et al.,* 1978; Pertovaara *et al.,* 1993). It is worthy of note that in addition to their abundance in the

central nervous system, α_2 AR's are abundant in many other body systems including the vascular system and the sympathetic system, which may also modulate peripheral inflammation and pain transmission (reviewed by Millan, 1998).

Purines

Purines [adenosine, adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP)] are present in all cells. There is mounting evidence adenosine may contribute to both physiological and pathophysiological central nociceptive transmission (reviewed by Sawnok & Sweeney (1989); Karlsten & Gordh, 2000). Adenosine is released from cells directly or via degradation of adenosine triphosphate (ATP), and is involved in many regulatory mechanisms in both physiological and pathophysiological conditions (reviewed by Pelleg & Porter, 1990; Brundege & Dunwiddie, 1997; Abbrachio & Burnstock, 1998). Multiple adenosine (A) receptors have been cloned (A1, $A2_A$, $A2_B$ and A3) (Fredholm *et al.,* 1994). A1 and A2 receptors occur in the spinal cord, with A1 receptors predominating in lamina II (Choca *et al.,* 1987; 1988) and in the brain (Goodman & Snyder, 1982; Braas *et al.,* 1986). Adenosine receptors are widely distributed throughout the central nervous system, concentrated in lamina II of the dorsal horn. Adenosine is released from small-diameter nociceptive fibres in the dorsal horn and modulates spinal nociceptive transmission through presynaptic inhibition of sensory nerve terminals, and through hyperpolarisation of the post-synaptic membrane by increasing K^+ conductance (Sawynok, 1998).

There is now abundant evidence that adenosine modulates central nociceptive transmission, principally through A1 receptors located in the superficial dorsal horn. Intrathecal administration of selective A1 and A2 receptor analogues demonstrated an antinociceptive effect of spinal A1 receptor activation (Karlsten, *et al.,* 1990; Sawynok, 1991). Activation of spinal A1 receptors inhibits C-fibre evoked responses, wind-up and post-discharge of dorsal horn neurons (Reeve & Dickenson,1995; Nakamura *et al.,* 1997). In addition to its potential tonic inhibitory influence on physiological nociceptive transmission, there is mounting evidence to support a role of adenosine in the modulation of various manifestations of pathophysiological nociceptive transmission, including hyperalgesia associated with neuropathic injury (Yamamoto & Yaksh, 1991; Lee & Yaksh, 1996; Cui *et al.,* 1998; Lavand'homme & Eisenach, 1999) and hyperalgesia induced by intrathecal prostaglandin F_2 administration (Minami *et al.,* 1992).

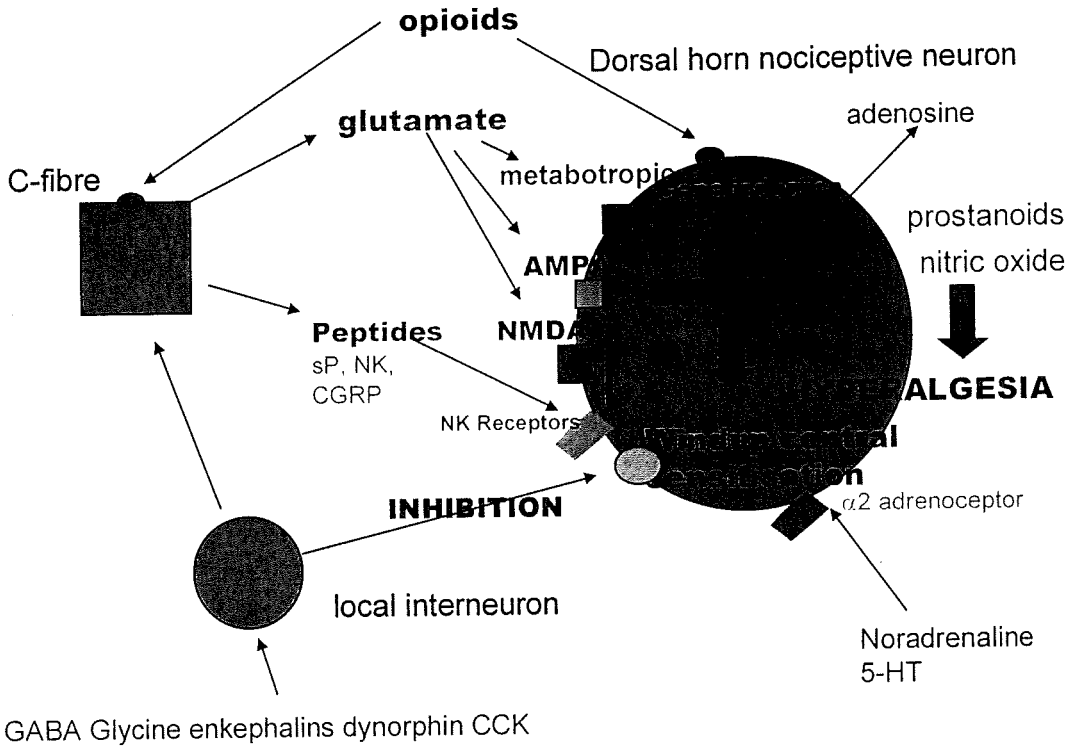
1.4 MOLECULAR PHARMACOLOGY OF PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL NOCICEPTIVE TRANSMISSION

The rapid response of nociceptive neurons in the central nervous system to peripheral noxious stimulation is mediated by transient processes, including receptor potentials in peripheral nociceptors, the conduction of action potentials, and rapid chemical transmission mediated by the opening of ligand-gated receptors coupled to ion channels (reviewed by Willis, 1996). However, noxious stimulation can also produce long-lasting modulation of central nociceptive neuronal activity. The intracellular signal transduction pathways activated by (for example) intracellular calcium influx or activation of receptors coupled to G-proteins or tyrosine kinase molecules, can produce intraneuronal metabolic changes which produce a prolonged alteration of neuronal excitability (Willis, 1996). Several types of signalling molecule have been implicated in the intracellular induction and/or maintenance of neuronal plasticity, including protein kinase enzymes, immediate early genes and their associated transcription factors and cytoskeletal proteins (reviewed by Morris, 1997). These are illustrated in Figure 1.5. Woolf & Costigan, (1999) and Woolf & Salter (2000) reviewed the molecular pathways which have been implicated in the induction and maintenance of central sensitisation of nociceptive transmission. Other signalling transduction pathways, such as those activated by opioidergic neurotransmission, reduce central nociceptive transmission (Willis, 1996). A greater understanding of the involvement of intracellular signal transduction mechanisms should lead to new targets for the therapy of persistent pain syndromes.

Calcium calmodulin kinase (CaMKII)

Many receptor-mediated events involve protein phosphorylation, which controls the functioning and binding properties of intracellular proteins. CaMKII is a protein kinase enzyme richly expressed in neurons, where it is a major constituent of post-synaptic density proteins (Kennedy *et al.,* 1983). It is a heteromultimer comprised of α and β subunits, 10 to 12 of which combine to form active CaMKII holoenzymes. Each subunit molecule contains an active site and a calmodulin binding site. The enzyme is calcium dependent, activated by the calcium-binding protein calmodulin which, when occupied by calcium, binds to a CaMKII subunit and elicits exposure of the catalytic domain of the enzyme. CaMKII phosphorylates numerous proteins involved in neurotransmitter synthesis and of dorsal root ganglia, presynaptic terminals (Ouimet *et al.,* 1984) and postsynaptic release, carbohydrate metabolism, and cytoskeleton

Figure 1.5: The pharmacology of central nociceptive transmission and central sensitisation.



assembly/dissociation, including $GABA_A$ receptors, phospholipase A_2, microtubule-associated protein 2 (MAP-2) and neurofilament proteins (reviewed by Hanson & Schulman, 1989).

The distribution of the α and β isoforms of CaMKII has been described by Terashima *et al.* (1994), who reported that CaMKIIα is strongly expressed in laminae I-IV of the dorsal horn (predominantly in presynaptic terminals and dendritic processes) but absent in laminae IV-X. CaMKIIβ is distributed homogeneously throughout spinal cord grey matter located more densely in laminae I/II, and is also present in ventral horn motorneurons.

CaMKII has intrinsic structural characteristics which make it a highly attractive candidate for the mediation of neuronal plasticity. The enzyme occupies a fixed position on the synaptic membrane which facilitates both its response to intracellular calcium influx and phosphorylation of ion channels (prerequisites for the induction of synaptic plasticity). CaMKII is the principal protein of the post-synaptic density (Kelly *et al.,* 1984), a sub-membrane structure physically linked to the post-synaptic membrane in a specific region in which both NMDA (notably the NR2B subunit) and non-NMDA receptors associated with modulation of synaptic activity are located (Carlin *et al.,* 1980; Kennedy *et al.,* 1983; Ocorr *et al.*,1991). CaMKII is transiently activated by phosphorylation but can also enter an autonomously activated state independent of calcium/calmodulin stimulation (reviewed by Lisman 1994; 1995). Following activation of a group of CaMKII molecules by calcium and calmodulin associated with significant neuronal stimulation, CAMKII autophosphorylates by an intersubunit intraholoenzyme reaction and becomes permanently 'switched on', remaining active independent of the continued presence of calcium and calmodulin and earning CaMKII the label of 'molecular switch' (Saitoh & Schwartz, 1985; Miller & Kennedy 1986). This is illustrated in Figure 1.6.

Lisman (1985) speculated that autophosphorylation potential maintains the active state of the kinase molecular group even if individual molecules are dephosphorylated or replaced by newly synthesised unphosphorylated molecules. Self-perpetuating CaMKII activation can persist throughout the lifetime of the neuron and this mechanism potentially provides a molecular pathway through which 'memory storage' of a triggering synaptic event may be mediated, thus maintaining long-term changes in neuronal excitability in the absence of subsequent alterations in gene expression (Lisman, 1994).

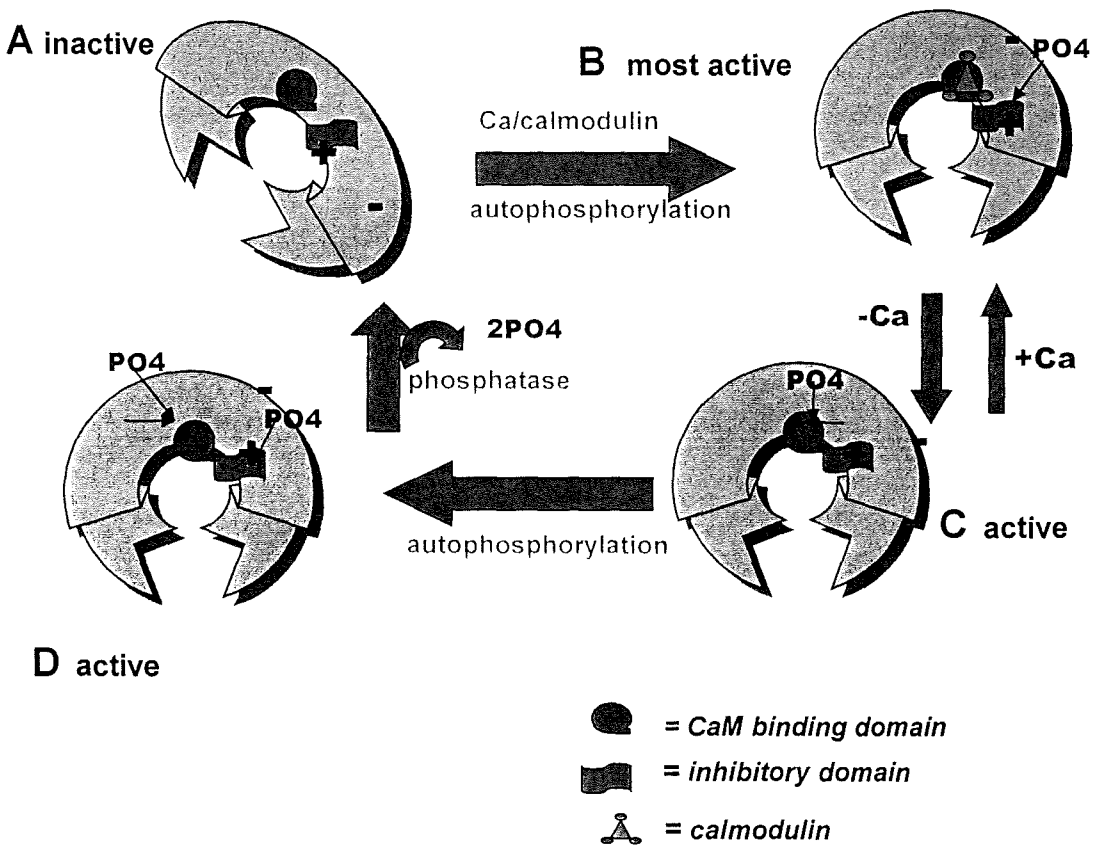
Figure 1.6: CaMKII as a molecular switch

A: In the absence of Ca^{2+}/calmodulin (CaM), the active site of CaMKII is inhibited by binding of the inhibitory domain.

B: Binding of CaM to its recognition site alters the enzyme's conformation, leading to phosphorylation of substrate proteins, including CaMKII itself.

C: When the intracellular Ca^{2+} level falls, CaM dissociates itself from the enzyme; however, the phosphorylated threonine prevents reassociation of the inhibitory domain with the active site. Kinase activity is now partially independent of Ca^{2+} (the switch is "on").

D: Autophosphorylation may now occur at a site within the CaM binding domain, which causes the enzyme to be completely insensitive to CaM. Finally, CaMKII is dephosphorylated by phosphatases, returning it to its inactive state.



The role of CaMKIIα in the induction of neuronal plasticity has been extensively studied using a variety of molecular techniques, including enzyme antagonist studies (Wang & Feng, 1992) gene-knockout studies (Silva *et al.,* 1992a, b; Stevens *et al.,* 1994) and direct assays of enzyme activity (Fukunaga *et al.,* 1993). In the hippocampus, electrophysiological studies using CaMKIIα antagonists indicate that CaMKIIα is involved in the induction of LTP (Malinow *et al.,* 1989; McGlade-McCulloch *et al.,* 1993), while in-vitro assays of CAMKIIα activity indicate that CAMKIIα is rapidly and persistently activated in response to LTP-inducing stimuli (Fukunaga *et al.,* 1993). Studies using CaMKIIα knockout mice (Stevens *et al.,* 1994) have demonstrated the involvement of CaMKIIα in three distinct forms of synaptic plasticity; CaMKII-α knockout mice are severely impaired in their ability to learn certain spatial tasks despite intact synaptic transmission pathways and normal NMDA receptor function. STP, LTP and LTD are absent or dramatically attenuated in these mice in comparison with CaMKIIα intact control animals. CaMKIIα induces persistent enhancement of synaptic transmission by phosphorylating post-synaptic density ion channel proteins (including, most critically, NMDA receptors (Leonard *et al.,* 1999; Shen & Meyer, 1999); AMPA receptors (Barria *et al.,* 1997; Mammen *et al.,* 1997); and other signalling proteins including immediate early genes (reviewed by Leonard *et al.,* 1999; Shen & Meyer, 1999). CaMKIIα-mediated phosphorylation of AMPA receptors is an important step in the induction of LTP (Leonard *et al.,* 1999). The calcium influx which activates CaMKIIα to produce LTP is commonly, but not exclusively, mediated by NMDA receptor activation (Leonard, 1999).

There is ample evidence that CaMKIIα regulates key excitatory neurotransmitter systems involved in nociceptive processing. Intracellular application of CaMKIIα enhances glutaminergic transmission and excitatory synaptic potentials in acutely isolated rat spinal dorsal horn neurons (Kolaj *et al.,* 1994; Pereda *et al.,* 1998). It has been proposed that NMDA-mediated activation of kinase enzymes following intraneuronal calcium influx may be one mechanism through which central sensitisation of nociceptive transmission is maintained (reviewed by Coderre & Yashpal, 1994); however, other studies suggest that CaMKIIα regulates 'general' inter-neuronal communication, rather than mediating specific modalities of synaptic transmission involved in the induction and/or maintenance of synaptic plasticity (Pereda *et al.,* 1998).

CaMKIIα also regulates activity in key inhibitory neurotransmitter systems, including $GABA_A$ receptor activity and glycinergic transmission. The $GABA_A$ receptor has specific phosphorylation sites for CaMKII (McDonald & Moss, 1994) and CaMKIIα has been shown to enhance $GABA_A$ receptor activity and inhibitory synaptic responses in dorsal

horn neurons *in-vitro* (Wang *et al.,* 1995a,b) suggesting that CaMKIIα may play a key role in the regulation of $GABA_A$ receptor activity and thus persistent enhancement of inhibitory synaptic responses. Basal kinase-mediated phosphorylation of either the $GABA_A$ receptor itself or a closely related protein is required to maintain $GABA_A$ receptor function in CA1 hippocampal neurons (Chen *et al.,* 1990). However, contradictory neuroanatomical data suggests that CaMKIIα is not involved in GABAergic neuronal responses or in presynaptic mechanisms within the axon terminals of dorsal horn neurons (Benson *et al.,* 1992; Liu & Jones 1996) following inflammatory injury. CaMKIIα also enhances glycine currents in acutely isolated rat spinal neurons (Wang & Randic, 1996). Neurons in the superficial dorsal horn co-express NMDA and glycine receptors in the post-synaptic membrane (Wang *et al.,* 1998); NMDA-mediated intracellular calcium influx can modulate glycine receptor function in the superficial dorsal horn through CaMKIIα activation (Xu *et al.,*1999; 2000). When excitatory (NMDA) and glycinergic inhibitory synaptic transmission coincide, activation of post-synaptic transmission by glutamate released from sensory afferents may in turn inhibit post-synaptic neurons via augmentation of glycinergic input. This 'feedback' enhancement of the glycine response by NMDA activation represents a potential 'adaptive' spinal signalling pathway which could attenuate excitatory nociceptive transmission. CaMKIIα has also has been implicated in the regulation of prodynorphin gene expression in cultured spinal cord cells (Ha *et al.,* 1997) and histamine (H_1 and H_2) receptor-stimulated anti-nociception (Chung *et al.,* 1998, 1999).

The factors and mechanisms which regulate CaMKIIα gene expression in the CNS appear to be complex and remain poorly defined; indeed, regulation of CaMKIIα gene expression in the spinal cord has not been investigated to date. In the brain, regulation of CaMKIIα mRNA expression has been investigated predominantly using models of specific electrical stimulation such as LTP induction, tetanic stimulation or seizure induction. CamKIIα mRNA expression is increased following reduction of afferent input into hippocampal neurons, suggesting that CaMKII gene expression may be regulated by normal physiological neuronal activity. In models of neuronal plasticity in the brain, upregulation, downregulation and biphasic regulation of CaMKIIα mRNA expression have been induced using differing modalities of electrical stimulation. Mackler *et al.* (1992), Thomas *et al.* (1994) and Ouyang *et al.* (1999) reported enhanced CaMKII mRNA expression in isolated hippocampal neurons following electrical stimulation, induction of LTP and tetanic stimulation respectively. Roberts *et al.* (1996) also reported upregulation of CaMKIIα mRNA expression in hippocampal neurons following the induction of LTP. In contrast, Liang *et al.* (1996) reported a reduction in CaMKIIα mRNA expression in neocortical

neurons for a short period (1-3 h) following tetanic stimulation, speculating that stimulus-dependent mechanisms underlying neocortical plasticity involved reciprocal changes in CaMKIIα expression which regulated the balance of excitation and inhibition. Murray *et al.,* (1995) reported reduced expression of CaMKIIα mRNA in hippocampal, neocortical and piriform cortical neurons following seizure activity.

The intracellular mechanisms which drive alterations in CaMKIIα mRNA expression are equally poorly defined. Johnston & Morris (1995) reported that NMDA and nitric oxide (NO) stimulation of hippocampal neurons induced initial enhancement of CaMKIIα mRNA expression for 6 hours followed by dramatic downregulation of CaMKIIα mRNA expression observed 24 h post-stimulation, speculating that biphasic regulation of CaMKII α mRNA expression could modulate long-term responses of hippocampal neurons to NMDA receptor stimulation or NO release. Another study similarly reported that high-dose NMDA stimulation of retinal neurons induced transient upregulation (for 2 h post-stimulation) followed by downregulation (at 24 h) of CaMKIIα mRNA expression. (Laabich & Cooper, 2000).

Evidently many questions regarding the involvement of CaMKIIα in nociceptive transmission and plasticity of central nociceptive processing remain unanswered. There is substantial evidence that other protein kinase enzymes, most notably protein kinase C (PKC) and protein kinase A (PKA) play pivotal roles in processes underlying neuronal sensitisation and enhancement of nociception (reviewed by Millan, 1999) and it would be of great interest to similarly identify and characterise a role for CaMKIIα in central nociceptive transmission and its modulation.

Immediate early genes and transcription factors

Immediate early genes (IEG's) are genes which are rapidly induced and/or expressed in neurons and other cells in response to an external stimulus. IEG's have been induced in cultured cells by growth factors, hormones, peptides and transmembranous ion fluxes. In neurons they can also be induced by after-discharges, electrical and chemical seizures, stimuli which share a 'common denominator', leading to or substituting for calcium influx through voltage-sensitive calcium channels (Greenberg *et al.,* 1985; Morgan *et al.,* 1989). IEG's play an important role in the initiation of cell division, differentiation and neoplastic processes as well as mediating plasticity of cellular responses (reviewed by Angel & Karin, 1991). Over 100 IEG's have now been identified, most of which encode transcription factors (TF's). The functional properties of IEG's and their role in the

mediation of sustained plasticity of neuronal activity in the brain and spinal cord have been reviewed by Morris (1997) and Herdegen & Zimmerman (1995).

Immediate early genes are characterised by certain key features:

1. They are transcriptionally activated: IEG's are expressed *de novo* in response to an external stimulus by 'stimulation-transcription coupling' (Morgan & Curran, 1991), activated by specific constitutively expressed trans-activating proteins through a mechanism which is independent of protein-synthesis; indeed, expression of these genes in the presence of protein-synthesis inhibitors is an important criterion for according IEG status to a particular gene (Curran & Morgan, 1987; Robertson, 1988).

2. They are rapidly induced: mRNA for IEG's is often detected within 15 minutes of suitable cellular stimulation, protein becoming detectable within 30 minutes. Intracellular IEG mRNA levels are also rapidly attenuated subsequent to rapid inhibition of transcription degradation of mRNA (Greenberg *et al.,* 1984; Krujer *et al.,* 1984; Lau *et al.,* 1987).

3. They are often transiently expressed: The half-life of mRNA is approximately 30 minutes, while that of transcription factor protein is 2 hours, (Greenberg *et al.,* 1984; Krujer *et al.,* 1984; Lau *et al.,* 1987; reviewed by Dragunow *et al.,* 1989). Rapidity of transcriptional activation implies that IEG promotors are poised for activation and transcription begins as soon as the second messenger is generated by the growth factor .

IEG's encode for proteins; inducible transcription factors (ITF's), secretory proteins, enzymes and membrane receptors. ITF's are the protein products of IEG translation, a group of nuclear proteins that bind to regulatory DNA promotor and enhancer sites and control transcription of specific target or effector genes, either enhancing or repressing gene expression. It has been hypothesised that ITF's play a crucial role in mediating long-lasting changes in CNS function and may be involved in establishing the neurological pathways responsible for learning, physiological adaptation to new environments, and certain persistent neurological diseases including forms of chronic pain (reviewed by Herdegen & Zimmermann, 1995; Morris 1997). ITF's activate or repress directly (on the promotor region) or indirectly the expression of other more durable genes which have been referred to as 'late effector genes' or LEG's, generally constitutively expressed in at higher

levels than IEG's (Curran & Morgan, 1987) which in turn are transcribed into proteins that mediate a prolonged change in cellular function.

The precise role of IEG's in the induction and maintenance of LTP, learning and memory in higher centres remains undetermined. In the hippocampus a close relationship has been identified between expression of certain IEG's and LTP (reviewed by Morris, 1997). Numerous 'families' of IEG's have been associated with LTP, notably the fos genes, the jun genes, the 'zinc finger genes' including zif/268 (krox-20) and NGF1-A (krox-24) (Cole *et al.,* 1989; Wisden *et al.,* 1990), the growth factor β-activin (Andreasson & Worley, 1995), protease tissue plasminogen activator (tPA) (Qian *et al.,* 1993), and cyclo-oxygenase-II (Yamagata *et al.,* 1993). The closest correlation between ITF expression and induction of LTP has been observed for zif/268. This relationship is not however constant. While some workers have observed a dramatic increase in zif/268 mRNA in post-synaptic neurons in the hippocampus following high-frequency stimulation (Cole *et al.,* 1989; Roberts *et al.,* 1996), others have observed LTP induction in the absence of zif/268 expression, and also zif/268 expression in the absence of LTP induction (Wisden *et al.,* 1990; Johnston & Morris, 1994). Recent evidence suggests that the principal role of IEG's in neuronal plasticity is in the maintenance and stabilisation of persistent LTP (associated with phenotypic alteration of the neuron), rather than in the induction of LTP (reviewed by Walton *et al.,* 1999). Strong induction of nerve growth factor 1A (NGF1-A) and zif/268 occurs in association with long-lasting LTP in the hippocampus, but not in association with less stable forms of LTP (Abraham *et al.,* 1993). Futhermore, increased expression of NGF1-A has been associated with some forms of long-term memory in primates and rats (Okuno & Miyashita, 1996; Richter-Levin *et al.,* 1998).

Herdegen & Zimmermann (1995) have reviewed evidence supporting the involvement of IEG's and ITF's in central sensitisation in the spinal cord. With the exception of zif/268 in lamina III of the lumbar spinal cord and constitutive expression of junD and COX-2, ITF's are absent in the dorsal horn under physiological conditions (Herdegen *et al.,* 1991; Tolle *et al.,* 1994). It has been proposed that a significant increase in levels of ITF's requires activation of both A-δ and C-fibres (Hunt *et al.,* 1987; Herdegen *et al.,* 1991; Molander *et al.,* 1992) and consequently it has been speculated that enhanced expression of IEG's in dorsal horn neurons induces central sensitisation. Some groups have mapped constitutive and induced ITF expression in the dorsal horn following noxious thermal stimulation (Wisden *et al.,* 1990), inflammatory injury (Lanteri-Minet *et al.,* 1993), and electrical nerve stimulation (Herdegen *et al.,* 1991).

Persistent noxious stimulation triggers dramatic alterations in dorsal horn neuronal expression of immediate early genes involved in transcriptional control (c-Fos, c-jun, zif/268) and of genes encoding neuropeptides (dynorphin, enkephalin, Substance P, NPY) and their receptors (Iadarola & Caudle 1997). The upregulation of IEG expression has been shown in some cases to parallel the time course of hyperalgesia associated with inflammatory injury (Ji *et al.,* 1994; Draisci *et al.,* 1991). It would appear that a 'transcriptional network' is activated in spinal pain-processing neurons, and if the stimulus is of a sufficient degree and duration, a network of target genes even further downstream can be activated resulting in long-term plasticity of the nociceptive response (Herdegen *et al.,* 1991).

A major challenge in our appreciation of the role of ITF's in synaptic plasticity remains identification of specific changes which they induce in neurotransmitter production or activity (reviewed by Sheng & Greenberg, 1990; Treisman, 1990; Herdegen & Zimmermann, 1995; Chaudhuri, 1997). Within the spinal cord, popular candidate targets for activation by ITF's include neuropeptides, glutamate, endogenous opioids and nitric oxide. Fos and Jun proteins have been linked with regulation of transcription of proenkephalin (Sonnenberg *et al.,* 1989; Noguchi *et al.,* 1992), prodynorphin (Naranjo *et al.,* 1991; Noguchi *et al.,* 1991) and nerve growth factor (Hengerer *et al.,* 1991). SP and its NK-1 receptor (Duggan *et al.,* 1988) have also been postulated as potential targets for ITF-mediated upregulation.

ZIF/ 268

Constitutive expression of zif/268 throughout the grey matter of the spinal cord has been previously demonstrated using *in-situ* hybridisation techniques by Wisden *et al.,* (1990). Upregulation of spinal zif/268 mRNA expression has been reported previously in association with numerous modalities of noxious stimulation. Wisden *et al.,* (1990) demonstrated that noxious thermal stimulation of the hindpaw induced dramatic upregulation of zif/268 expression in laminae I, II, V and X (measured 15-30 minutes after stimulation), while non-noxious thermal stimulation at 42 °C did not alter constitutive zif/268 expression. However Wisden *et al.,* (1990) did not quantify zif/268 up-regulation, nor comment on the unilateral or bilateral nature of upregulation. Herdegen *et al.* (1991) studied zif/ 268 mRNA expression following electrical stimulation of nociceptive fibres in the sciatic nerve, observing that selective stimulation of wide diameter A-fibres did not alter zif/268 mRNA expression beyond basal levels in the dorsal horn, while stimulation extended to include both Aδ and C-fibres induced ipsilateral upregulation of zif/268 mRNA expression in the dorsal horn. In this study, zif/268 protein expression was

detected 30-45 minutes after the onset of repetitive sciatic nerve stimulation and peaked 2-3h post stimulation. There are few reports of regulation of zif/268 expression associated with inflammatory disease; while Buritova *et al.* (1995) investigated zif/268 protein regulation associated with high-dose carrageenan treatment, and Lanteri-Minet *et al.* (1993) studied zif/268 protein expression (identified by immunocytochemistry techniques) associated with persistent CFA intraplantar inflammation, regulation of zif/268 expression following mild or repeated inflammatory injury has not been investigated to date.

Tissue plasminogen activator

Plasminogen activators (PA's) are serine proteases which convert plasminogen into the active protease plasmin and thus provide a source of proteolytic activity in many biological systems (Collen, 1980; Dano *et al.,* 1985). Two types of PA have been identified; tissue-type PA (tPA) (Astrup & Permin, 1947) and urokinase-type PA (uPA) (Williams, 1951). In the nervous system, PA's are involved in cell proliferation (Kalderon, 1984; Moonen *et al.,* 1985), migration (Moonen *et al.,* 1982) and differentiation in the developing brain tPA is secreted by neurons during neurite outgrowth and remodelling (Monard, 1988) and its substrates degrade many components of the extracellular matrix, enabling neurites to develop and sprout (Krystosek *et al.,* 1981; 1984). TPA has thus been proposed as an important regulatory molecule in neuron development, the modulation of neuronal architecture, and neuronal death (Tsirika *et al.,* 1996).

The role of tPA in the induction and maintenance of synaptic plasticity in the brain has been reviewed by Baranes *et al.*(1998). Qian *et al.* (1993) first reported that LTP induced an NMDA receptor-mediated increase in tPA gene expression in the dentate gyrus, suggesting that tPA played an important role in neuronal plasticity . TPA has since also been induced in cerebellar Purkinje neurons after cerebellar motor learning (Seeds *et al.,* 1995), a process associated with activity-dependent synaptic plasticity. TPA-gene-deficient mice show a selective defect in LTP (Frey *et al.,* 1996; Huang *et al.,* 1996) and spatial learning. Baranes *et al.* (1998) reported that tPA was critically involved in the production of LTP and in synaptic growth. TPA has also been induced in association with cerebellar LTD (Seeds *et al.,* 1995). There are no reports of investigations into spinal regulation of tPA mRNA expression associated with inflammatory injury.

Jun D

The involvement of jun D in plasticity of spinal nociceptive processing has been reviewed by Herdegen & Zimmermann (1995). Jun D protein is constitutively expressed at high levels in the brain (Lanterei-Minet *et al.,* 1993; Herdegen *et al.,* 1993; Herdegen *et al.,* 1995) and in the spinal cord (Herdegen *et al.,* 1991(a, b); 1994; Tolle, 1994). Numerous

studies have investigated the involvement of jun D in nociceptive transmission. Herdegen *et al.,* (1991a) reported upregulation of jun D protein expression in spinal neurons following noxious stimulation of C and Aδ fibres from 4h post-stimulation, persisting for 32 hours following stimulation. Lanteri-Minet *et al.,* (1993) reported upregulation of jun D protein expression when measured 7-15 days following the induction of CFA intraplantar inflammation, but not in the initial stages of CFA-induced inflammation. Herdegen *et al.,* (1994) reported upregulation of junD protein expression in the superficial dorsal horn in a formalin model of intraplantar inflammation, where jun D protein expression was maximal at 5h post-treatment, persisted at maximal levels for 10 hours post-injection (p.i). and remained significantly greater than constitutive levels at 24hours p.i. Tolle *et al.* (1994) reported significant upregulation of jun D protein expression from 4h following noxious thermal stimulation of the hindpaw.

The involvement of jun D in the induction of LTP in the spinal cord has been investigated previously. Wisden *et al.* (1990) reported that constitutive expression of junD mRNA in the spinal cord was not upregulated when measured 30 minutes after induction of LTP.

1.5 CONCLUSIONS

It is clear that the pain transmission system is subject to modulation at every level of the neuro axis. This system is inherently plastic, such that in any individual there is no constant clear relationship between the stimulus intensity and the conscious perception of pain. Pathophysiological pain transmission associated with inflammatory injury results from sensitisation of both peripheral and central nociceptive transmission, which may be attributed to alterations in excitatory and inhibitory systems involved in the induction and maintenance of central sensitisation.

1.6 AIMS OF STUDY

The purpose of the studies described in this thesis was to investigate plasticity of central nociceptive transmission associated with inflammatory injury. While ample experimental evidence suggests that the electrophysiological phenomenon of LTP is associated with central sensitisation of nociceptive transmission, to date there has been sparse experimental evidence to suggest that LTD of central nociceptive processing, the electrophysiological complement of LTP, may be related to hyposensitisation of central nociceptive processing. It was hypothesised that LTD-like phenomena may play an important role in the physiological response to mild inflammatory injury. On the basis that previous failures to observe a behavioural complement to central sensitisation might reflect

the high intensity of inflammatory challenge typically used in research into inflammatory pain (discussed in chapter 2), it was considered timely to study the pharmacological mechanisms associated with behavioural responses to low-intensity inflammatory challenge. Hypothesising that both the magnitude and the duration of inflammatory challenge critically influence central nociceptive transmission (discussed in chapter 2) the response to repeated inflammatory stimulation was studied, hypothesising that manipulation of the frequency of inflammatory challenge might provide further insights into mechanisms associated with physiological and pathophysiological responses to inflammatory injury. Clearly, mediators of depressed hyperalgesic responses would prove useful targets for the therapy of inflammatory hyperalgesia. Plasticity of nociceptive behaviour observed *in-vivo* was investigated further using *in-situ* hybridisation techniques to investigate regulation of key effector molecules in the spinal cord dorsal horn which have been implicated in the modulation of nociceptive transmission following inflammatory injury. In addition, an organotypic slice culture system for post-natal rat spinal cord was developed and characterised in order to provide a novel and useful technique for more detailed study of the molecular basis of central plasticity of nociceptive transmission.

CHAPTER 2: BEHAVIOURAL STUDIES

That which is static and repetitive is boring. That which is dynamic and random is confusing. In between lies art

John Locke

2.1 INTRODUCTION

2.1.1 Assessment of pain responses in animals

Contemporary concern over the ethical justification for the use of live animals in scientific research is of particular relevance to pain research. While advances in *in-vitro* techniques provide alternatives to the 'whole animal model', evaluation of new analgesic drugs and basic research into the neural mechanisms involved in the development and maintenance of persistent and chronic pain conditions still rely heavily on *in-vivo* models. Ethical guidelines, which pertain specifically to pain research, have been reviewed by Loew (1987) and Dubner (1987). Beecher (1957) provided recommendations that provide the ethical benchmark used to assess the integrity of 'painful stimuli' applied to animals in behavioural studies. His recommendations, designed to guide the selection of stimuli used to test pain responses in laboratory animals but applicable to all *in-vivo* studies on pain behaviour, were: that the chosen stimulus should be applied to a region of the body where the responses measured would accurately indicate a pain response; that the stimulus should produce minimal damage at the pain threshold level; that the potential hazard to the test subject at the highest testing intensity should be small; that there should be a definable relationship between the intensity of the stimulus and the intensity of perceived pain; that repeated testing using the same stimulus should not affect the quantifiable response; that the stimulus should induce a clear end-point (response); that the stimulus should be sufficiently sensitive to allow small alterations in threshold to be detected and should permit detection of different doses of analgesic and that the stimulus should be applicable to both humans and animals. Wood (1984) provided a further definition of 5 essential criteria for tests used to measure pain responses in animal, namely validity (ability of the test to predict the clinical value of known analgesic agents); reliability (reproducibility); simplicity; sensitivity; and facility for quantitative analysis of collected data.

A major hurdle in the use of animal models to investigate pain mechanisms is the recognition of alterations in stimulus-dependent pain behaviour in animals. Many studies use changes in nociceptive reflex behaviour as indicators of altered pain perception;

however such reflex behaviours often correlate poorly with stimulus-induced pain behaviour in humans. Certain behavioural parameters used as indicators of a 'pain response' in rodents - notably the tail flick response (also called the tail-immersion test) and limb withdrawal tests - are considered to be predominantly reflexive (Nolan *et al.,* 1987). Similar 'simple reflex' behavioural tests have been documented in other species, including skin flinch (Kamerling *et al.,* 1985) and jaw opening reflexes (Mitchell, 1964). These reflex behaviours are, however, subject to modulation; for example, response thresholds or latency measurements can change in association with alterations in motor function, potentially induced by unilateral inflammation in the hind limb; or with learned avoidance strategies. By definition, simple reflex behaviours cannot indicate any affective component of pain sensation. For this reason some workers prefer to use organised unlearned behaviour, such as paw licking, face rubbing (Chapman *et al.,* 1985) and vocalisation (Eddy, 1928; Kayser *et al.,* 1998) as response indicators on the premise that these are specific 'pain responses' which include an 'affective' component. Experimental methods of evaluating pain responses to thermal or mechanical stimulation commonly measure a threshold response or response latency. Such methods assume that pain thresholds (the intensity of noxious stimulation which is first perceived as painful) are similar in humans and animals (Vierk, 1976).

Mechanical stimulation

Mechanical hypersensitivity in the form of tactile allodynia is a hallmark of certain types of neuropathic pain (Wahren & Torebjork, 1992) but also develops as a consequence of inflammatory injury. In recognition of this, great efforts have been made to develop an accurate means of assessing cutaneous responsiveness to applied mechanical force resulting in many different quantitative techniques for assessment of alterations in sensitivity to mechanical pressure. Paw withdrawal or vocalisation responses are most commonly chosen to 'quantify' mechanical sensitivity. Many techniques use a method similar to that originally described by Randall & Selitto (1957), where animals are fully or partially restrained and incrementally increasing mechanical force is applied to the paw via a blunt piston or similar device (e.g. Stein *et al.,* 1988; Perrot *et al.,* 1993.) Commercially produced automated pressure gauges are available for this purpose. Such models assess responses to deep pressure rather than light touch, such that reported experimentally induced 'withdrawal thresholds' are typically some 100-fold higher than the force of light tactile stimulation required to provoke a withdrawal response in the allodynic animal (Ahlgren & Levine, 1993). Kayser & Guilbaud (1987) suggested that the vocalisation threshold represents a more integrated nociceptive response than paw withdrawal and may provide a superior method of assessing changes in mechanical sensitivity.

In 1897, Von Frey described the use of horse hairs of various diameter and length to exert a controlled mechanical force on human skin. He connected horse hairs to a lever, which enabled him to measure the force, which each hair exerted on the skin, and thus quantify the force required to evoke a 'pain' response. While modern Von Frey hairs are plastic filaments rather than horse hairs, the Von Frey test remains one of the most common methods used on human or animal subjects to assess and quantify responses to a constant mechanical stimulus.

Von Frey hairs are used in a variety of ways to assess sensitivity to mechanical pressure. Some measure a 'withdrawal response frequency' for the number of withdrawal responses evoked by repeated stimulation with hairs of different force (e.g. Gilchrist *et al.,* 1996); while others use a 'withdrawal threshold', the lowest force of Von Frey hair which elicits a consistent withdrawal response (e.g. Brennan *et al.,* 1996). Von Frey hairs are intrinsically limited in their capacity to assessing sensitivity to mechanical pressure. They provide a restricted categorical scale of measurement of applied force, and measure only one particular form of sensitivity to mechanical pressure, namely punctate hypersensitivity (reviewed by La Motte *et al.,* 1991). Commercially produced devices are available which attempt to overcome the categorical nature of Von Frey testing: for example, the Ugo-Basile analgesymeter (Ugo Basile, Comerio, Italy) generates a linearly increasing mechanical force applied by a small plastic tip. However such devices tend to produce a deep pressure stimulus rather than a light 'tactile' pressure stimulus. A distinct form of mechanical hypersensitivity to gentle swab stroking (in contrast to punctate stimulation) has been described which cannot be detected using Von Frey hairs, variously described as 'stroking' hyperalgesia (Koltzenberg *et al.,* 1992) and dynamic hyperalgesia (Ochoa *et al.,* 1993). There is substantial evidence to suggest that these two manifestations of mechanical hypersensitivity- punctate and stroking- are mediated through different mechanisms. The area of punctate hypersensitivity, which develops subsequent to cutaneous injury, is larger and develops more quickly than the zone of hypersensitivity to stroking stimuli (Cervero *et al.,* 1993). Electrophysiological studies suggest that while punctate hypersensitivity is mediated by small diameter fibres, presumably nociceptors (Cervero *et al.,* 1994), stroking hypersensitivity is conveyed by large diameter fibres, presumably mechanoreceptors (Torebjork *et al.,* 1992).

Thermal stimulation

Reflexive foot withdrawal in response to noxious radiant heat is a widely used method for assessing nociceptive behaviour in rodents. Hardy *et al.* (1940) first described a method of application of a radiant heat source as a means of measurement of thresholds to noxious

thermal stimulation in human subjects. Hardy used a 500-1000 watt projection lamp to focus radiant heat onto a 'target' area of blackened skin, controlling the intensity of radiant heat using a rheostat, and the duration of exposure using an electrically timed shutter. Variations of Hardy's method have been adapted for use in many animal species. Hargeaves *et al.* (1988) developed a custom-built plastic cage with a glass floor, elevated so that a thermal source could be applied to the hind foot of rodents. Hargreaves' design incorporated a removable and exchangeable glass plate floor, which provided a 'minimally invasive' technique of assessing the behavioural response to application of heat stimulus to the plantar surface of the rodent foot. Hargreaves' heat stimulus was a 50 watt projector lamp placed in a case, which allowed it to be manoeuvred and accurately focused on its target. A digital timer and electronic photocell apparatus were used to automatically measure withdrawal latency to thermal stimulation. The Hargreaves model is now the standard method used to apply a radiant heat stimulus in experimental studies on rats; and thermal devices for use in this model are commercially available (e.g.Ugo-Basile, Comerio, Italy, plantar test 7370) delivers a ramping infrared stimulus.

There are two commonly used alternatives to the radiant heat method described by Hardy which use conducted heat to stimulate nociceptive behaviour in rodents. These are the hot plate test, first described in 1944 by Woolf & Macdonald, and the tail immersion test, described in 1941 by D'Amour & Smith. Hot plates are commercially available and are typically set at a pre-determined temperature (commonly 50-55°C for behavioural tests conducted on rodents). Both of these methods of assessing nociceptive reflexes have numerous disadvantages in comparison to the radiant heat tests developed by Hardy and others. Neither method permits independent assessment of treatment effects in treated/untreated limbs, and the hot plate method does not permit immediate release from the noxious stimulus, unlike the Hargreaves' technique, in which the noxious stimulus stops at the instant when the test subject registers aversion. The tail-flick (TF) test, which measures response latency where the rodent's tail is submerged in water of pre-determined temperature, (believed to be a central spinal reflex) also has intrinsic limitations. The tail is a thermo-regulatory organ and tail skin temperature may vary considerably in response to environmental (Berge *et al.,* 1988) and experimental (Tjolsen *et al.,* 1988) conditions and also to drug treatment (Tjolsen *et al.*, 1990). Such variations in cutaneous temperature affect nociceptive thresholds and compromise the validity of experimental data. The foot withdrawal response to noxious radiant heating of the skin (characterised and reviewed by Yeomans & Proudfit, 1994) thus offers numerous advantages over alternative thermal methods of nociceptive testing, although there is evidence to suggest that the plantar

surface of the rodent foot may also play a role in thermo-regulation (Duggan *et al.,* 1978; Gordon, 1990).

Electrical stimulation

Noxious electrical stimulation has been used in both human (reviewed by Kattims, 1998) and animal (reviewed by Raffe, 1992) studies to assess nociceptive behaviour. Electrical tooth-pulp stimulation has been used in humans (Olausson & Sagvik, 2000), laboratory animals (Han *et al.,* 1999), companion animals and horses (Steffey *et al.,* 1977) to evaluate cranial nerve nociception and indicate the efficacy of analgesic agents administered during dental treatment. Electrical stimulation of the tail, footpad, cutaneous zones of the forearm, muscles and nerves have also been described in various species as a means of assessing nociceptive behaviour and evaluating analgesic agents (reviewed by Raffe, 1992).

2.1.2. Animal models of inflammatory pain

The ultimate test for an animal model is its value for leading to pain treatments in human and veterinary clinical practice (Koltzenberg, 1999). Pain models are broadly separated into three functional divisions based on current understanding of the underlying mechanisms and pharmacology of pain sensation (reviewed by Dickenson, 1999; Yaksh, 1999), namely: models of acute high-intensity pain- such as the hot plate test, the tail-flick test and paw pressure tests; models of pain resulting from inflammation and tissue damage of variable intensity and duration and models of pain associated with nerve damage- such as sciatic nerve ligation or compression.

The mechanisms of inflammatory and neuropathic pain differ markedly from those of acute pain and there is considerable plasticity in both the transmission and modulation of these prolonged pain states (Dickenson, 1999).

Inflammatory pain has been experimentally induced in animal models by numerous stimulus modalities, including thermal injury, mechanical injury, surgical incision and administration of endogenous or exogenous inflammatory agents (reviewed by Jasmin *et al.,* 1998). Administration of an exogenous inflammatory agent remains the most commonly used means of investigating mechanisms involved in inflammatory pain. Randall & Selitto (1957) first described a model where peripheral inflammation was induced by local injection of a chemical irritant. Many different agents have since been used in this model, most commonly carrageenan, complete Freund's adjuvant (CFA), capsaicin and formalin, but also kaolin, turpentine, elastase, trypsin, capsaicin, dextran, hyaluronidase, mustard oil and acid phosphatase (reviewed by Vinegar *et al.,* 1976;

Otterness & Bliven, 1985) and recently bee venom (Chen *et al.,* 1999). Each of these irritants induces an inflammatory response of characteristic nature and duration.

The carrageenan-oedema model was introduced by Winter *et al.* in 1962 as an alternative to the irritants originally described by Randall & Selitto (1957). Following the characterisation of indomethacin analgesia using this model (Winter *et al.,* 1963), carrageenan-induced footpad oedema became established as a dominant model for the evaluation of potential anti-inflammatory agents and research into inflammatory pain. The carrageenans (kappa, lambda and iota) are a family of polysulphated polysaccharides isolated from marine algae. The inflammatory response to an isolated high-dose intraplantar carrageenan injection has been well characterised. λ-carrageenan produces a characteristic biphasic inflammatory response when injected into the rat footpad (Vinegar *et al.,* 1968), with an 'early' phase of approximately 1 hour duration, and a 'late' phase from 2 hours post-injection (p.i.). Maximum hyperalgesia after carrageenan injection occurs 2-3 hours p.i. (Yamamoto *et al.,* 1993). The inflammatory response and hyperalgesia associated with carrageenan injection (2 -4 mg) subsides to control values within seven days of injection [Castro-Lopez *et al.,* 1994; Fletcher *et al.,* 1997; Kayser *et al.,* 1998), while vascular permeability and leucocyte accumulation following carrageenan injection into an air pouch return to control values by 7 days post-treatment (Hambleton & Miller, 1989). Carrageenan induces inflammation by activating pro-inflammatory cells and induces hyperalgesia by promoting the release of potent inflammatory mediators, including nitric oxide, histamine and 5-hydroxytryptamine (5-HT) (predominant mediators of the early phase response), kinins and cytokines (which maintain increased vascular permeability for up to 2.5 hours post-injection), and prostaglandins (predominant mediators of the late phase response) from 2.5 until at least 6 hours post-injection, closely associated with the migration of leucocytes into the inflamed site) (reviewed by Di Rosa *et al,* 1971). All of these mediators activate or sensitise nociceptive sensory neurons (Lang *et al.,* 1990). High doses of carrageenan have been shown to induce both peripheral sensitisation (Seibert *et al.,* 1994; Salvemini *et al.,* 1995) and central sensitisation (Kayser *et al.,* 1998; Perrot *et al.,* 1999) in behavioural and electrophysiological studies of nociceptive processing. In their characterisation of the 'carrageenan oedema assay', Winter *et al.* (1962; 1963) and Vinegar *et al.* (1968) administered doses of 1mg carrageenan in an injection volume of 50 μl. However, for reasons that are unexplained, contemporary models of inflammatory injury typically administer substantially higher doses of carrageenan [typically 100 - 200μl of 2 - 4%, equivalent to 2 - 8mg carrageenan] (e.g. Dirig *et al.,* 1998; Kayser *et al.,*1998; Pertovaara *et al.,* 1998; Perrot *et al.,* 1999)

with doses of 6mg or more commonly used (Castro-Lopez *et al.,* 1994; Ichitani *et al.,* 1997; Buritova & Besson, 1998; Honore *et al.,* 1998; Tsuruoka *et al.,* 1998).

Complete Freund's adjuvant (CFA) induces a chronic inflammatory focus and is employed in the investigation of mechanisms of persistent inflammatory pain. It is typically administered by intra-plantar or intra-articular injection (reviewed by Colpaert, 1987), producing a prolonged inflammatory response which peaks 8-24 hours after injection and is maintained at peak levels for at least 14 days following intraplantar injection (Hay *et al.,* 1997; Goff *et al.,* 1998; Stein *et al.,* 1988) and for up to 8 weeks following intra-articular injection (Colpaert, 1987).

Capsaicin, the pungent constituent of chilli peppers, produces acute pain and inflammation through selective excitation of C-polymodal nociceptors accompanied by depletion of substance P from the afferent terminals of these receptors (Kenins, 1982; LaMotte *et al.,* 1992). Capsaicin acts by binding to vanilloid receptors, non-selective cation channels which permit the influx of calcium and other ions into neurons to result in action potential generation leading to pain sensation, and release of substance P and other neuropeptides. In the rat, high doses of capsaicin desensitise neurons to subsequent capsaicin administration or other nociceptive stimulation (Lynn, 1992; Szallasi, 1994). Intradermal administration of capsaicin is commonly used in human as well as animal subjects (e.g. Gilchrist *et al.,* 1996) as an inflammatory stimulant, producing characteristic dose-dependent pain sensation of short duration accompanied by primary and secondary hyperalgesia (reviewed by Willis, 1997b). The capacity of topical or intradermal application of capsaicin to induce desensitisation of nociceptors has led to the development of capsaicin-derived products as anti-inflammatory and topical analgesic agents (reviewed by Wilcox, 1999).

Formalin, characterised by Dubuisson & Dennis (1977), induces a characteristic biphasic inflammatory and nociceptive response consisting of an early, brief painful response of 5-10 minutes duration and declining to a low level, produced by direct nociceptor stimulation, followed by a tonic (prolonged) period of persistent pain starting at 20 minutes following formalin administration and lasting for approximately 40 minutes. This second phase is a consequence of the release of endogenous inflammatory mediators (Dickenson & Sullivan, 1987b). In the 'formalin test', a standard method used to induce pain behaviour in rat models, 2.5-5% formalin is typically administered by intraplantar injection to produce dose-dependent pain responses for approximately 1 hour p.i. (reviewed by Carstens, 1992).

2.1.3 Plasticity of nociceptive transmission associated with inflammatory injury

Inflammatory injury induces peripheral and central sensitisation of nociceptive transmission. Ample evidence from diverse sources, including behavioural studies (e.g. Kaneko & Hammond, 1997), and electrophysiological studies (e.g. Aertsen *et al.,* 1989; Eblen-Zajjur & Sandkuhler, 1997) indicates that plasticity of nociceptive transmission associated with inflammatory injury is critically dependent upon the intensity of the inflammatory insult, and at least part of this plasticity is centrally mediated (reviewed by Treede *et al.,* 1992; Levine & Yaksh, 1994; Woolf, 1995 and in Chapter 1).

To date, few behavioural studies have investigated dose-dependent responses to mild inflammatory injury using standard inflammatory agents. The dose of 3-5mg formalin standardly used in the formalin test (e.g. Dirig & Yaksh, 1995) produces a 'ceiling effect' which has the potential to hinder investigations into molecular mechanisms of central plasticity. Kaneko & Hammond (1997) used a range of concentrations of formalin (0.25% to 2.5%) to report a significant dose-dependent change in pain responses to formalin treatment, proposing that an alteration in the intensity of formalin-induced peripheral inflammation could induce an alteration in the balance of central and peripheral nociceptive transmission to result in markedly different neuronal and behavioural responses to noxious challenge. With the exception of a study by Tabo & Eisele (1998) describing intraplantar doses of 1mg and 0.1mg carrageenan, few studies have investigated behavioural responses to low doses of carrageenan. The mild inflammatory response induced by low-dose carrageenan offers significant advantages to the behaviourist over the higher doses of carrageenan standardly used, over and above distinct animal welfare advantages. Low-dose carrageenan reduces the degree of inflammation-induced warming of the plantar skin towards a critical threshold at which artefactual facilitation of heat-evoked responses occurs (Hole & Tjolsen, 1993), and reduces the marked alterations in weightbearing and posture observed using higher doses of carrageenan (Tabo *et al.,* 1998). The Hargreaves model has been shown to be a highly sensitive means of measuring nociceptive behaviour relative to alternative methods of assessing pain in laboratory animals, including the hot-plate, tail-flick, weight-bearing and Randall-Selitto assays (reviewed by Tabo *et al.,* 1998; Pertovaara *et al.,* 1998).

Repetitive inflammatory stimulation

There have been similarly few studies of repeated acute inflammatory stimulation and its effects on nociceptive responses. Repeated carrageenan treatment has been reported to induce both hypersensitisation (Kayser *et al.,* 1998; Perrot *et al.,* 1999) and tachyphylaxis (Welsh & Nolan, 1994) of hyperalgesic responses. These differences may be related to the

dose of carrageenan used. It is worth noting that repeated intraplantar administration of 150 μl saline for 4 days has been reported to induce a progressive increase in post-injection swelling and transient hyperalgesic behaviour (Levine *et al.*, 1985).

2.1.4. Aims and objectives

The principal aim of the studies described in this chapter was to develop a behavioural model which could accurately detect plasticity of nociceptive transmission (potentially up regulation or down regulation) associated with mild repetitive inflammatory injury. Since the intensity and duration of inflammatory injury critically influence nociceptive transmission, it was hypothesised that this model could facilitate evaluation of both physiological and pathophysiological nociceptive responses and possibly assess the 'adaptive' nociceptive response to injury.

2.2 MATERIALS AND METHODS

Adult male Wistar rats [Harlan, U.K.](n=72) weighing 200-280g were used in all studies. Rats were acclimatised to behavioural apparatus for two weeks before behavioural testing began.

2.2.1 Intraplantar drug administration

All treatments were administered by intraplantar injection in a volume of 50 μl into the right (ipsilateral) hindpaw, by 27g, 0.5ml syringe. λ- carrageenan solutions (C3889, SIGMA, UK) were prepared and aliquoted 7 days prior to use and stored at 4°C. Emulsions (50% and 12.5%) of CFA(F5881, SIGMA, UK) used in pilot study 1 were prepared 2 hours before use. All solutions were warmed to room temperature prior to administration.

2.2.2. Behavioural testing

All measurements were made on ipsilateral (ipsi) and contralateral (contra) hindpaws.

Paw circumference

Paw circumference (PC) was recorded in mm using fine silk filament and was used as a measure of post-injection oedema.

Thermal withdrawal latency

The tail flick test was used to assess thermal withdrawal latency (TWL) in pilot study 1. Each animal was restrained on the recorder's forearm and its tail submerged in a water bath set at a constant water temperature of 48°C. The latency to aversion response (removing tail from water, in seconds) was measured (to 0.1s) using a hand-held stopwatch. In all subsequent studies, TWL was assessed using an adaptation of the behavioural model described by Hargreaves *et al.,* (1988). Animals were placed on an elevated glass plate in a clear plastic box of dimensions 28 cm by 18cm by 11.5cm. The thermal stimulus was a focused beam of light (50W projector bulb, OSRAM 5008-V) accurately directed onto the plantar surface of the footpad. Time to aversion was measured in seconds (to 0.1s) using a hand-held stopwatch.

Mechanical withdrawal threshold

Mechanical withdrawal threshold was assessed using an adaptation of the behavioural model described by Hargreaves *et al.,* (1988). The glass floor of the chamber described above was replaced with a wide wire mesh floor through which filaments could be passed. A staircase method (Chaplan *et al.,* 1994; Tabo *et al.,* 1998) was used to measure mechanical withdrawal threshold. (Table 2.1). In study 2.1, a ranked score (from 1-9) was assigned to Semmes-Weinstein Von-Frey filaments of force 1.17 g to 75.85 g [Table 2.1 (A)]. Filaments were presented in increasing order of stiffness, with an interval of 5 seconds between successive applications. Each filament was pushed to bending at various locations on the plantar surface of the paw. A positive response was noted if the paw was sharply withdrawn. In the absence of a positive response, a stronger stimulus was presented; following a stronger response, filaments of lower bending force were applied to confirm the minimum force which elicited a withdrawal response. This process was repeated 4 times. The mechanical withdrawal threshold (MWT) was defined as the 'score' which elicited a response. In study 2.2, no MWT responses were recorded at the 1.17 g. The scoring system was consequently revised such that a ranked score (from 1-8) was applied Semmes-Weinstein Von-Frey filaments of force 1.17 g to 75.85 g [Table 2.1 (B)]. Thermal and mechanical responses were assessed once daily for 3 days before test recording began. At each time point, TWL was measured twice in each subject, while the staircase method of assessing MWT was repeated three times.

2.2.3 STUDIES

PILOT STUDY 1: Selection of an inflammatory agent

Objective of study: To compare the inflammatory effects of low doses of carrageenan (Cx) and complete Freund's Adjuvant (CFA), administered by intraplantar injection.

Animals (n = 3/group) received an intraplantar injection of 50 µl of 0.9% saline (Group 1), 0.0625% carrageenan (Group 2), 0.6% Cx (Group 3), 50% CFA (Group 4), and 12.5% CFA (Group 5). Tail flick (TF) and paw circumference (PC) measurements were recorded prior to intraplantar treatments, at 0.5 h p.i., at hourly intervals for 7 h p.i., and again at 24 h p.i..

PILOT STUDY 2: Determination of peak inflammatory and hyperalgesic effect of intraplantar carrageenan treatment

Objective of study: To characterise the duration of the inflammatory response and associated hyperalgesia following low-dose intraplantar carrageenan treatment.

Animals (n = 3/treatment group) received an intraplantar injection of 50 µl of 0.6% Cx (Group 1) or 0.1% Cx (Group 2). Paw circumference (PC), thermal withdrawal latency (TWL) and mechanical withdrawal threshold (MWT) were measured pre-injection, at hourly intervals for 11 hours p.i. and at 24 hours p.i.

PILOT STUDY 3: Preliminary investigation of low-dose carrageenan intraplantar inflammation

Objective of study: To characterise further behavioural responses to intra-plantar injection of low-dose carrageenan.

Animals (n = 4/treatment group) received an intraplantar injection of 50 µl of saline (Group 1), 0.5% Cx (Group 2) or 0.1% Cx (Group 3). PC, TWL and MWT measurements were performed on ipsilateral (injected) and contralateral (non-injected) paws pre-injection and at 2 and 6 hours p.i.. Rats were euthanased 6 hours p.i. Photographs were taken of ipsilateral and contralateral paws at each time point.

Table 2.1

A: Conversion table of ranked scoring system applied to gramme force specification of Semmes-Weinstein Von-Frey filaments in study 2.1

Force (g)	1.17	2.04	3.63	5.50	8.51	11.54	15.14	23.83	75.86
Score	1	2	3	4	5	6	7	8	9

B: Conversion table of ranked scoring system applied to gramme force specification of Semmes-Weinstein Von-Frey filaments in studies 2.2 and 2.3

Force (g)	2.04	3.63	5.50	8.51	11.54	15.14	23.83	75.86
Score	1	2	3	4	5	6	7	8

STUDY 2.1: Weekly repeated carrageenan treatment

Objective of study: To study behavioural responses to weekly repeated intra-plantar injection of low-dose carrageenan.

Animals (72 in total) were randomly assigned to 3 treatment groups and received the following treatments (n=24/group): 0.5% Cx (Group 1); 0.1% Cx (Group 2); 0.9% saline (Group 3). Behavioural testing and assessment of inflammation were performed pre-injection and at 6 hours post-injection on each day. Six animals from each group were euthanased each week (by intraperitoneal injection of barbiturate (Euthatal, Intervet, UK) following completion of behavioural testing, such that within each treatment group there were 24 animals which received one carrageenan treatment, 18 animals which received 2 treatments, 12 animals which received three treatments and 6 animals which received 4 treatments.

STUDY 2.2: Daily repeated carrageenan injection

Objective of study: To study behavioural responses to daily repeated intra-plantar injection of low-dose carrageenan.

Animals (n = 12/ treatment group) received an intraplantar injection of 50 µl of 0.1% λ-carrageenan (Group 1), or 0.9% saline (Group 2) at 24 hour intervals for 5 days. On each day, behavioural testing and assessment of inflammation were performed pre-treatment and at 2 h, 4 h and 6 h p.i.

STUDY 2.3: Preliminary investigations into receptor systems involved in tachyphylaxis of hyperalgesia

Objective of study: To investigate the involvement of three pharmacological systems in the tachyphylaxis of hyperalgesia reported in study 2.2. It was hypothesised that the plasticity of nociceptive responses observed in study 2.2 could reflect modulation of NMDA-induced enhanced excitatory transmission; enhancement of opioid-mediated central inhibition of excitatory nociceptive transmission; or enhancement of α_2 adrenoceptor-mediated central inhibition of excitatory nociceptive transmission.

Experimental design: In study 2.2, tachyphylaxis of hyperalgesic behaviour occurred on days 3 and 4, followed by restoration of marked hyperalgesia on day 5. It was assumed, therefore, that specific events on days 1 and 2 induced attenuation of hyperalgesic responses on days 3 and 4. Specific receptor modulating agents were administered on days 3, 4 and 5.

STUDY 2.3(A)

Animals (n = 6/ treatment group) received an intraplantar injection of 50 μl of 0.1% λ-carrageenan (Group 1), or 0.9% saline (Group 2) at 24 hour intervals for 5 days. The NMDA receptor antagonist memantine hydrochloride (MEM)[TOCRIS, UK] was prepared in 0.9% normal saline and administered by s/c injection on days 3,4 and 5, at time 0 (immediately following carrageenan or saline treatment) at a dose of 20mg/kg (in a volume of 0.75ml), and again at 2h post-carrageenan/saline treatment at a dose of 5mg/kg (in a volume of 0.19 ml).

Study 2.3(B)

Animals received an intraplantar injection of 50 μl of 0.1% λ-carrageenan (Groups 1 and 2, n = 6/ treatmenr group), or 0.9% saline (Groups 3 and 4, n = 3/ treatment group) at 24 h intervals for 5 days. The opioid receptor antagonist naltrexone hydrochloride (NTX) [Research Biochemicals International, UK] was administered by s/c injection immediately following carrageenan or saline treatment on days 3, 4 and 5. Animals in groups 1 and 3 received a dose of 0.5mg/kg NTX, while animals in groups 2 and 4 received a dose of 0.05mg/kg NTX.

Study 2.3(C)

Animals (n = 6/ treatment group) received an intraplantar injection of 50 μl of 0.1% λ-carrageenan (Group 1), or 0.9% saline (Group 2) at 24 hour intervals for 5 days. The α2 receptor antagonist atipamezole hydrochloride (ATI) [Smithkline Beecham, UK] was prepared in 0.9% normal saline and administered by s/c injection on days 3,4 and 5, at time 0 (600μg/kg in a volume of 0.56ml) and at 2h post-carrageenan/saline treatment (400μg/kg in a volume of 0.37ml).

2.3.3. Behavioural testing

On each day, behavioural testing and assessment of inflammation were performed pre-treatment and at 2 h, 4 h and 6 h p.i. PCmax (the maximum PC, [mm]), TWLmin (the minimum TWL, [s]) and MWTmin, (the lowest MWT score) were noted in each subject on days 1-5. The maximal increase in PC, ΔPCmax (mm) induced by treatment was calculated on each day: ΔPCmax (mm) = PCmax - pre-treatment PC.

2.2.4 STATISTICAL ANALYSIS

Pilot studies 1,2,3

After confirmation of normality of distribution using Anderson-Darling normal probability plots, PC and TWL data were investigated using analysis of variance (ANOVA) techniques (general linear model analysis (GLM), one way ANOVA and balanced ANOVA analysis). Treatment and time were considered to be fixed factors, while individual animal variation was considered to be a random factor. Tukey's post-hoc test for multiple comparisons was used to assess time-related effects of treatment within each treatment group. In pilot studies 1, PC and TFL values in carrageenan-treated animals were compared to those of saline-treated animals at each time point. In pilot studies 2 and 3, PC and TWL values in carrageenan-treated animals were compared to those of saline-treated animals at each time point and the effect of time on ipsilateral and contralateral PC and TWL was investigated within each treatment group using GLM analysis. In pilot studies 2 and 3, median MWT scores provided an overview of the distribution of MWT responses associated with low dose carrageenan treatment.

Study 2.1

The number of animals included in each treatment group declined with each successive week, as 6 animals in each group were euthanased at the end of each test period on each week of the study. Thus 72 animals received a single treatment (n = 24/ treatment group); 54 animals received 2 treatments (n = 18/treatment group); 36 animals received 3 treatments (n = 12/ treatment group) and 18 animals received 4 treatments (n = 6/ treatment group).

PC and TWL data

The increase in PC (ΔPC, mm) induced by treatment was determined by subtracting pre-injection PC from post-injection PC (measured at 6 h p.i.) in ipsilateral and contralateral paws. The decrease in TWL (ΔTWL, s) associated with treatment was determined by subtracting post-injection TWL from pre-injection TWL in ipsilateral and contralateral paws. General linear model (GLM) analyses and Tukey-Kramer multiple comparison tests were performed on ipsilateral and contralateral ΔPC and ΔTWL. Treatment and time were considered as fixed factors. Animals were considered as a random factor. The effect of treatment (0.5% and 0.1% carrageenan compared with saline) on ipsilateral and contralateral ΔPC and ΔTWL was investigated. Within each treatment group, the effect of treatment was investigated by comparing ipsilateral and contralateral ΔPC and ΔTWL on

each week of the study. The effect of repeated treatment on ipsilateral/contralateral ΔPC and ΔTWL was also investigated.

MWT data

The change in MWT (ΔMWT) associated with treatment was determined by subtracting the post-treatment MWT score from the pre-injection MWT score in ipsilateral and contralateral paws. Kruskal-Wallis and Mann-Whitney tests were used to compare ΔMWT between treatment groups. Within each group, the Wilcoxon signed-rank test (hypothesising that the median ΔMWT= 0) was used to compare pre- and post-injection MWT, while Friedman analysis was used to investigate the effect of weekly repeated treatment on ΔMWT.

Study 2.2

PC and TWL data

The maximal increase in PC, ΔPCmax (mm) induced by treatment was calculated on each day: ΔPCmax (mm) = PCmax - pre-treatment PC. Data analysis was performed on baseline (pre-treatment) TWL (s), baseline (pre-treatment) PC (mm), TWLmin (s) and on TWL (s) at 2 h, 4 h and 6 h p.i. Data analysis was also performed on ΔPCmax and on median MWTmin scores.

General linear model (GLM) analyses and Tukey-Kramer multiple comparison tests were performed on ipsilateral and contralateral PCmax, TWLmin and TWL at 2 h, 4 h and 6 h p.i.. Treatment (carrageenan and saline) and repeated treatment (1, 2, 3, 4 and 5 daily repeated treatments) were considered as fixed factors. Animals were considered as a random factor.

The effect of treatment on ipsilateral and contralateral PC, TWL and TWLmin was investigated at each time point on each day of the study. Within each treatment group, the effect of treatment was investigated by comparing ipsilateral and contralateral PC, TWL and TWLmin at each time point on each day of the study. The effect of repeated treatment on ipsilateral/contralateral ΔPC and ΔTWL was also investigated using ANOVA techniques combined with Tukey's post-hoc test for multiple comparisons.

MWT data

Non-parametric tests were used to investigate changes in MWTmin. The effect of treatment on ipsilateral and contralateral MWT_{min} was studied using Friedman analysis. Within each treatment group, the Wilcoxon signed-rank test was used to compare MWT_{min}

in ipsilateral and contralateral paws on each day of the study. Friedman analysis was used to investigate the effect of daily repeated treatment on ipsilateral and contralateral MWT min in each treatment group.

Studies 2.3 (A,B,C)

Data analysis was performed on baseline TWL (s), baseline PC (mm), TWLmin (s), Δ PCmax and on median MWTmin scores.

PC and TWL data

The effect of repeated treatment on ipsilateral/contralateral ΔPC and ΔTWL was investigated using general linear model analysis combined with Tukey's post-hoc test for multiple comparisons. Within each treatment group, the effect of treatment was investigated using ANOVA techniques to compare ipsilateral and contralateral baseline PC, ΔPCmax and TWLmin at each time point on each day of the study.

General linear model (GLM) analyses and Tukey-Kramer multiple comparison tests were performed on ipsilateral (and contralateral) PC, TWL and TWLmin. Treatment and repeated treatment (1, 2, 3, 4 and 5 daily repeated treatments) were considered as fixed factors. Animals were considered as a random factor.

MWT data

Non-parametric tests were used to investigate changes in MWTmin. Friedman analysis was performed on ipsilateral and contralateral MWT_{min} to compare saline and carrageenan treatment. Within each treatment group, the Wilcoxon signed-rank test was used to compare MWT_{min} in ipsilateral and contralateral paws on each day of the study. Friedman analysis was used to investigate the effect of repeated treatment on ipsilateral and contralateral MWT min in each treatment group.

TWLmin and MWT data in study 2.3 were compared with TWLmin and MWT data from study 2.2. The same protocol for behavioural testing was used in studies 2.2 and 2.3. Due to slight differences in the intensity of the thermal stimulus, TWL measurements in study 2.3 (A) and 2.3 (B) were greater than those recorded in study 2.2 on days 1 and 2 (in the absence of receptor antagonist administration. Consistent differences in TWL were observed in both carrageenan and saline-treated animals. It was therefore considered inappropriate to compare TWL data from study 2.2 and studies 2.3 (A) and 2.3 (B) statistically. TWLmin data in study 2.3 (C) were compared with TWLmin data in study 2.2

MWTmin measurements in studies 2.3 (A), 2.3 (B) and 2.3 (C) did not differ significantly from those recorded in study 2.2, therefore MWTmin data from all studies were compared with those of study 2.2

2.3 RESULTS

2.3.1 Pilot study 1

Paw circumference

Baseline PC was 28.5 mm ± 0.5 mm in ipsilateral and contralateral paws (Figure 2.1 A,B). In 0.6% Cx-treated animals , ipsilateral PC was maximal 2 hours p.i. (33.2± 0.2 mm); in 0.0625% Cx-treated animals, ipsilateral PC was maximal at 7 hours p.i. (33.0 ± 1.5 mm). In 50% CFA-treated animals, ipsilateral PC was maximal 24 hours p.i. (34.7± 0.3 mm); in 12.5% CFA-treated animals, ipsilateral PC was maximal at 7 hours p.i. (32.3 ± 0.6 mm).

GLM analysis indicated a significant treatment effect on ΔPC [$F(4, 134) = 15.53$, $p < 0.01$]. Post-hoc analysis indicated that the change in PC measured in Cx-treated animals (groups 2 and 3) and 50 % CFA-treated animals (group 4), but not 12.5% CFA-treated animals (group 5) was significantly greater than group 1 (saline-treated) animals ($p < 0.05$).

In 0.6% Cx and 0.0625% Cx-treated animals, ipsilateral PC was significantly greater than that of group 1 (saline-treated) animals over time ($p < 0.001$). Ipsilateral PC in Cx-treated animals was significantly greater than that of saline-treated animals at 24 h p.i. (by 2.9 mm, $p < 0.01$ for group 2 and by 1.5mm, $p < 0.01$ for group 3).

In CFA-treated animals, ipsilateral PC increased over the duration of the test period and was maximal when measured 24 hours p.i. (34 ± 0.4 mm in 50% CFA-treated animals, 30.0 ±1.5 mm in 12.5% CFA-treated animals). Ipsilateral PC in 50 % CFA-treated animals was significantly greater than that of saline-treated animals over time ($p<0.01$). Ipsilateral PC in 12.5% CFA-treated animals did not differ significantly over time from saline-treated animals.

Figure 2.1

Change in mean ± S.E.M paw circumference (PC) measured over 24h (A) (and over 8 h p.i., B), following intra-plantar injection with 50 µl of saline (Group 1), 0.6% carrageenan [Cx] (Group 2), 0.06% carrageenan (Group 3), 50% complete Freunds Adjuvant [CFA] (Group 4) and 12.5% CFA (Group 5) (n=3/group). Pre-injection value = time 0.

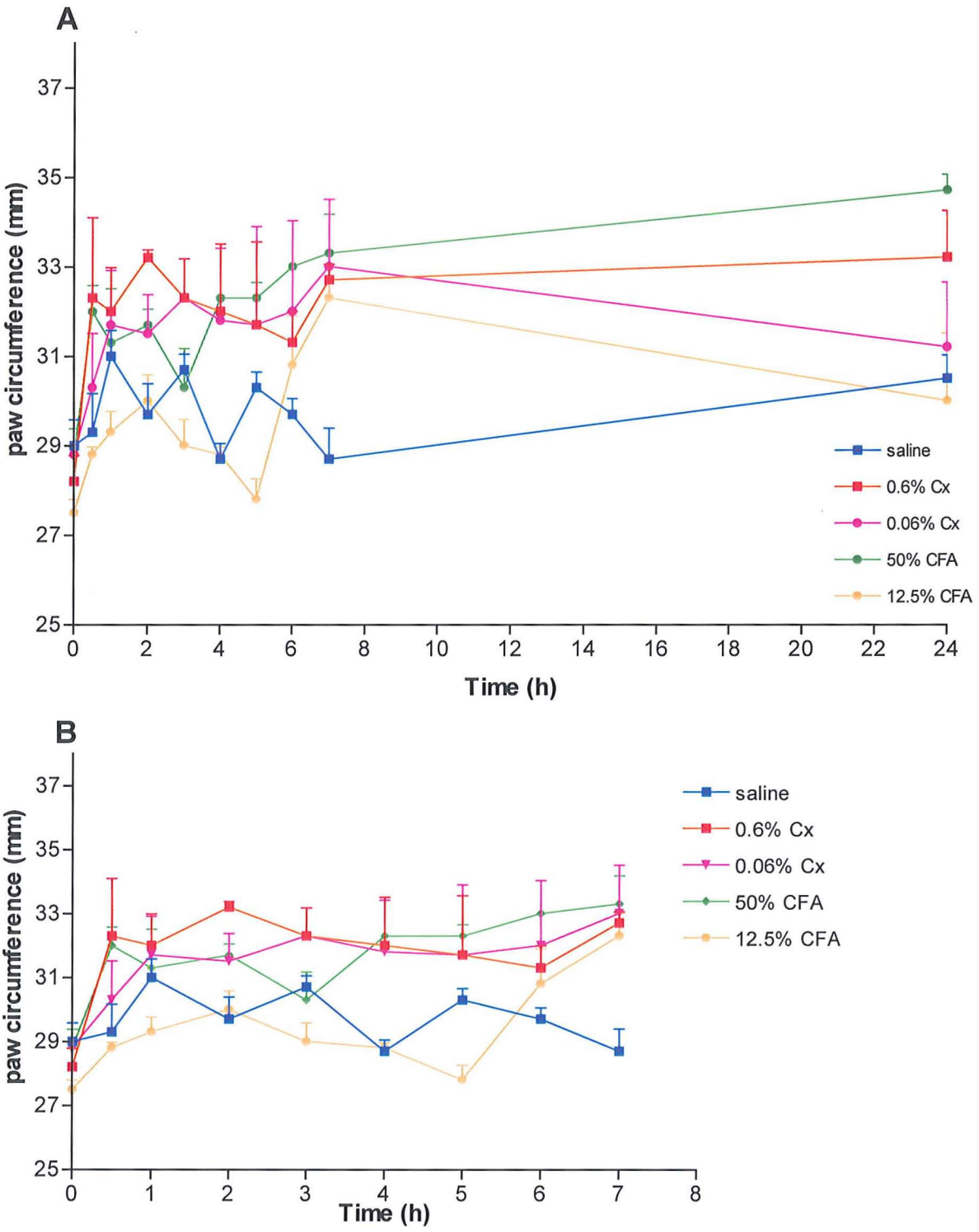
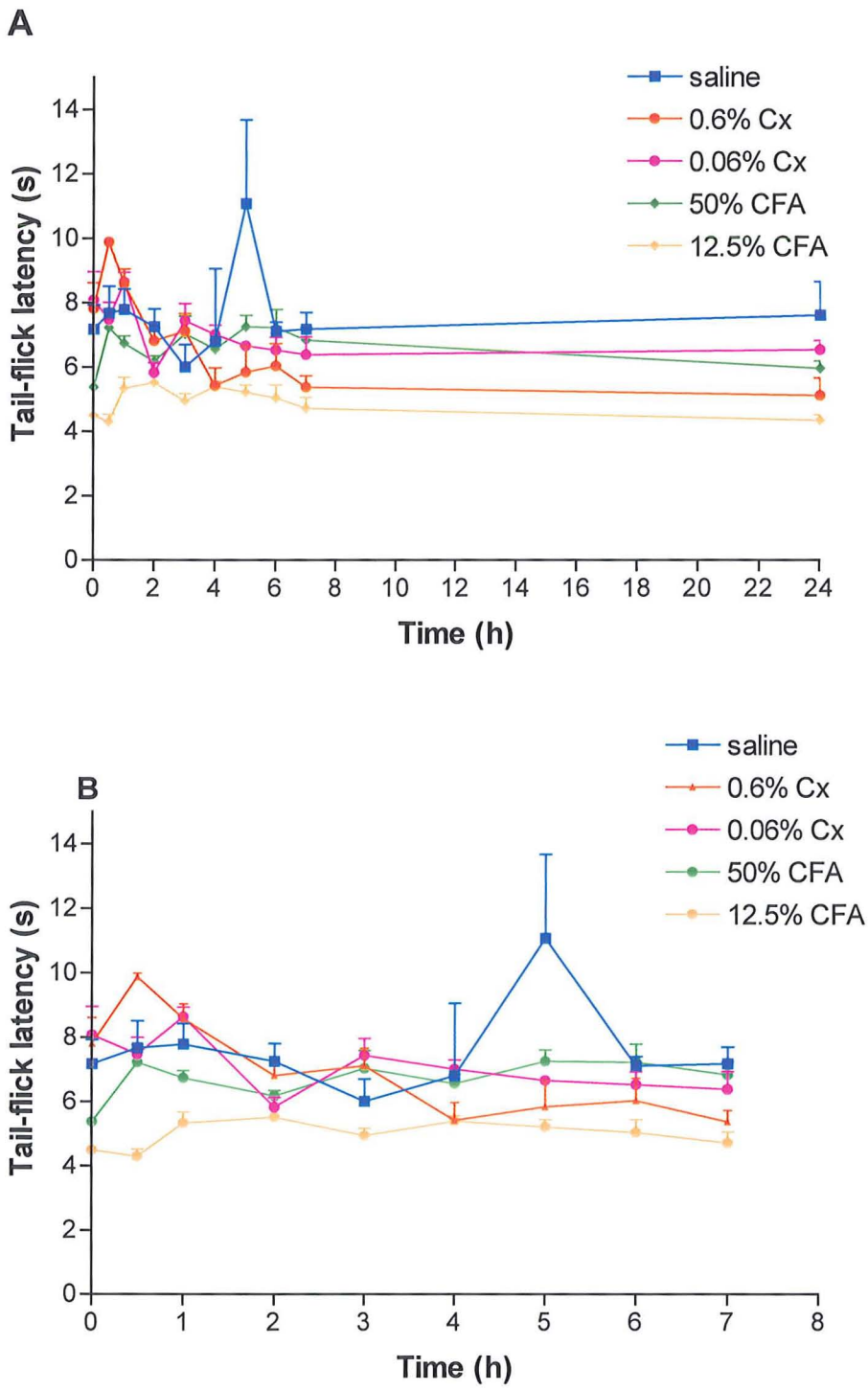


Figure 2.2

Change in mean ± S.E.M tail-flick latency (TF, s) measured over 24h (A) (and over 8h, B), following intra-plantar injection with 50 µl of saline (Group 1), 0.6% carrageenan [Cx] (Group 2), 0.0625% carrageenan (Group 3), 50% complete Freunds Adjuvant [CFA] (Group 4) and 12.5% CFA (Group 5) (n = 3/group). Pre-injection value = time 0.



Tail flick latency

There was considerable variation between treatment groups in baseline TFL (Figure 2.2). Pre-treatment TFL was 7.15 ± 1.30 s in group 1; 7.80 ± 1.40 s in group 2; 8.07 ± 1.48 s in group 3; 5.37 ± 0.13 s in group 4 and 4.49 ± 0.31 s in group 5.

GLM analysis indicated a significant difference between treatment groups in tail flick latency (TFL) measurements ($F(4,134) = 9.03$, $p < 0.01$); however, post-hoc analysis indicated that only TFL measurements in Group 5 (12.5% CFA-treated animals) were significantly shorter than those of all other groups. There was no treatment effect on TFL over time and no significant effect of time on TFL within any group.

2.3.2 Pilot study 2

Paw circumference

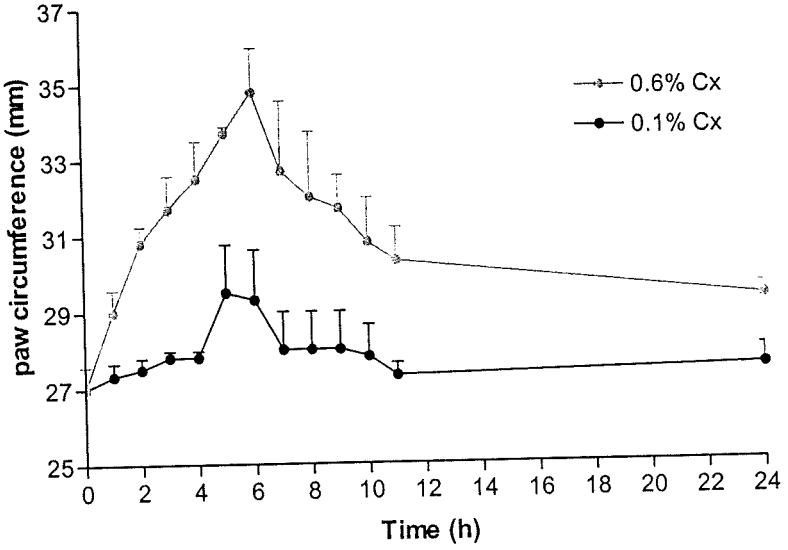
Paw circumference measurements recorded in pilot study 2 are shown in figure 2.3 (A). Baseline ipsilateral PC was 27.0 ± 0.58 mm in 0.6% Cx-treated animals and 27.0 ± 0.01 mm in 0.1% Cx-treated animals. In 0.6% Cx -treated animals, ipsilateral PC was maximal at 4 h p.i. (34.8 ± 1.2 mm). In 0.1% Cx-treated animals, ipsilateral PC was maximal at 5 h p.i. (29.5 ±1.3 mm) was measured at 5 hours p.i.

Ipsilateral PC in 0.6% Cx-treated animals was significantly greater than that of 0.1% Cx-treated animals over time ($F [2,62] = 104.62$, $p < 0.001$). Ipsilateral PC changed significantly over time in 0.6% Cx-treated animals ($F[12, 24] = 10.5$, $p < 0.001$) but not in 0.1% Cx-treated animals ($F[12, 24] = 1.58$, $p = 0.16$). In 0.5% Cx-treated animals at 24h p.i., ipsilateral PC (29.3 ± 0.3) was significantly greater ($p < 0.05$) than baseline ipsilateral PC. In 0.1% Cx-treated animals at 24h p.i., ipsilateral PC (27.5 ± 0.5) was not significantly different from baseline ipsilateral PC. Ipsilateral PC was significantly greater than contralateral PC over the duration of the study in both 0.6% Cx-treated animals ($F [1, 62] = 165.4$, $p < 0.001$) and 0.1% Cx- treated animals ($F [1, 62] = 28.34$, $p < 0.001$).

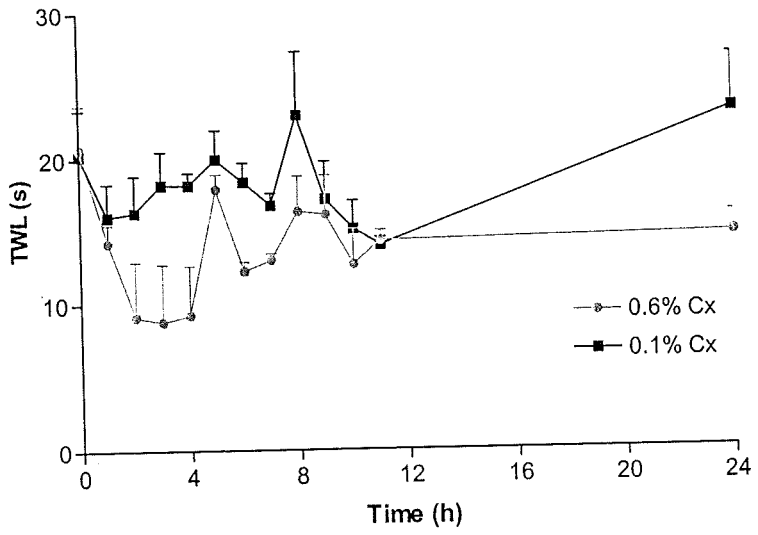
Thermal withdrawal latency (TWL)

Baseline ipsilateral TWL was 20.69 ± 3.00 s in 0.6% Cx-treated animals and 20.26 ± 3.09 s in 0.1% Cx-treated animals [Figure 2.3 (B)]. In 0.6% Cx -treated animals, the lowest ipsilateral TWL (TWLmin) occurred 3 h p.i. (8.75 ± 3.95s). In 0.1% Cx-treated animals, the lowest ipsilateral TWL (TWLmin) occurred 11 h p.i. (13.95 ± 0.53 s) [Figure 2.3 (B)].

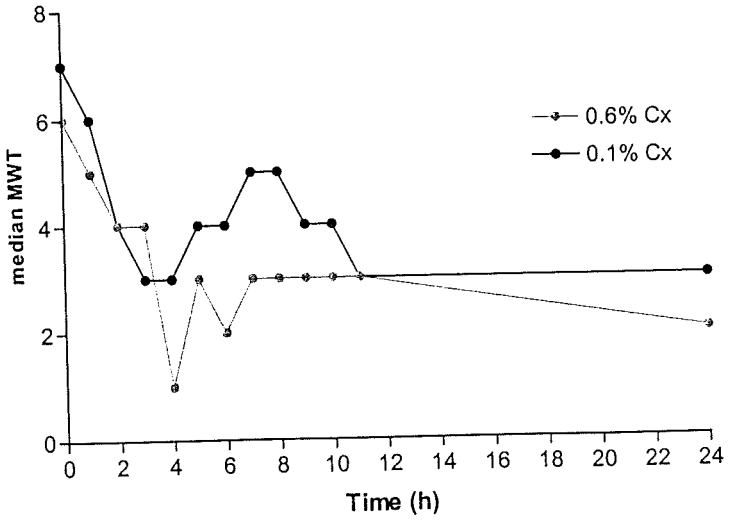
A


paw circumference (mm)
37
35
33
31
29
27
25
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)
0.6% Cx
0.1% Cx

B


TWL (s)
30
20
10
0
0
4
8
12
16
20
24
Time (h)
0.6% Cx
0.1% Cx

C


median MWT
8
6
4
2
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (h)
0.6% Cx
0.1% Cx

Ipsilateral TWL in 0.6% Cx-treated animals was significantly lower than that of 0.1% Cx-treated animals over time ($F [2, 62] = 18.54$, $p < 0.001$). Ipsilateral TWL changed significantly over time in 0.6% Cx-treated animals ($F[12, 24] = 2.15$, $p < 0.05$) but not in 0.1% Cx-treated animals ($F[12, 24] = 1.32$, $p = 0.27$). In 0.6% Cx-treated animals at 24h p.i., ipsilateral TWL (14.6 ± 1.4 s) was significantly greater ($p < 0.01$) than baseline ipsilateral PC. In 0.1% Cx-treated animals at 24h p.i., ipsilateral TWL (23.2 ± 3.7 s) was not significantly different from baseline ipsilateral TWL. Ipsilateral TWL was significantly lower than contralateral TWL over time in 0.6% Cx-treated animals ($F [1, 62] = 19.49$, $p < 0.001$) but not in 0.1% Cx- treated animals

Mechanical withdrawal threshold (MWT)

Baseline ipsilateral MWT was 6 (6,7) in 0.6% Cx-treated animals and 7(3,7) in 0.1% Cx-treated animals [Figure 2.3(C)]. In 0.6% Cx-treated animals, the lowest ipsilateral MWT(MWTmin) was 2.33 (1,3), recorded 5 h p.i. and was also 2.33 (2,3) at 11 h p.i. In 0.1% Cx-treated animals, the lowest ipsilateral MWT(MWTmin) was 2.33 (1,3) occurred 11 h p.i. Ipsilateral MWT in 0.6% Cx-treated animals did not differ significantly from that recorded in 0.1% Cx-treated animals.

2.3.3. PILOT STUDY 3

Paw circumference

Baseline ipsilateral PC did not differ between treatment groups (28.0 ± 0.5 mm in 0.5% Cx-treated animals, 27.75 ± 0.5 mm in 0.1% Cx-treated animals and 26.25 ± 0..25 mm in saline-treated animals) [Figure 2.4 (A)]. There was no effect of treatment on contralateral PC. In 0.5%-Cx-treated animals, ipsilateral PC at 6 h p.i. was 32.8 ± 0.5mm. In 0.1% Cx-treated animals. Ipsilateral PC at 6 h p.i. was 31.4 ± 0.9mm. In saline-treated animals, ipsilateral PC at 6 h p.i was 26.75 mm ± 0.25 mm.

There was a significant treatment effect on ipsilateral PC ($F[2, 26]=18.07$, $p < 0.001$). Ipsilateral PC in Cx-treated animals (both 0.5% and 0.1%) was significantly greater than saline-treated animals at 6 hours p.i.($p< 0.001$ for both 0.5% and 0.1% Cx). There was no significant difference in ipsilateral PC between 0.5% and 0.1% Cx- treated animals at 6h p.i. ($p=0.14$).

In 0.5% Cx-treated animals, ipsilateral PC was significantly greater than contralateral PC treated animals at 2 h p.i. ($p < 0.01$) and 6 h p.i. ($p < 0.001$). In 0.1% Cx-treated animals,

ipsilateral PC was significantly greater than contralateral PC at 2 h p.i. ($p < 0.05$) and 6 h p.i. ($p < 0.01$). In saline-treated animals, ipsilateral PC was not significantly different from contralateral PC at 2 h p.i. or 6 h p.i.

In 0.5% Cx -treated animals, ipsilateral PC was significantly greater than pre-treatment PC at 2h and at 6 h p.i. ($p < 0.001$ for both). In 0.1% Cx -treated animals, ipsilateral PC was significantly greater than pre-treatment PC at 2h p.i. ($p < 0.01$) and at 6 h p.i. ($p < 0.001$).

Thermal withdrawal latency

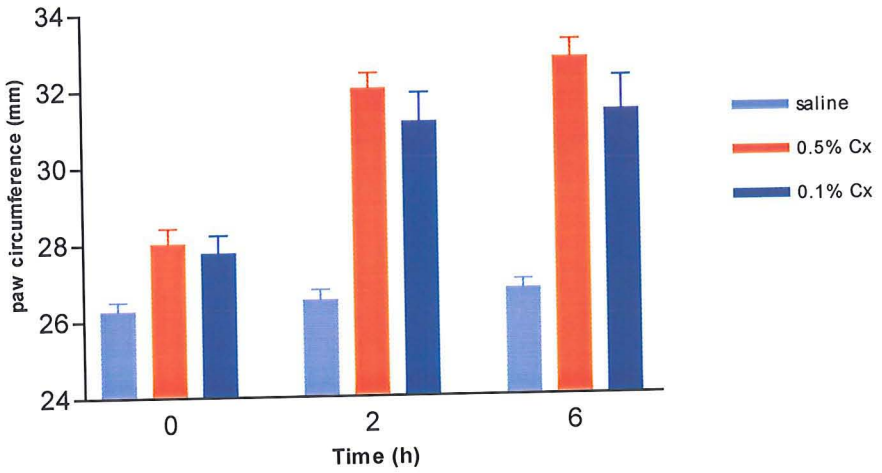
Baseline ipsilateral TWL was 13.67 ± 0.5 s in 0.5% Cx-treated animals, 12.50 ± 0.3 s in 0.1% Cx-treated animals and 14.10 ± 0.9 s in saline-treated animals (Figure 2.4 (B)]. There was no effect of treatment on contralateral TWL in any treatment group.

There was a significant treatment effect on ipsilateral TWL ($F [2,26] = 6.3$, $p < 0.01$). Ipsilateral TWL in 0.5% Cx-treated animals was significantly lower than that of saline-treated animals at both 2h p.i. ($p < 0.01$) and at 6h p.i. ($p < 0.05$) [Figure 2.4 (B)]. Ipsilateral TWL in 0.1% Cx-treated animals was significantly lower than that of saline-treated animals at both 2h p.i. ($p < 0.01$) and at 6h p.i. ($p < 0.05$).

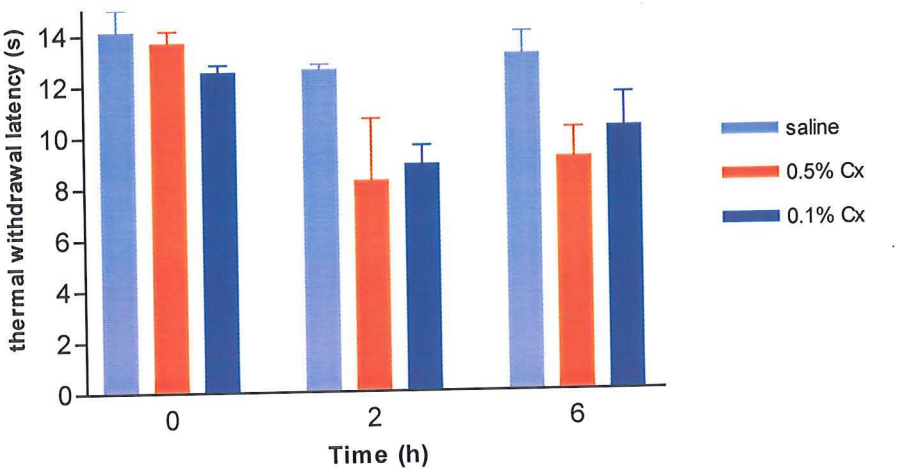
In saline-treated animals, ipsilateral TWL was not significantly different from contralateral TWL at 2h or 6 h p.i. However in 0.5% Cx and 0.1% Cx-treated animals, ipsilateral TWL was significantly lower than contralateral TWL at both 2 h p.i. ($p < 0.01$ for both) and 6 h p.i. ($p < 0.01$ and $p < 0.05$ respectively).

In saline-treated animals, ipsilateral post-treatment TWL(at 2 h and 6 h p.i.) was not different from pre-treatment TWL. In 0.5% Cx-treated animals, post-treatment ipsilateral TWL was significantly lower than pre-treatment TWL at 2h p.i. (8.23 ± 2.43 s, $p < 0.01$), and was close to significance at 6h p.i. (9.08 ± 1.16 s, $p = 0.052$). In 0.1% Cx-treated animals, ipsilateral TWL was significantly lower than pre-treatment TWL at 2h p.i. (8.85 ± 0.76s, $p < 0.01$) and was close to significance at 6h p.i. (10.25 ± 1.33 s, $p = 0.07$).

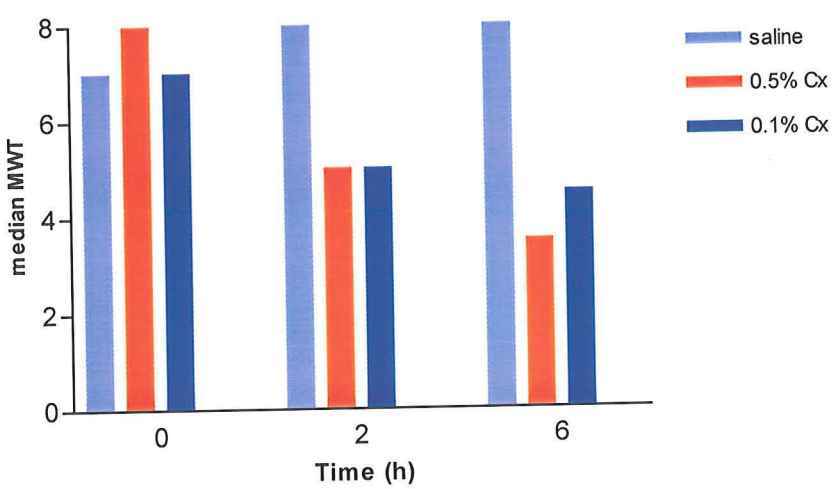
A


paw circumference (mm)
34
32
30
28
26
24
0
2
6
Time (h)
saline
0.5% Cx
0.1% Cx

B


thermal withdrawal latency (s)
14
12
10
8
6
4
2
0
0
2
6
Time (h)
saline
0.5% Cx
0.1% Cx

C


median MWT
8
6
4
2
0
0
2
6
Time (h)
saline
0.5% Cx
0.1% Cx

Mechanical withdrawal threshold

MWT data are shown in figure 2.4 (C). Baseline ipsilateral MWT was 8 (8,8) in 0.5% Cx-treated animals, 6.75 (5.25, 8) in 0.1% Cx-treated animals and 7.25 (7,7.75) in saline-treated animals. There was no effect of treatment on contralateral MWT in any treatment group. Saline treatment induced no change in ipsilateral MWT. There was a significant treatment effect on ipsilateral MWT at 2 h and 6 h p.i. ($p< 0.01$ for both). Ipsilateral MWT in carrageenan-treated animals (0.1% and 0.5) was significantly lower than that of saline-treated animals at both 2 and 6 hours p.i. ($p < 0.05$ for all). In saline-treated animals, ipsilateral MWT was not significantly different from contralateral MWT at any time. In 0.5% Cx-treated animals, ipsilateral MWT was significantly lower than contralateral MWT at both 2 h ($p < 0.05$) and 6 h p.i. ($p < 0.05$). In 0.1% Cx-treated animals, ipsilateral MWT was significantly lower than contralateral MWT at 6 h p.i. ($p < 0.05$) but not at 2 h p.i.

Ipsilateral MWT in carrageenan-treated animals (0.1% and 0.5) at 2 h and 6 h p.i. was significantly lower than pre-injection MWT ($p < 0.05$ for all).

2.3.4 STUDY 2.1: Inflammatory and nociceptive responses to weekly repeated carrageenan treatment

Baseline pre-injection PC, TWL and MWT in ipsilateral and contralateral paws did not differ between saline and carrageenan-treated animals on any week of the study. Carrageenan and saline treatment did not alter contralateral PC, TWL and MWT relative to baseline values on any week of the study. Ipsilateral baseline PC did not change significantly over the course of the study in any treatment group. Single and repeated saline treatment induced no change in ipsilateral PC, TWL or MWT.

Paw circumference

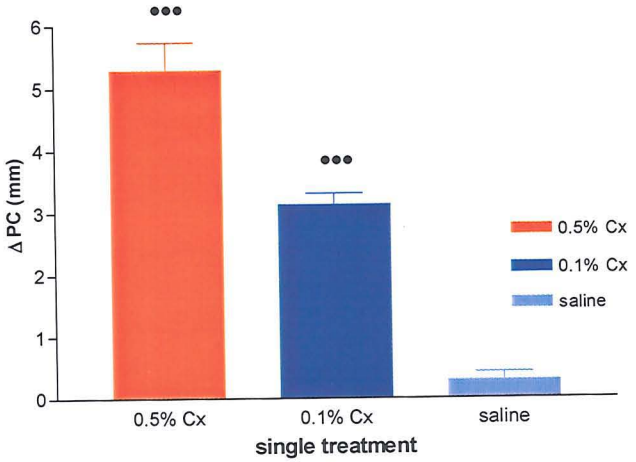
Changes in paw circumference measurements recorded in study 2.1 are shown in figure 2.5. Baseline PC pre-treatment on week 1 was 27.8 ± 0.1 mm for saline, 27.8 ± 0.1mm for 0.5% Cx and 28.8 ± 0.1 mm for 0.1% Cx-treated animals. At 6 h p.i. on week 1, ipsilateral PC was 28.04 ± 0.1 mm in saline-treated animals, 33.0 ± 0.2 mm in 0.5% Cx-treated animals and 31.0 ± 0.2 mm in 0.1% Cx-treated animals.

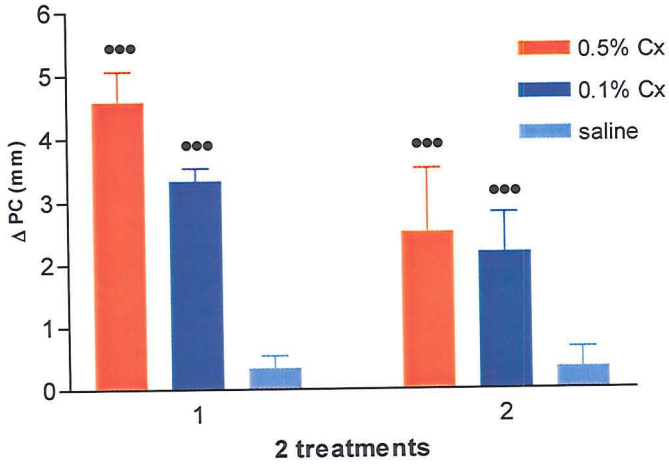
Treatment effect

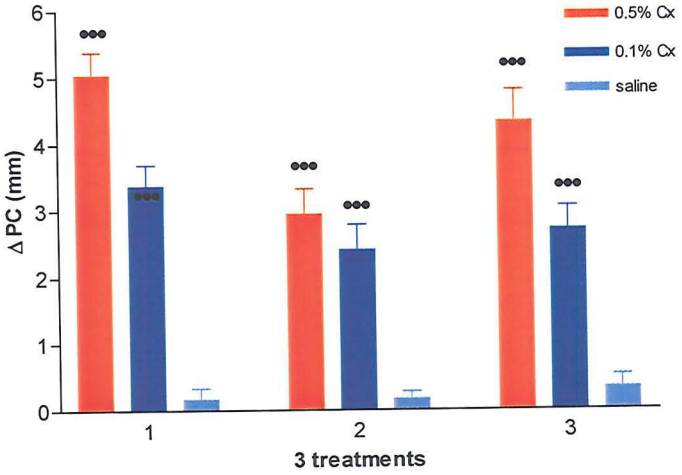
Statistical analysis of the effects of carrageenan treatment on ipsilateral ΔPC is summarised in Table 2.2

- Following a single treatment, there was a significant effect of treatment on ipsilateral ΔPC ($F [2,46] = 125.9$, $p < 0.001$). Carrageenan treatment (0.5% and 0.1%) significantly increased ΔPC relative to saline treatment ($p<0.001$ in both groups). Post-hoc analysis indicated 0.5% Cx treatment also induced a significantly greater increase in that ipsilateral ΔPC than 0.1% Cx treatment ($p < 0.001$).

- In the course of 2 treatments, a significant treatment effect was observed on both week 1($F[2,34]=102.23$, $p<0.001$) and week 2 ($F[2,34]=17.79$, $p<0.001$); 0.5% and 0.1% carrageenan induced a significantly greater ΔPC relative to saline control animals on both weeks 1 and 2 ($p<0.001$). 0.5% carrageenan also induced a significantly greater Δ PC relative to 0.1% carrageenan-treated animals on week 1 ($p < 0.001$) but not week 2 of the study].


●●●
●●●
6
5
4
3
2
1
0
Δ PC (mm)
0.5% Cx
0.1% Cx
saline
0.5% Cx
0.1% Cx
saline
single treatment


●●●
●●●
●●●
●●●
6
5
4
3
2
1
0
Δ PC (mm)
0.5% Cx
0.1% Cx
saline
1
2
2 treatments


●●●
●●●
●●●
●●●
●●●
6
5
4
3
2
1
0
Δ PC (mm)
0.5% Cx
0.1% Cx
saline
1
2
3
3 treatments

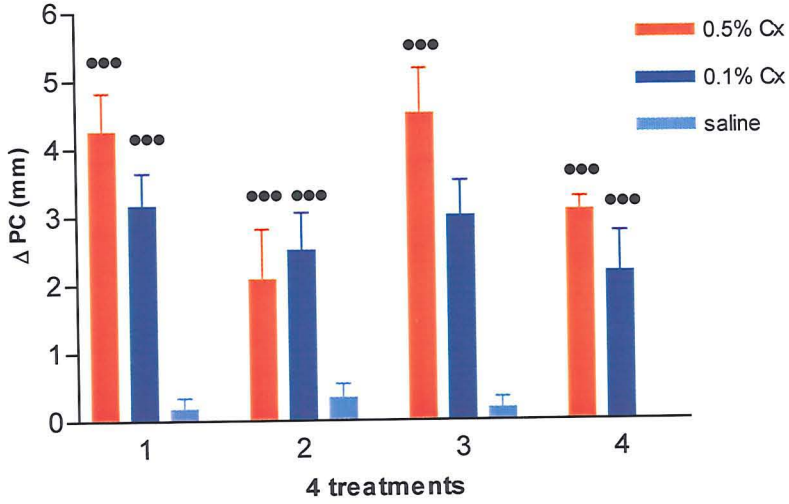

●●●
●●●
●●●
●●●
●●●
●●●
●●●
6
5
4
3
2
1
0
Δ PC (mm)
0.5% Cx
0.1% Cx
saline
1
2
3
4
4 treatments

Table 2.2

Study 2.1: Summary of statistical analysis of the effects of the effects of intraplantar injection of 50 μl, 0.5% carrageenan (Cx), 0.1% Cx and saline on ipsilateral paw circumference (PC, mm). PC was measured in ipsilateral (ipsi) paws pre-injection and at 6h post-injection. The effect of treatment on ipsilateral ΔPC (post-inection PC- pre-injection PC, mm) was investigated using ANOVA techniques combined with Tukey-Kramer multiple comparison tests. ΔPC in 0.5% and 0.1% carrageenan-treated animals was compared with ΔPC in saline-treated animals on each week of the study. ΔPC in 0.5% and 0.1% animals were also compared on each week of the study (n = 24 following 1 treatment, n = 18 following 2 treatments, n = 12 following 3 treatments and n = 6 following 4 treatments).

••• = $p<0.001$, •• = $p<0.01$, • = $p < 0.05$, NS = no significant difference between treatment groups.

Treatment		0.5% Cx/ saline	0.1% Cx/ saline	0.5% Cx/ 0.1% Cx
1 treatment (n = 24)		•••	•••	•••
2 treatments (n = 18)	week 1	•••	•••	•••
	week 2	•••	•••	NS
3 treatments (n = 12)	week 1	•••	•••	•••
	week 2	•••	•••	NS
	week 3	•••	•••	••
4 treatments (n = 6)	week 1	•••	•••	NS
	week 2	•	•	NS
	week 3	•••	•••	NS
	week 4	•••	•••	NS

•

- In the course of 3 treatments, a significant treatment effect was observed on week 1($F[2,22]=81.1$, $p < 0.001$), week 2 ($F[2,22]=19.57$, $p < 0.001$) and week 3 ($F[2,22]=37.65$, $p < 0.001$). ΔPC in 0.5% Cx-treated animals was significantly greater than that of saline-treated animals on all 3 weeks ($p < 0.001$); and was significantly greater than 0.1% Cx treatment on weeks 1 ($p < 0.001$) and week 3 ($p < 0.01$) but not on week 2. ΔPC in 0.1% Cx-treated animals was significantly greater than that of saline-treated animals on all 3 weeks ($p < 0.001$ on all three weeks).
- In the course of 4 treatments, a significant treatment effect was observed on all 4 weeks: week 1($F[2,10]=18.55$, $p < 0.001$), week 2 ($F[2,10]=4.5$, $p < 0.05$) week 3 ($F[2,10]=27.53$, $p < 0.001$) and week 4 $F[2,10]=19.41$, $p < 0.001$). ΔPC induced by 0.5% Cx was significantly greater than saline treatment on all 4 weeks ($p < 0.001$ on weeks 1, 2 and 4, $p < 0.05$ on week 2). ΔPC induced by 0.1% Cx was also significantly greater than saline treatment on each week ($p < 0.001$ on weeks 1, 2 and 4, $p< 0.05$ on week 3). There was no significant difference in ΔPC measured in 0.5% and 0.1% Cx-treated animals on any week of the study.

Ipsilateral/contralateral effect

Statistical analysis of the effects of carrageenan and saline treatment on ipsilateral (relative to contralateral) ΔPC is summarised in Table 2.3.

- Following a single treatment (n = 24/ group), a significant treatment effect on ipsilateral (relative to contralateral) ΔPC was observed in carrageenan-treated animals. Ipsilateral ΔPC was significantly greater than contralateral ΔPC in both 0.5% and 0.1% Cx-treated animals ($F[1,23]=447.7$, $p <0.001$) and ($F[1,23]=302.3$, $p<0.001$) respectively.
- In the course of 2 treatments (n = 18/group), in 0.5% Cx-treated animals, ipsilateral Δ PC was significantly greater than contralateral ΔPC on both week 1 ($F[1,17] = 224.5$, $p < 0.001$) and week 2 ($F[1,17] = 12.2$, $p < 0.01$). Similarly, in 0.1% Cx-treated animals, ipsilateral ΔPC was significantly greater than contralateral ΔPC on both week 1 ($F[1,17] = 148.5$, $p < 0.001$) and week 2 ($F[1, 17] = 52.06$, $p < 0.001$).

Table 2.3

Study 2.1: Summary of statistical analysis of the effects of intraplantar injection of 50 µl, 0.5% carrageenan (Cx), 0.1% Cx and saline on ipsilateral (relative to contralateral) paw circumference (PC, mm). PC was measured in ipsilateral and contralateral paws pre-injection and at 6h post-injection. Within each treatment group, on each week of the study, the effect of treatment on ipsilateral (relative to contralateral) ΔPC (post-inection PC- pre-injection PC, mm) was analysed using ANOVA techniques (n = 24 following 1 treatment, n = 18 following 2 treatments, n = 12 following 3 treatments and n = 6 following 4 treatments).

••• = $p<0.001$, •• = $p<0.01$, • = $p < 0.05$, NS = no significant difference between ipsilateral and contralateral ΔPC.

Treatment		0.5% Cx	0.1% Cx	saline
1 treatment (n = 24)		•••	•••	NS
2 treatments (n = 18)	week 1	•••	•••	NS
	week 2	••	•••	NS
3 treatments (n = 12)	week 1	•••	•••	NS
	week 2	•••	•••	NS
	week 3	•••	•••	NS
4 treatments (n = 6)	week 1	•••	••	NS
	week 2	•	••	NS
	week 3	•••	••	NS
	week 4	•••	•	NS

- In the course of 3 treatments (n = 12/group), a significant treatment effect on ipsilateral (relative to contralateral) ΔPC was observed in 0.5% and 0.1% carrageenan-treated animals PC ($F[1,57] = 324.5$, $p < 0.001$ and $F[1,57] = 206.4$, $p < 0.001$ respectively). Further analysis showed that in 0.5% Cx-treated animals, ipsilateral ΔPC was significantly greater than contralateral ΔPC on week 1 ($F[1,11] = 168.6$, $p < 0.001$), week 2 ($F[1,11] = 64.0$, $p < 0.001$) and week 3 ($F[1,11] = 90.8$, $p < 0.001$). In 0.1% Cx-treated animals, ipsilateral ΔPC was significantly greater than contralateral ΔPC on week 1 ($F[1,11] = 67.4$, $p < 0.001$) and week 2 ($F[1,11] = 40.8$, $p < 0.001$) and week 3 ($F[1,11] = 59.7$, $p < 0.001$).

- In the course of 4 treatments (n = 6/group), a significant treatment effect on ipsilateral (relative to contralateral) ΔPC was observed in 0.5% and 0.1% carrageenan-treated animals ($F[1,38] = 119.27$, $p < 0.001$ and $F[1,37] = 88.7$, $p < 0.001$ respectively). Further analysis showed that in 0.5% Cx-treated animals, ipsilateral ΔPC was significantly greater than contralateral ΔPC on week 1 ($F[1,5] = 54.9$, $p < 0.001$), week 2 ($F[1,5] = 8.03$, $p < 0.05$), week 3 ($F[1,5] = 49.7$, $p < 0.001$) and week 4 ($F[1,5] = 236.1$, $p < 0.001$. In 0.1% Cx-treated animals, ipsilateral ΔPC was significantly greater than contralateral ΔPC on week 1 ($F[1,5] = 20.3$, $p < 0.01$), week 2 ($F[1,5] = 19.7$, $p < 0.01$), week 3 ($F[1,5] = 33.8$, $p < 0.01$) and week 4 ($F[1,5] = 13.0$, $p < 0.05$).

Effect of repeated treatment

Statistical analysis of the effects of repeated carrageenan or saline treatment on ipsilateral ΔPC are summarised in Table 2.4.

- In animals which received 2 treatments, there was a significant effect of repeated treatment on ipsilateral ΔPC induced by 0.5% Cx ($F[1,17]=17.86$, $p<0.001$); ΔPC was significantly less on week 2 (2.8 ± 1.7 mm) than week 1 (4.9 ± 1.2 mm). Similarly, there was a significant effect of repeated treatment on ipsilateral ΔPC induced by 0.1% Cx treatment ($F[1,17] = 6.89$, $p<0.05$); ΔPC was significantly less on week 2 (2.3 ± 1.4 mm) than week 1 (3.4 ± 0.9 mm). Ipsilateral ΔPC did not change following single or repeated saline treatment.

Table 2.4

Study 2.1: Summary of statistical analysis of the effects of repeated intraplantar injection of 50 μl, 0.5% carrageenan (Cx), 0.1% Cx and saline on ipsilateral paw circumference (PC, mm). PC was measured in ipsilateral paws pre-injection and at 6h post-injection. Within each treatment group, on each week of the study, the effect of repeated treatment on ipsilateral ΔPC (post-inection PC- pre-injection PC, mm) was investigated using general linear model analysis combined with Tukey-Kramer multiple comparison tests (n = 18 following 2 treatments, n = 12 following 3 treatments and n = 6 following 4 treatments).

••• = $p<0.001$, •• = $p<0.01$, • = $p < 0.05$, NS = no significant effect of repeated treatment on ipsilateral ΔPC.

Treatment	0.5% Cx	0.1% Cx	saline
2 treatments (n = 18)	•••	•	NS
3 treatments (n = 12)	••	NS	NS
4 treatments (n = 6)	•	NS	NS

- In animals which received 3 treatments, there was a significant effect of repeated treatment on ipsilateral ΔPC induced by treatment with 0.5% carrageenan ($F[2,22] = 7.1$, $p < 0.01$); ΔPC was significantly less on week 2 (2.96 mm) than on weeks 1 (5.04 mm) and 3 (4.33 mm) ($p < 0.05$). In 0.1% Cx and saline-treated animals, there was no effect of repeated injection on the increase in change in ipsilateral PC induced by treatment.

- In animals which received 4 treatments, there was a significant effect of repeated treatment on ipsilateral ΔPC induced by treatment with 0.5% carrageenan ($F[3,15] = 4.26$, $p<0.05$); ΔPC was 4.3 ± 0.6 mm on week 1, 2.1 ± 0.7 mm on week 2 (significantly lower than week 3, $p<0.05$), 4.5 ± 0.7 mm on week 3 and 3.1 ± 0.2 mm on week 4. In 0.1% Cx (ΔPC of 2.2 ± 0.6 mm-3.2 ± 0.5 mm) and in saline-treated animals, there was no effect of repeated injection on ΔPC.

Thermal withdrawal latency

The change in TWL induced by carrageenan and saline treatment (ΔTWL) is shown in figure 2.6. Baseline ipsilateral TWL recorded pre-treatment on week 1 was 11.4 ± 0.4 s in saline-treated animals, 11.54 ± 0.42 s in 0.5% Cx-treated animals and 11.2 ± 0.38 s in 0.1% Cx-treated animals. Post- treatment (6 h p.i.) ipsilateral TWL on week 1 was 10.2 ± 0.5 s in saline, 6.26 ± 0.38 s in 0.5% Cx and 8.51 s ± 0.60 s in 0.1% Cx-treated animals. Ipsilateral baseline TWL did not change significantly over the course of the study in any treatment group. There was no significant difference between ipsilateral and contralateral baseline PC on any week of the study.

Treatment effect

Statistical analysis of the effects of carrageenan treatment on ipsilateral ΔTWL is summarised in Table 2.5

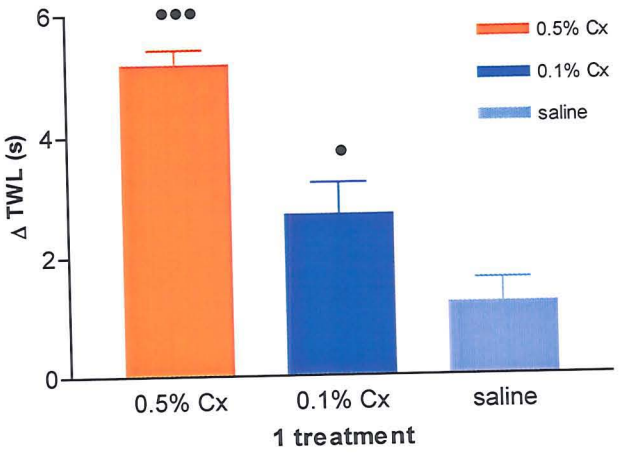
- Single treatment: ΔTWL induced by 0.5% Cx treatment was significantly greater than that induced by both saline treatment ($p < 0.001$) and 0.1% Cx treatment ($p < 0.001$). ΔTWL induced by 0.1% Cx treatment was significantly greater than that induced by saline treatment ($p < 0.05$).

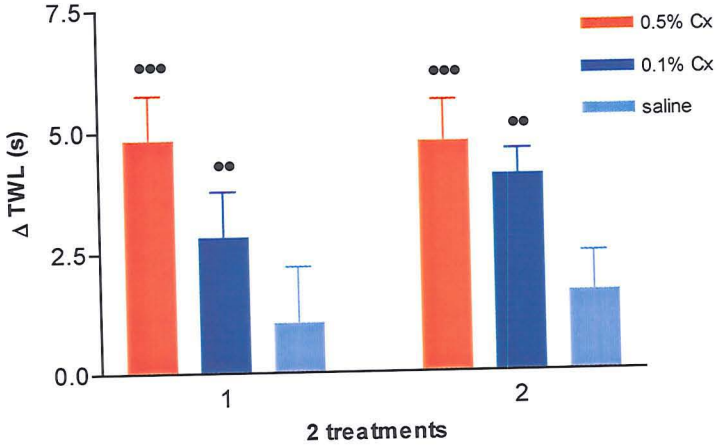
Table 2.5

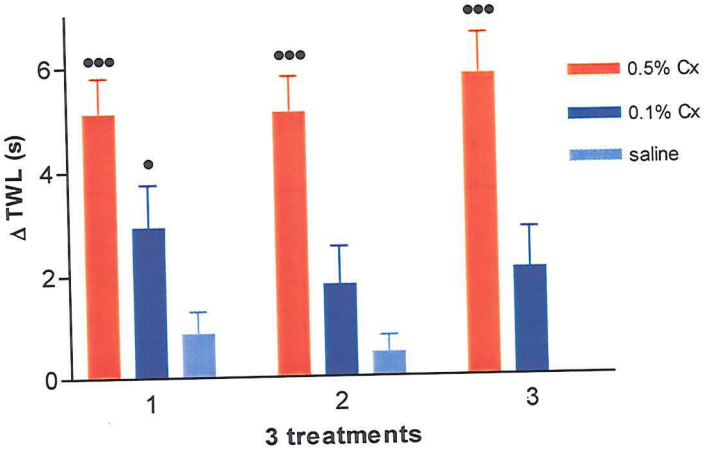
Study 2.1: Summary of statistical analysis of the effects of intraplantar injection of 50 µl, 0.5% carrageenan (Cx), 0.1% Cx and saline on ipsilateral thermal withdrawal latency (TWL, s). TWL was measured in ipsilateral (ipsi) paws pre-injection and at 6h post-injection. The effect of treatment on ipsilateral ΔTWL (pre-inection TWL- post-injection TWL, s) was investigated using ANOVA techniques combined with Tukey-Kramer multiple comparison tests. ΔTWL in 0.5% and 0.1% carrageenan-treated animals were compared with ΔTWL in saline-treated animals on each week of the study. ΔTWL in 0.5% and 0.1% animals were also compared on each week of the study (n = 24 following 1 treatment, n = 18 following 2 treatments, n = 12 following 3 treatments and n = 6 following 4 treatments).

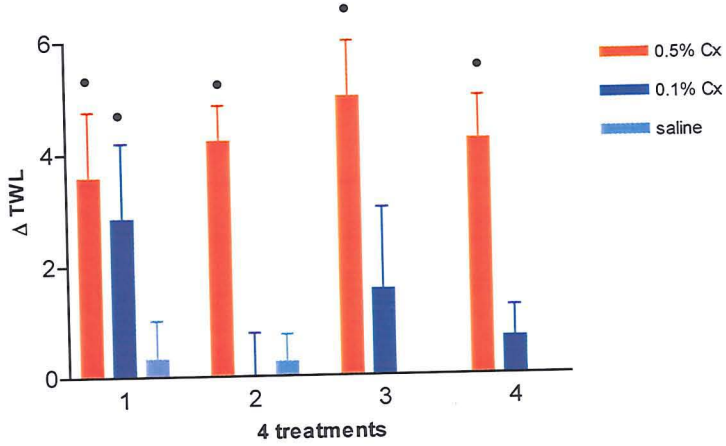
••• = $p<0.001$, •• = $p<0.01$, • = $p < 0.05$, NS = no significant difference between treatment groups.

Treatment		0.5% Cx/ saline	0.1% Cx/ saline	0.5% Cx/ 0.1% Cx
1 treatment (n = 24)		•••	•	•••
2 treatments (n = 18)	week 1	•••	•••	••
	week 2	•••	••	•••
3 treatments (n = 12)	week 1	•••	••	••
	week 2	•••	NS	••
	week 3	•••	NS	••
4 treatments (n = 6)	week 1	•	NS	NS
	week 2	••	NS	••
	week 3	••	NS	NS
	week 4	••	NS	••


Δ TWL (s)
0.5% Cx
0.1% Cx
saline
1 treatment


Δ TWL (s)
0.5% Cx
0.1% Cx
saline
2 treatments


Δ TWL (s)
0.5% Cx
0.1% Cx
saline
3 treatments


Δ TWL
0.5% Cx
0.1% Cx
saline
4 treatments

- In animals which received 2 treatments, a significant treatment effect on ipsilateral Δ TWL was observed on both week 1 ($F[2, 34] = 52.0$, $p < 0.001$) and week 2 ($F[2,34] = 34.1$, $p < 0.001$). 0.5% Cx treatment induced a significantly greater ΔTWL than saline treatment on both weeks 1 and 2 ($p < 0.001$). 0.1% Cx treatment also induced a significantly greater ΔTWL than saline treatment on both weeks 1 ($p < 0.001$) and week 2 ($p < 0.01$). 0.5% Cx treatment induced a significantly greater ΔTWL relative to 0.1% Cx treatment both week 1 ($p < 0.01$) and week 2 ($p < 0.001$).

- In animals which received 3 treatments, a significant treatment effect on ipsilateral Δ TWL was observed on both week 1 ($F[2,22] = 20.7$, $p < 0.001$), week 2 ($F[2,22] = 14.4$, $p < 0.001$) and week 3 ($F[2,22] = 20.7$, $p < 0.001$). 0.5% Cx treatment induced a significantly greater ΔTWL than saline treatment on weeks 1, 2 and 3 ($p < 0.001$). 0.1% Cx treatment induced a significantly greater ΔTWL than saline treatment on week 1 ($p < 0.01$), but not on week 2 ($p = 0.06$) or week 3 ($p = 0.30$). 0.5% Cx treatment induced a significantly greater ΔTWL relative to 0.1% Cx treatment on weeks 1,2 and 3 ($p<0.01$).

- In animals which received 4 treatments, a significant treatment effect on ipsilateral Δ TWL was observed on both week 1 ($F[2,10] = 3.8$, $p < 0.05$), week 2 ($F [2,10] = 9.86$, $p < 0.01$), week 3 ($F[2,10] = 6.6$, $p < 0.05$) and week 4 ($F[2,10] = 16.1$, $p < 0.01$). While 0.5% Cx induced a consistent reduction of 41 -46% in ipsilateral TWL on all 4 weeks of the study, 0.1% Cx induced a reduction of 29% on week 1, 1% on week 2, 15% on week 3 and 7% on week 4. Further analysis showed that 0.5% Cx treatment induced a significantly greater ΔTWL than saline treatment on all 4 weeks ($p < 0.05$ on week 1, $p < 0.01$ on weeks 2, 3 and 4); more detailed analysis did not indicate a significant difference in ΔTWL in 0.1% Cx-treated animals relative to saline-treated animals on any particular week of the study. 0.5% Cx treatment induced a significantly greater ΔTWL relative to 0.1% Cx treatment on weeks 2 and 4 ($p < 0.01$) but not on weeks 1 and 3.

Ipsilateral/contralateral effect

Statistical analysis of the effect of carrageenan and saline treatment on ipsilateral (relative to contralateral) ΔTWL is summarised in Table 2.6

Table 2.6

Study 2.1: Summary of statistical analysis of the effects of intraplantar injection of 50 µl, 0.5% carrageenan (Cx) , 0.1% Cx and saline on ipsilateral (relative to contralateral) thermal withdrawal latency (TWL,s. TWL was measured in ipsilateral and contralateral paws pre-injection and at 6h post-injection. Within each treatment group, on each week of the study, the effect of treatment on ipsilateral (relative to contralateral) ΔTWL (pre-inection TWL- post-injection TWL, s) was investigated using ANOVA techniques (n = 24 following 1 treatment, n = 18 following 2 treatments, n = 12 following 3 treatments and n = 6 following 4 treatments).

••• = $p<0.001$, •• = $p<0.01$, • = $p < 0.05$, NS = no significant difference between ipsilateral and contralateral ΔTWL.

Treatment		0.5% Cx	0.1% Cx	saline
1 treatment (n = 24)		•••	••	NS
2 treatments (n = 18)	week 1	•••	•••	NS
•••	week 2	•••	•••	NS
3 treatments (n = 12)	week 1	•••	NS	NS
	week 2	•••	•••	NS
	week 3	•••	•••	NS
4 treatments (n = 6)	week 1	••	•	NS
	week 2	•	NS	NS
	week 3	•	NS	NS
	week 4	•	NS	NS

- Following a single treatment, 0.5% Cx and 0.1% Cx treatment induced a significant reduction in ipsilateral TWL (relative to contralateral) TWL, from 11.54 ± 0.42 s to 6.26 ± 0.38 s ($F[1,23] = 86.3$, $p < 0.001$), a 45.7% reduction, and from 11.2 ± 0.38 s to 8.51 ± 0.6 s ($F[1,23) = 14.29$, $p < 0.01$), a 24% reduction, respectively .

- In the course of 2 treatments (n = 18/group), a significant treatment effect on ipsilateral (relative to contralateral) ΔTWL was observed in carrageenan, but not saline, treated animals TWL ($F[1,52] = 110.7$, $p < 0.001$ and $F[1,52] = 23.93$, $p < 0.001$ respectively). In 0.5% Cx-treated animals, ipsilateral ΔTWL was significantly greater than contralateral ΔTWL on both week 1 ($F[1,17] = 98.3$, $p < 0.001$) and week 2 ($F[1,17] = 20.0$, $p < 0.001$). In 0.1% Cx-treated animals, ipsilateral ΔTWL was significantly greater than contralateral ΔPC on both week 1 ($F[1,17] = 41.0$, $p < 0.001$) and week 2 ($F[1,17] = 6.0$, $p < 0.05$).

- In the course of 3 treatments, both 0.5% and 0.1% Cx, but not saline, treatment induced a significant reduction in ipsilateral (relative to contralateral)TWL ($F[1,57] = 144.59$, $p < 0.001$ and $F[1,57] = 14.79$, $p < 0.001$ respectively). Further analysis showed that in 0.5% Cx-treated animals, ipsilateral ΔTWL was significantly greater than contralateral ΔTWL on week 1 ($F[1,11] = 85.5$, $p < 0.001$), week 2 ($F[1,11] = 26.2$, $p < 0.001$) and week 3 ($F[1,11] = 33.5$, $p < 0.001$). In 0.1% Cx-treated animals, ipsilateral ΔTWL was significantly greater than contralateral ΔTWL on week 1 ($F[1,11] = 26.3$, $p < 0.001$), and week 3 ($F[1,11] = 12.8$, $p < 0.001$), but not on week 2 ($F[1,11] = 2.6$, $p < 0.13$).

- In the course of 4 treatments, both 0.5% and 0.1% carrageenan, but not saline, treatment induced a significant reduction in ipsilateral (relative to contralateral)TWL ($F[1,38] = 66.58$, $p < 0.001$ and $F[1,38] = 5.63$, $p < 0.05$ respectively). Further analysis showed that in 0.5% Cx-treated animals, ipsilateral ΔTWL was significantly greater than contralateral ΔTWL on week 1 ($F[1,5] = 21.1$, $p < 0.01$), week 2 ($F[1,5] = 9.1$, $p < 0.05$), week 3 ($F[1,5] = 12.9$, $p < 0.05$) and week 4 ($F[1,5] = 16.1$, $p < 0.01$). In 0.1% Cx-treated animals, ipsilateral ΔTWL was significantly greater than contralateral ΔTWL on week 1 ($F[1,5] = 12.8$, $p < 0.05$), and close to significance on week 4 ($F[1,11] = 5.03$, $p < 0.06$) but not on week 2 or 3.

Effect of repeated treatment

In animals which received 2, 3 or 4 treatments, repeated treatment did not alter ΔTWL associated with intraplantar injection of saline or carrageenan (0.5% and 0.1%).

Mechanical withdrawal threshold

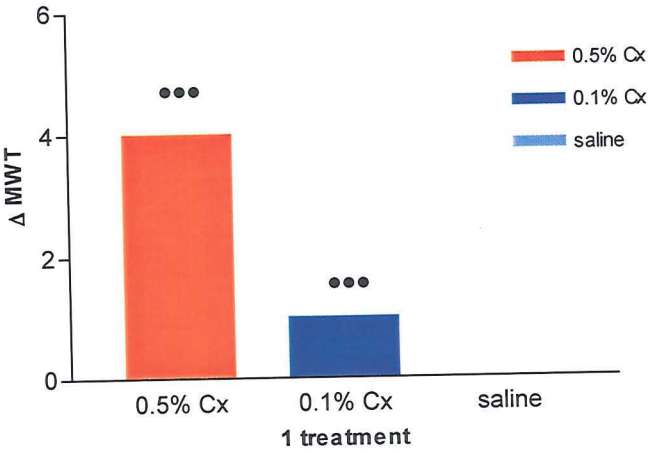
Mechanical withdrawal threshold measurements recorded in study 2.1 are shown in figure 2.7. Baseline ipsilateral MWT recorded pre-treatment on week 1 was 8 (8,9) in saline-treated animals, 8 (8,9) in 0.5% Cx-treated animals and 8 (7.25,9) in 0.1% Cx-treated animals. Ipsilateral baseline MWT did not change significantly over the course of the study in any treatment group. At 6 h p.i. on week 1, ipsilateral MWT was 8 (7,9) in saline-treated animals, 4 (3,5) in 0.5% Cx-treated animals and 6 (4,7) in 0.1% Cx-treated animals.

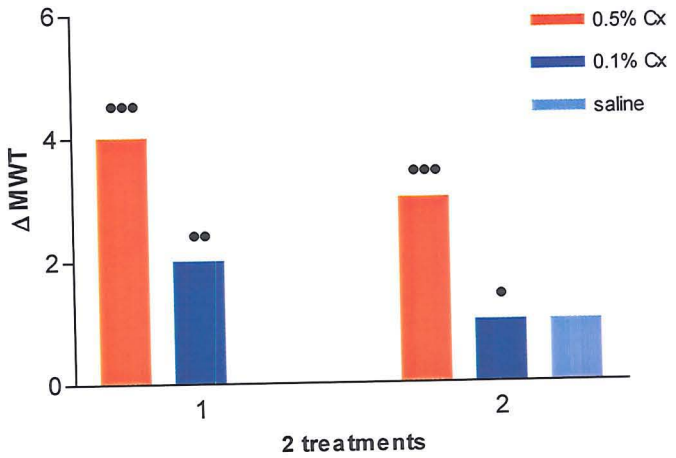
Treatment effect

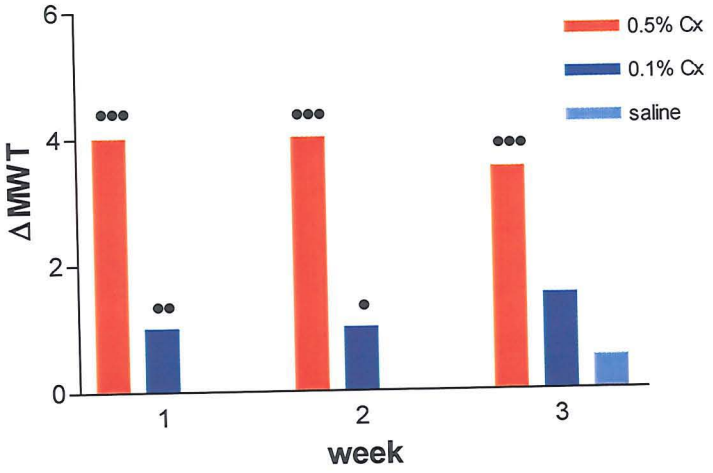
Statistical analysis of the effects of carrageenan treatment on ipsilateral MWT is summarised in Table 2.7.

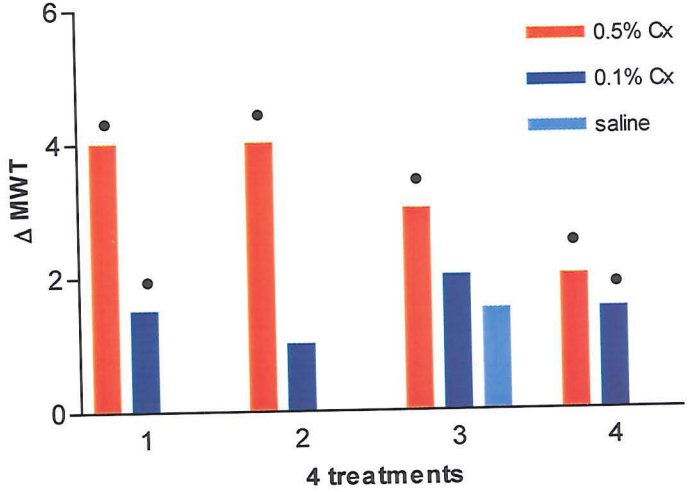
- Following a single treatment, a significant treatment effect on ipsilateral ΔMWT was observed. ΔMWT induced by 0.5% Cx was significantly greater than that induced by saline ($p < 0.001$) and 0.1% carrageenan ($p < 0.001$). ΔMWT induced by 0.1% Cx was significantly greater than that induced by saline ($p < 0.001$). A binary logistic regression model, applied to the distribution pattern of ipsilateral and contralateral MWT scores recorded on week 1, indicated that the distribution of ipsilateral MWT scores in 0.5% Cx-treated animals deviated strongly from the model fitted and that of ipsilateral MWT in 0.1% Cx-treated animals showed a small deviation from the model fitted. Ipsilateral MWT in saline-treated animals and contralateral MWT in all treatment groups fitted the binary logistic regression model closely.

- In animals which received 2 treatments, ΔMWT induced by 0.5% Cx was significantly greater than that induced by saline and 0.1% Cx on weeks 1 and 2 ($p < 0.001$ relative to saline on both week 1 and week 2, $p < 0.05$ relative to 0.1% Cx on both week 1 and week 2). ΔMWT induced by 0.1% Cx was significantly greater than that induced by saline on both weeks of the study ($p < 0.01$ on week 1, $p < 0.05$ on week 2). ΔMWT induced by 0.5% Cx was also significantly greater than that induced by 0.1% carrageenan ($p < 0.05$) on both weeks of the study ($p < 0.01$ on week 1 and $p < 0.001$ on week 2).


6
4
2
0
Δ MWT
●●●
●●●
0.5% Cx
0.1% Cx
saline
0.5% Cx
0.1% Cx
saline
1 treatment


6
4
2
0
Δ MWT
●●●
●●
●●●
●
0.5% Cx
0.1% Cx
saline
1
2
2 treatments


6
4
2
0
Δ MWT
●●●
●●
●●●
●
●●●
0.5% Cx
0.1% Cx
saline
1
2
3
week


6
4
2
0
Δ MWT
●
●
●
●
●
●
●
●
0.5% Cx
0.1% Cx
saline
1
2
3
4
4 treatments

- In animals which received 3 treatments, ΔMWT induced by 0.5% Cx was significantly greater than that induced by saline ($p < 0.001$) and 0.1% carrageenan ($p < 0.05$) on all three weeks of the study ($p < 0.001$ relative to saline on weeks 1, 2 and 3; $p < 0.05$ relative to 0.1% Cx on weeks 1,2 and 3). ΔMWT induced by 0.1% Cx was significantly greater than that induced by saline on weeks 1 and 2 of the study ($p < 0.01$ and $p < 0.05$ respectively), but not on week 3 .

- In animals which received 4 treatments, the ΔMWT associated with carrageenan treatment was significantly greater than that induced by saline on weeks 1 and 4 ($p<0.05$ on both weeks following 0.5% Cx treatment, and $p<0.05$ on both weeks following 0.1% Cx treatment). ΔMWT associated with 0.5% Cx was not significantly different from that induced by 0.1% Cx on week 1 or week 4.

Ipsilateral/contralateral effect

Statistical analysis of the effects of carrageenan treatment on ipsilateral MWT is summarised in Table 2.7

- Single treatment: both 0.5% and 0.1% Cx induced a significant increase in ipsilateral (relative to contralateral) ΔMWT on ($p < 0.001$ and $p < 0.01$ respectively). Saline injection did not alter ipsilateral (relative to contralateral) ΔMWT.

- In animals which received 2 treatments, both 0.5% and 0.1% Cx induced a significant increase in ipsilateral (relative to contralateral) ΔMWT on both week 1 ($p < 0.001$ and $p < 0.01$ respectively) and week 2 ($p < 0.001$ and $p < 0.01$ respectively). Saline injection induced a significant increase in ipsilateral (relative to contralateral) ΔMWT on week 1 ($p < 0.05$) (median change of 0.5), but no significant change in MWT on week 2.

Table 2.7

Study 2.1: Summary of statistical analysis of the effects of intraplantar injection of 50 μl, 0.5% carrageenan (Cx) and 0.1% Cx on ipsilateral mechanical withdrawal threshold (MWT). MWT was measured in ipsilateral (ipsi) paws pre-injection and at 6h post-injection. On each week of the study, the effect of treatment on ipsilateral ΔMWT (pre-inection MWT- post-injection MWT) was investigated using Friedman analysis. ΔMWT in 0.5% and 0.1% carrageenan-treated animals were compared with ΔMWT in saline-treated animals on each week of the study. ΔMWT in 0.5% and 0.1% animals were also compared on each week of the study (n = 24 following 1 treatment, n = 18 following 2 treatments, n = 12 following 3 treatments and n = 6 following 4 treatments).

••• = $p<0.001$, •• = $p<0.01$, • = $p < 0.05$, NS = no significant difference between treatment groups.

Treatment		0.5% Cx/ saline	0.1% Cx/ saline	0.5% Cx/ 0.1% Cx
1 treatment (n = 24)		•••	•••	•••
2 treatments (n = 18)	week 1	•••	••	••
	week 2	•••	•	•••
3 treatments (n = 12)	week 1	•••	••	•
	week 2	•••	•	•
	week 3	•••	NS	•
4 treatments (n = 6)	week 1	•	•	NS
	week 2	•	•	NS
	week 3	•	•	NS
	week 4	•	•	NS

- In animals which received 3 treatments, both 0.5% and 0.1% Cx, but not saline, treatment induced a significant increase in ipsilateral (relative to contralateral) ΔMWT on all three weeks ($p < 0.01$ on weeks 1, 2 and 3 following 0.5% Cx treatment, $p < 0.01$ on week 1 and $p < 0.05$ on weeks 2 and 3 following 0.1% Cx treatment).

- In animals which received 4 treatments, 0.5% Cx induced a significant reduction in ipsilateral ΔMWT on weeks 1, 2, 3 and 4 ($p < 0.01$). 0.1% carrageenan injection also induced a significant reduction in ipsilateral MWT (compared to pre-injection MWT) on weeks 1, 2, 3 and 4 ($p < 0.05$). Single or repeated saline injection induced no change in ipsilateral MWT. In all 3 treatment groups, the median change in ipsilateral and contralateral MWT did not alter with repeated injection.

Effect of repeated treatment

There was no effect of repeated treatment on ipsilateral or contralateral ΔMWT in carrageenan or saline-treated animals.

Table 2.8

Study 2.1: Summary of statistical analysis of the effects of intraplantar injection of 50 µl, 0.5% carrageenan (Cx) and 0.1% Cx and on ipsilateral (relative to contralateral) mechanical withdrawal threshold (MWT). MWT was measured in ipsilateral and contralateral paws pre-injection and at 6h post-injection. Within each treatment group, on each week of the study, the effect of treatment on ipsilateral (relative to contralateral) Δ MWT (pre-injection MWT- post-injection MWT) was investigated using the Wilcoxon Signed-Rank test (n = 24 following 1 treatment, n = 18 following 2 treatments, n = 12 following 3 treatments and n = 6 following 4 treatments).

••• = $p<0.001$, •• = $p<0.01$, • = $p < 0.05$, NS = no significant difference between ipsilateral and contralateral ΔTWL.

Treatment		0.5% Cx	0.1% Cx	saline
1 treatment (n = 24)		•••	••	NS
2 treatments (n = 18)	week 1	•••	••	•
•••	week 2	•••	••	NS
3 treatments (n = 12)	week 1	••	••	NS
	week 2	••	•	NS
	week 3	••	•	NS
4 treatments (n = 6)	week 1	••	•	NS
	week 2	••	•	NS
	week 3	••	•	NS
	week 4	••	•	NS

2.3.5 STUDY 2.2: Behavioural responses to daily repeated carrageenan treatment

Paw circumference

Paw circumference measurements recorded in study 2.2 are shown in figure 2.8. PCmax recorded on each day is shown in Table 2.9.

Treatment effect

Ipsilateral PCmax in Cx-treated animals was significantly greater than that of saline-treated animals on each day of the study ($F[1,11] = 50.0$, $p < 0.001$ on day 1; $F[1,11] = 96.8$, $p < 0.001$ on day 2; $F[1,11] = 317.3$, $p < 0.001$ on day 3; $F[1,11] = 451.0$, $p < 0.001$ on day 4 and $F[1,11] = 498.3$, $p < 0.001$ on day 5).

Effect of repeated treatment

Baseline PC

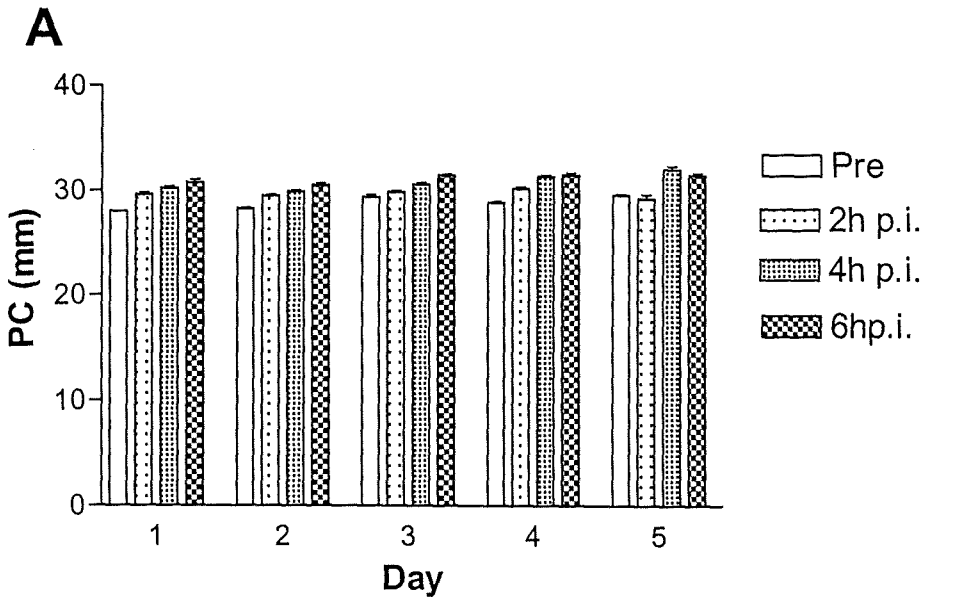
In saline-treated animals, baseline ipsilateral did not change over the duration of the study. In carrageenan-treated animals, baseline PC increased significantly over the study period ($F[4,44] = 48.8$, $p < 0.001$). Further analysis indicated that PC on day 1 was significantly lower than on all other days ($p < 0.001$); PC on day 2 was significantly lower than on days 3,4 and 5 ($p < 0.01$), while there was no significant difference in baseline ipsilateral PC recorded on days 3, 4 and 5.

PC max

There was no effect of repeated injection on ipsilateral pre-treatment PCmax or ΔPCmax in saline-treated animals.

In carrageenan-treated animals, ipsilateral PCmax increased significantly over the course of the study ($F[4,44] = 17.6$, $p < 0.001$). Further analysis showed that PCmax recorded on day 1 was significantly lower than that recorded on day 4 ($p < 0.05$) and day 5 ($p < 0.001$); PCmax recorded on day 2 was significantly lower than that recorded on days 3, 4 and 5 (all $p < 0.001$). PCmax recorded on day 5 was significantly greater than that recorded on all other days ($p < 0.001$).

ΔPC (PCmax- baseline PC) did not alter significantly over the course of the study.


A
40
30
20
10
0
PC (mm)
1
2
3
4
5
Day
Pre
2h p.i.
4h p.i.
6hp.i.

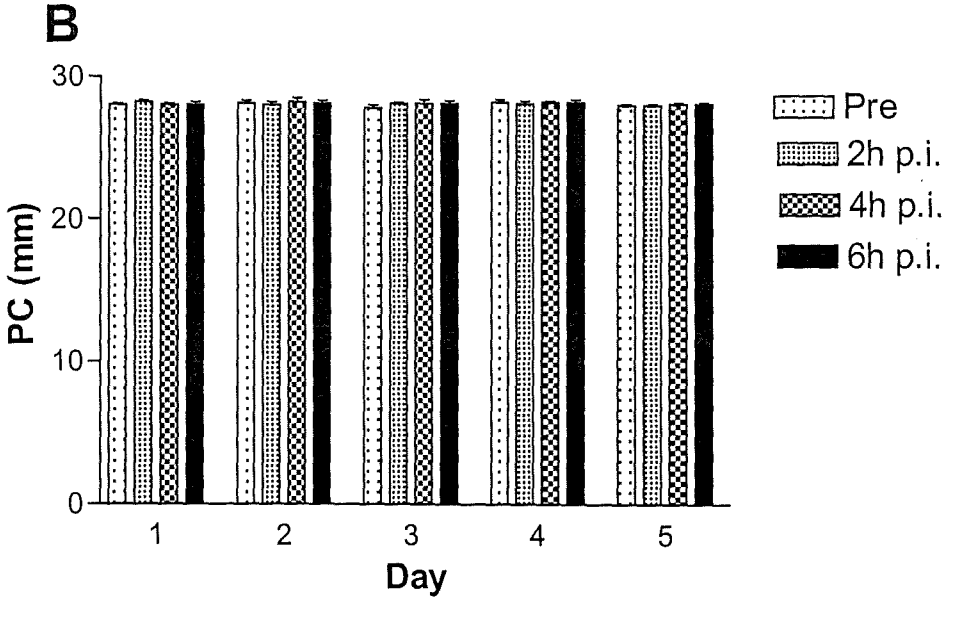

B
30
20
10
0
PC (mm)
1
2
3
4
5
Day
Pre
2h p.i.
4h p.i.
6h p.i.

Figure 2.9

Study 2.2: Mean ± S.E.M. ipsilateral PCmax (mm) measured on 5 consecutive days following daily repeated intaplantar injection of 50 μl, 0.1% carrageenan (n=12 in each group).

	PCmax (mm)	
Day	**carrageenan**	**saline**
1	30.8 ± 0.3	28.3 ± 0.1
2	30.5 ± 0.2	28.4 ± 0.1
3	31.3 ± 0.1	28.2 ± 0.1
4	31.5 ± 0.2	28.1 ± 0.1
5	32.3 ± 0.2	28.1 ± 0.1

Thermal withdrawal latency

Thermal withdrawal latency measurements recorded in study 2.2 are shown in figure 2.9. TWLmin recorded on each day is shown in Table 2.10.

Treatment effect

Baseline TWL

There was no treatment effect on contralateral baseline TWL on any day of the study. Ipsilateral baseline TWL was significantly lower on day 2 in carrageenan-treated animals ($F[1,11] = 7.1$, $p < 0.05$), but not on any other day.

Post-treatment TWL

Contralateral TWL was significantly lower in carrageenan-treated animals (relative to saline-treated animals) at 4h p.i. on days 1 and 4 ($p < 0.05$ on both days). There was no significant difference between treatment groups in contralateral TWL at 2h p.i. or at 6 h p.i. on any day of the study.

Ipsilateral TWL was significantly lower in carrageenan-treated animals (relative to saline-treated animals) at 2h p.i. on all days of the study, and at 4h p.i. and 6 h p.i. on days 1,2 and 5. On days 3 and 4, carrageenan treatment did not induce a significant reduction in ipsilateral TWL (relative to saline-treated animals) at 4h and 6h p.i.

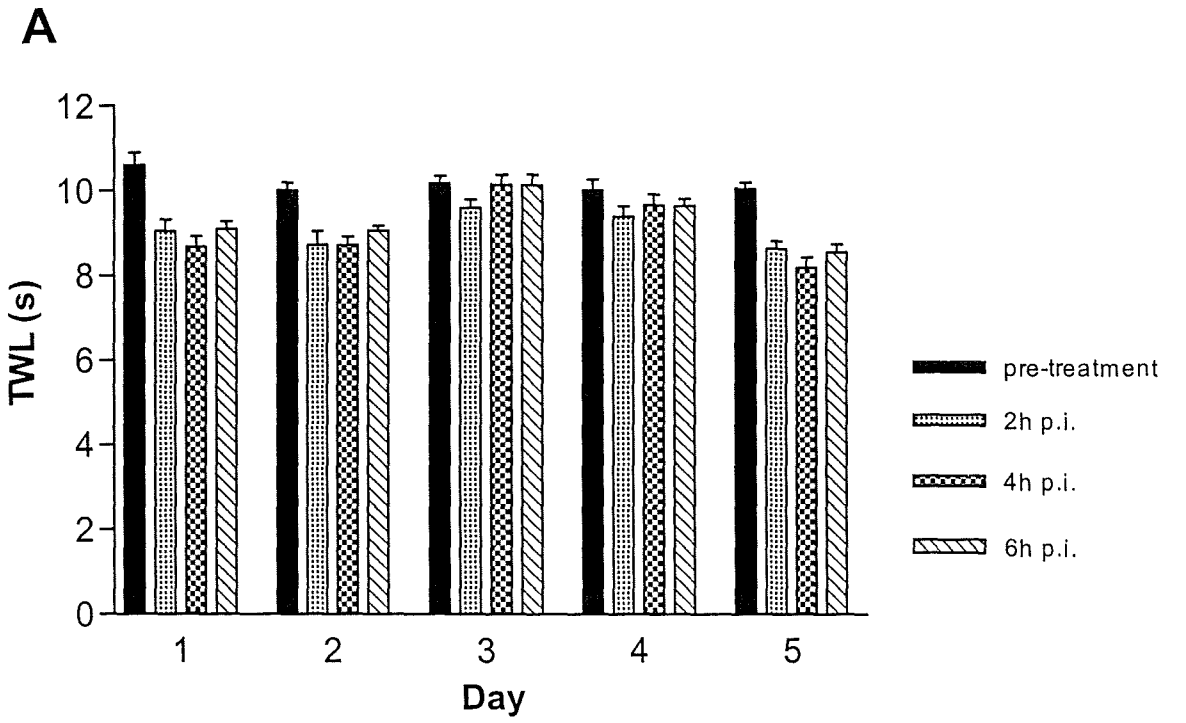
TWLmin

GLM analysis indicated a significant effect of carrageenan treatment (relative to saline treatment) on ipsilateral TWLmin on days 1,2 and 5 but not on days 3 and 4.($F[1,10] = 13.5$, $p < 0.01$ on day 1; $F[1,10] = 18.9$, $p < 0.001$ on day 2; $F[1,11] = 0.8$, $p = 0.4$ on day 3; $F[1,10] = 2.8$, $p = 0.1$ on day 4 and $F[1,10] = 37.7$, $p < 0.001$ on day 5. No treatment effect was observed on contralateral TWLmin on any day of the study.

Ipsilateral/contralateral effect

In saline-treated animals, there was no difference between ipsilateral and contralateral baseline TWL values on any day of the study and no difference between ipsilateral and contralateral post-treatment TWL values on any day of the study.

In carrageenan-treated animals, ipsilateral baseline TWL was significantly lower than contralateral baseline TWL on day 3 ($F[1,11] = 5.1$, $p < 0.05$) and day 5 ($F[1,11] = 7.8$, $p < 0.05$), but not on days 1, 2 and 4. Ipsilateral TWL was significantly lower than contralateral TWL at 2h, 4h and 6h p.i. on all 5 days of the study.


A
12
10
8
6
4
2
0
TWL (s)
1
2
3
4
5
Day
pre-treatment
2h p.i.
4h p.i.
6h p.i.

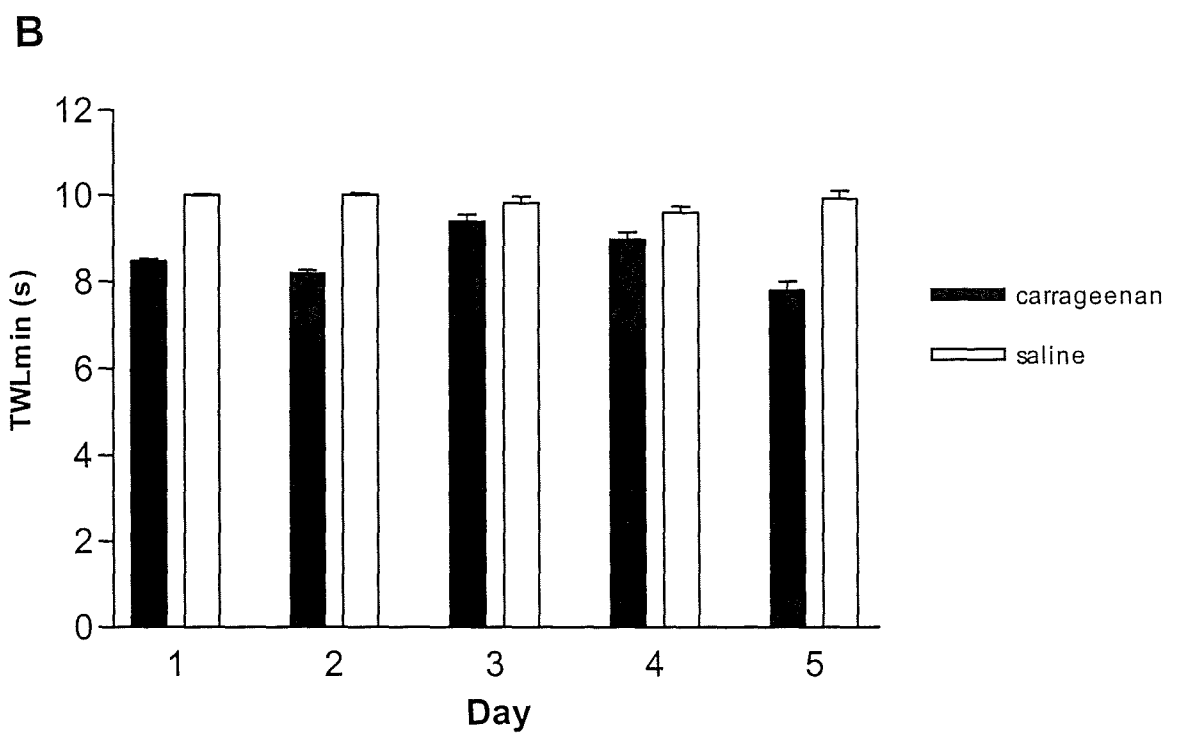

B
12
10
8
6
4
2
0
TWLmin (s)
1
2
3
4
5
Day
carrageenan
saline

Table 2.10

Mean ± SEM baseline TWL (s) and TWLmin (s) measured in ipsilateral (ipsi) and contralateral (contra) paws measured on 5 consecutive days following daily repeated intaplantar injection of 50 µl, 0.1% carrageenan (n=12 in each group).

Treatment	**carrageenan baseline TWL (s)**		**saline baseline TWL (s)**	
Day	***contralateral***	***ipsilateral***	***contralateral***	***ipsilateral***
1	10.8 ± 0.2	10.6 ± 0.3	10.7 ± 0.2	10.7 ± 0.3
2	10.6 ± 0.2	10.0 ± 0.2	10.6 ± 0.1	10.7 ± 0.1
3	10.8 ± 0.2	10.2 ± 0.2	10.7 ±0.3	10.4 ± 0.2
4	10.1 ± 0.2	10.0 ± 0.3	10.1 ± 0.2	10.3 ± 0.1
5	10.5 ±0.2	10.0 ± 0.2	10.6 ± 0.2	10.6 ± 0.1
Day	**carrageenan TWLmin (s)**		**saline TWLmin (s)**	
	contralateral	***ipsilateral***	***contralateral***	***ipsilateral***
1	10.0 ± 0.0	8.5 ± 0.1	10.1 ± 0.0	10.0 ± 0.0
2	9.84 ± 0.0	8.2 ± 0.1	10.0 ± 0.0	10.0 ± 0.0
3	10.2 ± 0.2	9.4 ± 0.2	10.0 ± 0.2	9.8 ± 0.2
4	9.6 ± 0.1	9.0 ± 0.2	9.2 ± 0.1	9.6 ± 0.1
5	9.8 ± 0.1	7.8 ± 0.2	10.0 ± 0.1	9.9 ± 0.2

TWLmin

In carrageenan-treated animals, ipsilateral TWLmin was significantly lower than contralateral TWLmin on all 5 days of the study ($F[1,10] = 18.3$, $p < 0.01$ on day 1; ($F[1,10] = 11.4$, $p < 0.01$ on day 2; ($F[1,10]$ 11.4, $p < 0.01$ on day 3; ($F[1,10] = 5.8$, $p < 0.05$ on day 4 and ($F[1,10] = 32.0$, $p < 0.001$ on day 5.

Effect of repeated treatment

There was no effect of repeated treatment on ipslateral or contralateral baseline TWL in saline or carrageenan-treated animals.

In saline-treated animals, a significant effect of repeated treatment on contralateral (but not ipsilateral) TWL was observed at 4h p.i. and 6h p.i. TWL recorded at 4h p.i. on day 4 (9.73 ± 0.1s) was significantly shorter than on all other days ($p<0.05$). TWL recorded at 6h p.i. on day 4 (9.86 ± 0.2) was significantly shorter than on days 1 and 2 ($p<0.03$ and $p<0.01$ respectively).

In Cx-treated animals a significant effect of repeated treatment on ipsilateral TWL was observed. Responses measured on days 3 and 4 were significantly longer than those measured on days 1,2 and 5 at various time points. This was most marked at 4h p.i., when TWL on day 3 was 10.16 ± 0.2s, compared to 8.69 ±0.2s on day 1, 8.73 ± 0.2s on day 2 and 8.2 ±0.2s on day 5. On day 4, ipsilateral TWL at 4 h was 9.67 ±0.3 s, significantly longer than days 1 ($p < 0.04$) and 5($p < 0.001$).

In Cx-treated animals, a significant effect of repeated treatment on contralateral TWL at 4h p.i. was also observed, where TWL recorded on day 3 (10.96 ± 0.2)s was significantly longer than on all other days ($p < 0.05$) [10.25 ± 0.1s on day 1; 10.3 ± 0.1s on day 2; 10.33 ± 0.2 s on day 4 and 10.32 ± 0.2 s on day 5]. There was no significant effect of repeated treatment on contralateral TWL at 2 h p.i. or 6 h p.i.

TWLmin

In saline-treated animals, a significant effect of repeated treatment on contralateral (but not ipsilateral) TWLmin was observed $F(4,44)=7.92$, $p<0.001$]. Contralateral TWLmin measured on day 4 was significantly lower than that recorded on all other days ($p<0.001$).

In carrageenan-treated animals, a significant effect of repeated treatment on ipsilateral (but not contralateral) TWLmin was observed ($F[4,44] = 27.5$, $p<0.001$). Further analysis showed that ipsilateral TWLmin on day 3 was significantly higher than that observed on days 1 ($p < 0.01$), 2 and 5 ($p < 0.001$). Ipsilateral TWLmin on day 4 was significantly higher than that observed on days 2 ($p < 0.05$) and 5 ($p < 0.001$).

Mechanical withdrawal threshold

Mechanical withdrawal threshold scores recorded in study 2.2 are shown in figure 2.10. Baseline MWT and MWTmin recorded on each day are shown in Table 2.11.

Treatment effect

Baseline MWT

There was no significant difference between carrageenan and saline-treated animals in ipsilateral or contralateral baseline MWT on any day of the study.

MWTmin

Carrageenan treatment produced a significant treatment effect on ipsilateral MWTmin (relative to saline treatment) on all 5 days of the study [day 1, $p < 0.001$; day 2, $p < 0.001$; day 3, $p < 0.05$; day 4, $p < 0.05$; day 5, $p < 0.001$].

Ipsilateral/contralateral effect

In saline-treated animals, there was no significant difference between ipsilateral and contralateral MWTmin on any day of the study.

In carrageenan-treated animals, ipsilateral MWTmin was significantly lower than contralateral MWTmin on days 1, 2 and 5 ($p < 0.01$), but not on days 3 and 4 ($p < 0.06$).

Effect of repeated treatment

In carrageenan-treated animals, Kruskal-Wallis analysis indicated a significant effect of repeated treatment on ipsilateral (but not contralateral) MWTmin. Further analysis using Mann-Whitney tests indicated that MWTmin on day 3 was significantly greater than on days 1,2 and 5 ($p < 0.001$), while MWTmin on day 4 was also significantly greater than MWTmin on days 1, 2 and 5 ($p < 0.002$ on days 1, 2 and 5).

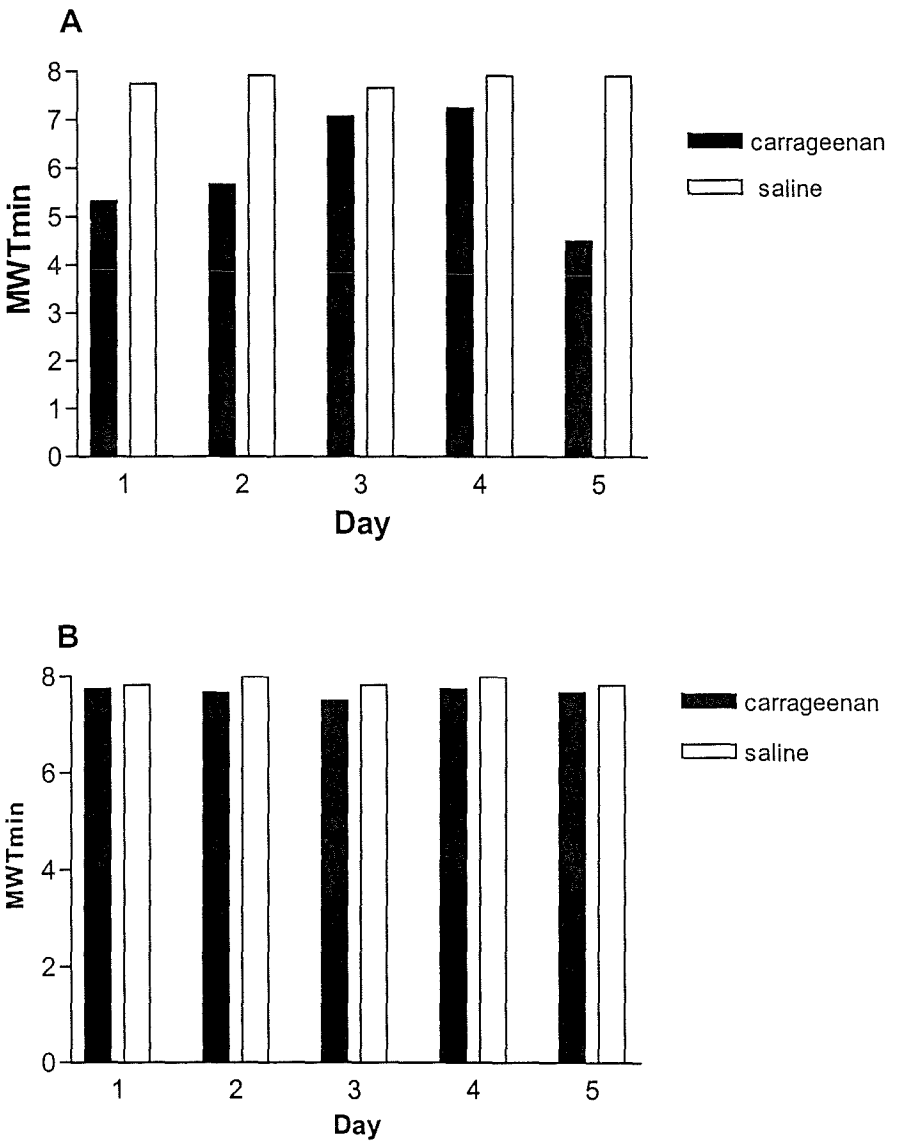
Table 2.11

Median (and interquartile ranges, Q_1 and Q_3) lowest MWT score (MWTmin) measured in ipsilateral and contralateral paws on each day of repeated injection of 0.1% carrageenan or 0.9% saline (50 μl) (n = 12/ group).

Treatment	**carrageenan baseline MWT**		**saline baseline MWT**	
Day	***contralateral***	***ipsilateral***	***contralateral***	***ipsilateral***
1	8 (7.75, 8)	8 (8,8)	8 (7.75, 8)	8 (7.75, 8)
2	8 (8, 8)	8 (7.75, 8)	8 (8, 8)	8 (8,8)
3	8 (8, 8)	8 (7.5, 8)	8 (8, 8)	8 (7.75, 8)
4	8 (7.75, 8)	8 (7.5, 8)	8 (8, 8)	8 (8, 8)
5	7 (7.75, 8)	8 (7.5, 8)	8 (8, 8)	8 (7.75, 8)
Treatment	**carrageenan MWTmin**		**saline MWTmin**	
Day	***contralateral***	***ipsilateral***	***contralateral***	***ipsilateral***
1	7.5 (7,8)	6 (4.25,6)	8 (8,8)	7.5(7,8)
2	8 (7,8)	6 (5,6)	8(8,8)	7.92(7.5,8)
3	8 (7,8)	7 (7,7)	8(8,8)	7.67(7,8)
4	8 (8,8)	7.67 (7,8)	8 (8,8)	7.67(7,8)
5	8 (7.25,8)	4 (3,6)	8 (8,8)	7.92(7,5, 8)

Figure 2.10

The effects of daily intraplantar injection of 50 µl 0.1% carrageenan and 50 µl 0.9% saline on median MWTmin. Median MWTmin (the lowest recorded MWT score) was recorded in the ipsilateral (A) and contralateral (B) paw on 5 consecutive days (n = 12/ group).



2.3.6 STUDY 2.3 (A): Preliminary investigations into the effects of the NMDA receptor antagonist, memantine, on inflammatory and nociceptive responses to daily repeated carrageenan treatment

Paw circumference

Baseline (pre-treatment) ipsilateral PC was 29.2 ± 0.2 mm in Cx-treated animals and 29.0 ± 0.1 mm in saline-treated animals. PCmax recorded in study 2.3 (A) is shown in Figure 2.11 (A)

Treatment effect

Ipsilateral PCmax in Cx-treated animals was significantly greater than that of saline-treated animals on each day of the study ($F[1,5] = 106.2$, $p < 0.001$ on day 1; $F[1,5] = 62.5$, $p < 0.001$ on day 2; $F[1,5] = 245.0$, $p < 0.001$ on day 3; $F[1,5] = 282.0$, $p < 0.001$ on day 4 and $F[1,5] = 605.0$, $p < 0.001$ on day 5).

Effect of repeated treatment

There was no effect of repeated injection on ipsilateral PCmax in saline-treated animals. In carrageenan-treated animals, PCmax increased significantly over the course of the study ($F[4,20] = 20.6$, $p < 0.001$). Further analysis showed that PCmax recorded on day 1 was significantly lower than that recorded on day 3 ($p < 0.05$), 4 ($p < 0.001$) and day 5 ($p < 0.001$); PCmax recorded on day 2 was significantly lower than that recorded on days 4 ($p < 0.001$) and 5 ($p < 0.001$). PCmax recorded on day 5 was significantly greater than that recorded on days 1,2 and 3 ($p < 0.001$). ΔPC (Pcmax - baseline PC) did not alter significantly over the course of the study.

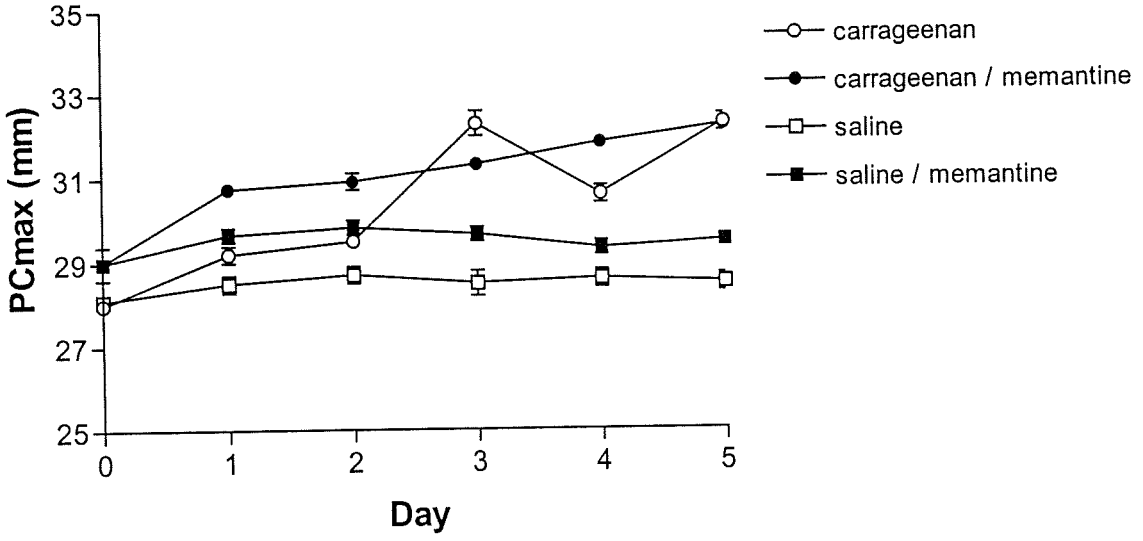
Thermal withdrawal latency

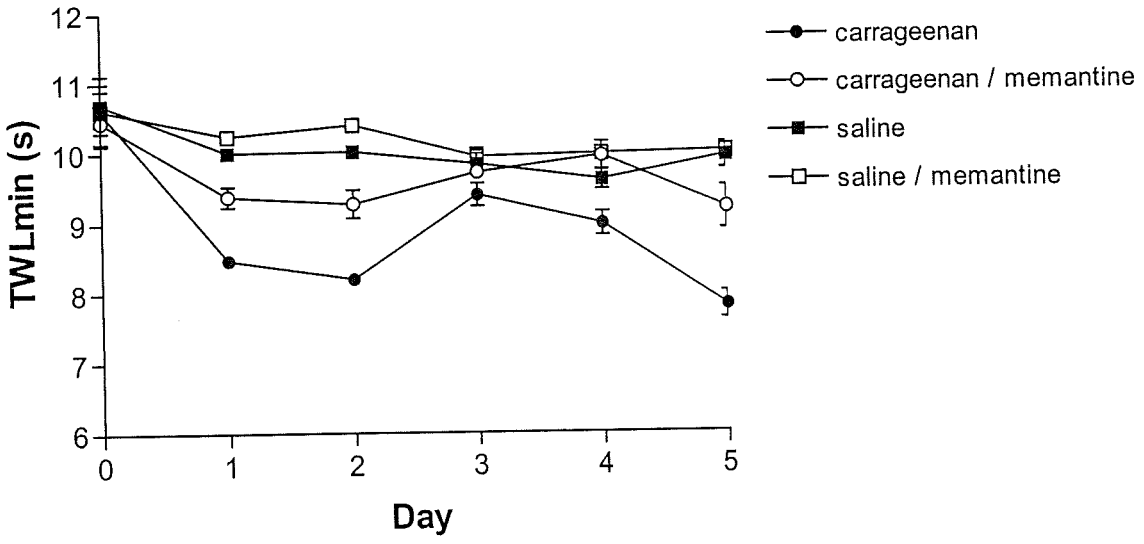
Baseline (pre-treatment) ipsilateral TWL was 10.45 ± 0.3 s in Cx-treated animals and 10.62 ± 0.5 s in saline-treated animals. TWLmin recorded in study 2.3 (A) is shown in Figure 2.11 (B) and Table 2.12.

Treatment effect

TWLmin

GLM analysis indicated a significant effect of carrageenan treatment (relative to saline treatment) on ipsilateral TWLmin on days 1,2 and 5 but not on days 3 and 4 ($F[1,5] = 69.4$, $p < 0.001$ on day 1; $F[1,5] = 22.6$, $p < 0.01$ on day 2; $F[1,5] = 4.4$, $p = 0.09$ on day 3; $F[1,5] = 0.2$, $p = 0.8$ on day 4 and $F[1,5] = 5.3$, $p < 0.05$ on day 5). No treatment effect was observed on contralateral TWLmin on any day of the study.


PCmax (mm)
35
33
31
29
27
25
0
1
2
3
4
5
Day
carrageenan
carrageenan / memantine
saline
saline / memantine


TWLmin (s)
12
11
10
9
8
7
6
0
1
2
3
4
5
Day
carrageenan
carrageenan / memantine
saline
saline / memantine

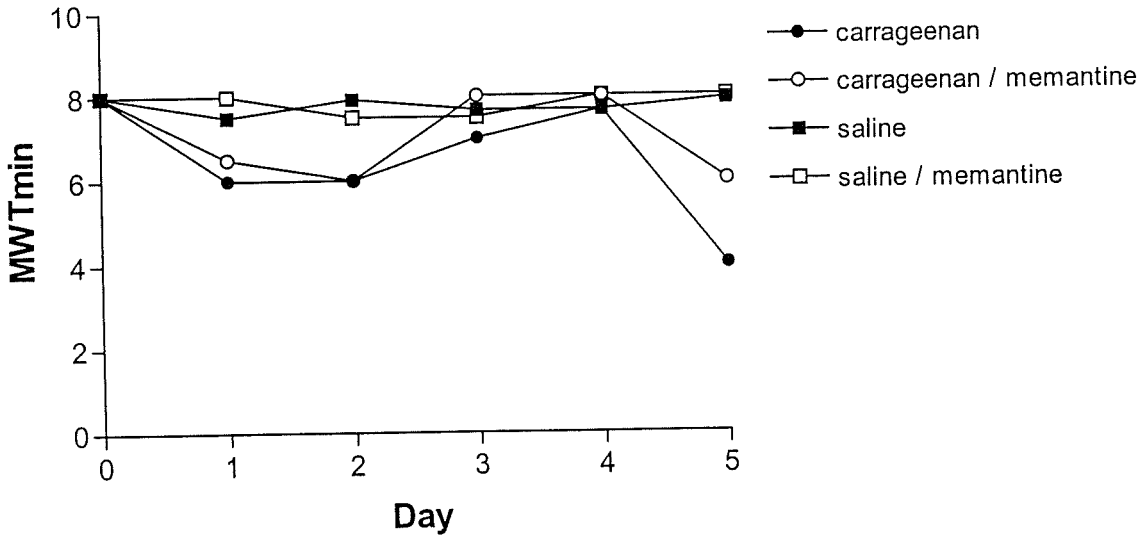

MWTmin
10
8
6
4
2
0
0
1
2
3
4
5
Day
carrageenan
carrageenan / memantine
saline
saline / memantine

Table 2.12

Mean ± SEM minimal thermal withdrawal latency (TWLmin, s) and median (first and third interquartile) minimal mechanical withdrawal threshold (MWTmin) measured in ipsilateral and contralateral paws following repeated intraplantar injection of 0.1% carrageenan or 0.9% saline (50 μl) in combination with systemic treatment with memantine (n = 6/group).

	MEMANTINE			
Treatment	**carrageenan TWLmin (s)**		**saline TWLmin (s)**	
Day	**contralateral**	**ipsilateral**	**contralateral**	**ipsilateral**
1	10.59 ± 0.05	9.38 ± 0.15	10.12 ± 0.07	10.24 ± 0.09
2	10.03 ± 0.07	9.27 ± 0.20	10.22 ± 0.04	10.39 ± 0.08
3	10.16 ± 0.09	9.71 ± 0.08	10.02 ± 0.06	9.93 ± 0.12
4	10.51 ± 0.07	9.94 ± 0.20	10.15 ± 0.10	9.97 ± 0.07
5	10.42 ± 0.06	9.2 ± 0.30	10.13 ± 0.09	10.00 ± 0.12
Treatment	**carrageenan MWTmin**		**saline MWTmin**	
Day	**contralateral**	**ipsilateral**	**contralateral**	**ipsilateral**
1	8(8,8)	6.5(5.75,7)	8(7.75,8)	8(7,8)
2	8(8,8)	6(5.75,7)	8(7.75,8)	7.5(7,8)
3	8(8,8)	8(8,8)	8(8,8)	7.5(8,8)
4	8(8,8)	8(8,8)	8(8,8)	8(8,8)
5	8(8,8)	6(6,7.25)	8(7.75,8)	8(8,8)

Ipsilateral/contralateral effect

TWLmin

In saline-treated animals, ipsilateral TWLmin was not significantly different from contralateral TWLmin on any day of the study.

In carrageenan-treated animals, ipsilateral TWLmin was significantly lower than contralateral TWLmin on days 1,2 and 5 of the study ($F[1,5] = 32.6$, $p < 0.01$ on day 1; ($F[1,5] = 21.2$, $p < 0.01$ on day 2; and ($F[1,5] = 8.9$, $p < 0.05$ on day 5. Ipsilateral TWLmin was not significantly different from contralateral TWLmin on day 3 ($F[1,5] = 0.14$, $p = 0.7$ or on day 4 ($F[1,5] = 1.07$, $p = 0.35$).

Effect of repeated treatment

TWLmin

In carrageenan-treated animals, there was no significant effect of repeated treatment on ipsilateral TWLmin ($F[4,29] = 2.37$, $p = 0.09$).

In saline-treated animals, there was a significant effect of repeated treatment on ipsilateral TWLmin [$F[4,29] = 3.15$, $p = 0.04$]; however post-hoc analysis did not identify particular days on which this effect occurred.

Mechanical withdrawal threshold (MWT)

Baseline (pre-treatment) ipsilateral MWT was 8 (7,8) in Cx-treated animals and 8 (8,8) in saline-treated animals. MWTmin recorded in study 2.3 (A) is shown in Figure 2.11 (C) and Table 2.12.

Treatment effect

Ipsilateral MWTmin in carrageenan-treated animals was significantly lower than that of saline-treated animals on days 1,2 and 5 ($p<0.05$ on days 1 and 2, $p<0.01$ on day 5), but not on days 3 and 4.

Ipsilateral/contralateral effect

In carrageenan-treated animals, ipsilateral MWTmin was significantly lower than contralateral MWTmin on days 1 and 2 ($p < 0.05$) and lay close to significance on day 5 ($p < 0.06$), but was not significantly lower than contralateral MWTmin on days 3 and 4.

Effect of repeated treatment

In carrageenan-treated animals there was a significant effect of repeated treatment on ipsilateral MWTmin (Friedman test, $p < 0.001$, adjusted for ties). MWTmin on day 3 was

significantly higher than that measured on days 1, 2 and 5 ($p < 0.05$). MWTmin on day 4 was significantly higher than that measured on days 1, 2 and 5 ($p < 0.05$).

In saline-treated animals, Friedman analysis indicated a significant effect of repeated treatment on ipsilateral MWTmin ($p < 0.02$, adjusted for ties), however more detailed analysis did not identify any day on which MWTmin differed significantly from other days.

2.3.7. STUDY 2.3(B): Preliminary investigations into the effects of the opioid receptor antagonist, naltrexone, on inflammatory and nociceptive responses to daily repeated carrageenan treatment

Paw circumference

Baseline (pre-treatment) ipsilateral PC was 29.0 ± 0.2 mm in group 1 (Cx-treated) animals, 29.8 ± 0.8 mm in group 3 (Cx-treated) animals, 29.0 ± 0.2 mm in group 2 (saline-treated) animals and 29.4 ± 0.5 mm in group 4 (saline-treated) animals. PCmax recorded in study 2.3 (B) is shown in Figure 2.12 (A).

Treatment effect

0.5mg/kg NTX (Groups 1 and 3): Ipsilateral PCmax in group 1 (carrageenan-treated) animals was significantly greater than that of group 3 (saline-treated) animals on each day of the study ($F[1,2] = 121.00$, $p < 0.01$ on day 1; ($F[1,2] = 100.0$, $p < 0.01$ on day 2; ($F[1,2] = 169.0$, $p < 0.01$ on day 3; ($F[1,2] = 51.6$, $p < 0.01$ on day 4 and ($F[1,2] = 243.0$, $p < 0.001$ on day 5).

0.05mg/kg NTX (Groups 2 and 4): Ipsilateral PCmax in group 1 (carrageenan-treated) animals was significantly greater than that of group 3 (saline-treated) animals on each day of the study ($F[1,2] = 27.0$, $p < 0.01$ on day 1; ($F[1,2] = 102.0$, $p < 0.01$ on day 2; ($F[1,2] = 196.0$, $p < 0.01$ on day 3; ($F[1,2] = 192.0$, $p < 0.01$ on day 4 and ($F[1,2] = 680.0$, $p < 0.001$ on day 5).

Repeated treatment

PCmax

In saline-treated animals (Groups 3 and 4), PCmax did not change over the course of the study.

In group 1 animals PCmax increased significantly over the course of the study ($F[4,20] = 104.9$, $p < 0.001$). Further analysis showed that PCmax recorded on day 1 was

significantly lower than that recorded on day 3 ($p < 0.001$), 4 ($p < 0.001$) and day 5 ($p < 0.001$); PCmax recorded on day 2 was significantly lower than that recorded on days3 ($p < 0.01$), 4 ($p < 0.001$) and 5 ($p < 0.001$). PCmax recorded on day 5 was significantly greater than that recorded on all other days 3 ($p < 0.001$). ΔPC (Pcmax - baseline PC) did not alter significantly over the course of the study.

In group 2 animals (0.05mg/kg NTX), PCmax increased significantly over the course of the study ($F[4,20] = 166.0$, $p < 0.001$). Further analysis showed that PCmax recorded on day 1 was significantly lower than that recorded on day 3 ($p < 0.01$), 4 ($p < 0.001$) and day 5 ($p < 0.001$); PCmax recorded on day 2 was significantly lower than that recorded on days3 ($p < 0.05$), 4 ($p < 0.001$) and 5 ($p < 0.001$). PCmax recorded on day 5 was significantly greater than that recorded on all other days 3 ($p < 0.001$). ΔPC (PCmax-baseline PC) did not alter significantly over the course of the study.

Thermal withdrawal latency (TWL)

Baseline (pre-treatment) ipsilateral TWL was 10.3 ± 0.1 s in group 1 (Cx-treated) animals, 10.7 ± 0.3 s in group 3 (Cx-treated) animals, 10.6 ± 0.4 mm in group 2 (saline-treated) animals and 10.8 ± 0.1 s in group 4 (saline-treated) animals. TWLmin recorded in study 2.3 (B) is shown in Figure 2.12 (B) and Table 2.13

Treatment effect

Baseline TWL

There was no difference in ipsilateral or contralateral baseline TWL measurements recorded in carrageenan and saline-treated animals on any day of the study.

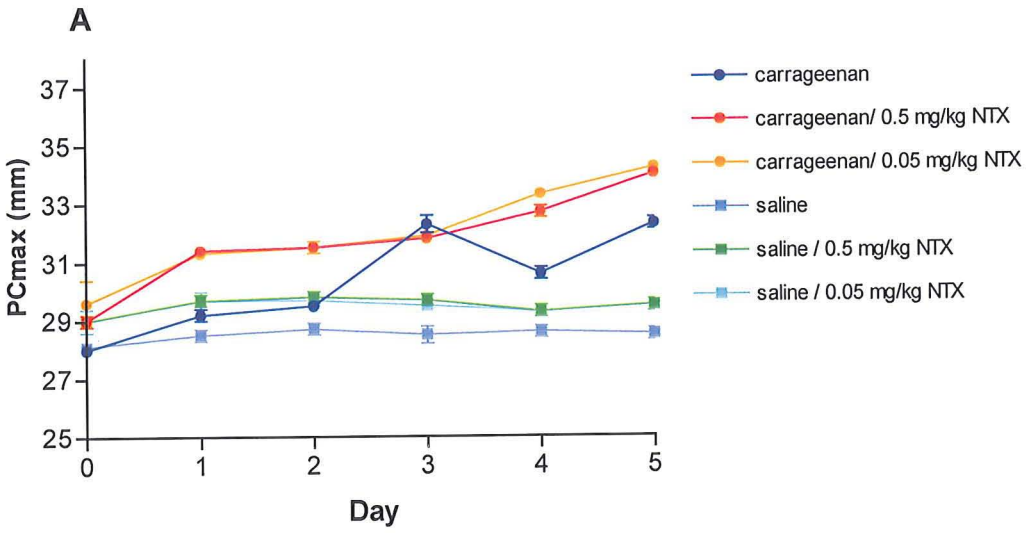
TWLmin

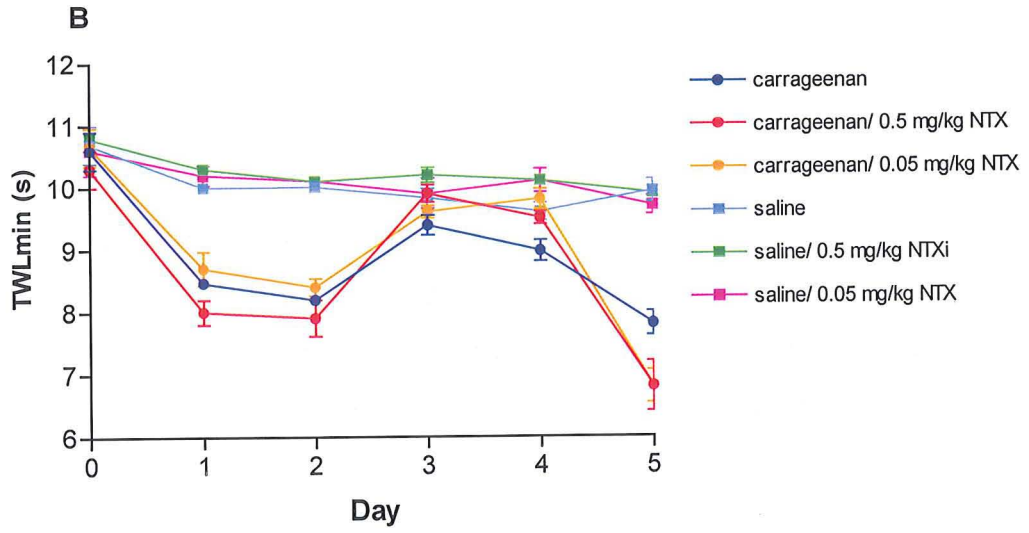
0.5 mg/kg NTX: Group 1 and group 3

GLM analysis indicated a significant effect of carrageenan treatment (relative to saline treatment) on ipsilateral TWLmin on days 1,2 and 5 but not on days 3 and 4.($F[1,2] = 99.8$, $p < 0.001$ on day 1; $F[1,2] = 228.9$, $p < 0.01$ on day 2; $F[1,2] = 5.1$, $p = 0.15$ on day 3; $F[1,2] = 1.8$, $p = 0.31$ on day 4 and $F[1,2] = 111.5$, $p < 0.01$ on day 5. No treatment effect was observed on contralateral TWLmin on any day of the study.

0.05 mg/kg NTX: Group 2 and group 4

GLM analysis indicated a significant effect of carrageenan treatment (relative to saline treatment) on ipsilateral TWLmin on days 1,2, 4 and 5 but not on days 3 and 4.($F[1,2] = 81.8$, $p < 0.05$ on day 1; $F[1,2] = 39.0$, $p < 0.05$ on day 2; $F[1,2] = 0.4$, $p = 0.60$ on day 3;


A
PCmax (mm)
37
35
33
31
29
27
25
0
1
2
3
4
5
Day
carrageenan
carrageenan/ 0.5 mg/kg NTX
carrageenan/ 0.05 mg/kg NTX
saline
saline / 0.5 mg/kg NTX
saline / 0.05 mg/kg NTX


B
TWLmin (s)
12
11
10
9
8
7
6
0
1
2
3
4
5
Day
carrageenan
carrageenan/ 0.5 mg/kg NTX
carrageenan/ 0.05 mg/kg NTX
saline
saline/ 0.5 mg/kg NTXi
saline/ 0.05 mg/kg NTX

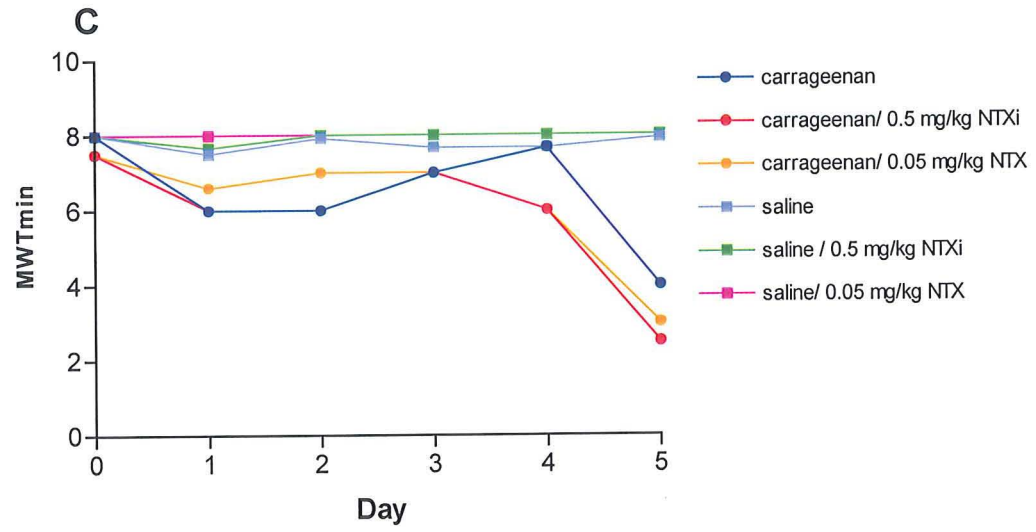

C
MWTmin
10
8
6
4
2
0
0
1
2
3
4
5
Day
carrageenan
carrageenan/ 0.5 mg/kg NTXi
carrageenan/ 0.05 mg/kg NTX
saline
saline / 0.5 mg/kg NTXi
saline/ 0.05 mg/kg NTX

Table 2.13

Mean ± SEM minimal thermal withdrawal latency (TWLmin) recorded following daily intraplantar injection of 50 µl, 0.1% carrageenan (n = 6/group) or 50 µl, 0.9% saline(n = 3/group) accompanied by systemic naltrexone (NTX) treatment on days 3,4 and 5.

0.1% carrageenan Day	**Group 1 0.5mg/kg NTX**		**Group 2 0.05mg/kg NTX**	
	contralateral	*ipsilateral*	*contralateral*	*ipsilateral*
1	10.1 ± 0.08	8.3 ± 0.20	10.2 ± 0.12	8.7 ± 0.27
2	10.3 ± 0.06	7.9 ±0.29	10.1 ± 0.10	8.4 ±0.14
3	10.1 ± 0.08	9.9 ± 0.14	10.1 ± 0.17	9.6 ± 0.10
4	10.0 ± 0 11	9.5 ± 0.10	10.0 ± 0.03	9.8 ± 0.16
5	10.2 ± 0.07	6.8 ± 0.40	10.0 ± 0.07	6.8 ± 0.26
saline Day	**Group 3 0.5mg/kg NTX**		**Group 4 0.05mg/kg NTX**	
	contralateral	*ipsilateral*	*contralateral*	*ipsilateral*
1	10.2 ± 0.13	10.3 ± 0.03	10.2 ± 0.16	10.2 ± 0.16
2	9.9 ± 0.27	10.1 ± 0.08	10.3 ±0.13	10.1 ± 0.07
3	10.1 ± 0.13	10.2 ± 0.12	10.1 ±0.11	9.9 ± 0.24
4	10.2 ± 0.17	10.1 ± 0.06	10.2 ±0.09	10.1 ± 0.19
5	10.0 ± 0.02	9.9 ± 0.04	10.2 ±0.08	9.7 ± 0.15

($F[1,2] = 39.8$, $p < 0.01$ on day 4 and ($F[1,2] = 31.3$, $p < 0.05$ on day 5. No treatment effect was observed on contralateral TWLmin on any day of the study.

Ipsilateral/contralateral effect

Baseline TWL

In all treatment groups, there was no difference between ipsilateral and contralateral baseline TWL values on any day of the study.

TWLmin

In saline-treated animals (Groups 3 and 4), ipsilateral TWLmin was not significantly different from contralateral TWLmin on any day of the study.

In group 1 (carrageenan/ 0.5 mg/kg NTX-treated) animals, ipsilateral TWLmin was significantly lower than contralateral TWLmin on days 1,2 and 5 of the study ($F[1,5] = 46.6$, $p < 0.001$ on day 1; ($F[1,5] = 127.0$, $p < 0.001$ on day 2; and ($F[1,5] = 155.9$, $p < 0.001$ on day 5. Ipsilateral TWLmin was not significantly different from contralateral TWLmin on day 3 ($F[1,5] = 4.6$, $p = 0.08$ or on day 4 ($F[1,5] = 1.7$, $p = 0.25$).

In group 2 (carrageenan/ 0.05 mg/kg NTX-treated) animals, ipsilateral TWLmin was significantly lower than contralateral TWLmin on days 1,2, 4 and 5 of the study ($F[1,5] = 150.9$, $p < 0.001$ on day 1; ($F[1,5] = 79.4$, $p < 0.001$ on day 2; ($F[1,5] = 18.3$, $p < 0.01$ on day 4 and ($F[1,5] = 67.8$, $p < 0.001$ on day 5. Ipsilateral TWLmin was not significantly different from contralateral TWLmin on day 3 ($F[1,5] = 3.3$, $p = 0.13$).

Effect of repeated treatment

In group 1 animals, there was a significant effect of repeated treatment on ipsilateral TWLmin [$F(4,25)=24.23$, $p < 0.001$]; more detailed analysis indicated that TWLmin on day 5 was significantly lower than all on other days of the study ($p < 0.001$); ipsilateral TWLmin on day 3 was significantly greater than that recorded on days 1 ($p < 0.01$), 2 and 5 (both $p<0.001$). Ipsilateral TWLmin on day 4 was also significantly greater than that recorded on days 1 ($p < 0.05$), 2 ($p <0.01$) and 5 ($p <0.001$).

In group 2 animals, there was also a significant effect of repeated daily treatment on ipsilateral TWLmin [$F(4,29) = 35.07$ $p < 0.001$]; more detailed analysis indicated that TWLmin on day 5 was significantly lower than all other days of the study ($p < 0.001$). Ipsilateral TWLmin on day 3 was significantly greater than that recorded on days 1 ($p < 0.05$), 2($p < 0.001$) and 5 ($p < 0.001$). Ipsilateral TWLmin on day 4 was also significantly greater than that recorded on days 1 ($p < 0.01$), 2 ($p < 0.001$) and 5 ($p < 0.001$).

Mechanical withdrawal threshold (MWTmin)

Baseline (pre-treatment) ipsilateral MWT was 8 (8,8) in group 1 (Cx-treated) animals, 8 (7,8) in group 3 (Cx-treated) animals, 8 (8,8) in group 2 (saline-treated) animals and 8 (7,8) in saline-treated animals. MWTmin recorded in study 2.3(B) is shown in Figure 2.12 (C) and Table 2.14.

Treatment effect

0.5 mg/kg NTX treatment (Groups 1 and 3): In group 1 animals, ipsilateral MWTmin was significantly lower than that of saline-treated animals on all 5 days of the study, ($p < 0.01$ on all days relative to both group 3 and group 4).

0.05 mg/kg NTX treatment (Groups 2 and 4): In group 2 animals, ipsilateral MWTmin was significantly lower than that of saline-treated animals on all 5 days of the study [day 1, $p < 0.001$; day 2, $p < 0.001$; day 3, $p < 0.05$; day 4, $p < 0.01$; day 5, $p < 0.001$].

Ipsilateral MWTmin in group 1 animals was not significantly different from group 2 animals on any day of the study.

Ipsilateral MWTmin in group 2 animals was not significantly different from group 4 animals on any day of the study.

Ipsilateral/ contralateral effect

In saline-treated animals (groups 3 and 4), there was no significant difference between ipsilateral and contralateral MWT min on any day of the study.

In group 1 animals, ipsilateral MWTmin was significantly lower than contralateral MWTmin on days 2 ($p<0.05$) and 5 ($p < 0.05$), while on days 1 ($p < 0.06$) and day 4 ($p < 0.06$), the score difference lay just outside accepted significance levels. On day 3 there was no significant difference between ipsilateral and contralateral MWTmin ($p=0.18$).

In group 2 animals, ipsilateral MWTmin was significantly lower than contralateral MWTmin on days 1 ($p < 0.05$), day 2 ($p < 0.05$), day 4 ($p < 0.05$) and day 5 ($p < 0.05$) but not on day 3 ($p = 0.10$).

Table 2.14

Median (with semi-interquartile ranges) minimum mechanical withdrawal threshold (MWTmin) recorded following daily intraplantar injection of 50 µl, 0.1% carrageenan (n=6/group) or 50 µl, 0.9% saline(n = 3/group) accompanied by systemic naltrexone (NTX) treatment on days 3, 4 and 5.

0.1% carrageenan Day	**Group 1 0.5mg/kg NTX**		**Group 2 0.05mg/kg NTX**	
	contralateral	***ipsilateral***	***contralateral***	***ipsilateral***
1	8 (7.75,8)	6.5 (5,7.25)	8(8,8)	6.5 (5.5, 7)
2	8 (7.75,8)	6 (5,7)	8(8,8)	7 (5.65, 7)
3	8 (7.75,8)	7 (5.75, 8)	8(7.75,8)	7 (6,8)
4	8 (7.75,8)	6 (5, 6.5)	8(8,8)	6 (4.75, 6.25)
5	8 (7.75,8)	2.5 (1,3)	8(8,8)	3(2.75, 3.25)
saline Day	**Group 3 0.5mg/kg NTX**		**Group 4 0.05mg/kg NTX**	
	contralateral	*ipsilateral*	*contralateral*	*ipsilateral*
1	7.67 (7,8)	7.67 (7,8)	8 (8,8)	8 (8,8)
2	8 (8,8)	8 (8,8)	8 (8,8)	8 (8,8)
3	8 (8,8)	8 (8,8)	8 (8,8)	8 (8,8)
4	8 (8,8)	8 (8,8)	8 (8,8)	8 (8,8)
5	8 (8,8)	8 (8,8)	8 (8,8)	8 (8,8)

2.3.8. STUDY 2.3(C): Preliminary investigations into the effects of the α-2 adrenoreceptor antagonist, atipamezole, on inflammatory and nociceptive responses to daily repeated carrageenan treatment

Paw circumference

Baseline (pre-treatment) ipsilateral PC was 29.0 ± 0.3 mm in Cx-treated animals and 29.2 ± 0.4 mm in saline-treated animals. PCmax recorded in study 2.3 (C) is shown in Figure 2.13 (A)

Treatment effect

Ipsilateral PCmax in Cx-treated animals was significantly greater than that of saline-treated animals on each day of the study ($F[1,5] = 20.9$, $p < 0.01$ on day 1; ($F[1,5] = 122.5$, $p < 0.001$ on day 2; ($F[1,5] = 48.1$, $p < 0.01$ on day 3; ($F[1,5] = 35.1$, $p < 0.01$ on day 4 and ($F[1,5] = 33.6$, $p < 0.01$ on day 5).

Effect of repeated treatment

There was no effect of repeated injection on ipsilateral pre-treatment PC or PCmax in saline-treated animals.

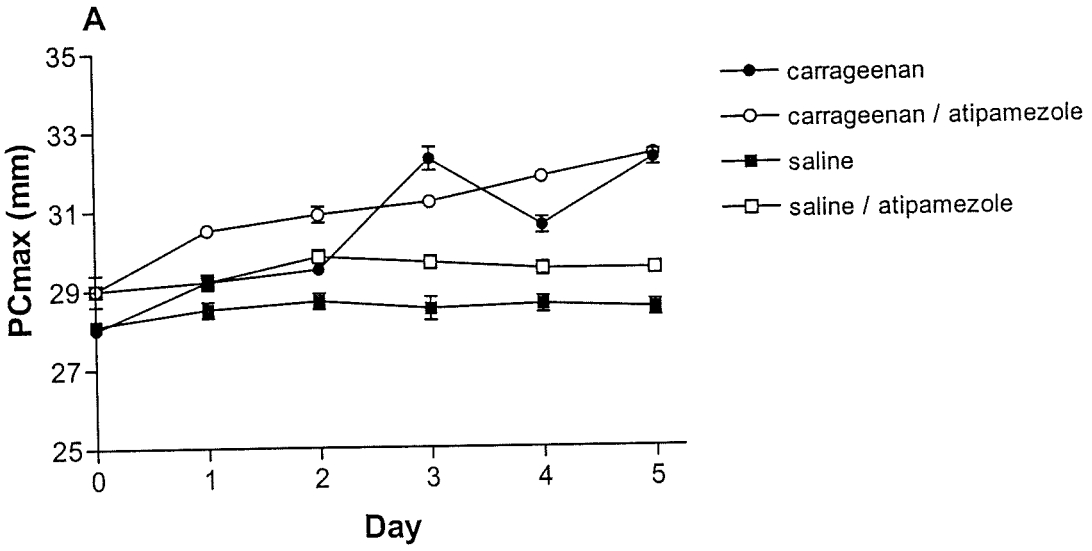
In carrageenan-treated animals, there was no effect of repeated treatment on baseline pre-treatment PC. However, PCmax increased significantly over the course of the study ($F[4,20] = 20.6$, $p < 0.001$). Further analysis showed that PCmax on day 1 was significantly lower than that on days 3 ($p < 0.05$), 4 ($p < 0.01$) and 5 ($p < 0.001$); PCmax on day 2 was significantly lower than that on days 4 ($p < 0.01$) and 5 ($p < 0.001$). PCmax on day 5 was significantly greater than that on days 1, 2 and 3 ($p < 0.001$). ΔPC (PCmax-baseline PC) did not alter significantly over the course of the study.

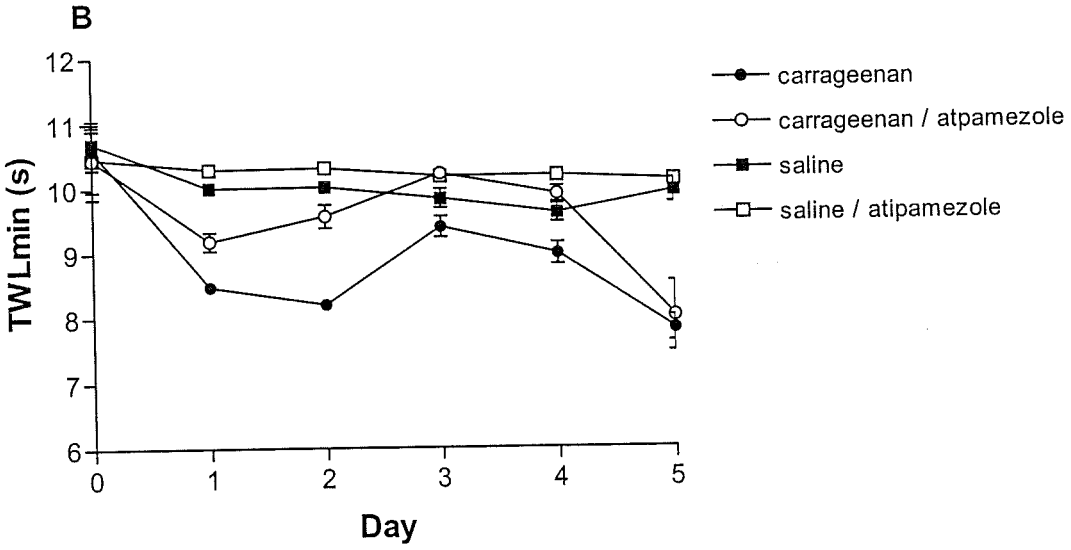
Thermal withdrawal latency (TWLmin)

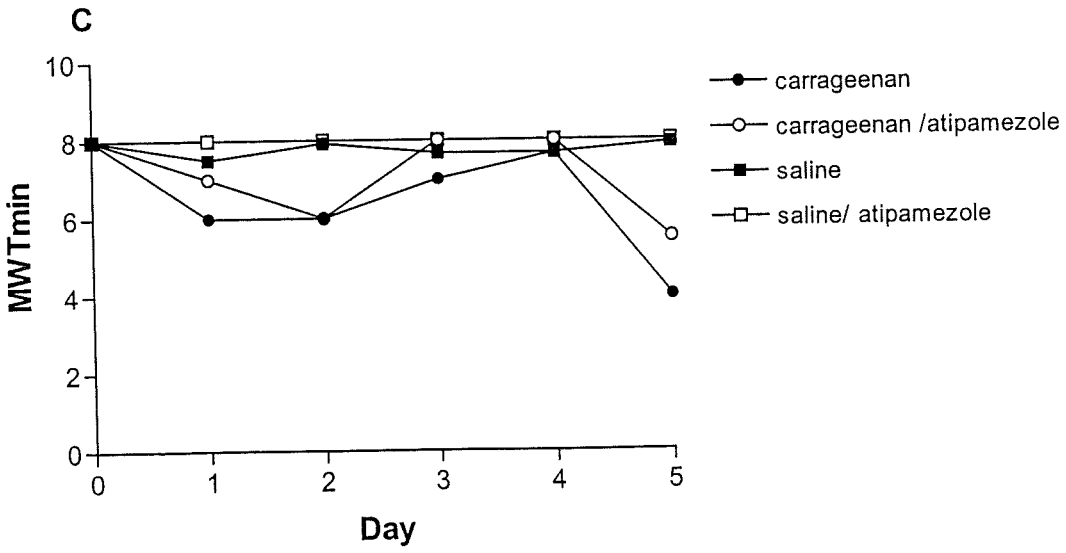
Baseline (pre-treatment) ipsilateral TWL was 10.5 ± 0.6 s in Cx-treated animals and 10.6 ± 0.5 s in saline-treated animals. TWLmin recorded in study 2.3 (C) is shown in Figure 2.13 (B) and Table 2.15.

Treatment effect

GLM analysis indicated a significant effect of carrageenan treatment (relative to saline treatment) on ipsilateral TWLmin on days 1, 2 and 5 but not on days 3 and 4 [($F[1,5] = 41.9$, $p < 0.001$ on day 1; ($F[1,5] = 20.2$, $p < 0.01$ on day 2; ($F[1,5] = 0.2$, $p = 0.67$ on day 3; ($F[1,5] = 5.29$, $p = 0.07$ on day 4 and ($F[1,5] = 14.3$, $p < 0.01$ on day 5]. Contralateral TWLmin did not change in response to carrageenan treatment (relative to saline treatment) on any day of the study.


A
PCmax (mm)
35
33
31
29
27
25
0
1
2
3
4
5
Day
carrageenan
carrageenan / atipamezole
saline
saline / atipamezole


B
TWLmin (s)
12
11
10
9
8
7
6
0
1
2
3
4
5
Day
carrageenan
carrageenan / atpamezole
saline
saline / atipamezole


C
MWTmin
10
8
6
4
2
0
0
1
2
3
4
5
Day
carrageenan
carrageenan /atipamezole
saline
saline/ atipamezole

Ipsilateral/contralateral effect

In saline-treated animals, ipsilateral TWLmin was not significantly different from contralateral TWLmin on any day of the study.

In carrageenan-treated animals, ipsilateral TWLmin was significantly lower than contralateral TWLmin on days 1, 2 and 5 of the study ($F[1,5] = 56.3$, $p < 0.001$ on day 1; ($F[1,5] = 26.4$ $p < 0.01$ on day 2; and ($F[1,5] = 14.9$, $p < 0.01$ on day 5. Ipsilateral TWLmin was not significantly different from contralateral TWLmin on day 3 ($F[1,5] = 3.2$, $p = 0.14$ or on day 4 ($F[1,5] = 3.3$, $p = 0.13$).

Effect of repeated treatment

In saline-treated animals there was a significant effect of repeated treatment on ipsilateral TWLmin ($F[4,29] = 10.35$, $p < 0.001$). TWLmin on day 5 was significantly lower than on all other treatment days ($p < 0.04$ compared with day 1, $p < 0.01$ compared with day 2, $p < 0.001$ compared with days 3 and 4). There was no effect of repeated treatment on ipsilateral TWLmin in carrageenan-treated animals.

Mechanical withdrawal threshold (MWT)

Baseline (pre-treatment) ipsilateral MWT was 8 (7,8) in Cx-treated animals and 8 (7,8) in saline-treated animals. MWTmin recorded in study 2.3 (C) is shown in Figure 2.13 (C) and Table 2.7

Treatment effect

Ipsilateral MWTmin in carrageenan-treated animals was significantly lower than that of saline-treated animals on days 1 ($p<0.05$), 2 ($p < 0.01$) and 5($p <0.01$), but not on days 3 and 4.

Ipsilateral/contralateral effect

In carrageenan-treated animals, ipsilateral MWTmin was significantly lower than contralateral MWTmin on days 1, 2 and 5 ($p < 0.05$). In saline-treated animals, ipsilateral MWTmin did not differ significantly from contralateral MWTmin on any day of the study.

Effect of repeated treatment

In carrageenan-treated animals there was a significant effect of repeated treatment on ipsilateral MWTmin (Friedman test, $p < 0.001$, adjusted for ties). MWTmin on day 3 was significantly higher than that measured on days 1, 2 and 5 ($p < 0.05$). MWTmin on day 4 was significantly higher than that measured on days 1, 2 and 5 ($p < 0.05$).

Table 2.15

Mean ± SEM minimal thermal withdrawal latency (TWLmin) and median (first and third interquartile) minimal mechanical withdrawal threshold (MWTmin) measured in ipsilateral and contralateral paws following repeated intraplantar injection of 0.1% carrageenan or 0.9% saline (50 µl) in combination with systemic treatment with atipamezole (n = 6/group).

	ATIPAMEZOLE			
Treatment	**carrageenan TWLmin**		**saline TWLmin**	
Day	**contralateral**	**ipsilateral**	**contralateral**	**ipsilateral**
1	10.18 ± 0.07	9.18 ± 0.14	10.29 ±0.04	10.29 ± 0.06
2	10.22 ± 0.08	9.56 ± 0.18	10.09 ±0.06	10.3 ±0.09
3	9.93 ± 0.13	10.21 ± 0.05	10.29 ±0.09	10.17 ± 0.09
4	10.21 ± 0.13	9.89 ± 0.12	10.12 ±0.08	10.18 ± 0.05
5	10.06 ± 0.02	8.00 ± 0.54	10.21 ±0.04	10.1 ± 0.06
Treatment	**carrageenan MWTmin**		**saline MWTmin**	
Day	**contralateral**	**ipsilateral**	**contralateral**	**ipsilateral**
1	8(8,8)	7(6.75,7)	8(8,8)	8(7,8)
2	8(8,8)	6(6,6.25)	8(8,8)	8(7,8)
3	8(8,8)	8(7.75,8)	8(8,8)	8(8,8)
4	8(8,8)	8(7,8)	8(8,8)	8(8,8)
5	8(8,8)	5.5(4.75,6)	8(8,8)	8(8,8)

2.3.9: Comparison of nociceptive responses in study 2.3 with those of study 2.2

Thermal withdrawal latency

Study 2.3(A): Comparison of the effect of memantine treatment on TWLmin responses on days 3,4 and 5 with those responses observed in study 2.2

Ipsilateral TWLmin recorded in memantine/carrageenan-treated animals in study 2.3 (A) was significantly higher than that recorded in carrageenan-treated animals in study 2.2 on all 5 days ($p < 0.001$ on days 1,2,4 and 5; $p < 0.05$ on day 3). Contralateral TWLmin recorded memantine/carrageenan-treated animals in study 2.3(A) was also significantly higher than that recorded in carrageenan-treated animals in study 2.2 on days 1-4 ($p < 0.01$ on days 1 and 4; $p < 0.05$ on days 2 and 3), but not on day 5.

Study 2.3(B): Comparison of the effect of 0.5 mg/kg NTX treatment on TWLmin responses on days 3,4 and 5 with those responses observed in study 2.2

Ipsilateral TWLmin recorded in group 1 animals in study 2.3(B) did not differ significantly from that recorded in carrageenan-treated animals in study 2.2 on days 1, 2, 3 or 5. However, TWLmin recorded in carrageenan/0.5 mg/kg NTX animals on day 4 was significantly lower than that recorded in animals in study 2.2 which received carrageenan treatment alone. Contralateral TWLmin in group 1 animals in study 2.3(B) was also significantly lower than that recorded in animals in study 2.2 on day 4, but not on any other day.

Study 2.3 (B): Comparison of the effect of 0.05 mg/kg NTX treatment on TWLmin responses on days 3,4 and 5 in comparison with those responses observed in study 2.2

Ipsilateral TWLmin recorded in group 2 animals in study 2.3(B) did not differ significantly from that recorded in carrageenan-treated animals in study 2.2 on days 1 or 2, but was significantly lower than that recorded in animals in study 2.2 which received carrageenan treatment alone on days 3,4 and 5. Contralateral TWLmin in group 2 animals in study 2.3(B) was also significantly lower than that recorded in animals in study 2.2 on days 2 and 4 .

Study 2.3 (C): Comparison of the effect of atipamezole treatment on TWLmin responses on days 3,4 and 5 in comparison with study 2.2

Ipsilateral TWLmin recorded in atipamezolee/carrageenan-treated animals in study 2.3(A) was significantly higher than that recorded in carrageenan-treated animals in study 2.2 on days 1-4 ($p < 0.001$ on each day), but not on day 5. Contralateral TWLmin recorded

atipamezole/carrageenan-treated animals in study 2.3 (A) was also significantly higher than that recorded in carrageenan-treated animals in study 2.2 on day 2 ($p < 0.05$) and day 4 ($p < 0.01$), but not on days 1,3 and 5.

Mechanical withdrawal threshold

Study 2.3(A): Comparison of the effect of memantine treatment on MWTmin responses on days 3, 4 and 5 with those responses observed in study 2.2

Mann-Whitney analysis indicated no significant difference between MWTmin recorded in carrageenan-treated animals in study 2.3(A) and study 2.2 on days 1-4 of repeated carrageenan treatment. On day 5, MWTmin in memantine/carrageenan treatment animals [6 (6, 7.25)] was significantly different ($p < 0.05$) from that recorded in animals which received carrageenan treatment alone [4.5 (4.25, 6)].

Study 2.3(B): Comparison of the effect of 0.5 mg/kg NTX treatment on MWTmin responses on days 3,4 and 5 with those responses observed in study 2.2

Mann-Whitney analysis indicated no significant difference between MWTmin recorded in carrageenan-treated animals in study 2.3(B) and study 2.2 on days 1-3 of repeated carrageenan treatment. On day 4, MWTmin in 0.5 mg/kg NTX-treated animals [6 (5,6.5)] was significantly different ($p < 0.05$) from that recorded in animals which received carrageenan treatment alone [7 (7,8)]. On day 5, MWTmin in 0.5 mg/kg NTX-treated animals [2.5 (1,3)] was significantly different ($p < 0.05$) from that recorded in animals which received carrageenan treatment alone [4.5 (4.25, 6)].

Study 2.3(B): Comparison of the effect of 0.05 mg/kg NTX treatment on MWTmin responses on days 3,4 and 5 with those responses observed in study 2.2

Mann-Whitney analysis indicated no significant difference between MWTmin recorded in carrageenan-treated animals in study 2.3 (B) and study 2.2 on days 1-3 of repeated carrageenan treatment. On day 4, MWTmin in 0.05 mg/kg NTX-treated animals [6(4.75, 6.25)] was significantly different ($p < 0.05$) from that recorded in animals which received carrageenan treatment alone [7 (7,8)]. On day 5, MWTmin in 0.05 mg/kg NTX-treated animals [3 (2.75, 3.25)] was significantly different ($p < 0.05$) from that recorded in animals which received carrageenan treatment alone [4.5 (4.25, 6)].

Study 2.3(C): Comparison of the effect of atipamezole treatment on MWTmin responses on days 3,4 and 5 in comparison with study 2.2

Mann-Whitney analysis indicated that MWTmin scores recorded in carrageenan-treated animals in study 2.3(A) were significantly different from those recorded in study 2.2 on days 1-4 but not on day 5. MWT scores were consistently higher in study 2.3(C) than in study 2.2, although by a very small factor.

2.4 DISCUSSION

2.4.1 Selection and characterisation of a suitable inflammatory agent

Pilot studies were used to select and characterise an appropriate inflammatory agent, which would permit investigation of behavioural responses to repeated mild acute inflammatory injury. Behavioural responses to low doses of carrageenan and complete Freund's adjuvant were assessed since these are the most common agents used in the investigation of pain responses associated with intraplantar inflammatory injury. Pilot studies indicated that carrageenan was a more suitable inflammatory agent than CFA for use in the investigation of nociceptive behavioural plasticity associated with mild inflammatory injury. In pilot study 1, while the inflammatory response (Δ PC) to low-dose (12.5%) CFA was not significantly different from that induced by saline treatment, 50% CFA induced an inflammatory response which persisted for at least 24 h p.i. suggesting that 50% CFA treatment was likely to produce an inflammatory response of similar persistence to that induced by treatment with 100% CFA (Colpaert, 1987). Pilot studies 1, 2 and 3 indicated that 0.6%, 0.5% and 0.1% carrageenan induced a significant inflammatory response (Δ PC) and hyperalgesic behaviour (Δ TWL, ΔMWT) relative to saline treatment, which was maximal between 2h p.i. and 6 h p.i. Following 0.6% carrageenan treatment, intraplantar inflammation and hyperalgesia was still present, but diminished, at 24 h p.i. Following 0.1% Cx treatment, residual intraplantar inflammation and hyperalgesia were no longer present at 24 h p.i. These observations thus showed that 0.5% and 0.1% carrageenan provided an inflammatory stimulus which was mild in nature relative to the chemical inflammatory stimuli standardly used to induce intraplantar inflammation for the purpose of investigations into inflammatory pain. Responses to 0.5% and 0.1% carrageenan were nonetheless significant, of predictable relatively short duration, and thus suitable for use in subsequent investigations into nociceptive responses induced by mild repetitive inflammatory injury. For subsequent studies, inflammatory and behavioural responses were recorded at 6h p.i. since the maximal inflammatory response (Δ PC) occurred at this

time point, and this was consistently accompanied by thermal and mechanical hyperalgesia.

Considerable variation in the time course of thermal and mechanical hyperalgesia associated with carrageenan treatment was observed in pilot studies. Tail flick latency was rejected as a method for assessing nociceptive thresholds due to the wide variation in response thresholds observed in pilot study 1. Baseline thermal withdrawal latency in pilot study 2, using a modified Hargreaves' method, was approximately 20 s. Hargreaves *et al.* (1988) reported a baseline TWL of 9.7-10.0 s following exposure to radiant heat, and baseline TWL of approximately 10 s is standardly reported by other studies which have used the Hargeaves model (e.g. Tabo & Eisele, 1998; Dirig *et al.,* 1998). Subsequently, the intensity of the radiant heat source was adjusted to produce a baseline TWL of approximately 10 s so that Δ TWL observed in these studies could be compared in a quantitative manner with previous studies that employed the method of Hargreaves *et al.* (1988).

2.4.2. Characterisation of hyperalgesia associated with low-dose carrageenan treatment

Behavioural responses to high-dose intraplantar carrageenan treatment (2-6 mg) in the rat have been characterised in detail (Vinegar *et al.,* 1962; Winter *et al.,* 1962; 1963; Castro-Lopez *et al.,* 1994; Ichitani *et al.,* 1997; Buritova & Besson 1998; Dirig *et al.,* 1998; Honore *et al.,* 1998; Kayser *et al.,* 1998; Pertovaara *et al.* 1998; Tsuruoka *et al.,* 1998; Perrot *et al.,* 1999a,b). Few studies, however, have investigated behavioural responses to low dose carrageenan stimulation.

Single administrations of 0.5% (0.25 mg) and 0.1% (0.05 mg) Cx produced a consistent inflammatory reaction accompanied by consistent mechanical and thermal hyperalgesia. The thermal hyperalgesia induced by a single administration of 0.25 mg Cx was similar quantitatively to that previously reported following higher doses of carrageenan (Dirig *et al.,* 1998; Tabo *et al.,* 1998). Administration of 0.25 mg Cx induced a mean reduction of 46% (relative to control values) in TWL, while treatment with 0.05 mg Cx induced a mean reduction of 24%. Tabo *et al.* (1998) reported a reduction of 48-54% in TWL following treatment with 1mg Cx, while Dirig *et al.* (1998) reported a reduction of 61% in TWL following injection of 4mg Cx. Tabo *et al.* (1998) also reported a significant reduction in TWL of 16% (relative to control values) following a single dose of 0.1 mg (100μl of 0.1%) carrageenan, observing that this dose did not induce any alteration in posture or weight-bearing in the ipsilateral limb.

While methods used to analyse MWT data in study 2.1 indicated a significant reduction in MWT induced by both 0.25 mg and 0.05 mg Cx administered on a single occasion, the categorical nature of these data (which thus required non-parametric statistical analysis) does not permit direct quantitative comparison with data analysed using parametric methods of statistical analysis. A reduction in MWT to 18-34% of baseline values following injection of 1mg carrageenan, and to 41.8% of baseline values following injection of 0.1mg carrageenan, was reported previously by Tabo *et al.* (1998). In study 2.1, 0.25 mg Cx induced a dramatic reduction in MWT from a score of 8 (8,9) to 4 (3,5) while 0.05mg Cx induced a less intense but consistent reduction in MWT from 8 (8,9) to 6 (4, 7). Thus strong and 'sub-maximal' thermal and mechanical hyperalgesic responses to intraplantar carrageenan treatment were determined in study 2.1

Plasticity of nociceptive transmission associated with inflammatory injury is well recognised, and it has been established that the intensity of injury critically influences the ultimate conscious sensation of pain. Nociceptive reponses ultimately result from an interaction between ascending 'excitatory' nociceptive transmission and descending or locally mediated 'anti-nociceptive' inhibitory transmission; inflammatory injury activates both 'pro' nociceptive/hyperalgesic and 'anti' nociceptive/hyperalgesic mechanisms at every level of the nociceptive transmission system. Identification of the mechanisms responsible for the transformation of nociceptive transmission pathways from 'physiological' to 'pathophysiological' requires proper appreciation of the interaction between pro-nociceptive/hyperalgesic and anti-nociceptive/hyperalgesic pathways. However, research into plasticity of nociceptive processing associated with inflammatory injury has tended to focus on peripheral and central sensitisation associated with high-intensity models of inflammatory injury. Such models provide the means to investigate excitatory pathways associated with the induction of central sensitisation of nociceptive processing, but may not permit investigation of complementary compensatory 'anti-hyperalgesic' (inhibitory) pathways induced as an adaptive response to injury, since any adaptive 'anti-hyperalgesic' response is likely to be over-ridden by the intensity of the inflammatory stimulus.

While few studies have investigated the effects of differing intensities of carrageenan-induced inflammatory injury, studies using the formalin model have highlighted the intrinsic limitations of high-intensity models of inflammatory injury in investigations into central plasticity of nociceptive processing (Kaneko & Hammond, 1997, 1998; John *et al.*, 1998; Kaneko *et al.*, 2000). In a series of studies into the role of $GABA_A$ receptors in formalin-induced nociception in the rat using a range of doses of formalin from 0.25-2.5%,

Kaneko & Hammond (1997a; b; 1998) and John *et al.,* 1998) identified marked differences in $GABA_A$ receptor-mediated responses associated with incremental doses of formalin, administered by intraplantar injection. They observed that the effect of $GABA_A$ receptor blockade on pain responses (recorded as flinching behaviour) changed in direct association with the dose of formalin, speculating that the change in effect was associated with dose-dependent antagonism of tonic GABA-mediated inhibition of excitatory nociceptive transmission by inflammatory stimulation. There have been conflicting reports on the ability of high-dose formalin treatment to induce central sensitisation; while Xu *et al.* (1995) reported that a single injection of 5% formalin did not induce prolonged sensitisation to nociceptive input in spinalised rats, other reports have demonstrated the induction of central sensitisation by 2.5-5% formalin (Dickenson & Sullivan, 1987 (b); 1987 (c); Ji *et al.,* 1997; 1999). Kaneko & Hammond (1997) speculated that the inability to detect central sensitisation after high-dose formalin treatment under certain experimental conditions could reflect extensive release of inhibitory neurotransmitters (such as GABA) into the spinal cord leading to dominance of inhibition over excitation of dorsal horn neuronal responses.

It is highly likely that the central response to carrageenan treatment varies in a similar fashion to the central response to formalin treatment, highlighting the need for investigations into central plasticity of nociceptive processing following inflammatory injury to be performed over a range of stimulus intensities, if the interaction of excitatory and inhibitory pathways in the induction of central nociceptive plasticity is to be elucidated. In his review of the induction of pain, Millan (1999) suggested that the dominance of research into mechanisms underlying the induction of pain rather than the inhibition of pain reflected frustration with the development of novel analgesics based on the principle of reproducing endogenous mechanisms of antinociception. An improved understanding of endogenous anti-nociceptive and anti-hyperalgesic mechanisms may become possible through the exploitation of low-intensity models of inflammatory pain.

2.4.3. Characterisation of nociceptive plasticity associated with repeated low-dose carrageenan treatment

Few studies have investigated the consequences of repeated mild acute inflammatory stimulation, although repetitive stimulation with high doses of carrageenan has been reported previously (Kayser & Guilbaud 1987; Kayser *et al.,* 1998; Perrot *et al.,* 1999). In study 2.1, baseline (pre-treatment) nociceptive responses and hyperalgesic responses induced by carrageenan treatment (0.25 mg and 0.05 mg) were not clearly altered by weekly repeated treatment. In contrast, in study 2.2, while baseline nociceptive responses

were not altered by daily repeated treatment with low-dose carrageenan (0.05 mg), daily repeated treatment (0.05 mg Cx) induced tachyphylaxis of thermal and mechanical hyperalgesia. Significant attenuation of the maximal thermal hyperalgesic response (TWLmin) and of the maximal mechanical hyperalgesic response (MWTmin) to carrageenan treatment was observed on days 3 and 4, an effect which was abolished when the stimulus was repeated on day 5. TWL responses were significantly longer at various time points post-treatment (for up to 6 h p.i.) on days 3 and 4 relative to days 1, 2 and 5. This effect was limited to the ipsilateral foot and was absent in control animals that received daily repeated doses of saline, where no change in the magnitude of transient paw swelling or nociceptive behaviour occurred.

Tachyphylaxis of nociceptive processing associated with repeated low-dose carrageenan treatment has been reported previously in sheep (Welsh & Nolan, 1994) observed at a distant site (the ipsilateral ear) following repeated intradermal injection of 0.0625% carrageenan in the forelimb at a 14-day inter-treatment interval. However, in contrast to the tachyphylaxis of hyperalgesic responses observed in study 2.2 and reported by Welsh & Nolan (1994), there have been numerous reports of enhanced nociceptive transmission following repeated carrageenan treatment. Ferreira *et al.* (1978) reported that while unilateral hyperalgesia developed following an isolated carrageenan treatment, a subsequent carrageenan treatment into the contralateral paw after a 2 hour interval reduced the time to maximal mechanical hyperalgesia in both the ipsilateral and contralateral paws, suggesting that this change was centrally mediated. Repeated intraplantar administration of 2mg carrageenan at a 7 day interval induced sensitisation of nociceptive processing characterized by an enhanced inflammatory response and an enhanced bilateral behavioural response following the second treatment (Kayser *et al.,* 1998; Perrot *et al.,* 1999). Kayser *et al.* (1998) assessed pain behaviour at 15, 30, 60, 120 minutes and 7 days following carrageenan treatment, observing that repeated unilateral carrageenan injection induced bilateral enhancement of pain-related behaviour at all time points following the second treatment, while Perrot *et al.* (1999) reported bilateral enhancement of pain behaviour assessed at 3h following repeated carrageenan treatment. Moreover, while tachyphylaxis of nociceptive transmission was observed following daily administration of 0.05 mg Cx in study 2.2, daily repeated administration of saline (150 μl) over 4 days has been previously reported to induce a progressive increase in post-injection swelling, transient hyperalgesic behaviour (maximal at 3 hours and absent by 7 h p.i., nociceptive thresholds reduced by 18-22% using the Randall-Selitto test) which was bilateral (Levine *et al.,* 1985). Similarly, previous studies which investigated plasticity of nociceptive behaviour associated with repeated high dose carrageenan treatment reported enhanced

nociceptive processing following repeated treatment, which may extend beyond the site of injury.

These contrasting reports of both enhanced and attenuated nociceptive processing associated with differing frequency and intensity of inflammatory stimulation demonstrate the enormous inherent capacity for plasticity- both enhancement and attenuation - of central nociceptive transmission, critically dependent upon the nature, the magnitude and the frequency of inflammatory challenge.

The tachyphylaxis of nociceptive processing observed in study 2.2 may have been mediated through modulation of activity at any or all levels of the pain transmission system, from the periphery to the cerebral cortex. Melzack & Wall (1965), in their exposition of the theory of 'gate control', demonstrated that spinal nociceptive transmission is subject to modulation both segmentally (within the cord itself), and by supraspinal influences.

Modulation of peripheral nociceptive transmission

As was discussed in chapter 1, hyperalgesic behaviour is classically subdivided into a 'primary' component, associated with peripheral sensitisation of nociceptors (Meyer & Campbell, 1981), and a 'secondary' component, associated with modulation of central nociceptive transmission. Thus the tachyphylaxis observed in study 2.2 may have been mediated, at least in part, through modulation of peripheral nociceptor sensitivity. Peripheral sensitisation is mediated through multiple mechanisms (reviewed by Millan, 1999). These include:

Modulation of receptor activity: primary afferent nociceptors normally express a combination of excitatory and inhibitory receptors, including opioid receptors, GABA, vanilloid, histamine, bradykinin, neurokinin and excitatory amino acid receptors (reviewed by Dray, 1994). Injury modulates the activity of many of these receptors: for example, opioid receptors, normally ineffective in modulating normal activity in articular nociceptors, become effective following intense inflammatory stimulation (reviewed by Stein, 1994 and in chapter 1).

Modulation of receptor expression: for example, adrenoreceptors are expressed *de novo* following peripheral nerve injury (Sato & Perl, 1991, Bossut & Perl, 1995).

Modulation of neurotransmitter release and synergistic interaction of sensitising agents: In addition to modulation of receptor activity and expression, peripheral injury modulates the synthesis of a multitude of excitatory and inhibitory agents. Many of the sensitising

agents that excite nociceptor terminals following tissue injury synergise to enhance peripheral terminal sensitivity to other pro-nociceptive agents for example, prostaglandins sensitise peripheral nociceptor terminals to the actions of bradykinin, as well as other stimuli (reviewed by Dray & Urban, 1997 and Millan, 1999). Moreover, these agents subsequently alter the activity of key ion channels such as the TTX-resistant sodium channel, altering the intrinsic excitability of the nociceptor or activate intracellular mediators such as protein kinase enzymes (Gold *et al.,* 1996, Dray & Urban, 1996).

It is worthy of note that numerous endogenous peripheral anti-nociceptive mechanisms exist, the enhancement of which could attenuate hyperalgesia associated with inflammatory injury. Opioid peptides are synthesised in peripheral tissues [for example, β -endorphin is synthesised by immune cells (Lyons & Blalock, 1997)] and can inhibit nociceptive transmission through activation of receptors located on nociceptors and sympathetic terminals (Catheline *et al.,* 1997; Stein & Yassouridis, 1997). Disruption of the perineurial blood-nerve barrier in inflamed tissue, a potential consequence of repetitive inflammatory stimulation, could also improve access of circulating pools of opioids (and other agents) to peripheral nociceptor terminals (Antonijevic *et al.,* 1997; Coggeshall *et al.,* 1997).

While intense inflammatory injury modulates both excitatory and inhibitory receptors and systems to ultimately produce excitatory dominance resulting in hyperalgesic behaviour, it is possible that a less intense inflammatory stimulus could alter the balance of excitatory and inhibitory transmission to ultimately produce downregulation of hyperalgesia, through attenuation of excitatory dominance or enhancement of inhibition. It would be of great interest to evaluate all of the mechanisms through which peripheral nociceptor behaviour is modulated in a model of mild repetitive inflammatory injury. An understanding of physiological 'adaptation' of peripheral nociceptive responses to mild injury, could provide a strategy that could be exploited clinically to prevent the development of persistent pathophysiological hyperalgesia.

Modulation of central (spinal) nociceptive transmission

Changes in spinal nociceptive transmission following intense inflammatory injury that result in central sensitisation – reflecting enhanced dorsal horn neuronal excitability and leading to behavioural hyperalgesia were discussed in chapter 1. Pronociceptive and antinociceptive mechanisms induced in the spinal cord by inflammatory injury have been extensively investigated and reported (reviewed by Basbaum & Fields, 1984). However a fundamental deficiency persists in our understanding of the factors that transform the

nociceptive system from a 'physiological' state, in which pronociceptive and antinociceptive mechanisms are balanced, to a 'pathophysiological' state in which pro-nociceptive mechanisms become dominant, potentially for a prolonged period or even permanently.

Many parallels have been drawn between afferent stimulation-induced central sensitisation of dorsal horn neurons following inflammatory injury, and the electrophysiological phenomenon of long-term potentiation of dorsal horn neuronal responses [reviewed by Pockett, 1995 (b)]. Afferent stimulation-induced central sensitisation of dorsal horn neurons has been recognised since 1965, when Melzack and Wall demonstrated that peripheral afferent stimulation could also induce spinally mediated antinociception. They demonstrated that conditioning stimulation of large-diameter Aβ and Aα fibres could excite small interneurons in the superficial dorsal horn to exert inhibitory effects on presynaptic terminals of Aδ and C fibres, or could produce post-synaptic inhibition of spinal nociceptive projection neurons. On the basis of the gate control theory, transcutaneous electrical nerve stimulation (TENS) techniques were developed for the relief of acute and chronic pain. Electrical stimulation at an intensity and frequency which is selective for Aβ and Aα fibres (100 Hz, low intensity) produces analgesia or hypoalgesia that does not persist beyond the duration of stimulation (Johnson *et al.,* 1991). Conditioning stimulation at low-frequency, moderately painful intensity which is selective for Aδ fibres (1-10 Hz), administered repetitively, can produce a long-lasting or even permanent analgesic effect which is not explained by the gate-control theory (Thorsen & Lumsden, 1997). It has been proposed that this form of afferent-induced antinociception may represent long-term depression of excitatory nociceptive transmission in the spinal dorsal horn (reviewed by Sandkuhler, 2000).

LTD of spinal excitatory nociceptive transmission has been thoroughly characterised (Cerne *et al.,* 1991; Randic *et al.,* 1993; Pockett & Figurov, 1993; Pockett, 1995). However, while the efficacy of specific techniques of electrical stimulation for the treatment of acute or persistent pain (including painful TENS, electroacupuncture and certain forms of needle acupuncture) have been attributed to the induction of spinal LTD (reviewed by Sandkuhler), the factors which induce LTD, as opposed to LTP, of central spinal nociceptive transmission, have not been identified and no 'physiological' or adaptive role for spinal LTD has been proposed to date. The development of tachyphylaxis of central nociceptive transmission in response to repeated 0.1% carrageenan treatment could represent LTD of spinal nociceptive transmission in the dorsal

horn as an 'anti-hyperalgesic' adaptive response to mild repetitive injury. This hypothesis correlates well with observations of LTD in the hippocampus, which is similarly induced by milder patterns of stimulation than those which produce LTP of synaptic transmission (Malenka, 1993; Malenka & Nicoll, 1993; Pockett, 1995), and with indirect evidence of LTD of synaptic transmission induced by specific patterns of electrical stimulation which produce analgesia through the use of TENS techniques (Sandkuhler, 2000).

Existing knowledge about the molecular basis of plasticity of nociceptive transmission induced by inflammatory injury supports the potential for LTD-like attenuation of spinal nociception as a mechanisms for the tachyphylaxis of hyperalgesia observed in study 2.2. In addition to the enhancement of excitatory nociceptive transmission (via EAA's, tachykinins and prostaglandin systems amongst others), multiple 'antinociceptive' systems, including GABAergic, adrenergic and opioidergic transmission, are enhanced in the spinal cord in response to injury (reviewed by Millan, 1999 and in chapter 1). Analgesia induced by electrical conditioning stimuli (including electroacupuncture) is associated with enhanced release of endogenous opioids (Sjolund *et al.,* 1977, 1979). Recognising that spinal nociceptive transmission represents a complex balance of excitatory and inhibitory nociceptive transmission, each of which may be enhanced in response to inflammatory injury, it is clearly possible that, depending upon the nature and intensity of injury, inhibitory rather than excitatory neurotransmission may dominate. These complementary transmitter systems are interdependent and interconnected. For example, tonic NMDA receptor-mediated modulation of central opioid antinociceptive systems- or vice versa- has been reported previously (Dickenson, 1997; Mao, 1999). A more complete appreciation of the molecular basis of the transformation from excitatory towards inhibitory dominance, which developed in study 2.2 between days 2 and 3 and was maintained on day 4, but abolished on day 5, may help identify key molecular targets for therapeutic agents designed to combat or prevent post-injury hyperalgesia.

2.4.3.3. Modulation of supraspinal nociceptive transmission - modulation of descending facilitation and inhibition

Enhanced descending inhibition, or indeed, attenuated descending facilitation, may have contributed to the tachyphylaxis of hyperalgesic behaviour observed in study 2.2. Descending inhibitory pathways arise in a number of structures, including the periaqueductal grey matter, the loci coerulus and subcoerulus, the nucleus raphe magnus and several nuclei of the bulbar reticular formation. The cerebral cortex and various

limbic structures, including the hypothalamus, contribute to descending modulation (reviewed by Willis & Westlund, 1997, and in chapter 1). Many studies have suggested that inflammation induces transient or sustained activation of descending inhibitory pathways, which dampen down the activity of sensitised projection neurons in the dorsal horn (Basbaum & Fields, 1984; Cervero & Laird, 1996a,b; Ren & Dubner, 1996; Sandkuhler, 1996; Traub, 1997). It is believed that descending inhibitory pathways operate through direct inhibition of peripheral neurons, suppression of neurotransmitter release from nociceptor terminals, and engagement of inhibitory interneurons (Reviewed by Millan, 1999). Descending inhibitory pathways use several different neurotransmitter systems, including opioids (Millan, 1993), catecholamines (Millan, 1997), serotonin (Millan, 1997) and GABA (Hammond, 1997). Plasticity in all of these pathways has been demonstrated following intense inflammatory stimulation (reviewed in chapter 1). Certain of these pathways (notably adrenergic but not opioidergic) exert tonic inhibitory influence on nociception; for example, via activation of α_2 adrenoreceptors located in the dorsal horn (Schaible *et al.,* 1991).

Attenuation of descending facilitatory pathways could also have contributed to the attenuation of hyperalgesia. It has been suggested that descending facilitation originates in the rostro-medial medulla (Wiertelak, 1994 (a), (b), (c), (d); Watkins *et al.,* 1994) and in the anterior pretectal area of the brainstem (Rees & Roberts, 1993; Rees *et al.,* 1995). Descending facilitatory mechanisms contribute to the induction of dorsal horn sensitisation and hyperalgesia (Pertovaara *et al.,* 1996; Mansikkka & Pertovaara, 1997), through the recruitment of nitric oxide and NMDA receptors in the dorsal horn (Watkins *et al.,* 1994; 1995; Wiertelak *et al.,* 1994 (a), (b), (c), (d)). While agents associated with engagement of these facilitatory pathways have been identified, the mechanisms which control their activation are not well appreciated. Several agents, including the 'anti-opioidergic' agents neuropeptide FF and cholecystokinin, have been implicated in the engagement of descending facilitation (reviewed by Millan, 1999). It has been proposed that GABA mediates tonic inhibition of descending facilitatory pathways (Hammond, 1997).

In higher centres as elsewhere in the nociceptive system, the ultimate dominance of pro-nociceptive, or anti-nociceptive, transmission depends upon the balance of interacting excitatory and inhibitory pathways. Enhancement of descending inhibitory dominance induced by mild repetitive inflammatory injury may represent a physiological adaptive mechanism which prevents the development of maladaptive hyperalgesia.

'Stress-induced analgesia' represents a manifestation of supraspinal descending modulation of spinal nociceptive transmission mediated through opioidergic and non-

opioidergic mechanisms (reviewed in chapter 1)- essentially an 'adaptive response'of the nociceptive transmission system to potential continuous or repetitive insult. Since 'stress' has been defined as anything related to an alteration in psychological homeostatic processes (Burchfield, 1979), the repetitive nature of inflammatory stimulation employed in study 2.2 provided ample potential for stimulation of conditioned psychological or fear-induced endogenous hypoalgesic behaviour. However, while 'stress-induced' analgesic responses could account for the tachyphylaxis of hyperalgesia observed on days 3 and 4 of study 2.2, they do not explain the return of marked hyperalgesia on day 5 in association with repetitive inflammatory stimulation of constant magnitude.

2.4.4. Preliminary investigations into the pharmacology of nociceptive plasticity induced by repetitive mild inflammatory stimulation

2.4.4.1. Study Design

Since a host of peripheral and central mechanisms, mediated through the modulation or excitatory and/or inhibitory neurotransmission, may have been implicated in tachyphylaxis of hyperalgesic behaviour, preliminary studies were undertaken to identify candidate mechanisms likely to contribute to the observed nociceptive plasticity. It was considered of interest in the first instance to identify the existence and relative importance of 'excitatory' and 'inhibitory' modulation to the observed nociceptive plasticity. Excitatory nociceptive transmission in the central nervous system is driven predominantly by neuropeptides (including substance P, NKA, CGRP and NPY); excitatory amino acids (EAA's), and prostaglandins. The role of the NMDA receptor was of particular interest due to the key role of this receptor in both the induction of synaptic plasticity in the central nervous system and the induction of post-inflammatory central sensitisation of nociceptive processing in the dorsal horn (reviewed by Dickenson, 1990; Coderre & Yaksh, 1996; and in Chapter 1). Many inhibitory transmitter systems contribute to spinal and supraspinal modulation of nociceptive transmission, including GABA, endogenous opioids and adrenergic pathways. The roles of opioid and α_2 adrenoreceptor-mediated plasticity of nociceptive processing were of particular interest in study 2.3, since these are key transmitter systems implicated in both segmental spinal and supraspinal descending inhibition, and their activity is modulated by inflammatory injury. Moreover, the NMDA receptor, opioid and α_2 adrenoreceptors are clinically effective anti-hyperalgesic and analgesic therapeutic agents (reviewed by Millan, 1999).

NMDA receptors, opioid receptors and α_2 adrenoreceptors are expressed throughout the peripheral and nervous system and in non-neuronal sites (discussed in chapter 1). Specific evaluation of the central effects of NMDA, opioid and α_2 adrenoreceptor antagonism can thus only be achieved through local (intrathecal) administration of appropriate antagonists. While providing an invaluable means of assessing spinal activity of specific agents, intrathecal drug delivery was not considered to be appropriate for the purpose of a preliminary screening study. Intrathecal drug delivery may fundamentally alter central mechanisms of nociceptive plasticity through the necessity for spinal surgery in small rodents to enable insertion of intrathecal catheters.

Receptor antagonists were selected which would achieve effective active concentrations within the central nervous system following subcutaneous administration. Specific antagonists were not administered on days 1 and 2 in order to allow the induction of behavioural plasticity, since tachyphylaxis of hyperalgesia developed only on days 3 and 4 of repetitive low-dose carrageenan administration. Antagonists were also administered on day 5 in order to evaluate the role of each receptor system in the restoration of hyperalgesia.

2.4.4.2. Study 2.3(A)

Selection of an NMDA receptor antagonist

Since glutamate is the principal and ubiquitous excitatory neurotransmitter in the central nervous system, NMDA receptor antagonism is likely to produce depression or modulation of general sensory transmission (reviewed by Eide, 1999). However, the role of NMDA in nociceptive plasticity is primarily due to its induction of plasticity of central excitatory nociceptive transmission (reviewed in chapter 1). The psychomimetic and psychomotor side-effects of NMDA receptor antagonists in humans are well documented (reviewed by Olney & Farber, 1995). Thus, in order to study the role of NMDA receptors in the behavioural plasticity observed in study 2.2, an agent was required which would effectively achieve central spinal NMDA blockade while avoiding side-effects which would distort accurate assessment of hyperalgesic behaviour.

Memantine is a non-competitive NMDA antagonist with a half-life of 4.6h following subcutaneous administration in the rat (Parsons *et al.*, 1993). Due to its moderate potency, rapid channel blocking/unblocking kinetics and strong voltage dependency, memantine effectively blocks NMDA receptors with minimal adverse psychomimetic and psychomotor effects (reviewed by Parsons *et al.*, 1999). Reports of memantine antagonism of 'physiological' nociceptive transmission, as opposed to hyperalgesia,

remain somewhat inconclusive. Memantine has been reported to attenuate spinal neuronal responses to noxious (but not innocuous) peripheral stimulation (Neugebauer *et al.,* 1993). Olivar & Laird (1999) reported that memantine (ID50 = 17± 12 mg/kg) inhibits responses to pinch stimuli in anaesthetised rats. However, a study on human subjects reported that memantine administration (30 mg p.o.) did not alter flexor reflex thresholds or magnitude, pain and tolerance thresholds (Schepelmann, *et al.,* 1998).

Systemic memantine administration attenuates hyperalgesic behaviour associated with inflammatory injury. Neugebauer *et al.*(1993) and Eisenberg *et al.*(1994) reported that memantine (10-15 mg/kg) attenuated hyperalgesic behaviour in high-dose carrageenan models of peripheral inflammatory injury in rats. Eisenberg *et al.*(1993) reported that memantine (2.5-10 mg/kg delivered by intraperitoneal injection) attenuated pain behaviour induced by high-dose intraplantar formalin treatment. Systemic memantine administration also attenuates hyperalgesic behaviour associated with neuropathic injury; Carlton & Hargett (1995) and Eisenberg *et al.* (1995) reported that memantine blocked and reversed thermal hyperalgesia and mechanical allodynia without obvious effects on motor reflexes at doses of 10-20mg/kg in models of neuropathic pain. Side-effects in animals characteristic for NMDA receptor antagonists (including ataxia, myorelaxation and amnesia) only develop at higher doses of 20-30 mg/kg (Danysz *et al.,* 1994), reflecting the relatively low affinity of memantine for NMDA receptors relative to more potent NMDA antagonists such as MK-801, dextromethorphan and dextromethorphan (Chaplan *et al.,* 1997). Memantine thus fulfilled the specifications of an NMDA receptor antagonist which could be used to investigate the effect of NMDA receptor blockade on tachyphylaxis of hyperalgesic behaviour. Two doses of memantine were administered (20 mg/ kg at 0 h and 5 mg/ kg at 2 h p.i.) in order to maintain serum memantine at or above 10 mg/kg for the duration of behavioural testing, a dose which was chosen in order to achieve antagonism of central NMDA receptors without induction of adverse side-effects.

It is worthy of note that NMDA receptor activation has also been implicated in peripheral mechanisms of hyperalgesia. Intraplantar memantine administration has been shown to attenuate formalin-induced lifting and licking behaviour, without affecting central pain responses (Davidson & Carlton, 1998), suggesting that part of the antihyperalgesic effect of memantine may be achieved through blockade of NMDA receptors on peripheral cutaneous nociceptors.

Inflammatory and hyperalgesic responses associated with carrageenan/memantine treatment

Memantine administration did not alter baseline PC, TWL or MWT responses in saline-treated control animals on days 3, 4 and 5. Memantine administration did not alter contralateral TWL or MWT responses. Memantine administration did not alter the magnitude of the inflammatory response to repeated carrageenan treatment on days 3,4 and 5 relative to that observed in study 2.2 (Figure 2.11). In animals which were treated with carrageenan alone, PCmax was 32.3 ± 0.3 mm on day 3, 30.6 ± 0.2 mm on day 4 and 32.3 ± 0.2 mm on day 5. In memantine-carrageenan-treated animals, PCmax was 32.3 ± 0.3 mm on day 3, 30.6 ± 0.2 mm on day 4 and 32.3 ± 0.2 mm.

While the inflammatory response to daily repeated carrageenan treatment in study 2.3 (A) was not significantly different from that observed in study 2.2, significant thermal and mechanical hyperalgesia were not observed on days 1 and 2 in study 2.3 (A), so that the effect of memantine treatment on plasticity of nociceptive behaviour could not be assessed.

2.4.4.3. Study 2.3(B)

Selection of an opioid receptor antagonist

A host of highly selective agents have now been described which have been of great use in defining the pharmacology of the three major opioid receptor classes (reviewed by Pasternak, 1993). For example, β-funaltrexamine selectively antagonises μ-opioid receptors (Ward *et al.,* 1982), while naltrindole selectively blocks δ-opioid receptors (Abdelhamid *et al.,*1991) and nor-binaltorphinimine acts against κ receptors (Portoghese *et al.,* 1991). Other agents have been described that can further differentiate receptor subtypes (reviewed by Pasternak, 2000).

Study 2.3(B) was a preliminary study, which aimed to investigate the potential involvement of opioidergic pathways in the mediation of nociceptive plasticity observed following repeated carrageenan treatment. It was recognised that thorough characterisation of the role of the separate opioid receptors would require systematic administration of subtype-specific receptor antagonists, which was beyond the intended scope of a preliminary study. The three major opioid receptor types, μ, δ and κ, differ in their pharmacological effects on nociceptive transmission (reviewed by Dickenson, 1997a; Pasternak, 2000, and in chapter 1). It is well established that the opioidergic response to inflammatory injury depends critically upon the intensity and duration of injury, but the factors which regulate this plasticity remain undetermined and may differ between the different opioid receptor types (reviewed by Dickenson, 1997). μ- opioid receptor

activation consistently inhibits excitatory nociceptive transmission at peripheral, spinal and supraspinal sites (Pasternak, 2000). Similarly, δ receptor activation produces strong analgesic and anti-hyperalgesic effects through actions at both spinal (Heyman *et al., 1987;* Stewart & Hammond, 1993; 1994) and supraspinal sites (Ossipov *et al.,* 1995; Thosipov & Hammond, 1997; 1991). The effects of κ opioid receptor activation are less well defined, with both pro-and antinociceptive effects attributed to κ opioid receptor activation (reviewed in chapter 1). Moreover, κ opioid agonists have been shown to functionally antagonise the μ-receptor in the spinal cord (Stanfa *et al.,* 1992). It was therefore of interest to differentiate between the potentially antagonistic effects of μ- and κ receptor activation.

Naltrexone is a non-selective opioid receptor antagonist with a 10-fold preference for μ-receptors (Yoshimura *et al.,* 1982; Millan *et al.,* 1989), which has been characterised as a 'long-acting' opioid antagonist in the rat (Ragavan *et al.,* 1983; Zukin *et al.,* 1982), reaching peak serum levels at 1h p.i. following subcutaneous administration with an elimination half-life of 4.6h. (Yoburn *et al.,* 1986). Millan (1989) reported that naltrexone attenuated μ-receptor (morphine)-mediated antinociception by 50% at doses of 0.016 [0.009-0.029]mg/kg, while higher doses of 0.16 [0.062-0.422] mg/kg) attenuate κ receptor (U50488H) mediated responses by 50%. Naltrexone administration (1-3 mg/kg) has no effect on physiological nociceptive thresholds (Bienkowski *et al.,* 1999). NTX thus fulfilled the specifications of an opioid receptor antagonist suitable for investigation of potential opioidergic mediation of tachyphylaxis of hyperalgesic behaviour. NTX was suitable for administration by subcutaneous injection, while the relatively prolonged half-life of NTX in the rat ensured effective opioid receptor blockade for the duration of behavioural testing on each day of the study.

In the absence of data relating to specific NTX dose rates required for δ-opioid receptor blockade, it was considered that high dose NTX administration would produce general opioid receptor blockade. Two doses of NTX were thus administered to differentiate μ-selective antagonism (0.05 mg/kg NTX) from general (μ, δ and κ) receptor antagonism (0.5 mg/kg NTX).

Naltrexone administration did not alter the magnitude of the inflammatory response to repeated carrageenan treatment on days 3,4 and 5 relative to that observed in study 2.2.(Figure 2.12(A). It is worthy of note that baseline PC in both carrageenan and saline-treated animals was slightly higher in study 2.3(B) than in study 2.2., but the difference

between PCmax in carrageenan-treated animals in study 2.3 and study 2.2 remained constant .

Naltrexone administration did not alter thermal or mechanical response thresholds in saline-treated control animals [Figure 2.12(B)], consistent with previous evidence that opioid receptors do not affect 'physiological' nociceptive transmission (Bienkowski *et al.*, 1999). Naltrexone administration (0.5 mg/kg and 0.05 mg/kg) did not alter the pattern of nociceptive responses to thermal stimulation associated with repeated carrageenan administration and did not affect TWLmin on days 3 and 4. This suggests that suggesting that opioidergic mechanisms did not play a dominant role in the mediation of tachyphylaxis of thermal hyperalgesia observed in study 2.2. However, TWLmin on day 5 was significantly lower in NTX-treated animals (6.8 ± 0.4 s following 0.5 mg/kg NTX and to 6.8 ± 0.3 s following 0.05 mg/kg NTX) than in animals which received carrageenan treatment alone (7.8 ± 0.2 s).

Naltrexone administration (0.5 mg/kg and 0.05 mg/kg) did not alter the pattern of nociceptive responses to mechanical stimulation associated with repeated carrageenan administration and did not affect MWTmin on days 3 and 4. This suggests that opioidergic mechanisms did not play a dominant role in the mediation of tachyphylaxis of mechanical hyperalgesia observed in study 2.2. However, MWTmin was significantly lower in NTX-treated animals on day 5 (2.5 (1, 3) in 0.5 mg/kg NTX-treated animals, and 3 (2.75, 3.25) in 0.05 mg/kg NTX animals) than in animals which received carrageenan treatment alone [4.5 (3, 6)].

The enhancement of both thermal and mechanical hyperalgesic responses on day 5 by opioid antagonism suggests that tonic engagement of opioid inhibitory pathways was present on day 5 of repeated mild inflammatory stimulation. The lack of dramatic change in tachyphylaxis of thermal hyperalgesia on days 3 and 4 associated with naltrexone treatment suggest that opioid mechanisms were not prime mediators of this tachyphylaxis. The partial restoration of mechanical hyperalgesia on day 4 (but notably not day 3) associated with naltrexone treatment suggests that opioidergic mechanisms may have played some role in the tachyphylaxis of mechanical hyperalgesia induced by repeated carrageenan treatment.

Differential modulation of thermal and mechanical nociceptive responses associated with selective opioid receptor antagonism has been reported previously. Millan (1989)

investigated behavioural responses to non-noxious and noxious thermal and mechanical stimulation following administration of selective opioid receptor agonists. He reported that at moderate matched intensities, both μ- receptor agonists and κ-receptor agonists were equipotent against thermal and mechanical stimuli, while at noxious intensities of thermal and mechanical stimulation, and in distinction to μ- receptor activation, the potency of κ-receptor agonists against noxious thermal (but not mechanical) stimulation was intensity dependent. While the objectives of Millan's study were quite distinct to those of the present study, these data indicate a precedent for differential modulation of thermal and mechanical nociceptive responses, potentially mediated through κ-receptor activity.

In study 2.3(B), no significant difference was observed in the pattern of thermal and mechanical nociceptive responses recorded in low-dose NTX (relative μ-selective opioid antagonism) and high-dose NTX (general opioid receptor antagonism). This would suggest that at least a component of the endogenous opioidergic attenuation of hyperalgesia on day 5 of repeated carrageenan treatment is mediated by δ and/or κ receptors, rather than exclusively by μ opioid receptors. More precise definition of the roles of δ and κ receptors in the attenuation of hyperalgesic responses associated with repeated mild inflammatory stimulation will require systematic administration of more selective δ and κ receptor antagonists. Furthermore, it was not possible to distinguish between antagonism of spinal and supraspinal opioid receptors and it is therefore possible that the observed effects were due to blockade of opioid receptors located peripherally, spinally or in higher centres. Intrathecal and intracerebroventricular administration of selective agonists and antagonists should permit identification of the precise location of opioidergic modulation of nociceptive transmission.

2.4.4.4. Study 2.3(C)

Selection of an α_2 adrenoreceptor antagonist

Atipamezole is a potent and highly selective α_2 adrenoreceptor (AR) antagonist (Scheinin *et al.,* 1989; Virtanen *et al.,* 1989; Winter & Rabin 1992; Haapalina *et al.,* 1997) which penetrates rapidly into the CNS following subcutaneous injection (Biegon *et al.,* 1992) with an elimination half-life of 2 h in the rat (Haapalina *et al.,* 1998). A dose of 300 μ g/kg administered by subcutaneous injection induces complete central α_2AR blockade and stimulates central noradrenaline release in the rat (Haapalinna *et al.,* 1997). At this dose,

atipamezole has been reported to have no adverse effects on general central sensory transmission in rats (Jakala *et al.,* 1992; Sirvio *et al.,* 1993).

Atipamezole has been reported to produce variable and conflicting effects on pain responses in rats, associated with habituation of the subject to test conditions, the nature and intensity of noxious stimulation, the dose of atipamezole administered and the response parameter measured (Mansikka & Pertovaara, 1995; Mansikka *et al.,* 1996; Kaupilla *et al.,*1999). Mansikka & Pertovaara (1995) reported that systemic administration of 10 μg /kg and 1 mg/kg atipamezole did not alter mechanical hyperalgesia (assessed using Von Frey hairs) following unilateral topical application of mustard oil to the hind paw, while a dose of 100 μg /kg atipamezole significantly attenuated mustard-oil induced hyperalgesia. Mansikka *et al.*(1996) reported that intrathecal administration of atipamezole (2.5 μg) to the lumbar spinal cord, or intracerebral administration into the nucleus raphe magnus did not attenuate hyperalgesia associated with intraplantar application of mustard oil, although the same dose applied directly to the lateral reticular nucleus of the medulla completely reversed hyperalgesia. Kaupilla *et al.*(1999) similarly reported variation in pain responses associated with differing doses of systemic atipamezole, administered in combination with noxious mechanical stimulation or intraplantar formalin administration. They observed that doses of 30μg/kg and 1.5 mg/kg atipamezole (administered by intraperitoneal injection) did not significantly alter paw withdrawal latency to noxious mechanical stimulation or pain behavior in the formalin test, but did increase licking latency in the hot-plate test and reduce response latencies in the tail-flick test. Our study shows that a dose of 400-600μg/kg atipamezole administered by subcutaneous injection, which produces complete central α_2- adrenoreceptor blockade (Haapalina *et al.,* 1998) permits investigation of α_2-adrenergic regulation of nociceptive transmission without producing motor dysfunction which would obscure behavioural responses.

Atipamezole was selected in preference to other α_2- adrenoreceptor antagonists due to the pharmacological characteristics of the drug discussed above, which proposed to provide a good level of central α_2- adrenoreceptor antagonism for the duration of behavioural testing. The two doses of atipamezole administered, 600 μg/kg at 0 h and 400 μg/kg at 2 h p.i., were used in order to achieve serum levels of close to 300 μg/kg for as long as possible over the period of behavioural testing.

Atipamezole administration did not alter the magnitude of the inflammatory response to repeated carrageenan treatment on days 3,4 and 5 relative to that observed in study

2.2.(Figure 2.13). It is worthy of note that baseline PC in both carrageenan and saline-treated animals was slightly higher in study 2.3(C) than in study 2.2.

Atipamezole administration did not alter nociceptive behaviour associated with repeated carrageenan administration, suggesting that α_2-adrenergic mechanisms did not play a dominant role in the mediation of plasticity of nociceptive responses which occurred in study 2.2.

The modulation of central nociceptive transmission by central α_2-adrenergic pathways following inflammatory injury remains a contentious issue (reviewed by Kingery *et al.*, 1997). Some behavioural (Hylden *et al.*, 1991) and electrophysiological (Stanfa & Dickenson, 1994) studies using high-dose intraplantar carrageenan inflammation have reported that intense inflammatory injury enhances the analgesic effects of α_2-adrenergic agonists, thus suggesting that endogenous α_2-adrenergic (antinociceptive) neurotransmission may be enhanced in response to injury. However, a study by Idanpaan-Heikkila *et al.*(1994) did not identify any enhancement of α_2-adrenergic responses following high-dose carrageenan treatment. It is possible that engagement or enhancement of endogenous α_2-adrenergic antinociceptive transmission is critically influenced by the nature and severity of inflammatory injury.

In study 2.3(C), systemic administration of atipamezole administration meant that the potentially conflicting peripheral, spinal and supraspinal effects of α_2-adrenenoreceptor antagonism could not be distinguished. Mansikka *et al.*(1996) reported that intrathecal administration of atipamezole (2.5 μg) to the lumbar spinal cord, or intracerebral administration into the nucleus raphe magnus did not attenuate hyperalgesia associated with intraplantar application of mustard oil, although the same dose applied directly to the lateral reticular nucleus of the medulla completely reversed hyperalgesia. In the same study it was reported that medetomidine (an α_2-adrenoceptor agonist) administration to the lumbar cord, reversed ipsilateral mechanical hyperalgesia without altering contralateral mechanical withdrawal thresholds, and attenuated nociceptive responses in control rats following application, while medetomidine administration into the nucleus raphe magnus or into the lateral reticular nucleus of the medulla did not affect nociceptive or hyperalgesic responses. Mansikka *et al.*(1996) thus elegantly demonstrated that α_2-adrenergic blockade antagonises mechanical hyperalgesia through action on the medulla, whereas α_2-adrenoceptor stimulation antagonises mechanical hyperalgesia through direct action on the spinal cord. Study 2.3.2 demonstrates the complexity of α_2-adrenergic modulation of central nociceptive transmission following acute injury and highlights the

need for reappraisal of the involvement of α_2-adrenergic mechanisms in the central response to moderate, as opposed to intense, inflammatory injury.

2.5 CONCLUSIONS

Mild inflammatory injury induced by 0.5% and 0.1% carrageenan treatment induced consistent hyperalgesic behaviour which was not altered by weekly repetitive stimulation. Temporary attenuation of hyperalgesia developed following daily repeated mild inflammatory injury induced by 0.1% carrageenan treatment, but hyperalgesia returned when this repetitive inflammatory stimulation was maintained. These studies demonstrate that the intensity and the frequency of mild inflammatory challenge critically regulate nociceptive transmission and the development of hyperalgesic behaviour. Identification of differences in the expression of pro- nociceptive and anti-nociceptive agents induced in association with 'maximal' ('ceiling') and 'sub-maximal' thermal and mechanical hyperalgesic responses, may lead to greater understanding of the mechanisms which transform nociceptive transmission from 'physiological' to 'pathophysiological'. LTP of neuronal transmission in the dorsal horn has been reported following high-intensity inflammatory injury (Liu & Sandkuhler 1995, 1996; Sandkuhler & Liu, 1998) and associated with central sensitisation of nociceptive transmission. In contrast, LTD of neuronal transmission in the spinal dorsal horn has been reported as a purely electrophysiological phenomenon induced through specific patterns of electrical stimulation and while it has been speculated that LTD of synaptic transmission in the dorsal horn may provide an alternative mechanism for suppression of excitatory nociceptive transmission, this speculation is not supported by evidence produced in studies using natural noxious stimulation or injury. It is possible that the tachyphylaxis of hyperalgesia observed in study 2.2 may represent LTD of dorsal horn nociceptive responses.

The tachyphylaxis observed in study 2.2 may represent a 'physiological' pathway, directed towards restoration of functional 'homeostasis' of nociceptive processing, which functions until a critical level of inflammatory challenge is surpassed, whereupon adaptive mechanisms are overridden and persistent hyperalgesia develops. Manipulation of the intensity and frequency of inflammatory challenge in standard models of inflammatory pain may provide further insights into mechanisms associated with physiological and pathophysiological responses to inflammatory injury. Clearly, improved understanding of

physiological mechanisms which prevent the onset of persistent hyperalgesia offers huge potential for the development of new therapeutic strategies to pre-empt the development of persistent post-injury hyperalgesia, and to provide novel targets for the treatment of hyperalgesia associated with inflammatory injury.

Preliminary study 2.3 (A), investigating the role of NMDA receptors, gave inconclusive results. Repetition of this study in a population of test subjects with demonstrable thermal and mechanical hyperalgesia is indicated. Preliminary studies on the role of opioid receptors and α_{2A} adrenoreceptors in the mediation of tachyphylaxis of hyperalgesic behaviour induced by daily repeated low-dose carrageenan treatment suggest that these receptor systems did not play a major role in the observed tachyphylaxis. However opioid receptors and α_{2A} adrenoreceptors appeared to influence, to a degree, the development of pronounced thermal and mechanical hyperalgesic behaviour on day 5 of repeated carrageenan treatment. These observations demonstrate once again the constant interplay between excitatory (hyperalgesic) and inhibitory (antihyperalgesic) nociceptive transmission following inflammatory injury. In study 2.3 it was not possible to identify the location -peripheral, spinal or supraspinal- at which receptor antagonists induced their effects on day 5. Further studies using intrathecal and supraspinal administration of the same antagonists will identify specific sites at which the observed modulation of nociceptive behaviour occurred. For example, it remains possible that tachyphylaxis of hyperalgesia was mediated by descending inhibitory mechanisms originating in higher centres; in order to investigate this, it shall be necessary to perform more specific studies which can target those key regions associated with descending inhibition of nociceptive transmission, including the periaqueductal grey matter (PAG) and the ventromedial medulla. The mechanisms underlying the observed tachyphylaxis of hyperalgesia associated with mild repetitive inflammatory injury remain unresolved, but are likely to involve a combination of spinal and supraspinal mechanisms and multiple molecular mediators. Clearly, greater appreciation of endogenous mechanisms which attenuate hyperalgesia following inflammatory injury should lead to improved management of persistent pain associated with inflammatory injury.

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CHAPTER 3: AN IN-SITU HYBRIDISATION STUDY OF POTENTIAL MOLECULAR MEDIATORS OF PLASTICITY OF NOCICEPTIVE PROCESSING IN THE SPINAL CORD FOLLOWING INFLAMMATORY INJURY

Not everything that counts can be counted, and not everything that can be counted counts

Albert Einstein

3.1 INTRODUCTION

Pathophysiological pain sensation is an expression of plasticity of nociceptive transmission, potentiated at multiple sites and mediated by diverse mechanisms, located both peripherally and centrally. Central sensitisation of nociceptive transmission, a common denominator of numerous pathological pain states, is a manifestation of transient or persistent enhancement of dorsal horn neuronal responses to noxious stimulation (reviewed by Millan, 1999). Persistent enhancement of dorsal horn nociceptive transmission may be an expression of long-term potentiation or long term depression of synaptic transmission in the spinal cord, associated with phenotypic changes in dorsal horn nocisponsive neurones (reviewed in Chapter 1). Mechanisms of dorsal horn sensitisation share many common characteristics with long-term potentiation, but are not invariably comparable, as was discussed in chapter 1. Rapid and reversible sensitisation of dorsal horn neuronal responses can occur in the absence of morphological changes, and may require peripheral input for its maintenance (Millan, 199). In contrast, LTP or LTD in higher centres can persist for prolonged periods in the absence of stimulation , and are associated with phenotypic changes in sensitised neurons (reviewed in Chapter 1). The association of synaptic plasticity in the dorsal horn with central sensitisation and pathophysiological pain states remains poorly defined.

The molecular basis of stimulus-dependent neuronal plasticity has been extensively studied in higher centres, where LTP and LTD of synaptic transmission are believed to express the synaptic basis of memory. Stimulus-induced intraneuronal calcium influx through excitatory amino acid receptors, transduced by protein kinase enzymes to activate immediate early genes has been strongly implicated in the induction and/or maintenance of LTP in higher centres (reviewed by Roberson *et al.*,1996, and in chapter 1). Many other signalling molecules and neurotransmitter molecules have been implicated in the induction and/or maintenance of synaptic plasticity in higher centres, including GABA (Kanter &

Haberly, 1993; Yasui *et al.,* 1993; Tomasulu *et al.,* 1993; Watanebe *et al.,* 1995), and endogenous opioids (Satoh *et al.,* 1989; Bramham *et al.,* 1992; Xie & Lewis, 1995a; Williams & Johnston 1996; Ishihara *et al.,* 1990; Bramham *et al.,* 1991; Derrick *et al.,* 1991; 1992; Xie *et al.,* 1991; Francesconi *et al.,* 1997; Ikeda *et al.,* 1999). The molecular basis of LTP and LTD in the spinal cord remains poorly defined, although it has been postulated that similar mechanisms mediate synaptic plasticity in the spinal cord and in higher centres. Excitatory amino acid receptors (Sandkuhler *et al.,* 1996), immediate early genes (Wisden *et al.,* 1990), CaMKII α (Kolaj *et al.,* 1994) and endogenous opioids (Pockett, 1995), amongst other agents, have been implicated in the induction of persistent synaptic plasticity in the spinal cord dorsal horn.

In studies 3.1 and 3.2, *in-situ* hybridisation techniques were used to investigate the expression of key neurotransmitters and intracellular signalling pathways implicated in nociceptive transmission, many of which have also been implicated in the induction and maintenance of LTP and LTD in higher centres (discussed in chapter 1). Since it has been speculated that persistent changes in central nociceptive transmission may represent LTP and LTD of dorsal horn neurotransmission, leading to the development of a 'memory of pain' in the dorsal horn as a mechanism for persistent pain sensation, it was of interest to correlate expression of genes known to mediate synaptic plasticity in higher centres, with the plasticity of nociceptive behaviour observed in studies 2.1 and 2.2.

The agents investigated were: the kinase enzyme CaMKIIα, an intracellular mediator activated by intraneuronal calcium ion influx which phosphorylates (and thus covalently modifies) key proteins involved in neurotransmitter production and release; the immediate early genes zif/268, tPA and jun D, which are rapidly and independently activated by neuronal stimulation; the cyclo-oxygenase enzymes COX-1, and COX-2, which catalyse prostaglandin synthesis, and the precursor genes for key 'inhibitory' neurotransmitters, proenkephalin, prodynorphin and glutamic acid decarboxylase [GAD, which catalyses synthesis of γ-amino-butyric acid (GABA) and is entirely restricted to GABAergic neurons]. Gene expression was investigated in the superficial laminae (I/II) and in lamina V of the lumbar spinal dorsal horn, key laminae involved in central processing of nociceptive information and the major targets of investigations into plasticity of central nociceptive transmission (reviewed in chapter 1).

Identification of specific regional and cellular sites of gene expression within nervous tissue using *in-situ* hybridisation (ISH) techniques was first described by Gee *et al.*(1983), and subsequently characterised and developed in greater detail by Lewis *et al.*(1985; 1988)

and Young *et al.*(1986 a;b). ISH provides a highly sensitive method of identification of mRNA expression. A nucleic acid probe tagged with radiolabelled nucleotides (or other molecules which permit colorimetric or light detection) is hybridised directly onto a tissue section where it recognises its cognate mRNA to form a stable probe-RNA duplex (reviewed by Wisden & Morris, 1992). After removing surplus probe, tissue sections are either exposed for autoradiography or processed histochemically to reveal specific sites of gene expression. Synthetic deoxyribonucleotides are now the predominant form of nucleic acid probe used in ISH studies, having been proven to be generally more specific, more reliable and more resilient to repeated use than alternative forms of complementary DNA and RNA nucleic acid probes (Wisden & Morris, 1992).

3.2 MATERIALS AND METHODS

In-situ hybridisation was performed according to the method described by Morris *et al.* (1986) and reviewed by Wisden & Morris (1994).

3.2.1. Probe design

Base sequences for probes were obtained from the U.S. National Centre for Biotechnology (USA). Synthetically produced probes were commercially synthesised on an Applied Biosystems DNA synthesiser by Genosys Biotechnologies (Europe) Ltd.. Probes were stored at -20° C and used at a working concentration of 5ng/ µl. The following sequences were used in *in*-situ hybridisation studies (5'-3'):

Tubulin T26 (45-mer) complementary to a section of the 3' non-coding region: (Miller *et al.*, 1987)

Preproenkephalin (45mer) complementary to bases 388-432 of the predicted gene sequence (Howells *et al.*, 1984; Yoshikawa *et al.*, 1984)

Prodynorphin (45-mer) complementary to base sequences 862-906 of the predicted gene sequence (Civelli *et al.*, 1985)

Cyclo-oxygenase-1 (COX-1) (45mer), complementary to nucleotides spanning amino acids 13-28 (Feng *et al.*, 1993; Yamagata *et al.*, 1993)

Cyclo-oxygenase-2 (COX-2) (45mer) (Feng *et al.*, 1993; Yamagata *et al.*, 1993)

Calcium calmodulin kinase II, α-subunit (45mer) complementary to nucleotides spanning amino acids 408-422 (Lin *et al.*, 1987)

Zif/268 (45-mer), complementary to nucleotides spanning amino acids 2-16 (Millbrand, 1987)

Tissue plasminogen activator (TPA) (45mer) complementary to nucleotides spanning amino acids 265-280 (Ny *et al.*, 1988)

GAD 67 (45mer) complementary to base sequence published by Julien *et al.*(1990) CCTGCACACATCGGTTGCATCCTTGGAGTATACCCTTTCCTTG

Jun-D (45mer) complementary to nucleotides corresponding to the last 20 amino acids of the predicted protein (Ryder *et al.*, 1989)

3.2.2. Probe labelling protocol

0.9 µl. diethylpyrocarbonate (DEPC)-treated water
1.25 µl. 10x terminal deoxynucleotidyl transferase(TdT) tailing buffer (Pharmacia)
1.5 µl. oligonucleotide probe (5 ηg/ µl)
2.0 µl. ^{35}S d-ATP
0.6 µl. terminal deoxynucleotidyl transferase (TdT) [Pharmacia]

Reagents were dispensed in the order listed above into a 1.5ml eppendorf tube, pipette-mixed and incubated at 35-37 °C for approximately 2 hours. Following incubation, 60 µl. of sterile DEPC-treated water was added to the incubation mixture to arrest TdT enzyme activity.

3.2.3. Purification of labelled oligonucleotide probes

The radiolabelled oligonucleotide solutions were transferred into biospin-6 chromatography spin columns (Biorad Ltd., U.K.) in order to remove non-incorporated nucleotides from the reaction mixture. The columns were inverted several times to resuspend settled gel. The tip was removed from the 1.5ml gel tube and excess buffer drained into an eppendorf tube positioned below it. Spin columns were centrifuged for 2 minutes at 1000rpm in a swinging bucket centrifuge until all buffer had drained from the gel. The reaction mixture was carefully applied drop-wise to the centre of the column and the column re-centrifuged for 4 minutes at 1000 r.p.m.

3.2.4. Analysis of purified probes

2 µl. of purified eluate was mixed into 4ml of scintillation fluid and specific radioactivity measured using a liquid scintillation counter. The oligonucleotide probe was considered 'labelled' and suitable for use if a count of between 100,000 and 300,000 counts per µl was obtained. Having confirmed the success of the labelling reaction, 2 µl of 1 molar dithiothreitol (DTT, Sigma) was added to the probe to provide protection against oxidative damage during storage. Labelled probes were stored at -20°C and freeze-thawed for repeated use for up to a month.

3.2.5. Tissue preparation

Preparation of poly-l-lysine coated slides

Slides were coated in a 0.1mg/ml solution of poly-L-lysine hydrobromide (P-1524, SIGMA, U.K.) in DEPC-treated water to optimise adhesion of tissue sections. Slides were wrapped in aluminium foil and baked for 4-6 hours at 180°C then immersed in poly-L-lysine solution, air-dried and stored in aluminium foil at 4 °C.

Spinal cord ejection

Fresh spinal cord tissue was prepared using a method described by Meikle *et al.*(1981). Following euthanasia, the vertebral column was dissected and the vertebral canal exposed. A pipette tip was inserted into the caudal end of the canal and sterile 0.9% saline injected at constant pressure to expel the spinal cord from the cervical extremity of the canal. The lumbar enlargement of the cord was excised, snap-frozen in liquid nitrogen then stored at -70° C. 20 µm sections of lumbar spinal cord were cryostat-cut (Reichert-Jung) and thaw-mounted onto poly-L-lysine coated slides. Slides were air dried for 20 minutes, fixed in ice-cold 4% depolymerised paraformaldehyde (PFA) dissolved in 1X PBS buffer solution for 5 minutes; rinsed in 1x phosphate-buffered saline (PBS) for 10 minutes and dehydrated in serial dilutions of ANALAR ethanol. Slides were stored in 100% ANALAR ethanol at 4°C prior to use.

3.2.6. Hybridisation of radionucleotide probes onto tissue sections

Labelled probes were diluted in hybridisation buffer for application onto slides. The buffer was prepared according to the protocol described below:

10ml of 20x stock sodium citrate (SSC) and 25ml of 100% de-ionised formamide, de-ionised using 'Amberlite' MB-1 mixed resin beads, were gently mixed in a sterile graduated polypropylene (Falcon) tube. To this mixture was added:

2.5ml 0.5M sodium phosphate

0.5ml sodium pyrophosphate

1ml 5mg/ml polyadenylic acid

5g dextran sulphate

DEPC-treated water was added to give a total volume of 50 ml. The buffer mix was gently mixed on a rocking platform overnight and stored at 4°C.

Radiolabelled probe was diluted in hybridisation buffer in a ratio of 200µl of probe/hybridisation buffer. 1µl of probe and 4µl of 1M DTT were added to each 100µl of hybridisation buffer and mixed thoroughly to overcome the tendency of the high-viscosity hybridisation buffer to displace the low-density probe. 200µl of labelled hybridisation mixture was applied to each slide. Slides were covered with a parafilm coverslip and air bubbles removed by gentle pressure, then placed on an elevated 'platform' in a petri dish. 4X SSC-saturated tissue was placed beside the slides to prevent dehydration during incubation. Petri dishes were then sealed and incubated overnight at 42° C. Fifty-times excess concentration of unlabelled probe was added to hybridisation buffer and applied to selected sections to assess non-specific binding of probe to tissue.

mRNA present in tissue sections strongly hybridised to the probe during incubation to form a region of double stranded nucleic acid, which was resistant to single-stranded RNA'ase attack. Deionised water was used for all washing procedures. Coverslips were removed in a solution of 1 X stock sodium citrate (SSC) and sections were washed in pre-warmed SSC at 55-60° C for 30 minutes. Sections were finally passed through a series of room temperature rinses, spending a few seconds in each, of 1XSSC, 0.1X SSC, 70% analytical grade (AR) ethanol, 95% (AR) ethanol and air dried before exposure to radiographic film.

3.2.7. Exposure of hybridised tissue to radiographic film

Sections were stabilised on blank paper using double-sided adhesive tape and exposed to radiographic film (Kodak Bio-Max MR 20.3 x 25.4cm) for 3-20 days depending on the labelling-intensity of the probe and the predicted abundance of specific mRNA in tissue sections. Exposed film was developed in D-19 (Kodak) developer for 3 minutes, rinsed in water for 1 minute and fixed in UNIFIX solution for 3 minutes.

3.2.8. Photographic emulsion coating of slides

Emulsion coating was performed under safelight in a water bath set at 45° C. Ilford K5 photographic emulsion was dissolved in 0.5% Glycerol at 45° C, in a ratio of 1:1. Each slide was dipped into emulsion and then allowed to air dry for 6 hours. Dry slides were placed in a light-resistant box containing a few grains of silica gel to protect against oxidation and stored at 4 °C for 5 times as long as the length of exposure time required to produce a visible signal on radiographic film.

3.2.9. Development and counterstaining of emulsion-dipped slides

Exposed slides were developed in D-19 Kodak developer for 2 minutes, rinsed in water for 30 seconds, fixed in freshly-prepared 30% sodium thiosulphate solution for 2 minutes and rinsed in distilled water for 20 minutes. Slides were then immersed in neutral red solution for 45 minutes, rinsed, and dehydrated in serial dilutions of ethanol. Counterstained slides were transferred to histoclear solution (National Diagnostics) overnight and mounted using 'Histomount' solution and glass coverslips.

3.2.10 Quantitative analysis of labelled tissue sections

Study 3.1: Weekly repeated carrageenan treatment

Semi-quantitative densitometry was performed on the counterstained cells under bright-field microscopy using a Macintosh image analysis system (Image v.1.52 software, [NIH]). Following tissue processing, 4-6 tissue sections from each treatment group at each time point were analysed. For each tissue section, measurements were made bilaterally in laminae I/II and lamina V of the spinal dorsal horn. The method of analysis used depended on the abundance of labelled cells in the tissue section. There was strong expression of CamKIIα and proenkephalin mRNA in the superficial laminae of the dorsal horn such that labelling of individual cells could not be measured. Thus CaMKIIα and proenkephalin mRNA expression in laminae I/II were quantified by measuring the mean relative optical density (M.R.O.D.) of two regions of fixed size within the superficial laminae in each lumbar section (n = 4-6 in each group).

mRNA expression of all other oligonucleotide probes in laminae I/II and lamina V, with the exception of COX-1 and COX-2, was performed by analysing six regions corresponding to counterstained cells within each lamina on each side, measuring the mean relative optical density of silver-grain clustering (M.R.O.D.), indicating labeling intensity, and the major axis of each cell, indicating cell size. COX-1 and COX-2 mRNA expression in study 1 were analysed using the method used for all oligonucleotide probes in study 2.

Study 3.2: Daily repeated carrageenan treatment

Spinal cord was harvested from saline-treated animals euthanased at 1h p.i. and 6 h p.i. on days 1 and 5 (n = 4 at each time point on each day). In each section, the density of silver grains clustered over individual counterstained cells was measured under bright-field microscopy using a KS400 image analysis system (USA). Measurements were made bilaterally in the superficial laminae (I-II) and lamina V of the dorsal horn. Six counter-stained cells were measured in both ipsilateral and contralateral laminae I/II and V, producing a total silver-grain count and diameter (in μm) for 6 positively labelled cells. The mean silver-grain count/cell, mean cell diameter and mean silver-grain count/unit area were thus calculated in ipsilateral and contralateral laminae I/II and V.

3.2.11 Statistical analysis

In studies 3.1 and 3.2, analysis of variance techniques supplemented by the Tukey-Kramer test for multiple comparisons were used to investigate the effects of repeated carrageenan treatment on mRNA expression of labelled probes in the laminae I/II and in lamina V. GLM analysis and Tukey-Kramer multiple comparison tests were used to compare mRNA expression (quantified as M.R.O.D). in study 3.1, and total silver-grain count/cell in study 3.2) and cell diameter between treatment groups at each time point. Within each treatment group, GLM analysis was used to compare ipsilateral and contralateral mRNA expression and cell diameter at each time point. GLM analysis and Tukey-Kramer multiple comparison tests were used to investigate the effect of weekly (study 3.1) or daily (study 3.2) repeated treatment on ipsilateral and contralateral mRNA expression.

3.3 RESULTS

3.3.1. CALCIUM CALMODULIN KINASE IIα

Basal CaMKIIα mRNA expression

Preliminary studies of CaMKIIα mRNA expression in spinal cord tissue taken from untreated control animals demonstrated constitutive expression of CaMKIIα mRNA in spinal cord grey matter. In studies 3.1 and 3.2, CaMKIIα positive cells were observed throughout the grey matter of the lumbar spinal cord and in the hippocampus. The absence of CaMKIIα mRNA expression in tissue sections labelled with fifty-times excess unlabelled probe confirmed probe specificity. Cells in the superficial laminae (I/ II) expressing CaMKIIα mRNA were of uniform 8-15 μm diameter. CaMKIIα positive cells

in laminae V varied in diameter from 8- 32 μm, although the majority of cells counted were 25-32 μm. There was no significant difference between treatment groups in the diameter of CaMKIIα positive cells. CaMKIIα labelled cells in the lumbar spinal cord of carrageenan and saline-treated animals are shown in Figure 3.1.

Study 3.1: Weekly repeated carrageenan treatment

Laminae I/II

CaMKIIα mRNA expression in laminae I/II in study 3.1 is shown in Figure 3.2.

Treatment effect

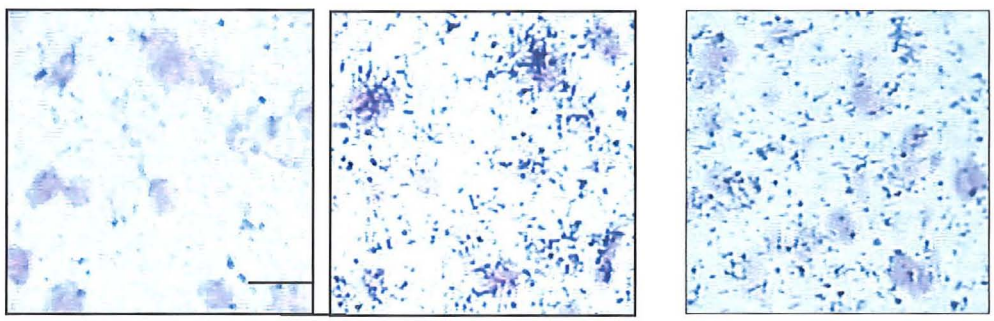
GLM analysis indicated a significant treatment effect on ipsilateral, but not contralateral, CaMKIIα mRNA expression in laminae I/II ($F[2,58] = 3.42$, $p < 0.05$) and $F[2,58] = 1.36$, $p = 0.26$ respectively). Further analysis indicated that ipsilateral CaMKIIα mRNA expression was significantly upregulated in 0.5% carrageenan-treated animals relative to saline-treated animals ($p < 0.01$) and relative to 0.1% carrageenan-treated animals ($p < 0.05$). CaMKIIα mRNA expression in 0.1% carrageenan animals was not significantly different from that of saline-treated animals. More detailed analysis did not indicate a significant treatment effect on ipsilateral mRNA expression on any particular week of the study.

Ipsilateral/contralateral effect

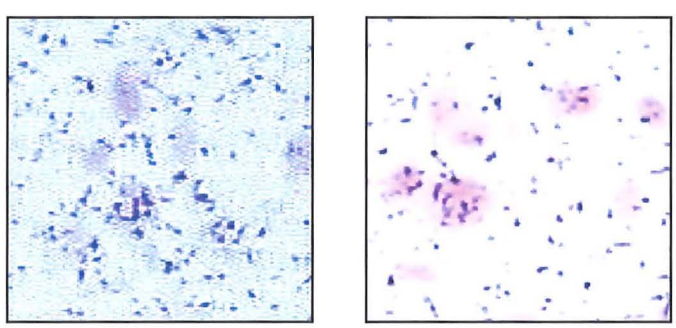
Saline treatment did not alter ipsilateral CaMKIIα mRNA expression (relative to contralateral expression) on any week of the study. In 0.5% carrageenan-treated animals, CaMKIIα mRNA was up-regulated ipsilaterally on all 4 weeks of the study but only significantly so on weeks 2 ($F[1,11] = 7.59$, $p < 0.05$) and week 3 ($F[1,11] = 6.27$, $p < 0.05$); considerable variation in ipsilateral CamKIIα mRNA expression was observed on week 1. In 0.1% carrageenan-treated animals, CaMKIIα mRNA was up-regulated ipsilaterally on week 2 ($F[1,11] = 6.88$, $p < 0.05$) and week 4 ($F[1,11] = 8.1$, $p < 0.05$).

Figure 3.1 : CaMKIIα mRNA expression

Laminae I/II

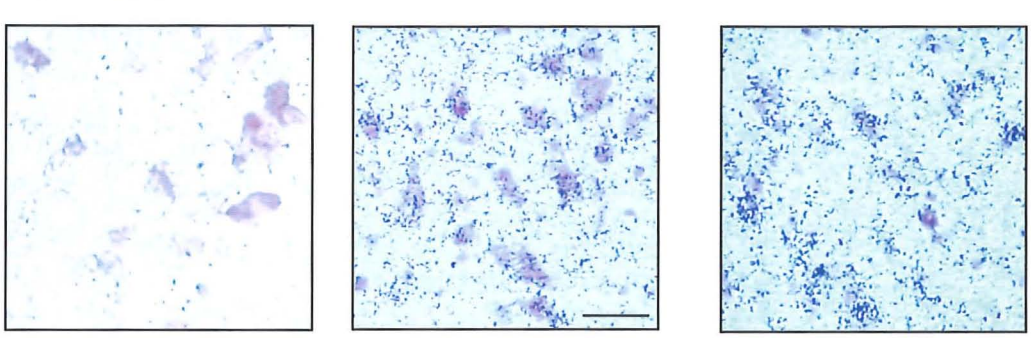


A: control **B**: carrageenan(ipsi) **C**: carrageenan(contra)



D: saline (ipsi) **E**: saline (contra)

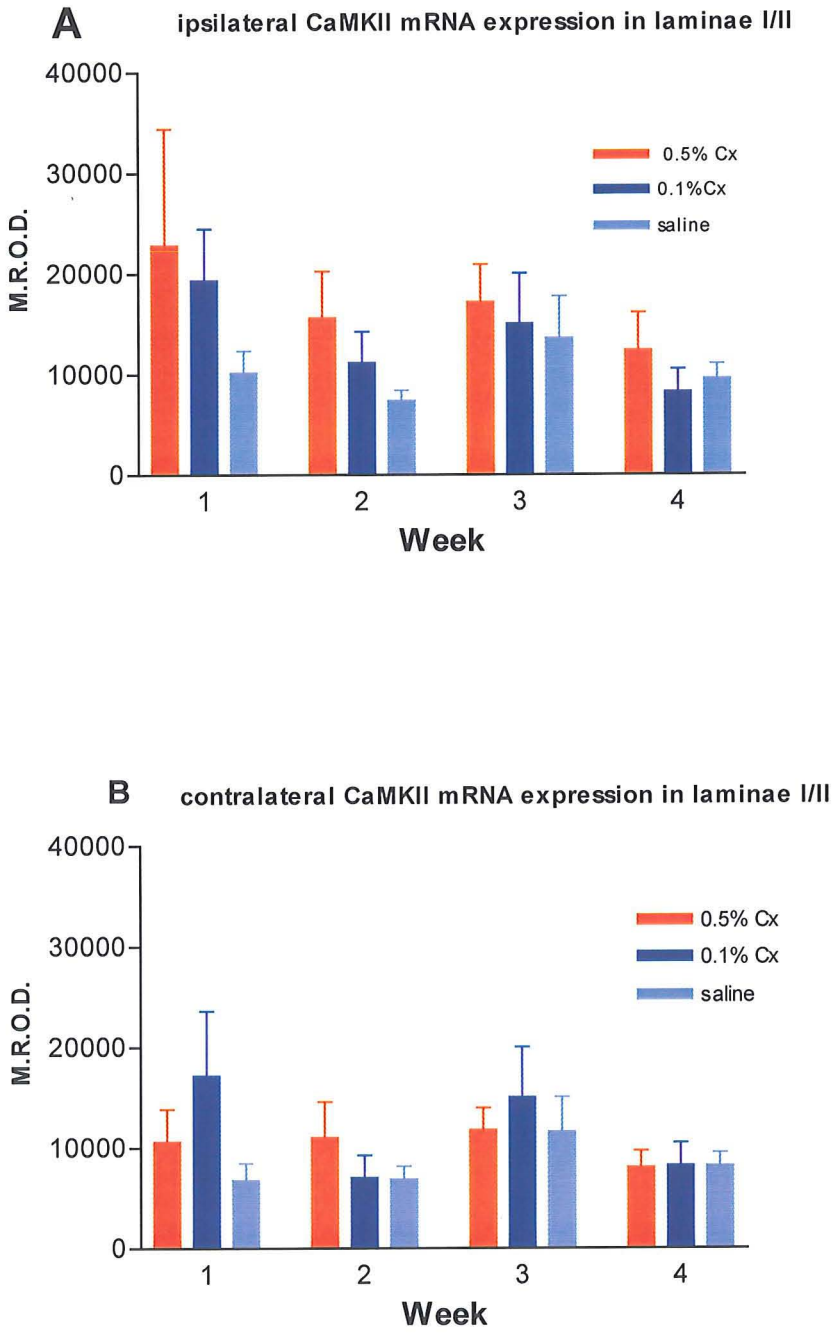
Lamina V



F: control **G**: carrageenan **H**: saline

Figure 3.2: Study 3.1: CaMKIIα mRNA expression in laminae I/II

Expression of CaMKIIα mRNA, expressed as the mean ± SEM mean relative optical density (M.R.O.D.) of silver-grain expression in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% carrageenan (0.1% Cx) or 0.9% saline (n=4-6/group).



Effect of repeated treatment

Weekly repeated treatment did not alter the magnitude of ipsilateral or contralateral CaMKIIα mRNA expression in any treatment group.

Lamina V

In lamina V there was no significant treatment effect, no significant ipsilateral/ contralateral effect and no effect of repeated treatment on CaMKIIα mRNA expression.

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

CaMKIIα mRNA expression in laminae I/II in study 3.2 is shown in Figure 3.3

Treatment effect

No significant treatment effect was observed in ipsilateral or contralateral laminae I/II at 1 h p.i. or at 6 h p.i. on day 1 or day 5.

Ipsilateral/contralateral effect

In carrageenan-treated animals, CaMKIIα mRNA expression was not upregulated ipsilaterally (relative to contralateral expression) at 1h p.i. on any day of the study. At 6h p.i., CaMKIIα mRNA expression was upregulated ipsilaterally on each day of the study: day 1 ($F[1,3] = 11.58$, $p < 0.05$), day 2 ($F[1,3] = 48.25$, $p < 0.01$, day 3 ($F[1,3] = 119.86$, $p < 0.01$) and day 5 ($F[1,3] = 80.43$, $p < 0.01$).

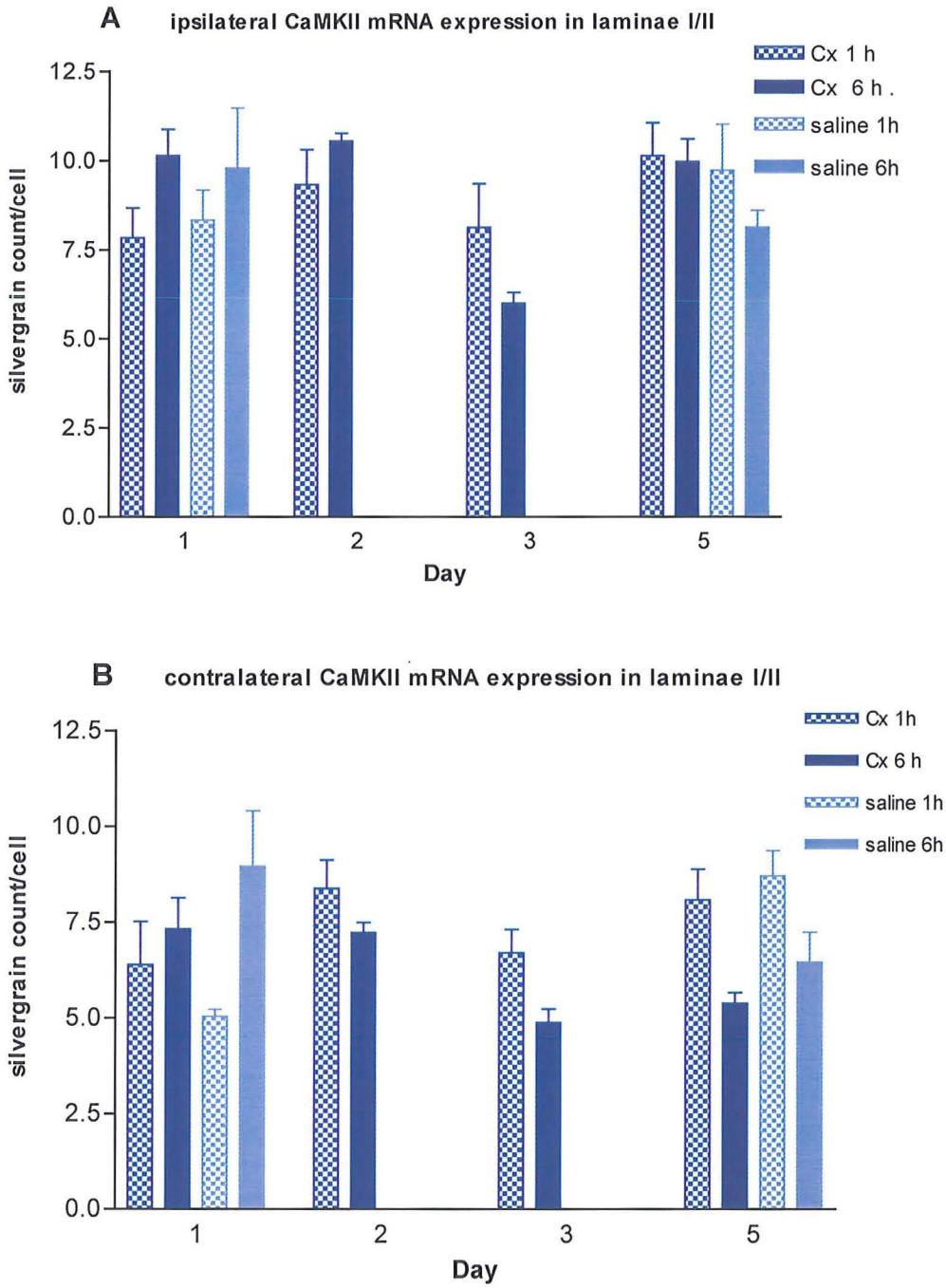
In saline-treated animals, CaMKIIα mRNA expression was upregulated ipsilaterally at 1h p.i. on day 1 ($F[1,3] = 12.99$, $p < 0.05$), but not day 5 ($F[1,3] = 0.47$, $p = 0.54$). At 6 h p.i., CaMKIIα mRNA expression was not upregulated ipsilaterally (relative to contralateral expression) on day 1 ($F[1,3] = 7.41$, $p = 0.07$) or day 5 ($F[1,3] = 3.51$, $p = 0.16$).

Effect of repeated treatment

In carrageenan-treated animals at 1 h p.i., daily repeated treatment did not alter ipsilateral ($F[3,9] = 1.31$, $p = 0.33$) or contralateral ($F[3,9] = 0.98$, $p = 0.45$) CaMKIIα mRNA expression. At 6h p.i. there was a significant effect of repeated treatment on both ipsilateral ($F[3,9] = 13.83$, $p < 0.001$) and contralateral ($F[3,9] = 7.88$, $p < 0.01$) CaMKIIα mRNA expression. Ipsilateral expression was significantly lower on day 3 than on days 1 ($p < 0.01$), 2 ($p < 0.01$) and day 5($p < 0.01$). Contralateral mRNA expression was

Figure 3.3: Study 3.2: CaMKIIα mRNA expression in laminae I/II

Expression of CaMKIIα mRNA, expressed as the mean ± SEM mean silvergrain count/cell of silver-grain expression in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord, measured in animals euthanased 1h or 6h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/group).



significantly higher on day 1 than on day 3 ($p < 0.02$) and day 5 ($p < 0.05$). Contralateral expression was significantly higher on day 2 than on day 3 ($p < 0.05$).

In saline-treated animals at 1 h p.i. there was a significant effect of repeated treatment on contralateral ($F [1,3] = 32.46$, $p < 0.05$) but not ipsilateral ($F[1,3] = 1.16$, $p = 0.36$) CaMKIIα mRNA expression. At 6h p.i., repeated saline treatment did not alter ipsilateral or contralateral CaMKIIα mRNA expression ($F[1,6] = 6.63$, $p = 0.08$) and $F[1,6]=1.16$, $p = 0.36$ respectively).

Lamina V

CaMKIIα mRNA expression in lamina V in study 3.2 is shown in Figure 3.4.

Treatment/time effect

On day 2, ipsilateral CaMKIIα mRNA expression was significantly higher at 1h p.i. than at 6h p.i. in carrageenan-treated animals ($p < 0.05$). There was no other significant treatment/ time effect on ipsilateral or contralateral CamKIIα mRNA expression on days 1, 3 or 5.

Ipsilateral/contralateral effect

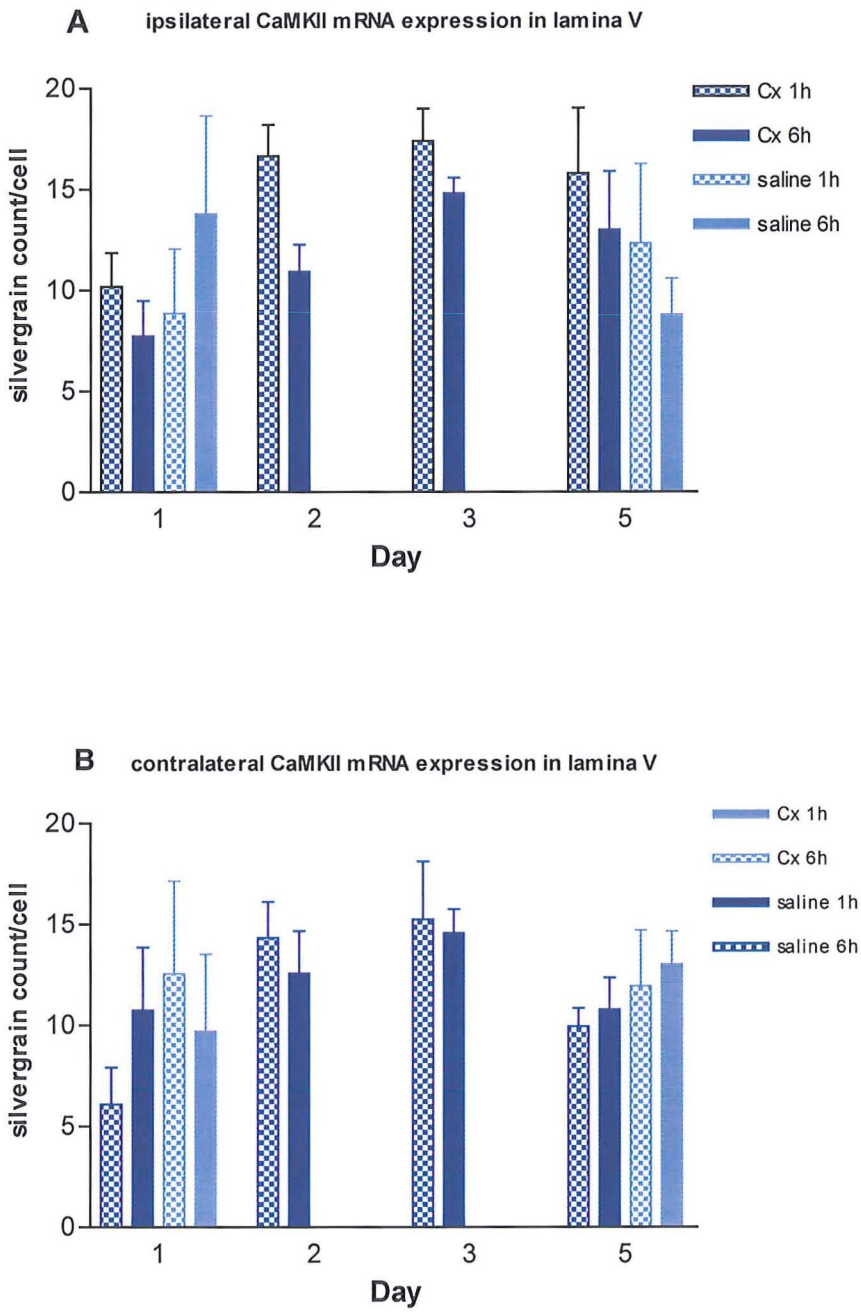
Ipsilateral CaMKIIα mRNA expression was not upregulated (relative to contralateral expression) at 1h or 6h p.i. following carrageenan or saline treatment on any day of the study.

Effect of repeated treatment

In carrageenan-treated animals at 1 h p.i., there was a significant effect of repeated treatment on contralateral, ($F[3,9] = 3.87$, $p < 0.05$) but not ipsilateral, CaMKIIα mRNA expression. Contralateral CaMKIIα mRNA expression was significantly higher on day 5 than day 1 ($p < 0.05$). At 6h p.i., there was a significant effect of repeated treatment on ipsilateral CaMKIIα mRNA expression ($F[3,9] = 3.75$, $p < 0.05$). Ipsilateral expression was significantly higher on day 3 than day 1 ($p < 0.05$). There was no effect of repeated saline treatment on ipsilateral or contralateral CaMKIIα mRNA expression.

Figure 3.4: Study 3.2: CaMKIIα mRNA expression in lamina V

Expression of CaMKIIα mRNA, expressed as the mean ± SEM mean silvergrain count/cell of silver-grain expression in ipsilateral (A) and contralateral (B) lamina V of lumbar spinal cord, measured in animals euthanased 1 h or 6 h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n = 4/group).



3.3.2 ZIF/268

Basal Zif/268 mRNA expression

There was no significant difference between groups in the diameter of cells expressing zif/268 mRNA , nor in the number of zif/268 mRNA positive cells. There was considerable inter-individual variation in the intensity of zif/268 mRNA expression. Zif/268 labelled cells in the lumbar spinal cord of carrageenan and saline-treated animals are shown in Figure 3.5.

Study 3.1: Weekly repeated carrageenan treatment

Laminae I/II

Zif/268 mRNA expression in laminae I/II in study 3.1 is shown in Figure 3.6

Treatment effect

GLM analysis indicated no significant treatment effect on ipsilateral or contralateral zif/268 mRNA expression in laminae I/II.

Ipsilateral/contralateral effect

Zif/268 mRNA expression was upregulated in the ipsilateral cord (relative to the contralateral cord) in animals treated with 0.5% Cx ($F[1,19] = 5.12$, $p < 0.05$); further analysis indicated that this effect occurred on week 1 ($p < 0.05$) and week 3 ($p < 0.05$) and was close to significance on week 2 ($p = 0.07$). Zif/268 mRNA expression was upregulated in the ipsilateral cord (relative to the contralateral cord) in animals treated with 0.1% Cx on week 1 ($p < 0.05$) and was close to significance on week 2 ($p = 0.067$).

Zif/268 mRNA expression was upregulated in the ipsilateral cord (relative to the contralateral cord) in saline-treated animals ($F[1,27]=4.29$, $p < 0.05$), although post-hoc analysis did not indicate a particular week on which this effect was observed.

Effect of repeated treatment

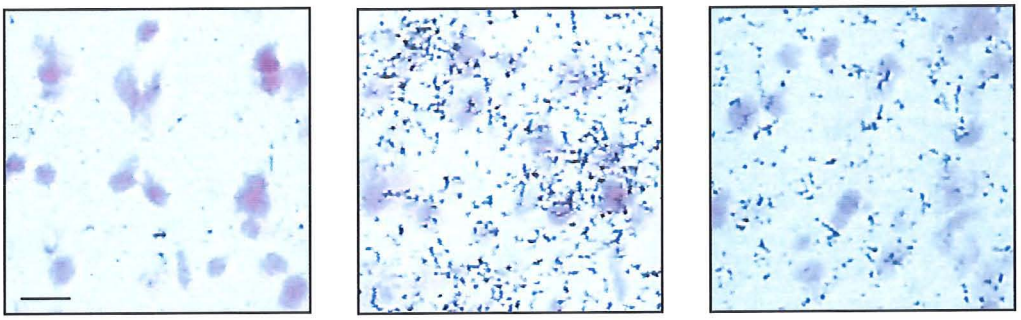
Weekly repeated treatment did not alter the intensity of zif/268 mRNA expression in ipsilateral or contralateral laminae I/II in carrageenan or saline-treated animals.

Lamina V

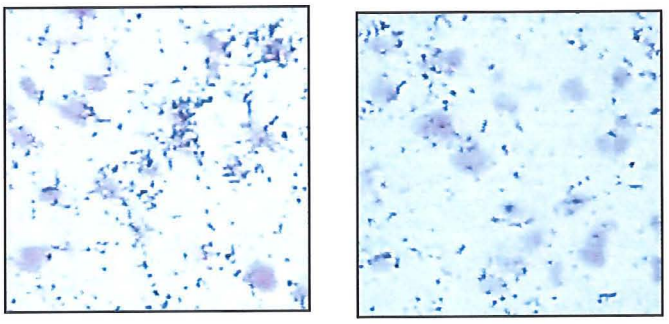
Zif/268 mRNA expression in lamina V in study 3.1 is shown in Figure 3.7.

Figure 3.5: zif/268 mRNA expression

Laminae I/II

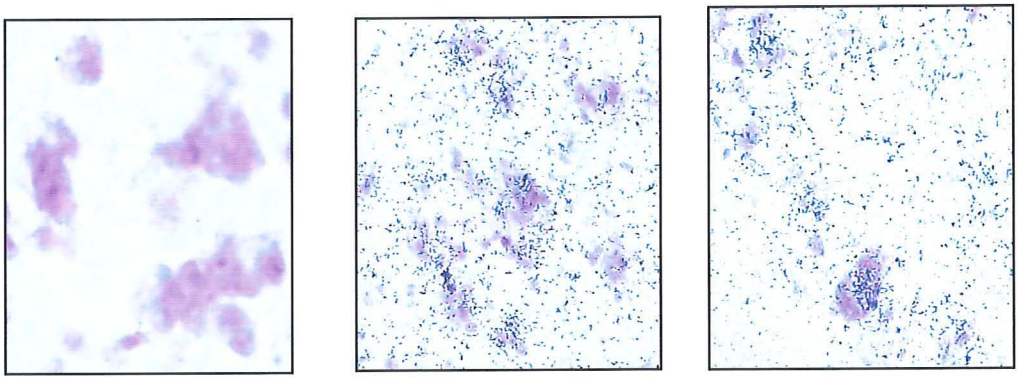


A: control **B**: carrageenan(ipsi) **C**: carrageenan(contra)



D: saline (ipsi) **E**: saline (contra)

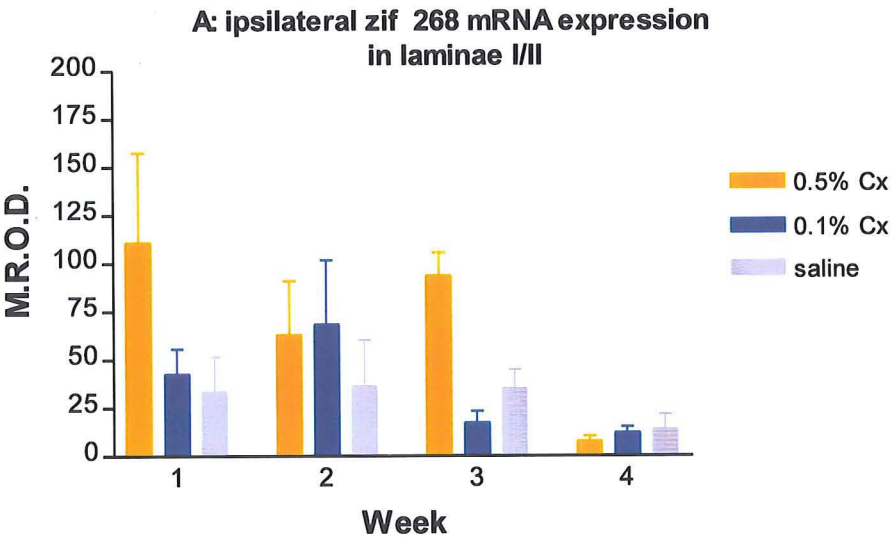
Lamina V



F: control **G**: carrageenan **H**: saline

Figure 3.6: Study 3.1: Zif/268 mRNA expression in laminae I/II

Expression of zif/268 mRNA, expressed as the mean ± SEM mean relative optical density (M.R.O.D./ cell in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% carrageenan (0.1% Cx) or 0.9% saline (n=4-6/group).



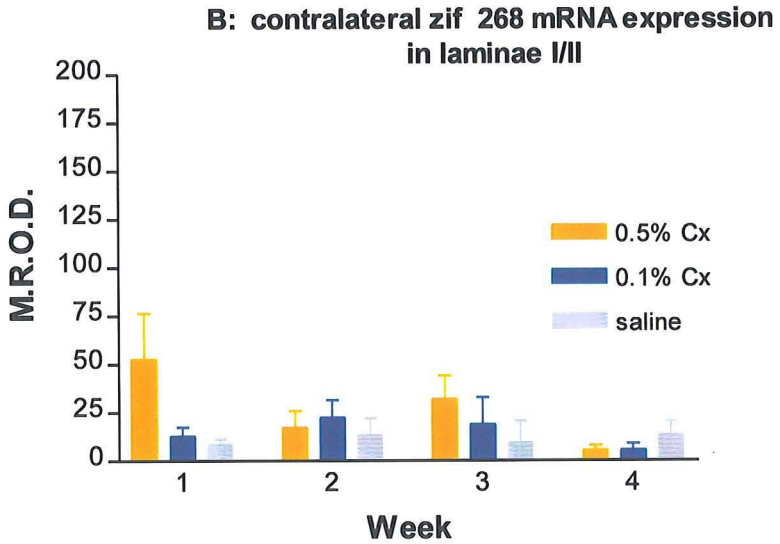
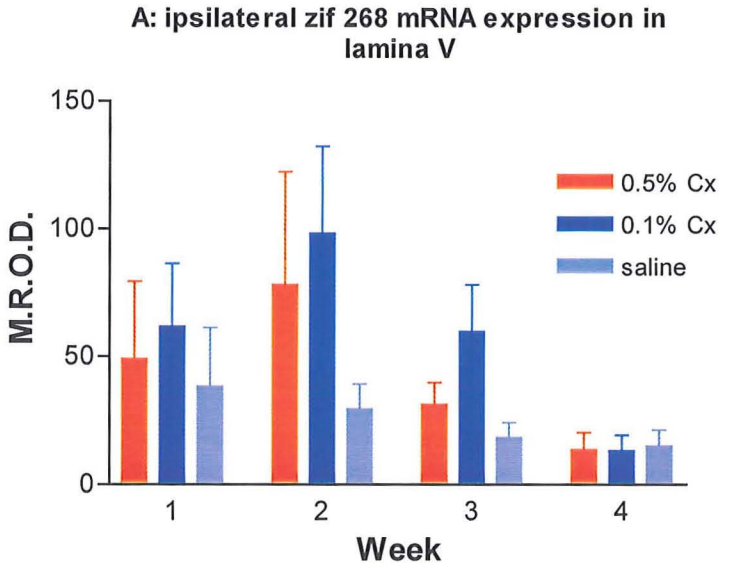
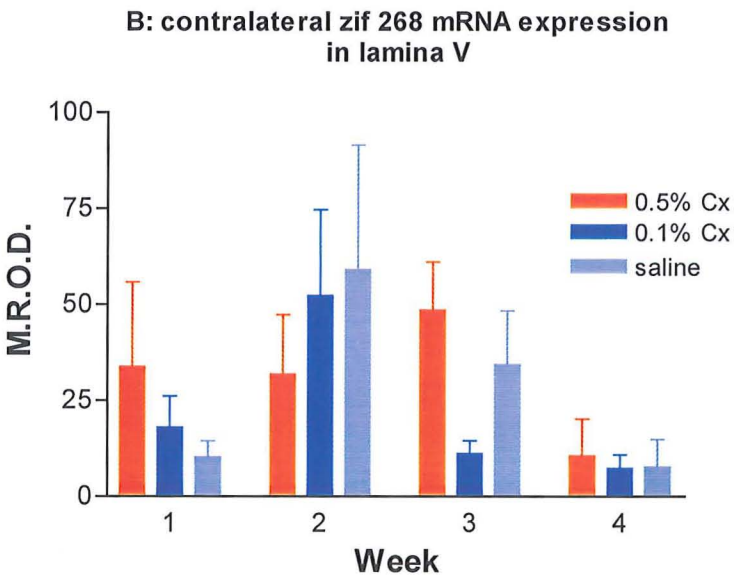


Figure 3.7: ***Study 3.1: Zif/268 mRNA expression in lamina V***

Expression of zif/268 mRNA, expressed as the mean ± SEM mean relative optical density (M.R.O.D./ cell in ipsilateral (A) and contralateral (B) lamina V of lumbar spinal cord , measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% carrageenan (0.1% Cx) or 0.9% saline (n=4-6/group). following intraplantar injection of 50 µl of 0.5% carrageenan (Cx), 0.1% Cx or 0.9% saline.





Treatment effect

There was no treatment effect on the intensity of ipsilateral or contralateral zif/268 mRNA expression on any week of the study.

Ipsilateral/contralateral effect

Zif/268 mRNA expression was upregulated in the ipsilateral cord (relative to the contralateral cord) in animals treated with 0.1% carrageenan ($F[1,27] = 7.23$, $p < 0.01$), although more detailed analysis did not indicate any particular week on which this effect was observed. Zif/268 mRNA expression was not upregulated in the ipsilateral cord (relative to the contralateral cord) observed in 0.5% carrageenan or saline-treated animals.

Effect of repeated treatment

Weekly repeated treatment did not alter ipsilateral or contralateral Zif268 mRNA expression in lamina V in any treatment group.

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

Zif/268 mRNA expression in laminae I/II in study3.2 is shown in Figure 3.8.

Treatment effect

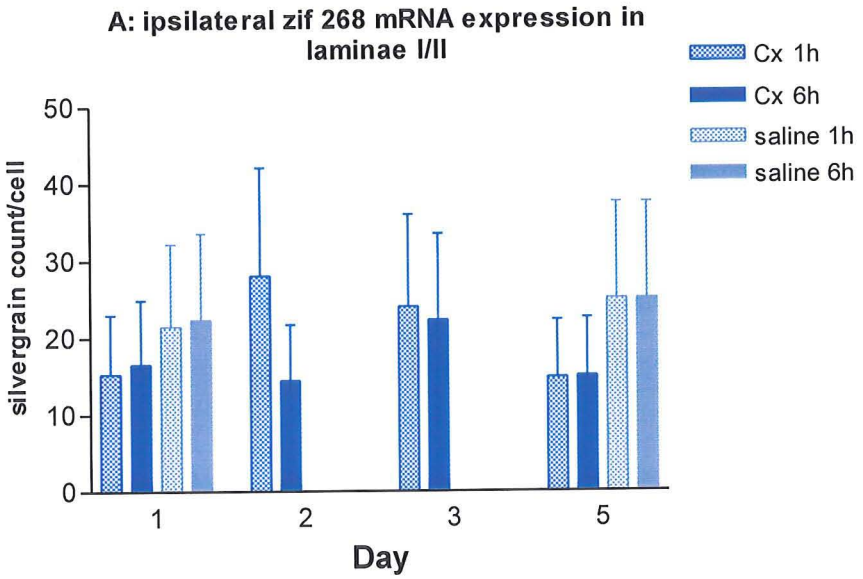
There was no significant effect of treatment on ipsilateral or contralateral zif/268 mRNA expression at 1 h p.i. or 6 h p.i. on day 1 or day 5. In carrageenan-treated animals, contralateral zif/268 mRNA expression was significantly enhanced at 1 h p.i. relative to 6 h p.i. on day 2 ($F[3,7] = 17.39$, $p < 0.05$) but this effect was not observed on any other day.

Ipsilateral/contralateral effect

In carrageenan-treated animals, ipsilateral zif/268 mRNA expression was upregulated (relative to contralateral expression) at 1h p.i. on day 1 ($F[1,3] = 32.17$, $p <0.01$) and day 3 ($F [1,3] = 14.27$, $p < 0.05$), and was close to significance on day 2 ($F [1,3] = 8.67$, $p = 0.06$) but not on day 5 ($F[1,3] = 3.79$, $p= 0.15$. Ipsilateral zif/268 mRNA expression was upregulated (relative to contralateral expression) at 6 h p.i. expression on day 2 ($F[1,3] = 8.42$, $p <0.05$), day 3 ($F[1,3] =34.28$, $p < 0.001$) and day 5 ($F[1,3] = 29.34$, $p < 0.01$), and was close to significance on day 1($F [1,3] = 5.37$, $p = 0.06$).

Figure 3.8: Study 3.2: Zif 268 mRNA expression in laminae I/II

Expression of zif/268 mRNA, expressed as the mean ± SEM mean silvergrain count/cell of silver-grain expression in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord, measured in animals euthanased 1 h or 6 h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n = 4/group).



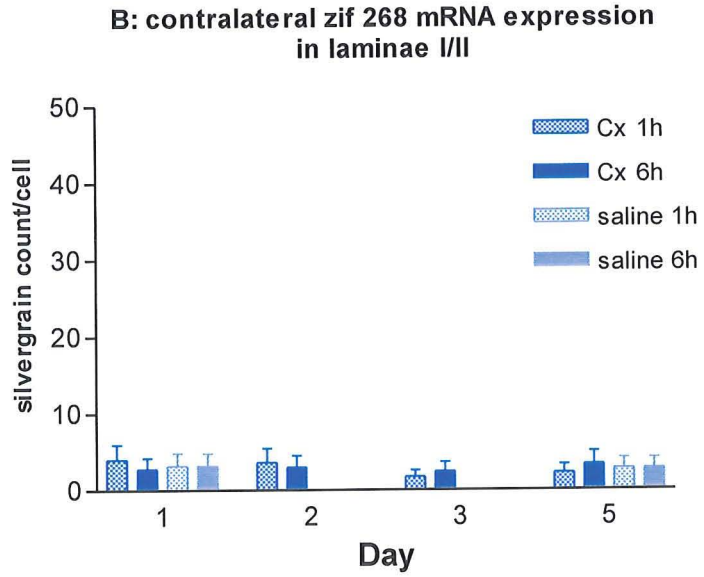
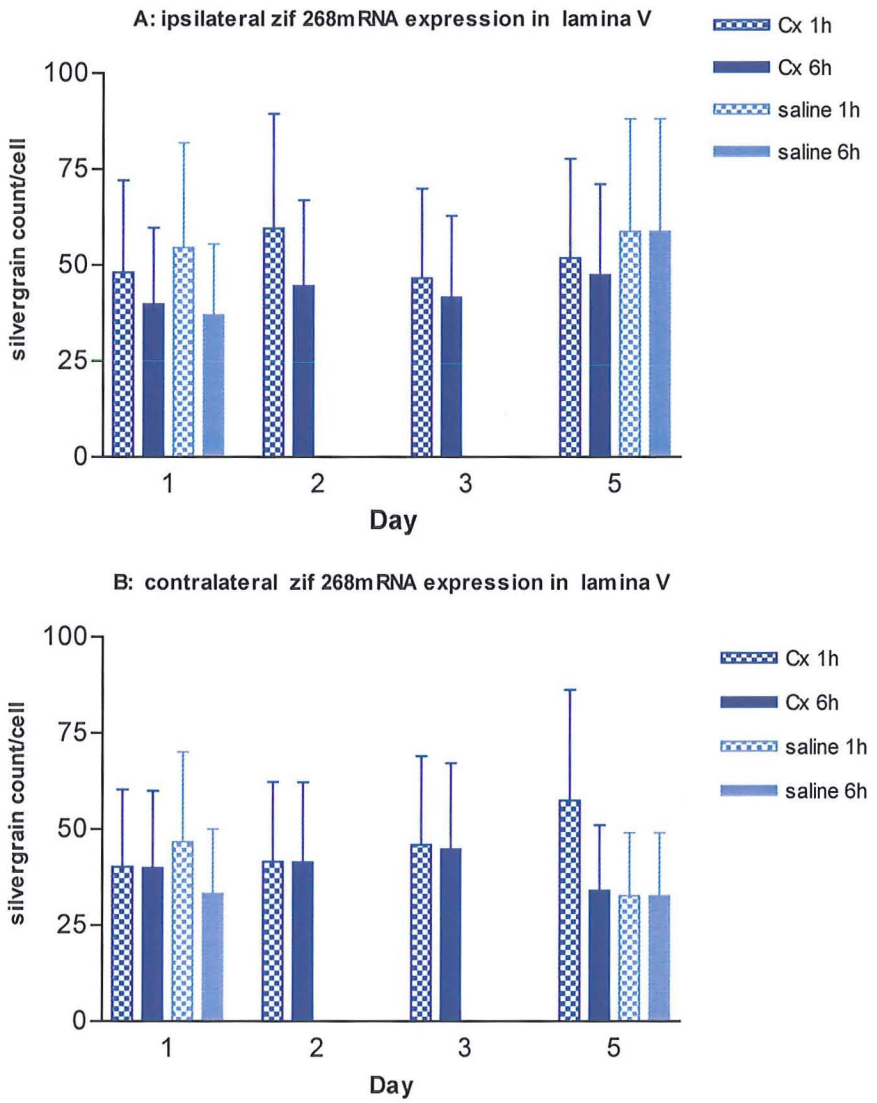


Figure 3.9: Study 3.2: zif/268 mRNA expression in lamina V

Expression of zif/268 mRNA, expressed as the mean ± SEM mean silvergrain count/cell of silver-grain expression in ipsilateral(A) and contralateral(B) lamina V of lumbar spinal cord, measured in animals euthanased 1 h or 6 h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n = 4/group).



In saline treated animals, ipsilateral zif/268 mRNA expression was upregulated (relative to contralateral expression) at 1h p.i. on day 1 ($F[1,3] = 16.87$, $p < 0.05$) and day 5 ($F[1,3] = 15.75$, $p < 0.05$). Ipsilateral zif/268 mRNA expression was also upregulated (relative to contralateral expression) at 6h p.i. on day 1 ($F[1,3] = 108.38$, $p < 0.01$ and day 5 ($F[1,3]=39.12$, $p < 0.01$).

Effect of repeated treatment

In carrageenan treated animals, there was a significant effect of daily repeated treatment on contralateral (but not ipsilateral) zif/268 mRNA expression at 1 h p.i. ($F[3,9] = 5.03$, $p < 0.05$); post-hoc analysis did not indicate a particular day on which this effect occurred although the difference was close to significance on day 1 ($p = 0.07$) and day 3 ($p = 0.07$). At 6h p.i. there was no effect of repeated carrageenan treatment on ipsilateral or contralateral zif/268 mRNA expression. In saline treated animals there was no effect of daily repeated treatment on contralateral zif/268 mRNA expression at 1h p.i. or 6 h p.i. Ipsilateral zif/268 mRNA expression at 1h p.i. was significantly higher on day 5 than day 1 ($F[1,3] = 12.63$, $p < 0.05$). Daily repeated treatment did not alter the intensity of ipsilateral zif/268 mRNA expression at 6h p.i.

Lamina V

Zif/268 mRNA expression in lamina V in study 3.2 is shown in Figure 3.9.

Treatment effect

There was no significant treatment effect on ipsilateral or contralateral zif/268 mRNA expression at 1 h p.i. or 6h p.i. on day 1 or day 5.

Ipsilateral/contralateral effect

In carrageenan treated animals, the intensity of ipsilateral zif/268 mRNA expression did not differ from contralateral expression at 1h p.i. or 6 h p.i. on any day of the study. In saline treated animals, ipsilateral zif/268 mRNA was upregulated (relative to contralateral expression) at 1 h p.i. and 6 h p.i. on day 5 ($F[1,3]=21.08$ and $p < 0.05$ and $F[1,3]=24.09$, $p < 0.05$ respectively) but not on day 1.

Effect of repeated treatment

There was no effect of repeated carrageenan or saline treatment on ipsilateral or contralateral zif/268 mRNA expression at 1h or 6h p.i.

3.3.3. TISSUE PLASMINOGEN ACTIVATOR

Basal tPA mRNA expression

tPA mRNA expression was detected in a very small number of cells in laminae I/II and lamina V. Small numbers of tPA positively labelled cells were observed throughout the grey matter of the lumbar spinal cord and in the hippocampus. The absence of tPA mRNA expression in tissue sections labelled with fifty-times excess unlabelled probe confirmed probe specificity. Cells in laminae I/II expressing tPA mRNA were of uniform 8-15μm diameter. tPA positive cells in laminae V varied in diameter from 8-32μm. There was no significant difference between treatment groups in the diameter of tPA positive cells. TPA labelled cells in the lumbar spinal cord of carrageenan and saline-treated animals are shown in Figure 3.10.

Study 3.1: Weekly repeated carrageenan treatment

Laminae I/II

TPA mRNA expression in laminae I/II in study 3.1 is shown in Figure 3.11.

Treatment effect

Carrageenan treatment (relative to saline treatment) did not alter the number of tPA mRNA positively labelled cells in ipsilateral or contralateral laminae I/II on any week of the study.

Ipsilateral/contralateral effect

In 0.5% carrageenan-treated animals, more tPA positive cells were identified in ipsilateral (relative to contralateral) laminae I/II on week 1 ($F[1,6]=6.76$, $p<0.05$) but not on weeks 2, 3 or 4.

Effect of repeated treatment

Weekly repeated carrageenan or saline treatment did not alter the number of tPA mRNA positively labelled cells in ipsilateral or contralaterallaminae I/II.

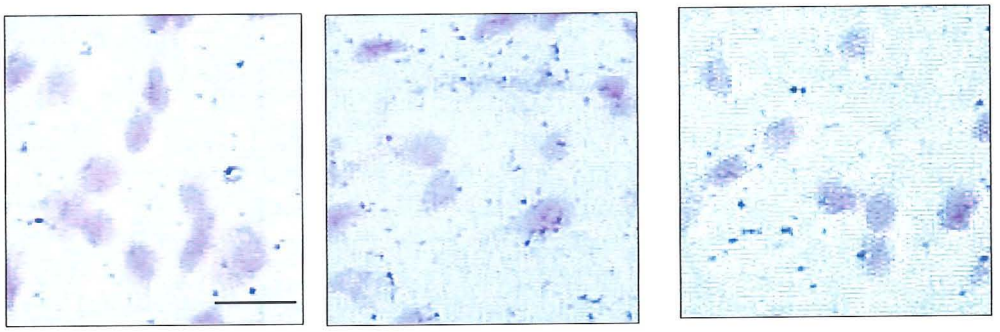
Lamina V

Treatment effect

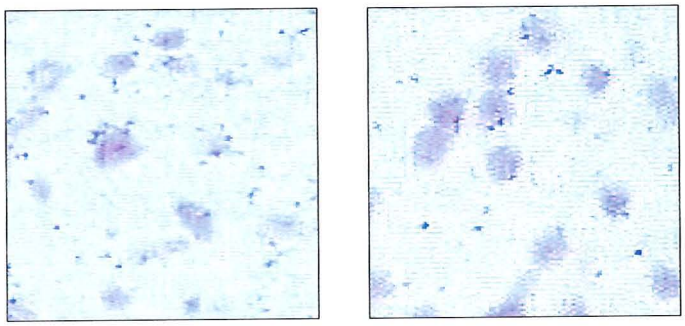
Carrageenan treatment did not alter the tPA mRNA positive cell count (relative to saline treatment) in ipsilateral or contralateral lamina V on any week of the study.

Figure 3.10 tPA mRNA expression

Laminae I/II

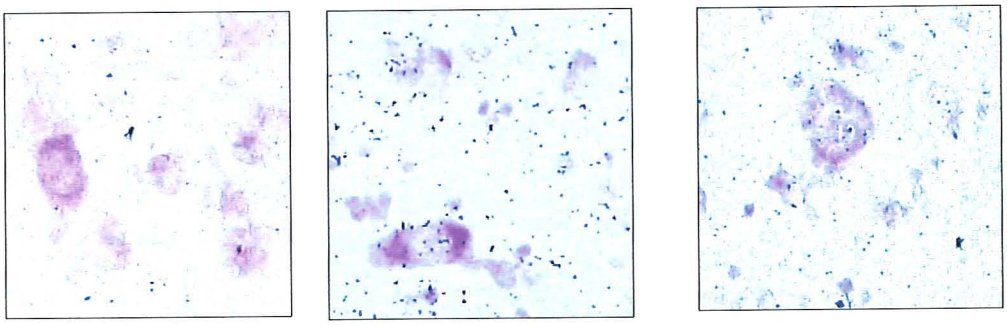


A: control **B:** carrageenan(ipsi) **C:** carrageenan(contra)



D: saline (ipsi) **E:** saline (contra)

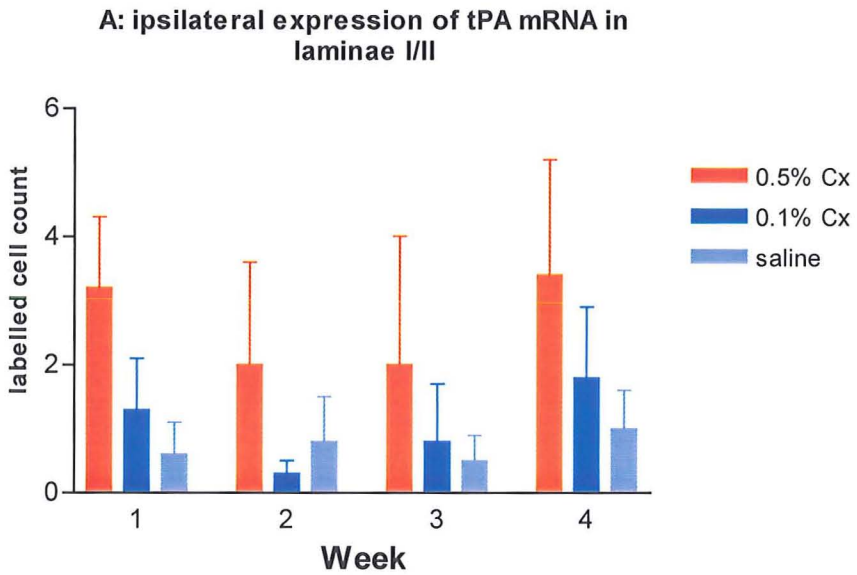
Lamina V

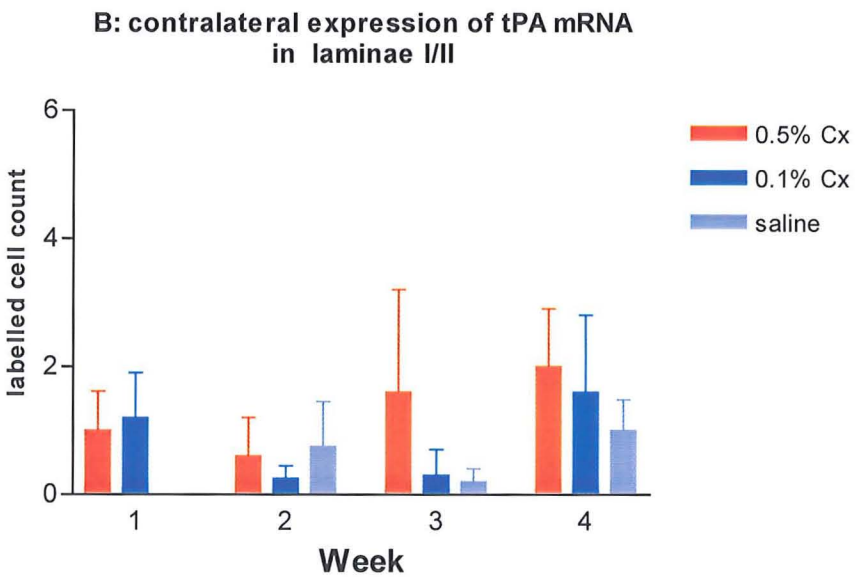


F: control **G**: carrageenan **H**: saline

Figure 3.11: Study 3.1: Expression of tPA mRNA expression in laminae I/II

Expression of tissue plasminogen activator (tPA) mRNA, expressed as the mean ± SEM number of cells expressing tPA in ipsilateral (Graph A) and contralateral (Graph B) laminae I/II of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% carrageenan (0.1% Cx) or 0.9% saline (n = 4-6/group).





Ipsilateral/contralateral effect

Carrageenan and saline treatment did not alter the ipsilateral (relative to contralateral) tPA positive cell count in lamina V on any week of the study.

Effect of repeated treatment

There was no effect of weekly repeated carrageenan or saline treatment on the ipsilateral or contralateral tPA positive cell count.

3.3.4. JUN D

Basal Jun D mRNA expression

Jun D mRNA positive cells were observed throughout the grey matter of the lumbar spinal cord and in the hippocampus. The absence of Jun D mRNA expression in tissue sections labelled with fifty-times excess unlabelled probe confirmed probe specificity. Cells in the superficial laminae (I/ II) expressing Jun D mRNA were of uniform 8-15μm diameter. Jun D positive cells in laminae V varied in diameter from 8-32μm. There was no significant difference between treatment groups in the diameter of Jun D positive cells. JunD 268 labelled cells in the lumbar spinal cord of carrageenan and saline-treated animals are shown in Figure 3.12.

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

Jun D mRNA expression in laminae I/II in study 3.2 is shown in Figure 3.13.

Treatment effect

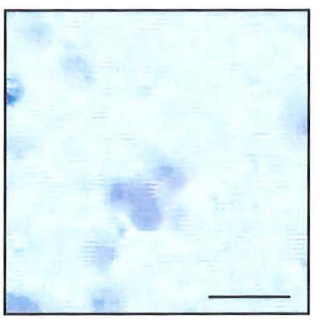
There was no significant treatment effect on the intensity of ipsilateral or contralateral Jun D mRNA expression at 1h p.i. or 6h p.i. on day 1 or day 5.

Ipsilateral/contralateral effect

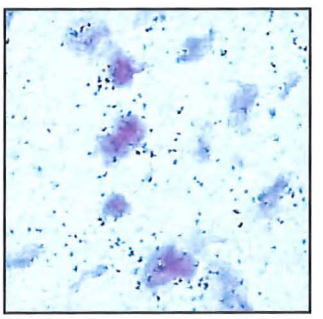
In carrageenan-treated animals, Jun D mRNA was significantly upregulated in ipsilateral (relative to contralateral) laminae I/II at 1h p.i. on day 1 ($F[1,3] = 24.82$, $p < 0.05$), and was close to significance on day 2 ($F[1,3] = 9.28$, $p = 0.06$), day 3 ($F[1,3] = 9.58$, $p = 0.06$) and day 5 ($F[1,3] = 8.75$, $p = 0.06$). At 6h p.i., ipsilateral Jun D mRNA expression was upregulated (relative to contralateral expression) on day 1 ($F[1,3] = 12.48$, $p< 0.05$) and day 5 ($F[1,3] = 11.21$, $p < 0.05$) and was close to significance on days 2 ($F[1,3] = 8.88$, $p =$

Figure 3.12 jun D mRNA expression

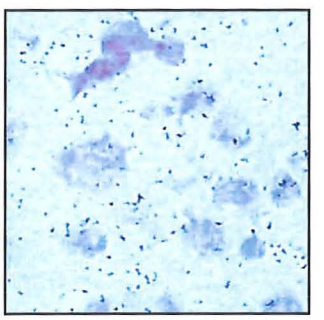
Laminae I/II



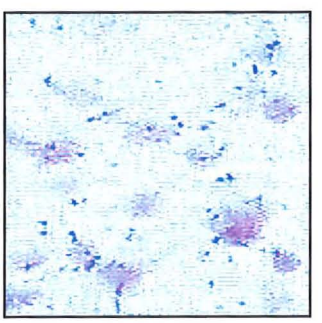
A: control



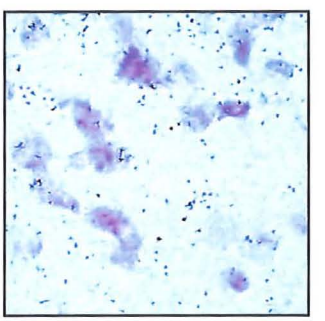
B: carrageenan(ipsi)



C: carrageenan(contra)

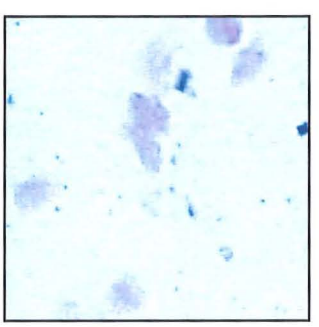


D: saline (ipsi)

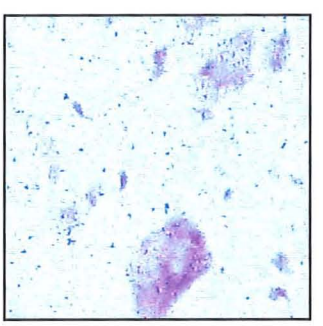


E: saline (contra)

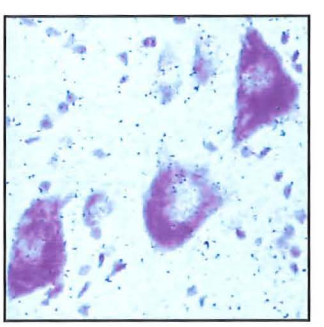
Lamina V



F: control



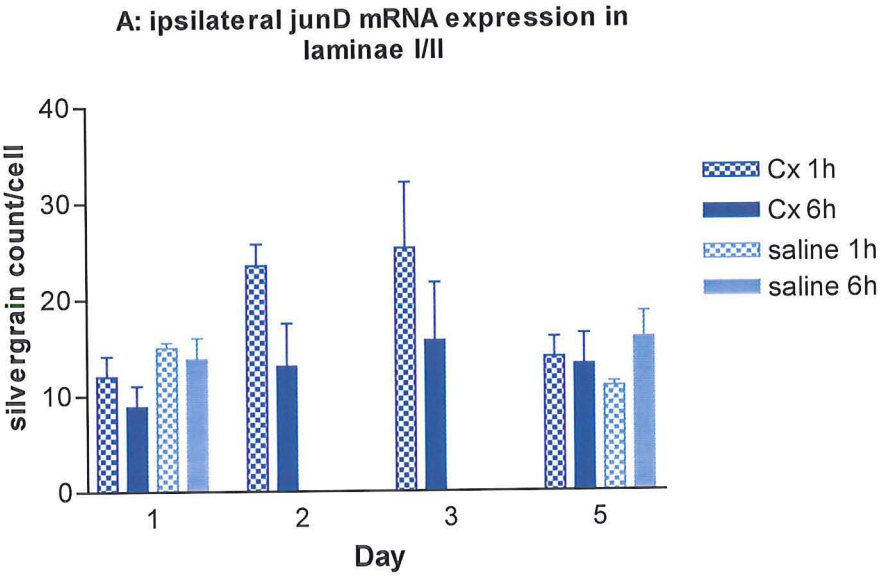
G: carrageenan



H: saline

Figure 3.13: Study 3.2: Jun D mRNA expression in laminae I/II

Expression of Jun D mRNA, expressed as the mean ± SEM mean silvergrain count/cell of silver-grain expression in ipsilateral(A) and contralateral(B) laminae I/II of lumbar spinal cord, measured in animals euthanased 1h or 6 h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n = 4/group).



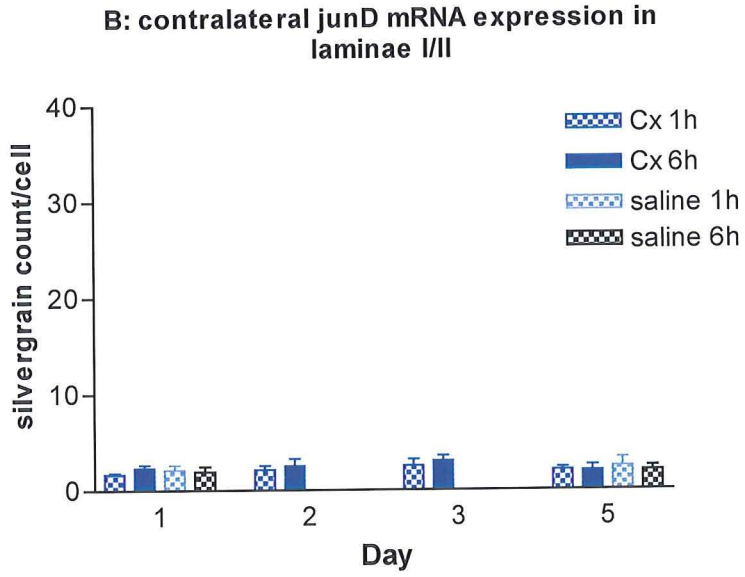
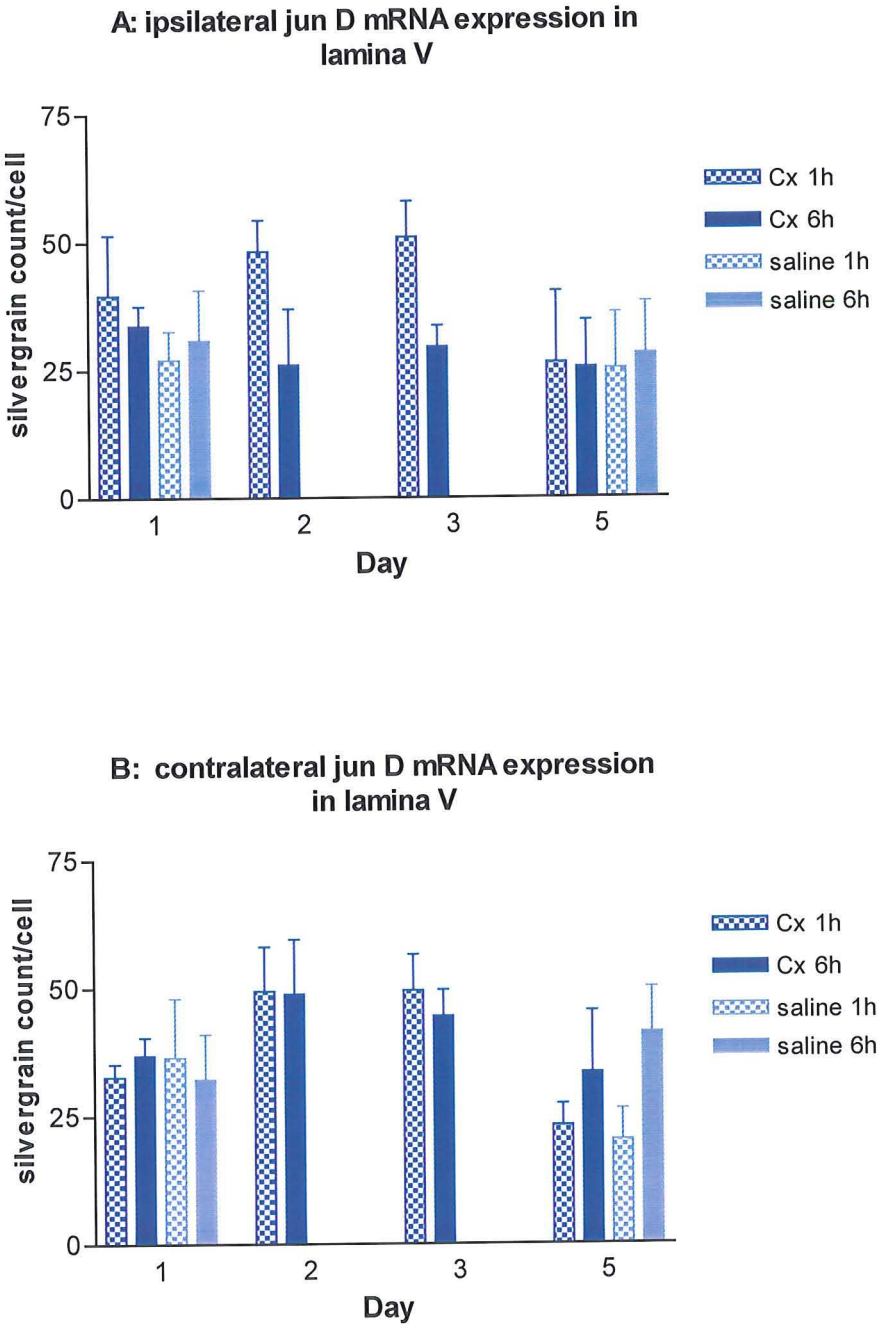


Figure 3.14: Study 3.2: Jun D mRNA expression in lamina V

Expression of jun D mRNA, expressed as the mean ± SEM mean silvergrain count/cell of silver-grain expression in ipsilateral(A) and contralateral(B) lamina V of lumbar spinal cord, measured in animals euthanased 1h or 6 h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n = 4/group).



0.06) and day 3 ($F[1,3] = 7.98$, $p = 0.07$). In saline-treated animals, Jun D mRNA was significantly upregulated in ipsilateral (relative to contralateral) laminae I/II at 1 h p.i. on day 1 ($F[1,3] = 21.2$, $p < 0.02$) and day 5 ($F[1,3] = 42.01$, $p < 0.01$) and also at 6h p.i. on day 1 ($F[1,3] = 26.31$, $p < 0.01$) and day 5 ($F[1,3] = 40.67$, $p < 0.01$).

Lamina V

Jun D mRNA expression in laminae V in study 3.2 is shown in Figure 3.14.

Treatment effect

There was no significant treatment effect on ipsilateral or contralateral Jun D mRNA expression at 1 h p.i. or 6 h p.i. on day 1 or day 5.

Ipsilateral/contralateral effect

Significant downregulation of ipsilateral (relative to contralateral) Jun D mRNA expression was observed in carrageenan-treated animals at 6h p.i. on day 2 ($p < 0.05$) and day 3 ($p < 0.05$). In carrageenan-treated animals at 1h p.i. and in saline-treated animals at 1h p.i. and 6 h p.i., treatment did not alter the intensity of ipsilateral (relative to contralateral) Jun D mRNA expression on any day of the study.

Effect of repeated treatment

There was no effect of repeated treatment on ipsilateral or contralateral Jun D mRNA expression in carrageenan or saline-treated animals at 1h or 6h .

3.3.5. CYCLO-OXYGENASE ENZYMES

Basal COX mRNA expression

Both COX-1 and COX-2 positive cells were observed throughout the grey matter of the lumbar spinal cord and in the hippocampus but not in other brain regions, including thalamus and hypothalamus. COX-1 labelled cells in the lumbar spinal cord are shown in fugure 3.15. COX-2 labelled cells in the lumbar spinal cord are shown in figure 3.19.

The absence of COX-1 or COX-2 mRNA expression in tissue sections labelled with fifty-times excess unlabelled probe further confirmed probe specificity for COX-1 and COX-2 mRNA. Cells in the superficial laminae (laminae I and II) expressing COX isoenzyme mRNA were of uniform diameter, range 8-15μm. There was greater variation in the diameter of cells expressing COX-1 and COX-2 mRNA in laminae V, from 8- 32μm

diameter. There was no significant difference between treatment groups in the diameter of the cell population expressing mRNA for either enzyme.

3.3.5.1. COX-1

Laminae I/II

COX-1 mRNA expression in laminae I/II in study 3.1 is shown in Figure 3.16.

Treatment effect

Carrageenan treatment did not alter the intensity of ipsilateral COX-1 mRNA expression relative to saline treatment. There was a significant treatment effect on contralateral COX-1 mRNA expression ($F[2,43] = 3.6$, $p < 0.05$), where 0.5% Cx induced significantly greater expression than saline treatment ($p < 0.05$), however this was not associated with any individual week of the study.

Ipsilateral/contralateral effect

There was significant ipsilateral (relative to contralateral) upregulation of COX-1 mRNA in all treatment groups: 0.5% Cx ($F[1,29] = 5.22$, $p < 0.05$, 0.1% Cx ($F[1,27] = 13.74$, $p < 0.001$ and saline ($F[1,29] = 77.00$, $p < 0.01$), however this was not associated with any individual week of the study.

Effect of weekly repeated treatment

W eekly repeated treatment did not alter COX-1 mRNA expression.

Lamina V

In lamina V, there was no significant treatment effect, no significant ipsilateral/ contralateral effect and no effect of repeated treatment on COX-1 mRNA expression.

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

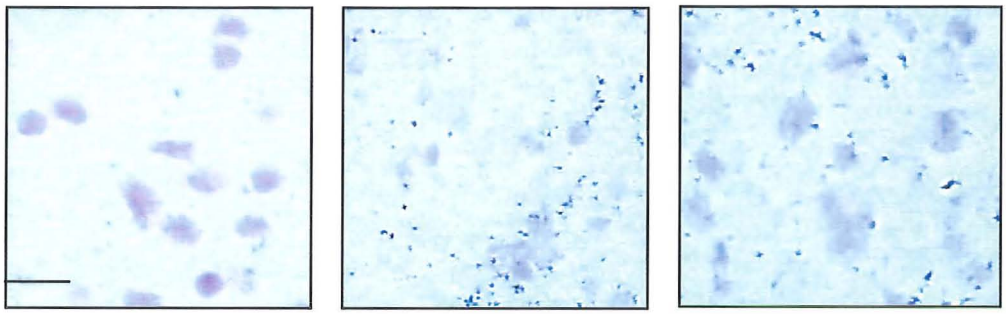
COX-1 mRNA expression in lamina I/II in study 3.2 is shown in Figure 3.17.

Treatment effect

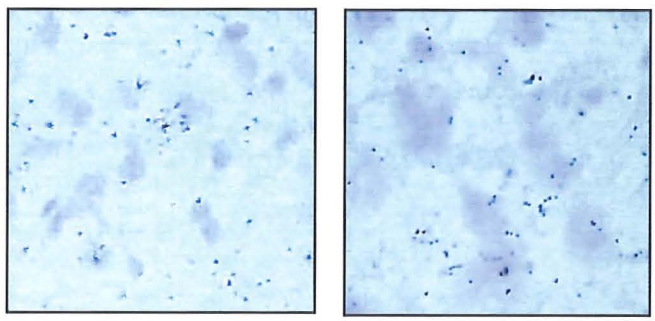
There was no significant treatment effect on ipsilateral or contralateral COX-1 mRNA expression on day 1 or day 5.

Figure 3.15 COX-1 mRNA expression

Laminae I/II

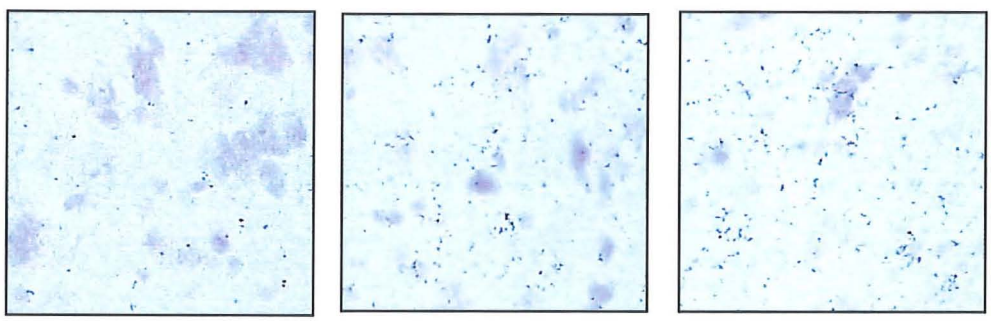


A: control **B**: carrageenan(ipsi) **C**: carrageenan(contra)



D: saline (ipsi) **E**: saline (contra)

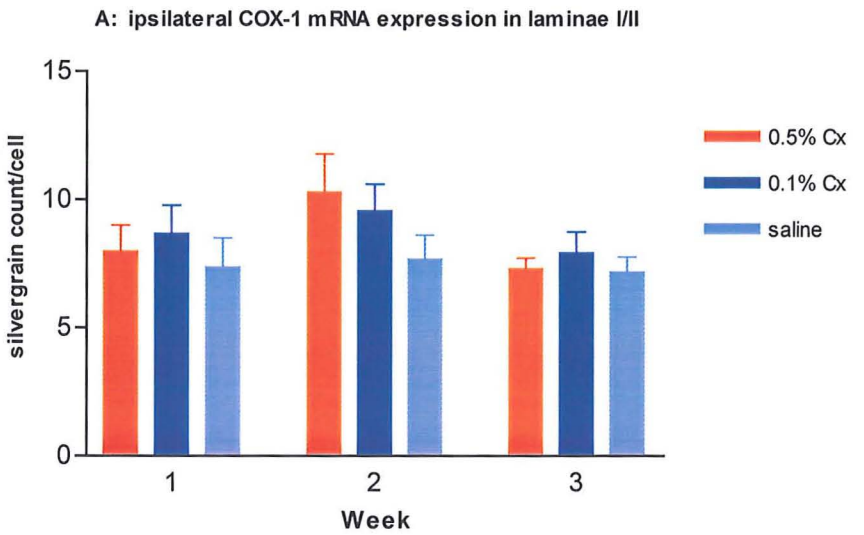
Lamina V



F: control **G**: carrageenan **H**: saline

Figure 3.16: Study 3.1: COX-1 mRNA expression in laminae I/II

Expression of COX-1 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (ipsi) and contralateral (contra) laminae I/II of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% carrageenan (0.1% Cx) or 0.9% saline (n=4-6/group). COX-1 mRNA expression in ipsilateral laminae I/II was significantly greater in 0.5% carrageenan-treated animals (relative to saline-treated animals) on week 2 of the study ($p < 0.05$).



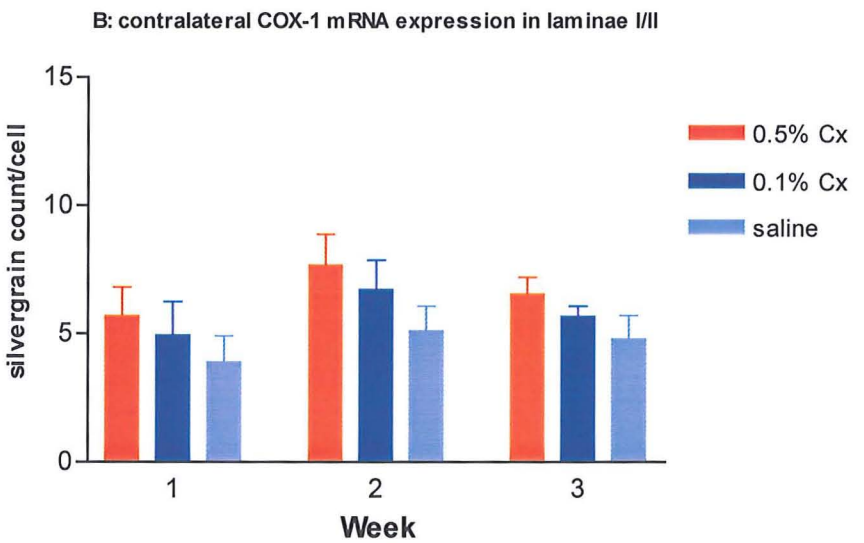
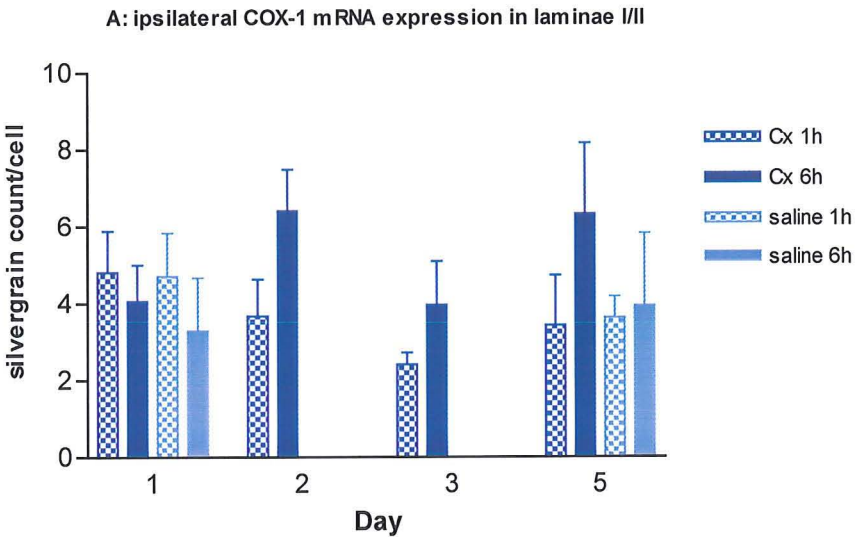


Figure 3.17: Study 3.2: COX-1 mRNA expression in laminae I/II

Expression of COX-1 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord, measured in animals euthanased 1h and 6h following injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/ group).



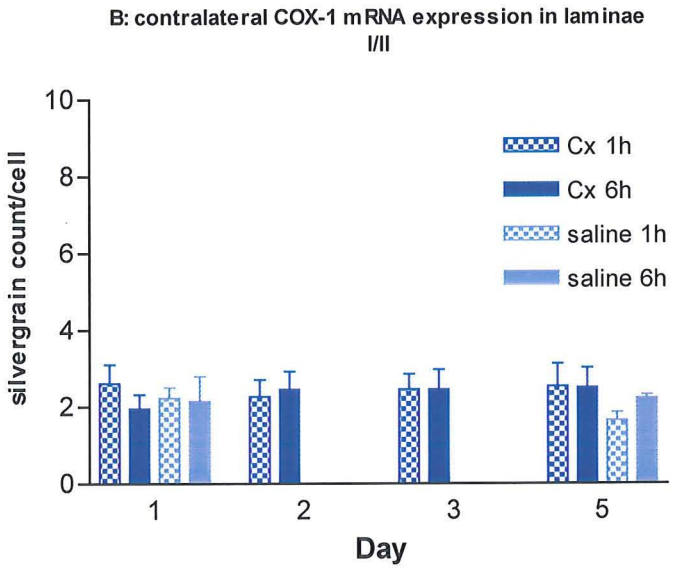
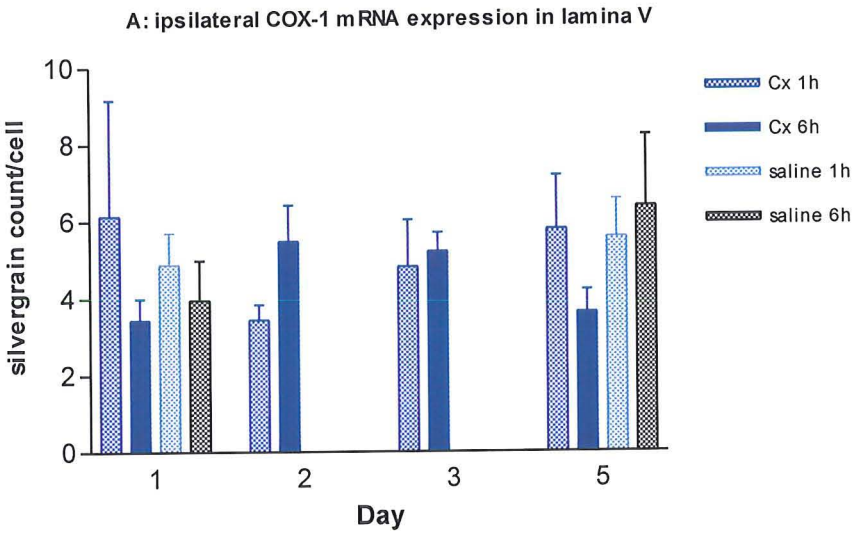
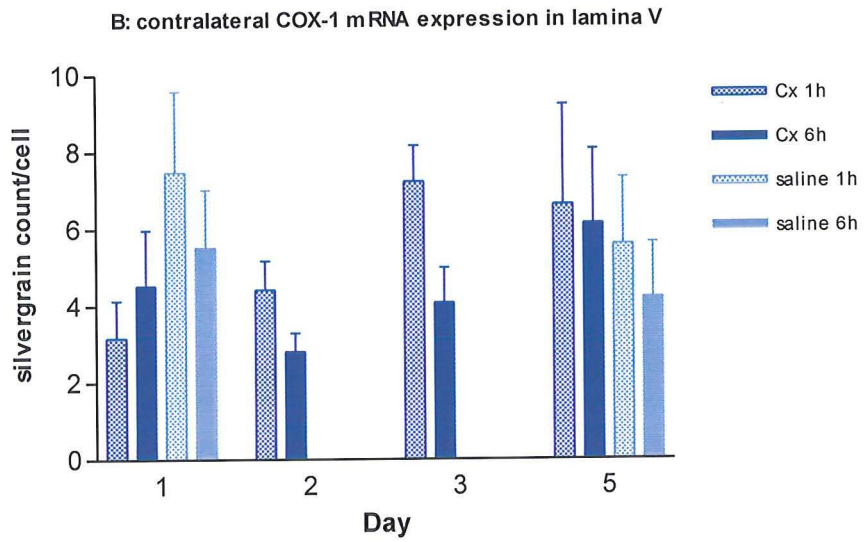


Figure 3.18: Study 3.2: COX-1 mRNA expression in lamina V

Expression of COX-1 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) lamina V of lumbar spinal cord, measured in animals euthanased 1h and 6h following injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/ group).





Ipsilateral/contralateral effect

Carrageenan treatment did not alter the intensity of ipsilateral (relative to contralateral) COX-1 mRNA expression at 1 h p.i. or 6 h p.i. on any day of the study, although close to significance at 6h p.i. on day 2 ($F [1,3] = 9.71$ $p = 0.053$). Saline treatment did not alter the intensity of ipsilateral (relative to contralateral) COX-1 mRNA expression at 1 h p.i. or 6h p.i. on any day of the study.

Effect of repeated treatment

Daily repeated treatment did not alter the intensity of ipsilateral or contralateral COX-1 mRNA expression in carrageenan or saline-treated animals.

Lamina V

COX-1 mRNA expression in lamina V in study 3.2 is shown in Figure 3.18.

Treatment effect

On day 1 and day 5, carrageenan treatment did not alter the intensity of ipsilateral or contralateral COX-1 mRNA expression relative to saline treatment. In both carrageenan and saline-treated animals, ipsilateral and contralateral COX-1 mRNA expression at 6h p.i. were not significantly different from that observed at 1 h p.i.

In carrageenan-treated animals, contralateral (but not ipsilateral) COX-1 mRNA expression was significantly higher at 1h p.i. than at 6h p.i. on day 2 ($F[1, 3] = 18.00$, $p< 0.05$) and day 3 ($F[1,3] = 26.2$, $p < 0.01$).

Ipsilateral/contralateral effect

Carrageenan treatment did not alter the intensity of ipsilateral (relative to contralateral) COX-1 mRNA expression at 1h p.i. or 6h p.i. on any day of the study. Saline treatment did not alter the intensity of ipsilateral (relative to contralateral) COX-1 mRNA expression at 1 h p.i. or 6 h p.i. on day 1 or day 5 of the study.

Effect of repeated treatment

Daily repeated treatment did not alter the intensity of ipsilateral or contralateral COX-1 mRNA expression in carrageenan or saline-treated animals.

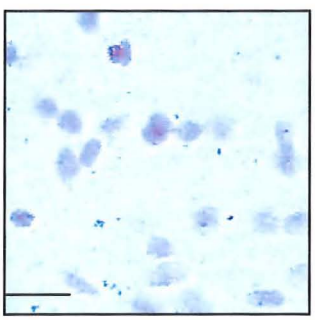
3.3.5.2. COX-2

Laminae I/II

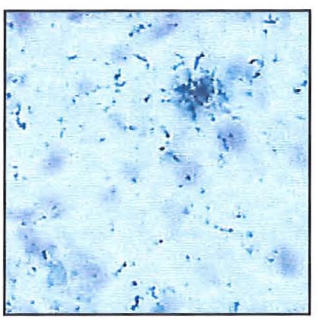
COX-II mRNA expression in laminae I/II in study 3.1 is shown in Figure 3.20.

Figure 3.19 COX-2 mRNA expression

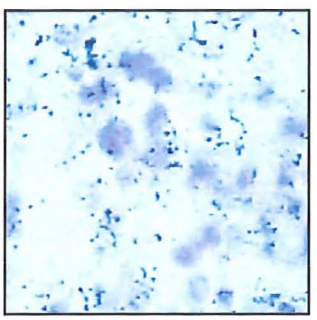
Laminae I/II



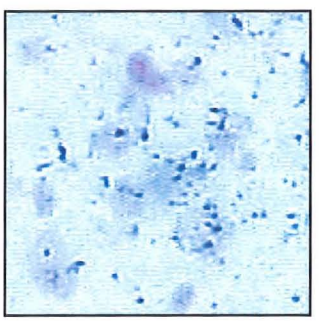
A: control



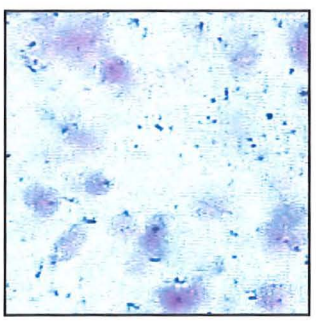
B: carrageenan(ipsi)



C: carrageenan(contra)

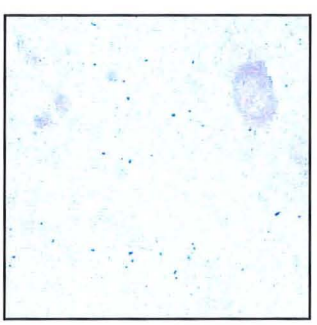


D: saline (ipsi)

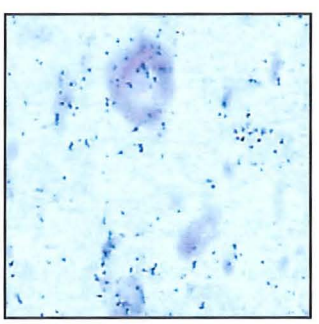


E: saline (contra)

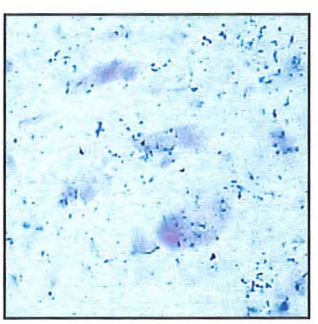
Lamina V



F: control



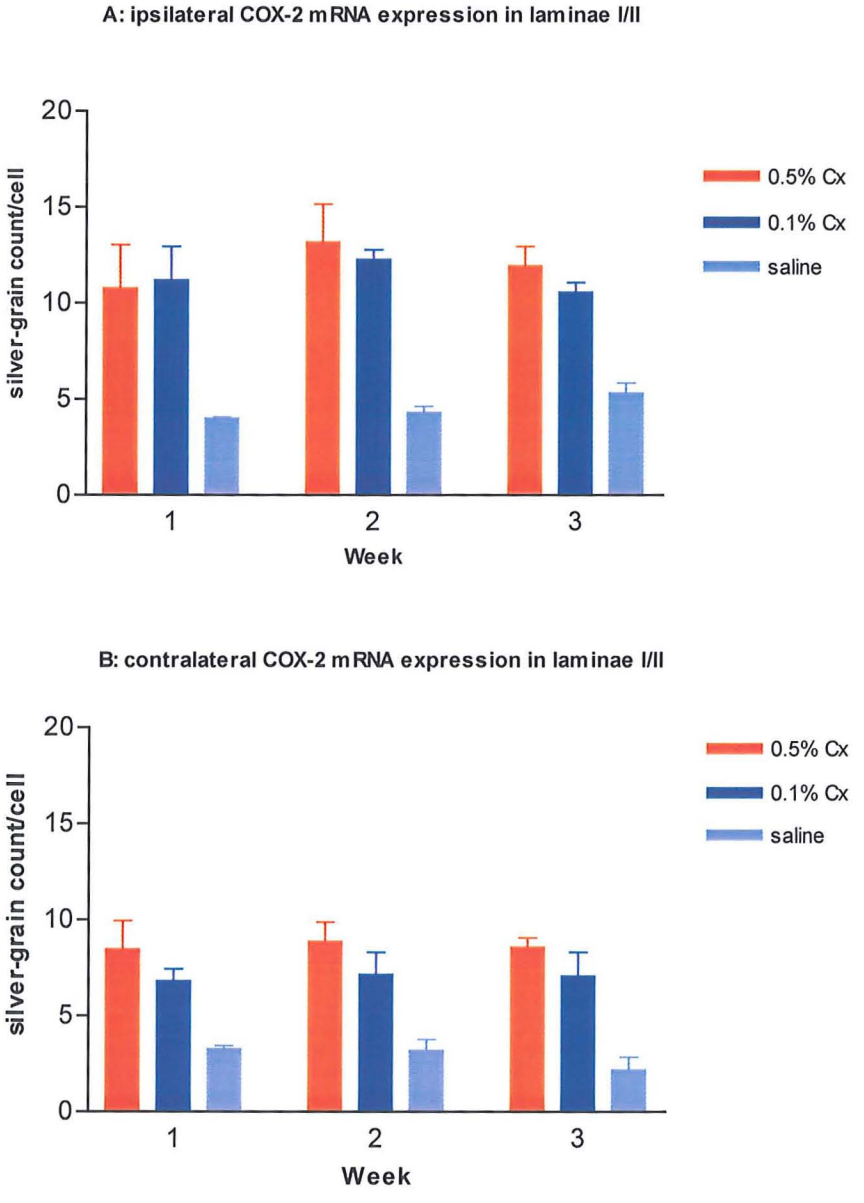
G: carrageenan



H: saline

Figure 3.20: Study 3.1: COX-2 mRNA expression in laminae I/II

Expression of COX-2 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (ipsi) and contralateral (contra) laminae I/II of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% carrageenan (0.1% Cx) or 0.9% saline (n=4-6/group). Both ipsilateral and contralateral COX-2 mRNA expression were significantly greater in carrageenan-treated animals (relative to saline-treated animals) on all three weeks of the study.



Treatment effect

A significant treatment effect on COX-2 mRNA expression was observed in both ipsilateral and contralateral laminae I/II ($F[2,43] = 43.9$, $p < 0.001$) and $F[2,43] = 36.4$, $p < 0.001$ respectively). Ipsilateral COX-2 mRNA expression was significantly higher in carrageenan-treated animals (both 0.5% and 0.1%) than saline-treated animals ($p < 0.001$ for both groups), but there was no significant difference in COX-2 mRNA expression in 0.5% and 0.1% carrageenan-treated animals. Detailed week-by-week analysis indicated carrageenan treatment induced significantly greater ipsilateral COX-2 mRNA expression than saline treatment on all 3 weeks of the study [$p < 0.05$ on week 1, $p < 0.001$ on weeks 2 and 3 in 0.5% Cx-treated animals, $p < 0.05$ on week 1, $p < 0.01$ on week 2 and $p < 0.001$ on week 3 in 0.1% Cx-treated animals). Contralateral COX-2 mRNA expression was also significantly higher in carrageenan-treated animals (both 0.5% and 0.1%) than saline-treated animals ($p < 0.001$ for both groups), while there was no significant difference in the level of COX-2 mRNA expression in 0.5% Cx and 0.1% Cx-treated animals .

Ipsilateral/contralateral effect

GLM analysis indicated significant ipsilateral (relative to the contralateral) upregulation of COX-2 mRNA expression in all treatment groups (0.5% Cx, $F[1,29] = 8.19$, $p < 0.01$); 0.1% Cx, $F[1,27] = 35.4$, $p < 0.001$); saline, $F[1,27] = 14.72$, $p < 0.001$). More detailed analysis did not indicate significant ipsilateral upregulation of ipsilateral (relative to contralateral) COX-2 mRNA expression in saline-treated animals on any particular week of the study, while in both 0.5% Cx and 0.1% Cx-treated animals, significant ipsilateral upregulation of ipsilateral (relative to contralateral) COX-2 mRNA was observed on weeks 1, 2 and 3 ($p < 0.05$ on each week).

Effect of weekly repeated treatment

Weekly repeated treatment did not alter ipsilateral or contralateral COX-2 mRNA expression.

Lamina V

In lamina V, there was no significant treatment effect, no significant ipsilateral/ contralateral effect and no effect of repeated treatment on COX-2 mRNA expression.

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

COX-II mRNA expression in laminae I/II in study 3.2 is shown in Figure 3.21.

Figure 3.21: Study 3.2: COX-2 mRNA expression in laminae I/II

Expression of COX-2 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord measured in animals euthanased 1h and 6h following injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/ group).

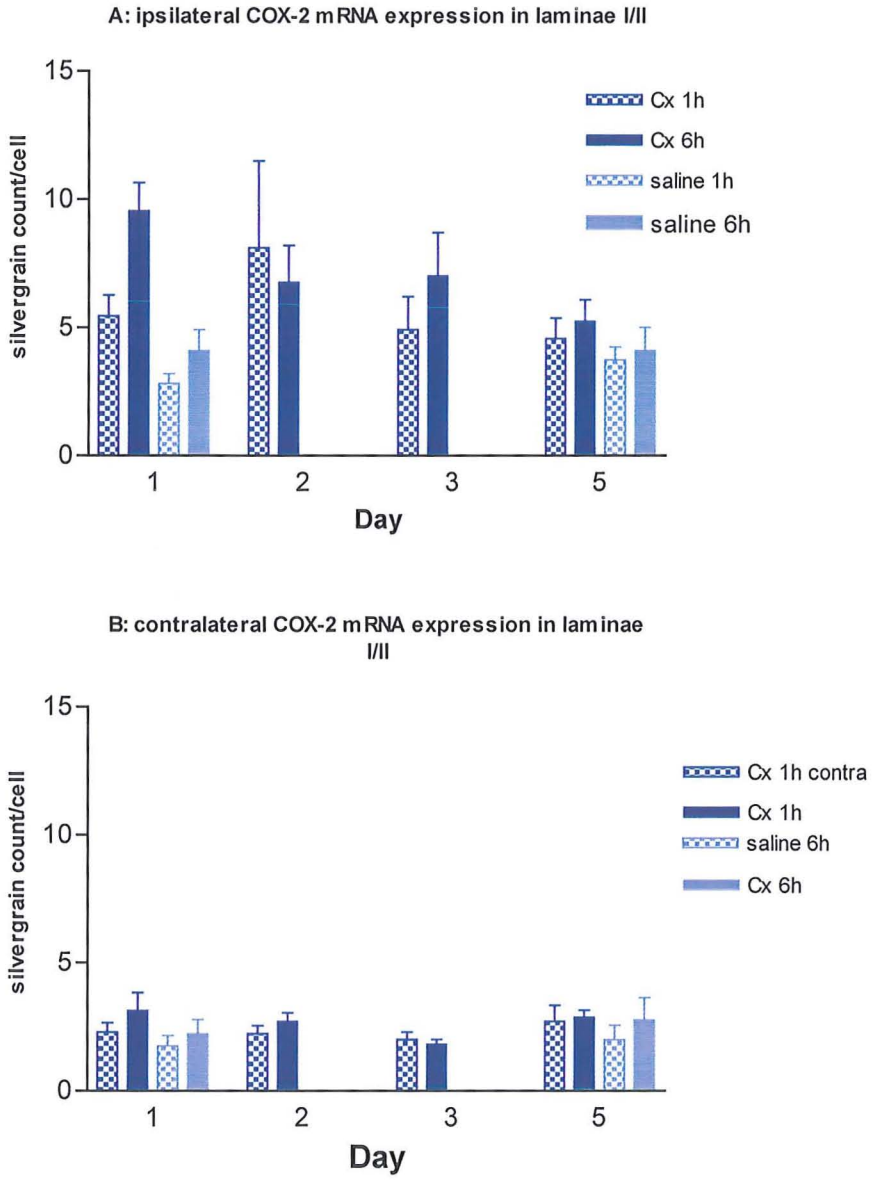
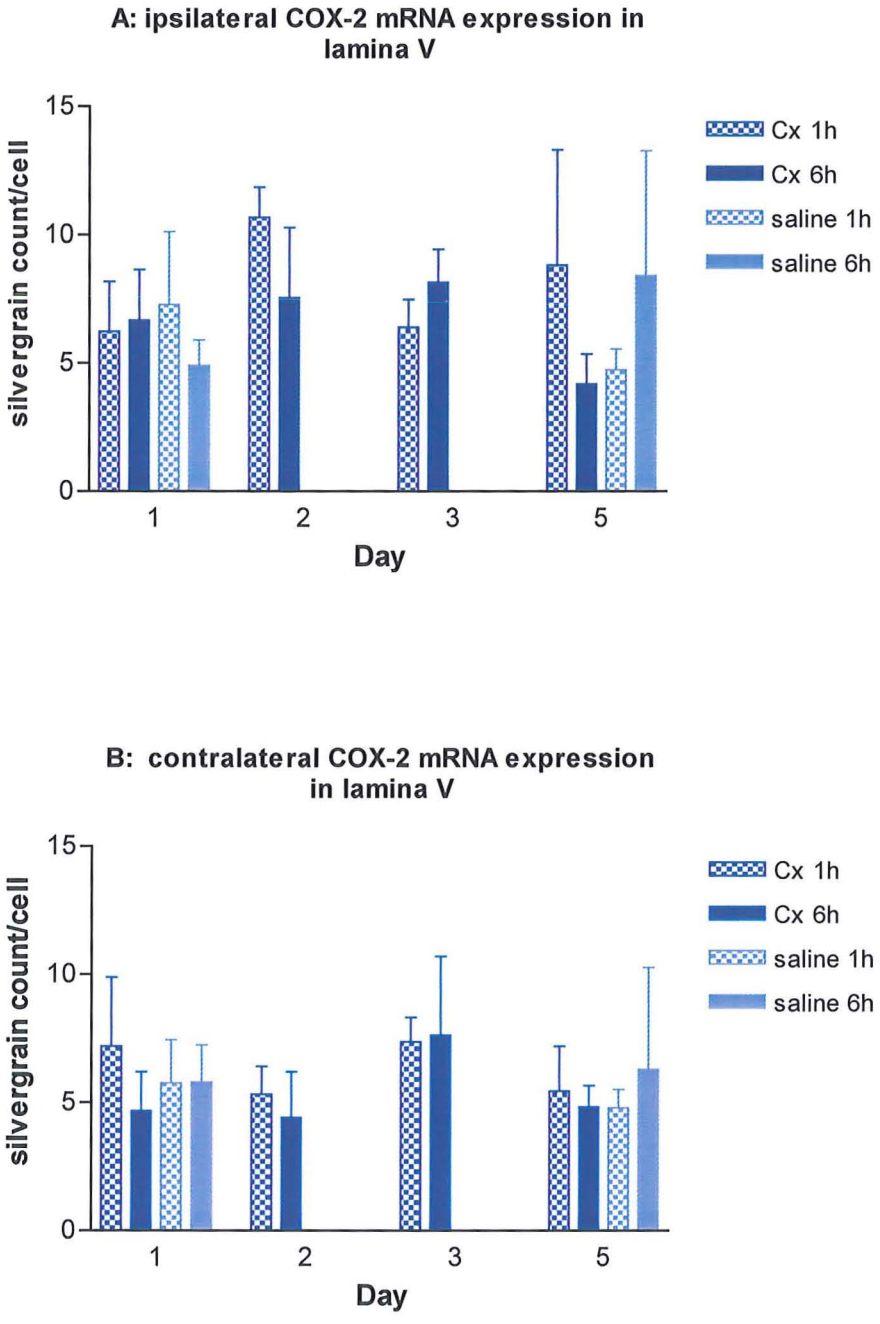


Figure 3.22: Study 3.2: COX-2 mRNA expression in lamina V

Expression of COX-2 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral(A) and contralateral(B) lamina V of lumbar spinal cord measured in animals euthanased 1h and 6h following injection of 50 µl of 0.1% carrageenan or 0.9% saline (n = 4/ group).



Treatment effect

On day 1, ipsilateral COX-2 mRNA expression was significantly upregulated in carrageenan-treated animals (relative to saline-treated animals) at 1 h p.i. ($p < 0.01$) and at 6 h p.i. ($p < 0.01$). Ipsilateral COX-2 mRNA expression was significantly higher at 6h p.i. than at 1hp.i. on day 1 ($p< 0.05$). On day 5, ipsilateral COX-2 mRNA expression was not significantly upregulated in carrageenan-treated animals (relative to saline-treated animals) at 1 h p.i. or 6 h p.i.

On days 2 and 3, the intensity of ipsilateral and contralateral COX-2 mRNA expression at 6h p.i. was not significantly different from that observed at 1h p.i.

Ipsilateral/contralateral effect

In carrageenan-treated animals, ipsilateral COX-2 mRNA expression was upregulated (relative to contralateral mRNA expression) at 1 h p.i. on day 2 ($F [1,3] = 26.94$, $p < 0.02$) and was close to significance on day 1 and day 3. At 6h p.i., ipsilateral COX-2 mRNA expression was upregulated (relative to contralateral mRNA expression) on day 1 $F[1,3] = 45.05$, $p < 0.01$, but not on any other day, although close to significance on day 3 ($F[1,3] = 8.92$, $p = 0.058$) and day 5 ($F[1,3] = 7.9$, $p = 0.067$). In saline-treated animals ipsilateral COX-2 mRNA expression was upregulated (relative to contralateral mRNA expression)at 1h p.i. on day 1($F [1,3] =30.3$, $p <0.01$) but not on day 5. At 6h p.i., ipsilateral COX-2 mRNA expression was upregulated (relative to contralateral) on day 1 ($F [1,3] = 10.64$, $p < 0.05$) but not on day 5.

Effect of repeated treatment

There was no effect of daily repeated treatment on ipsilateral or contralateral COX-2 mRNA expression in any treatment group.

Lamina V

COX-2 mRNA expression in lamina V in study 3.2 is shown in Figure 3.22.

Treatment effect

There was no effect of treatment on ipsilateral or contralateral COX-2 mRNA expression on day 1 or day 5.

Ipsilateral/contralateral effect

In carrageenan-treated animals, COX-2 mRNA was significantly upregulated in ipsilateral (relative to contralateral) lamina V at 1h p.i. on day 2 ($F[1,3] = 14.74$, $p < 0.05$) but not on

any other day. COX-2 mRNA was not significantly upregulated in ipsilateral (relative to contralateral) lamina V at 6 h p.i. on any day of the study.

In saline-treated animals, COX-2 mRNA was not significantly upregulated in ipsilateral (relative to contralateral) lamina V at 1 h p.i. or 6 h p.i. on any day of the study.

Effect of repeated treatment

There was no effect of daily repeated carrageenan or saline treatment on ipsilateral or contralateral COX-2 mRNA expression at 1h or 6h p.i. on any day of the study.

3.3.6. PROENKEPHALIN

Basal proenkephalin mRNA expression

Preliminary studies of proenkephalin mRNA expression in spinal cord tissue taken from untreated control animals demonstrated constitutive expression of proenkephalin mRNA in spinal cord grey matter. Proenkephalin mRNA positive cells were observed in superficial laminae and elsewhere in the dorsal horn of the lumbar spinal cord, and in the hippocampus. The absence of proenkephalin mRNA expression in tissue sections labelled with fifty-times excess unlabelled probe confirmed probe specificity. Cells in the superficial laminae (I/II) expressing proenkephalin mRNA were of uniform 8-15μm diameter, while positively-labelled cells in laminae V varied in diameter from 8- 32μm. There was no significant difference between treatment groups in the diameter of proenkephalin positive cells. Proenkephalin labelled cells in the lumbar spinal cord are shown in figure 3.23.

Study 3.1: Weekly repeated carrageenan treatment

Laminae I/II

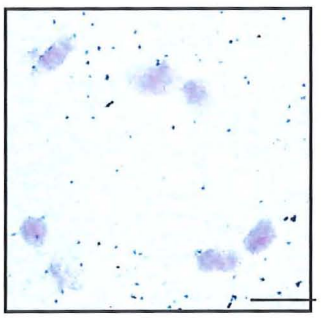
Proenkephalin mRNA expression in laminae I/II in study 3.1 is shown in Figure 3.24.

Treatment effect

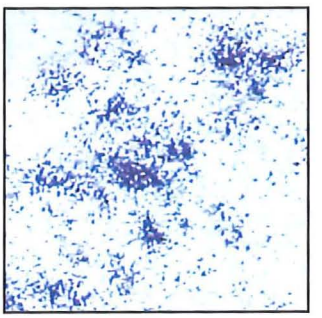
There was a significant treatment effect on proenkephalin mRNA expression in ipsilateral superficial laminae ($F [2,51] = 5.44$, $p < 0.01$). Proenkephalin mRNA expression was significantly upregulated in 0.5% carrageenan-treated animals relative to saline-treated animals, ($p < 0.01$), but not relative to 0.1% carrageenan-treated animals ($p < 0.14$). Proenkephalin mRNA expression in 0.1% carrageenan-treated animals was not significantly different from that of saline-treated animals ($p < 0.06$). Post-hoc analysis did not indicate a significant treatment effect on any individual week of the study. There was no significant treatment effect on contralateral proenkephalin mRNA expression.

Figure 3.23: proenkephalin mRNA expression

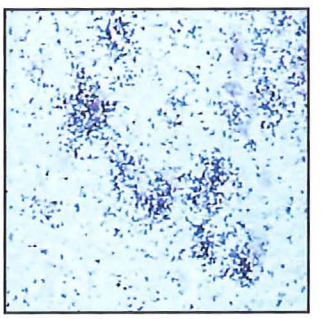
Laminae I/II



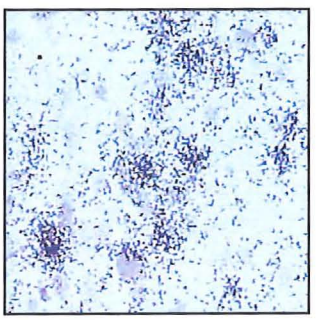
A: control



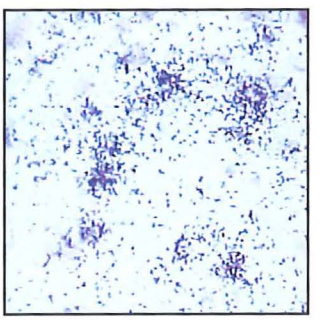
B: carrageenan(ipsi)



C: carrageenan(contra)

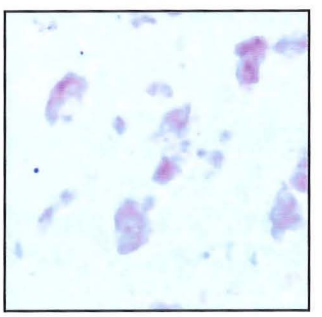


D: saline (ipsi)

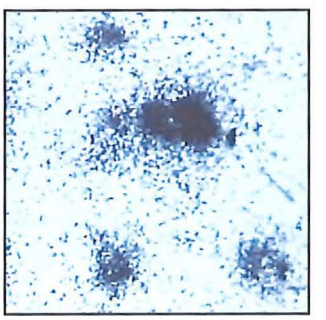


E: saline (contra)

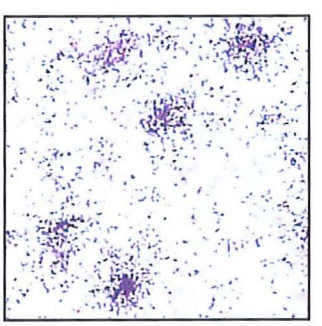
Lamina V



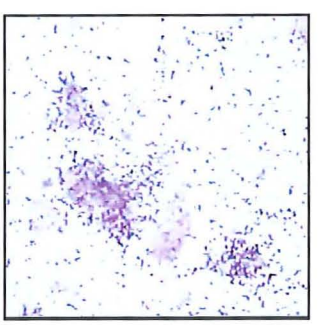
F: control



G: carrageenan



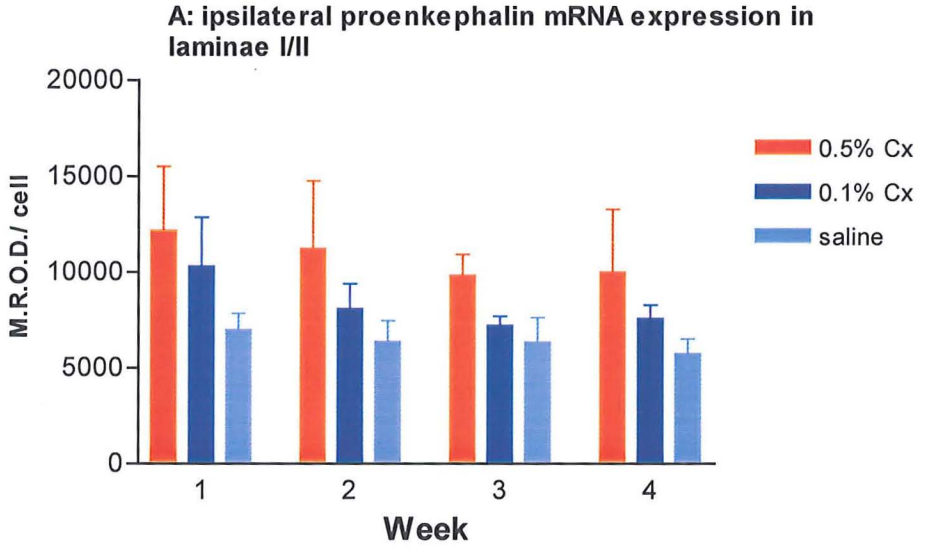
H: carrageenan

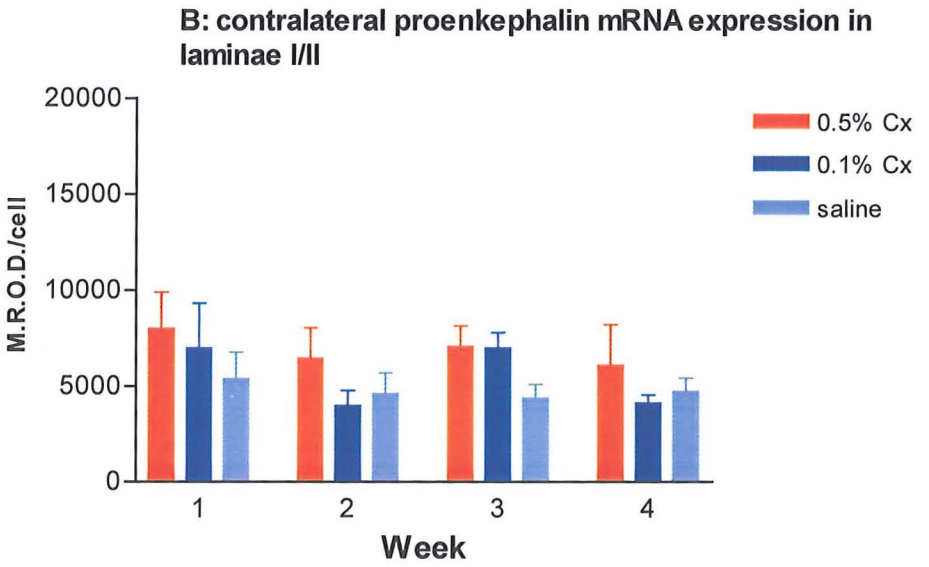


I: saline

Figure 3.24 Study 3.1: Proenkephalin mRNA expression in laminae I/II

Expression of proenkephalin mRNA, expressed as the mean ± SEM mean relative optical density (M.R.O.D.)/ cell in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% carrageenan (0.1% Cx) or 0.9% saline (n = 4-6/group).





Ipsilateral/contralateral effect

Proenkephalin mRNA expression was significantly upregulated in ipsilateral (relative to contralateral) superficial laminae in all three treatment groups. In 0.5% carrageenan - treated animals ($F[1,32] = 17.47$, $p < 0.001$), post-hoc analysis indicated significant upregulation of ipsilateral (relative to contralateral) proenkephalin mRNA expression only on week 3 ($F[1,5] = 30.02$, $p < 0.01$), although close to significance on week 2 ($F[1,5] = 5.45$, $p < 0.08$). In 0.1% carrageenan -treated animals ($F[1,32] = 8.53$, $p < 0.01$), post-hoc analysis indicated significant upregulation of ipsilateral (relative to contralateral) proenkephalin mRNA expression on week 2 ($F[1,9] = 12.14$, $p < 0.03$) and week 4 ($F[1,9] = 15.04$, $p < 0.02$). In saline-treated animals ($F[1,32]= 7.68$, $p<0.01$), post-hoc analysis indicated significant upregulation of ipsilateral (relative to contralateral) proenkephalin mRNA on week 2 ($F[1,5] = 26.86$, $p < 0.01$) and week 4 ($F[1,5] = 6.84$, $p < 0.05$).

Effect of repeated treatment

There was no significant effect of weekly repeated treatment on proenkephalin mRNA expression in ipsilateral or contralateral laminae I/II in carrageenan or saline-treated animals.

Lamina V

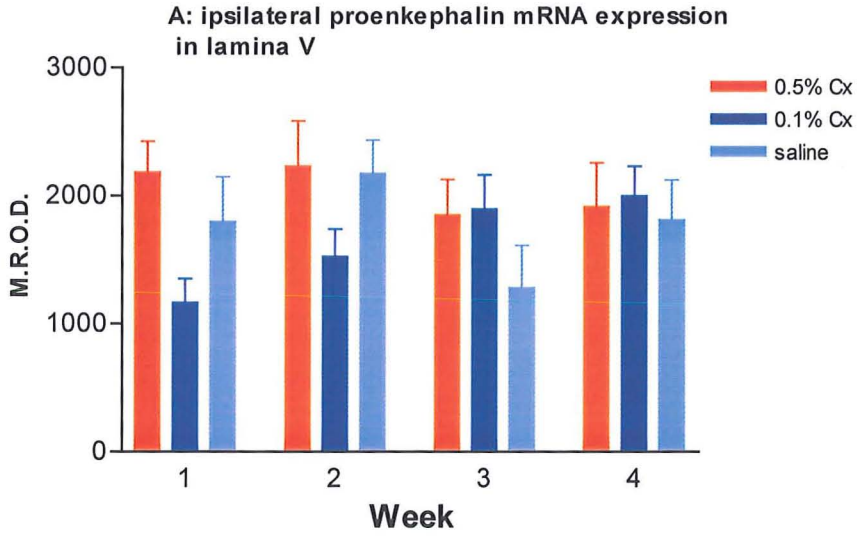
Proenkephalin mRNA expression in lamina V in study 3.1 is shown in Figure 3.25.

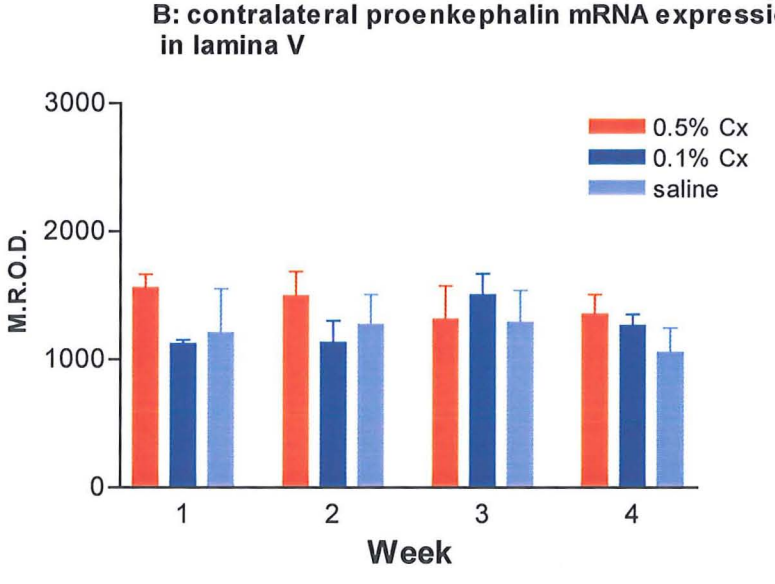
Treatment effect

There was no effect of carrageenan treatment on ipsilateral or contralateral proenkephalin mRNA expression in lamina V (relative to that observed following saline treatment).

Figure 3.25 ***Study 3.1: Proenkephalin mRNA expression in lamina V***

Expression of proenkephalin mRNA, expressed as the mean ± SEM mean relative optical density (M.R.O.D.)/ cell in ipsilateral (A) and contralateral (B) lamina V of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% Cx or 0.9% saline.





Ipsilateral/contralateral effect

Proenkephalin mRNA expression was significantly upregulated in ipsilateral (relative to contralateral) lamina V in all three treatment groups. In 0.5% carrageenan -treated animals ($F[1,32] = 13.92$, $p < 0.001$), post-hoc analysis indicated significant upregulation of ipsilateral (relative to contralateral) proenkephalin expression on week 1 $F[1,9] = 6.25$, $p < 0.05$) and week 3 ($F[1,9] = 28.53$, $p < 0.01$). In 0.1% carrageenan-treated animals ($F[1,32] = 8.53$, $p < 0.01$), post-hoc analysis indicated significant upregulation of ipsilateral (relative to contralateral) proenkephalin expression on week 3 $F[1,9] = 6.60$, $p < 0.05$) and week 4 ($F[1,9] = 7.1$, $p < 0.05$). In saline-treated animals ($F[1,32] = 7.68$, $p < 0.01$)], post-hoc analysis did not indicate any particular week on which significant upregulation of ipsilateral (relative to contralateral) proenkephalin expression occurred.

Effect of repeated treatment

Weekly repeated treatment did not alter the intensity of ipsilateral or contralateral proenkephalin mRNA expression in carrageenan or saline-treated animals.

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

Proenkephalin mRNA expression in laminae I/II in study 3.2 is shown in Figure 3.26.

Treatment effect

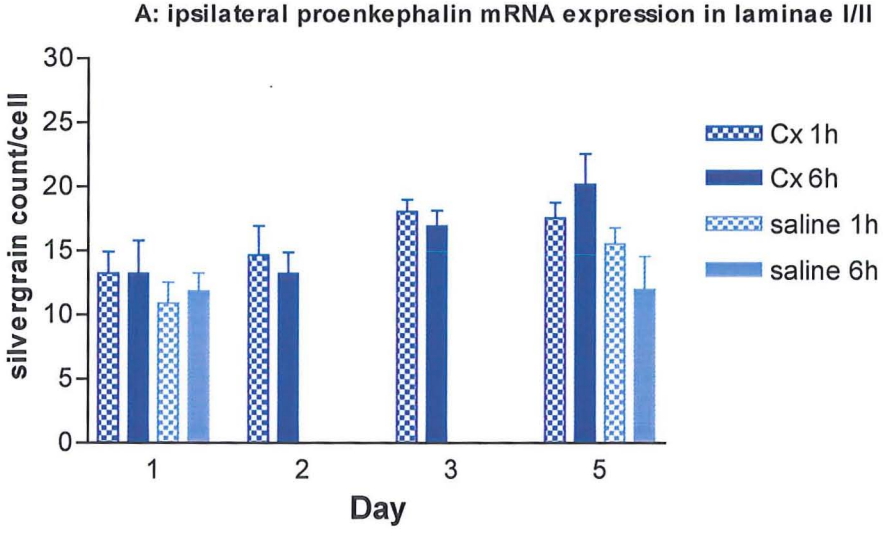
Carrageenan treatment did not alter the intensity of ipsilateral proenkephalin mRNA expression (relative to saline treatment) at 1 h p.i. or 6 h p.i. on day 1 or day 5. However, on day 5 at 6 h p.i., contralateral proenkephalin mRNA expression in carrageenan-treated animals was significantly upregulated relative to saline-treated animals ($F [1,6]] = 5.36$, $p < 0.05$).

Ipsilateral/contralateral effect

In carrageenan-treated animals, ipsilateral proenkephalin mRNA expression was upregulated (relative to contralateral) at 1 h p.i. on day 3 ($F [1,3] = 28.32$, $p < 0.01$) and day 5 ($F[1,3] = 95.82$, $p < 0.01$). At 6h p.i., ipsilateral proenkephalin mRNA expression was upregulated (relative to contralateral) on day 2 ($F[1,3] = 38.74$, $p < 0.01$), day 3 ($F[1,3] = 29.85$, $p < 0.02$) and day 5 ($F[1,3] = 11.17$, $p < 0.05$). In saline-treated animals,

Figure 3.26 Study 3.2: Proenkephalin mRNA expression in laminae I/II

Expression of enkephalin mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord, measured in animals euthanased 1h or 6h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/group).



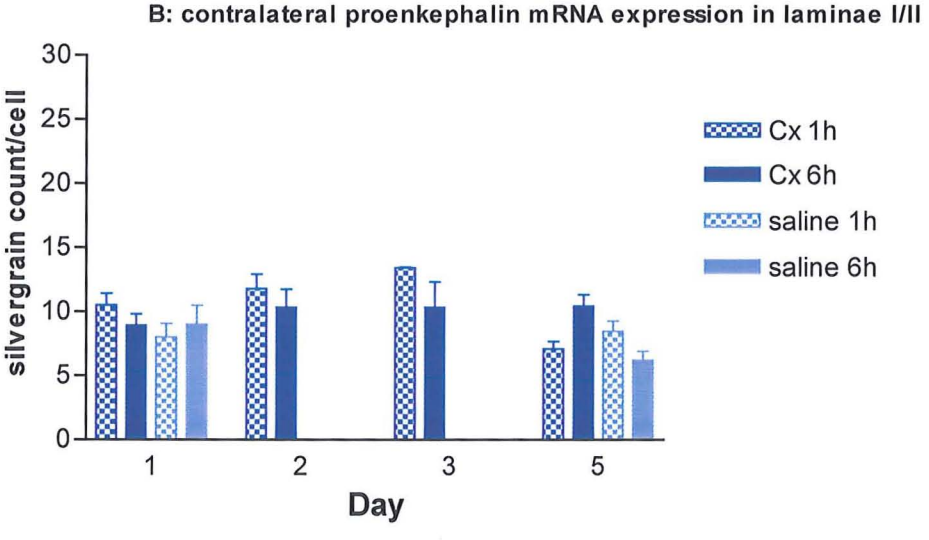
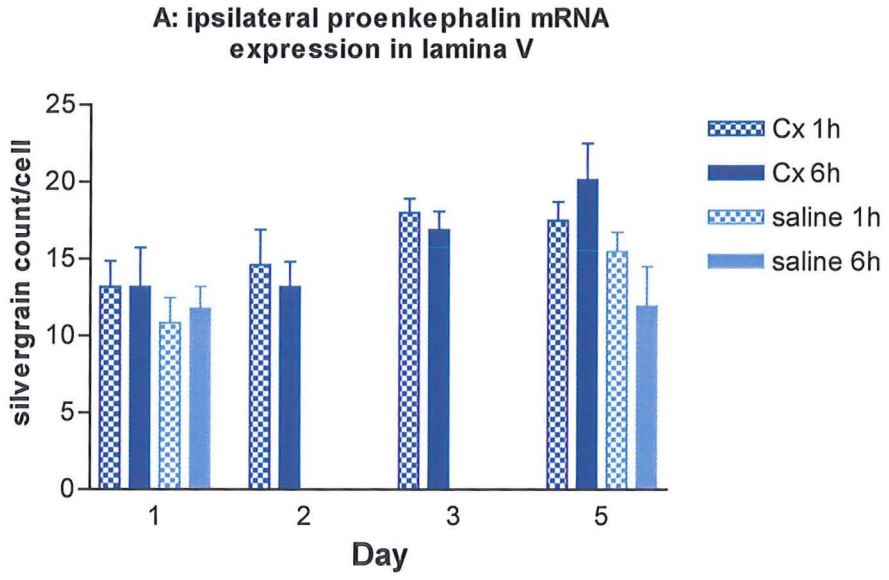
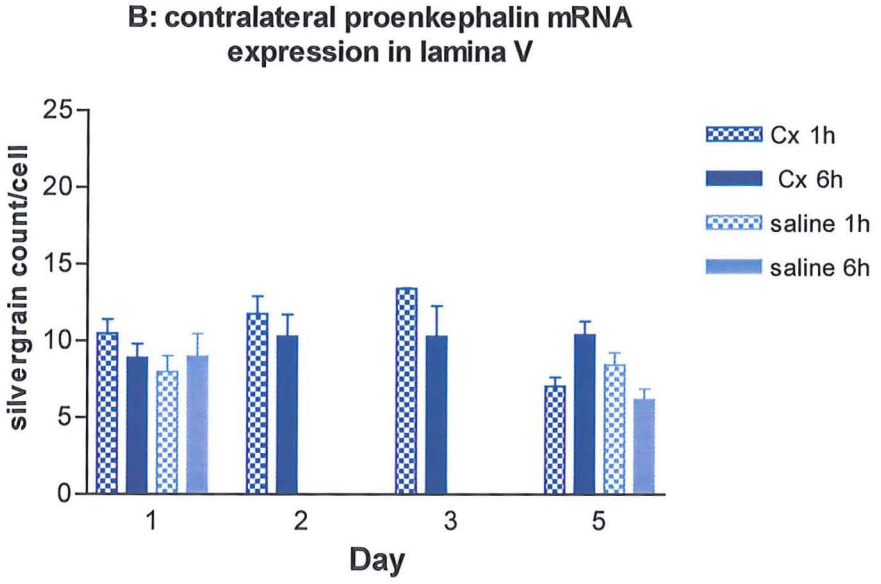


Figure 3.27 Study 3.2: Proenkephalin mRNA expression in lamina V

Expression of enkephalin mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) lamina V of lumbar spinal cord , measured in animals euthanased 1h or 6h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/group).





ipsilateral proenkephalin mRNA expression was upregulated (relative to contralateral) at 1 h p.i. on day 5 ($F[1,3] = 37.24$, $p < 0.01$) but not on day 1 ($F[1,3] = 6.59$, $p = 0.08$). At 6h p.i. , ipsilateral proenkephalin mRNA expression was upregulated (relative to contralateral expression) on day 1 ($F[1,3] = 24.49$, $p < 0.05$) but not on day 5 ($F[1,3] = 4.02$, $p = 0.14$).

Effect of repeated treatment

In carrageenan-treated animals there was a significant effect of daily repeated treatment on contralateral (but not ipsilateral) proenkephalin mRNA expression at 1h p.i. ($F[1,6] = 10.97$, $p < 0.01$); contralateral mRNA expression was significantly higher on day 3 than on day 5 ($p < 0.05$). There was no effect of repeated treatment on ipsilateral or contralateral proenkephalin mRNA expression in carrageenan-treated animals at 6h p.i. or in saline-treated animals at 1 h and 6h p.i.

Lamina V

Proenkephalin mRNA expression in lamina V in study 3.2 is shown in Figure 3.27.

Treatment effect

Carrageenan treatment did not alter the intensity of ipsilateral proenkephalin mRNA expression (relative to saline treatment) at 1 h p.i. or 6 h p.i. on day 1 or day 5. In carrageenan-treated animals on day 5, ipsilateral proenkephalin mRNA expression was significantly higher in carrageenan-treated animals at 6h p.i. than at 1h p.i. ($F[1,6] = 25.0$, $p < 0.05$).

Ipsilateral/contralateral effect

Proenkephalin mRNA expression was not significantly upregulated in ipsilateral (relative to contralateral) lamina V in carrageenan or saline-treated animals at 1 h p.i. or 6 h p.i. on any day of the study.

Effect of repeated treatment

Daily repeated carrageenan or saline treatment did not alter the intensity of ipsilateral or contralateral proenkephalin mRNA expression at 1 h p.i. or 6 h p.i.

3.3.7. DYNORPHIN

Basal prodynorphin mRNA expression

Small numbers of prodynorphin mRNA positive cells were observed in laminae I/II and V of the lumbar spinal dorsal horn and in the hippocampus, but not in other brain regions. The absence of prodynorphin mRNA expression in tissue sections labelled with fifty-times excess unlabelled probe confirmed probe specificity. Positively labelled cells in laminae I/II were of uniform 8-15 μm diameter, while positively-labelled cells in laminae V varied in diameter from 8- 32μm. There was no significant difference between treatment groups in the diameter of prodynorphin positive cells. Prodynorphin labeled cells in the lumbar spinal cord are shown in Figure 3.28.

Study 3.1: Weekly repeated carrageenan treatment

Laminae I/II

Treatment effect

Carrageenan treatment did not alter the intensity of ipsilateral or contralateral prodynorphin mRNA expression on any week of the study.

Ipsilateral/contralateral effect

Prodynorphin mRNA expression was not upregulated in ipsilateral (compared to contralateral) superficial laminae in carrageenan or saline-treated animals on any week of the study.

Effect of repeated treatment

There was no significant effect of weekly repeated treatment on ipsilateral or contralateral prodynorphin mRNA expression in carrageenan or saline-treated animals.

Lamina V

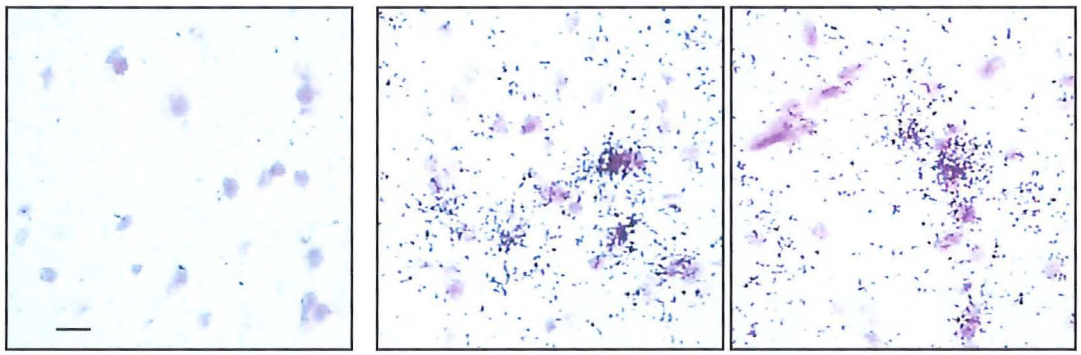
Prodynorphin mRNA expression in lamina V in study 3.1 is shown in Figure 3.29.

Treatment effect

There was no significant difference between treatment groups in mRNA expression in either ipsilateral or contralateral lamina V on any week of the study.

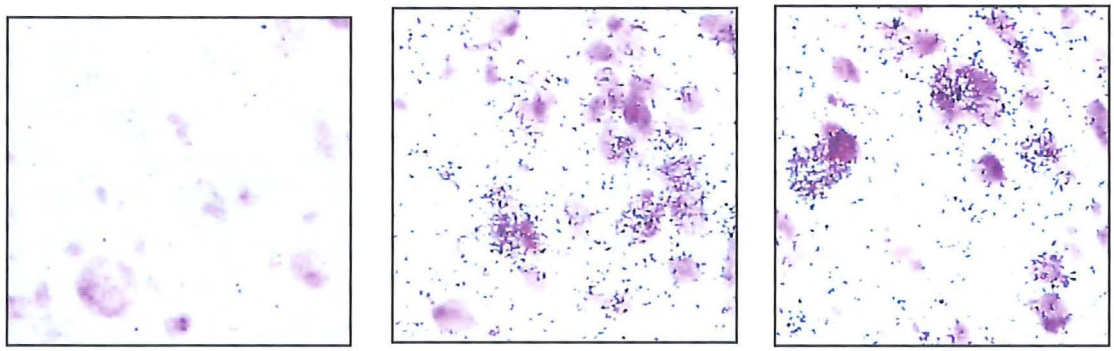
Figure 3.28 prodynorphin mRNA expression

Laminae I/II



A: control **B**: carrageenan(ipsi) **C**:saline (ipsi)

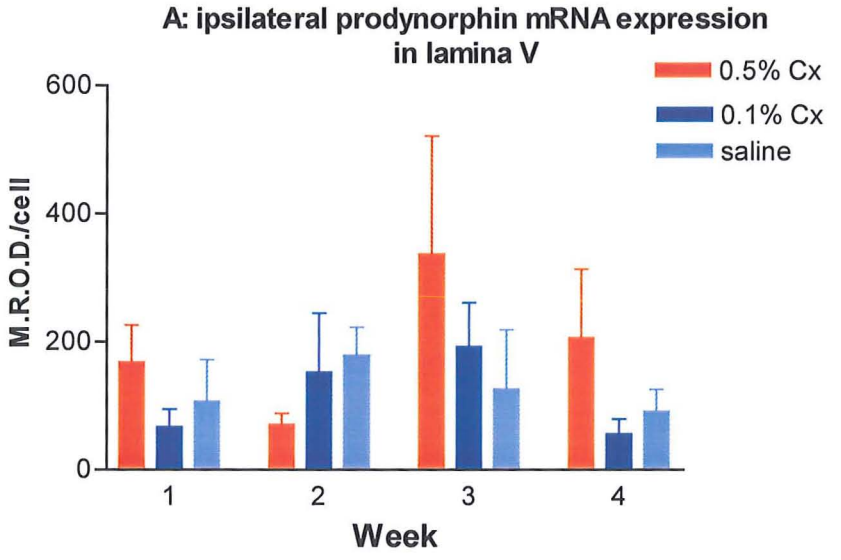
Lamina V

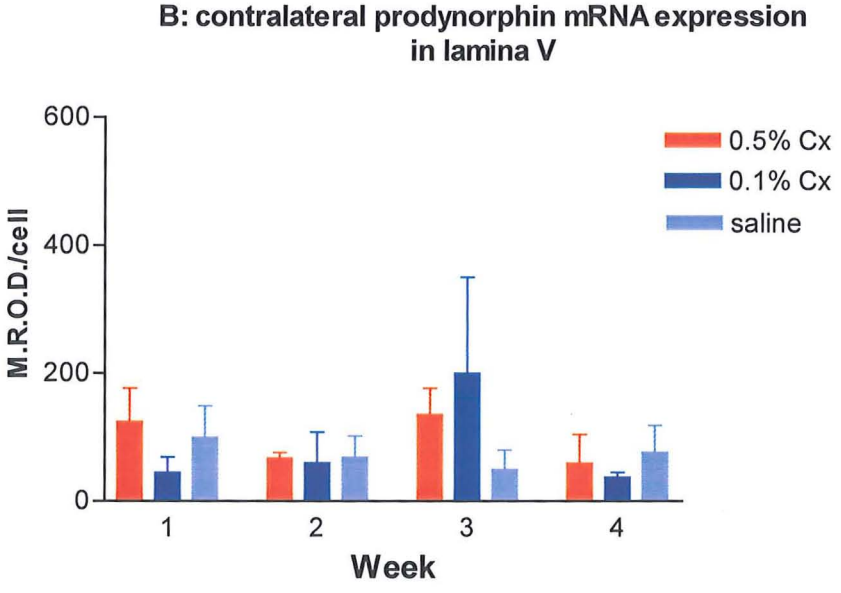


D: control **E**: carrageenan **F**: saline

Figure 3.29: Study 3.1: Prodynorphin mRNA expression in lamina V

Expression of prodynorphin mRNA, expressed as the mean ± SEM mean relative optical density (M.R.O.D.)/ cell in ipsilateral (A) and contralateral (B) lamina V of lumbar spinal cord, measured in animals euthanased 6 hours following intraplantar injection of 50 µl of 0.5% carrageenan (0.5% Cx), 0.1% Cx or 9.9% saline.





Ipsilateral/contralateral effect

Prodynorphin mRNA expression was upregulated in ipsilateral (compared to contralateral) lamina V in 0.5% Cx-treated animals ($F[1,38]=5.4$, $p<0.05$) but not in other treatment groups. More detailed week-by-week analysis using Turkey's post-hoc test indicated a significant difference in ipsilateral/contralateral mRNA expression on week 3 ($F[1,5] = 8.37$, $p < 0.01$) and week 4 ($F[1,5] = 2.25$, $p < 0.05$)

Effect of repeated treatment

There was no significant effect of weekly repeated treatment on prodynorphin mRNA expression in any treatment group.

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

Prodynorphin mRNA expression in laminae I/II in study 3.2 is shown in Figure 3.30.

Treatment effect

There was no significant difference between treatment groups in mRNA expression in either ipsilateral or contralateral laminae I/II on day 1 or day 5.

Ipsilateral/contralateral effect

Prodynorphin mRNA expression was not upregulated in ipsilateral (compared to contralateral) superficial laminae in carrageenan or saline-treated animals at 1 h p.i. or 6 h p.i. on any day of the study. However, near-significant upregulation of ipsilateral mRNA expression occurred in both carrageenan and saline-treated animals.

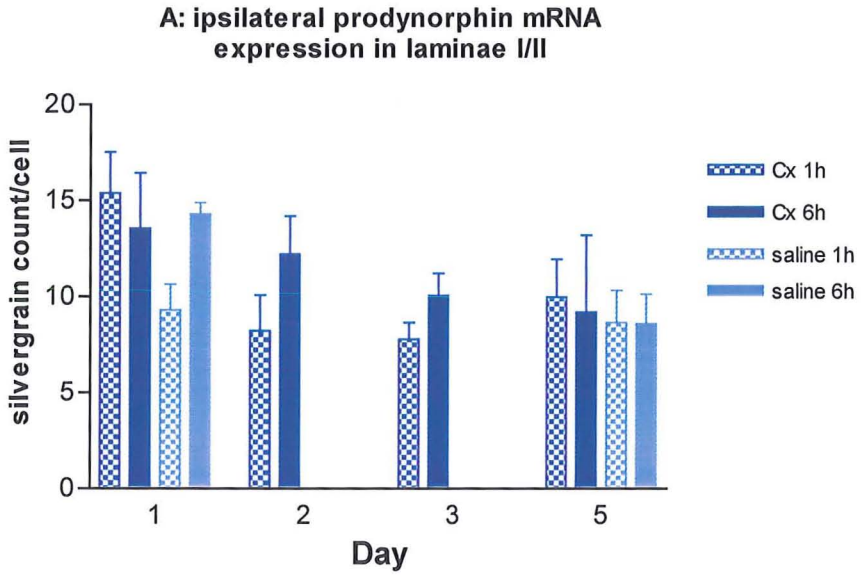
In carrageenan-treated animals at 6 h p.i., ipsilateral prodynorphin mRNA expression was upregulated (relative to contralateral) on day 1 ($F[1,3] = 7.51$, $p < 0.07$) and day 2 ($F[1,3] = 9.33$, $p < 0.06$). In saline-treated animals, ipsilateral prodynorphin mRNA expression was upregulated (relative to contralateral) at 1 h p.i. on day 1 ($F [1,3] = 7.58$, $p < 0.07$) and day 5 ($F[1,3] = 9.39$, $p < 0.06$) and at 6 h p.i. ($F[1,3] = 8.57$, $p < 0.06$).

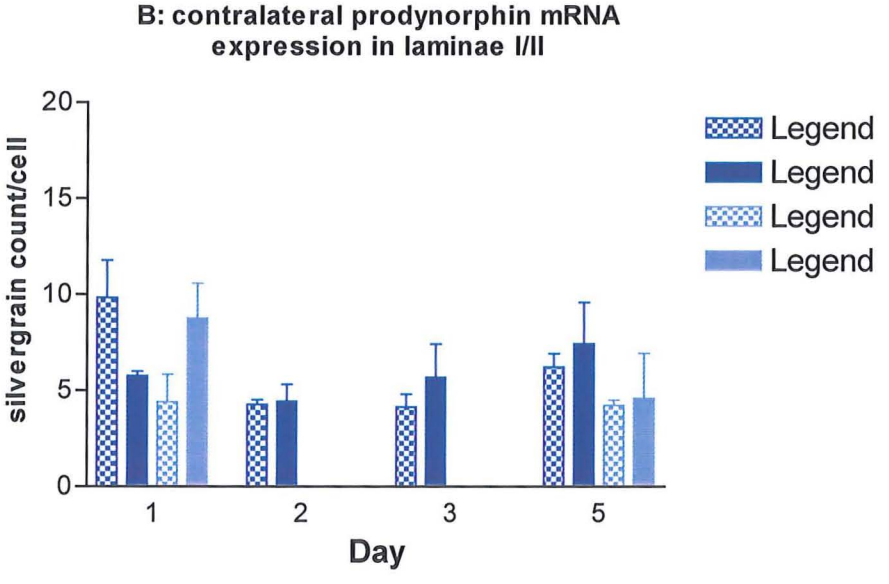
Effect of repeated treatment

In carrageenan-treated animals, the intensity of ipsilateral prodynorphin mRNA expression at 1 h p.i. and 6 h p.i. was not altered by daily repeated treatment. In saline-treated

Figure 3.30: Study 3.2: Prodynorphin mRNA expression in laminae I/II

Expression of prodynorphin mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord , measured in animals euthanased 1h or 6h following intraplantar injection of 50 µl of 0.1% carrageenan or 0.9% saline (n = 4/group).





animals, ipsilateral prodynorphin expression was significantly higher on day 1 than on day 5 at 6h p.i. ($F [1,3] = 14.55$, $p < 0.05$). There was no significant effect of daily repeated treatment on contralateral prodynorphin mRNA expression at 6h in carrageenan or saline-treated animals. There was a significant effect of daily repeated treatment on contralateral prodynorphin expression at 1 h p.i. in carrageenan-treated animals ($F[3,9] = 6.15$, $p < 0.05$; mRNA expression on day 1 was significantly higher than on day 2 ($p < 0.02$) and day 3 ($p < 0.02$).

Lamina V

Treatment effect

Carrageenan treatment did not alter the intensity of ipsilateral or contralateral prodynorphin mRNA expression (relative to saline treatment) at 1 h p.i. or 6 h p.i. on day 1 or day 5.

Ipsilateral/contralateral effect

Carrageenan or saline treatment did not alter the intensity of ipsilateral (relative to contralateral) prodynorphin mRNA expression at 1 h p.i. or 6 h p.i. on any day of the study.

Effect of repeated treatment

Daily repeated carrageenan or saline treatment did not alter the intensity of ipsilateral or contralateral prodynorphin mRNA expression at 1 h p.i. or 6 h p.i.

3.3.8. GAD 67

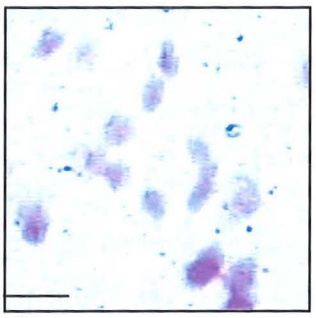
Basal GAD 67 mRNA expression

GAD 67 mRNA positive cells were observed throughout the grey matter of the lumbar spinal cord and in the hippocampus. The absence of GAD 67 mRNA expression in tissue sections labelled with fifty-times excess unlabelled probe confirmed probe specificity. Cells in the superficial laminae (I/II) expressing GAD 67 mRNA were of uniform 8-15μm diameter. GAD 67 positive cells in laminae V varied in diameter from 8-32μm. There was no significant difference between treatment groups in the diameter of GAD 67 positive cells.

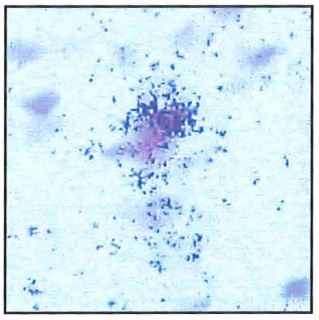
GAD 67 labelled cells in the lumbar spinal cord are shown in Figure 3.31.

Figure 3.31 GAD 67 mRNA expression

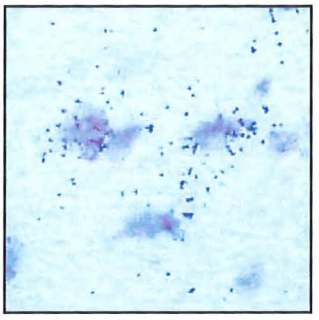
Laminae I/II



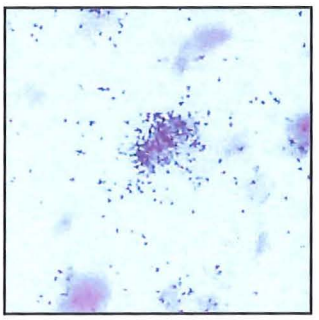
A: control



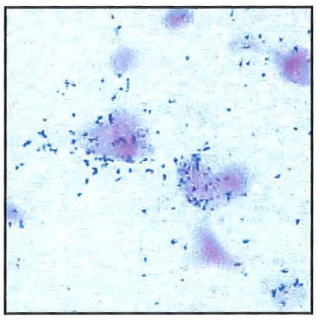
B: carrageenan(ipsi)



C: carrageenan(contra)

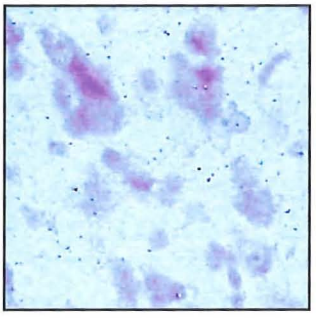


D: saline (ipsi)

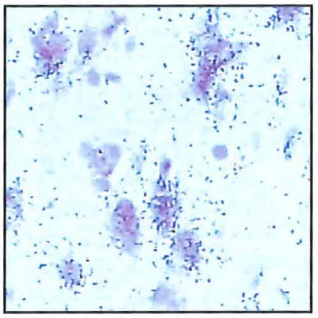


E: saline (contra)

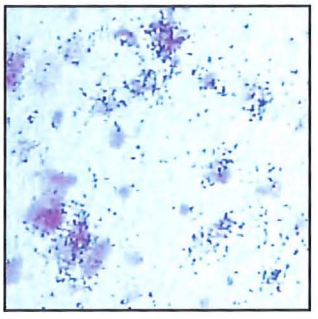
Lamina V



F: control



G: carrageenan



H: saline

Study 3.2: Daily repeated carrageenan treatment

Laminae I/II

GAD 67 mRNA expression in laminae I/II in study 3.2 is shown in Figure 3.32.

Treatment effect

Carrageenan treatment did not alter the intensity of ipsilateral or contralateral GAD 67 mRNA expression (relative to saline treatment) at 1 h p.i. or 6 h p.i. on day 1 or day 5.
In carrageenan-treated animals, ipsilateral GAD mRNA expression was significantly higher at 1h than at 6h p.i. on day 3 ($F[1,6] = 34.17$, $p < 0.01$), but not on any other day.

Ipsilateral/contralateral effect

In carrageenan-treated animals, ipsilateral GAD 67 mRNA expression was upregulated (relative to contralateral) at 1 h p.i on day 1 ($F[1,3] = 20.44$, $p < 0.05$), day 2 ($F[1,3] = 18.78$, $p < 0.05$) and day 3 ($F [1,3] = 28.91$, $p < 0.01$), but not on day 5. There was no difference in the intensity of ipsilateral and contralateral GAD 67 mRNA expression at 6 h p.i. on any day of the study. In saline-treated animals, ipsilateral GAD 67 mRNA expression was upregulated (relative to contralateral) at 1 h p.i on day 1 ($F[1,3] = 42.41$, $p < 0.01$) and day 5 ($F[1,7]=37.27$, $p < 0.01$, and at 6 h p.i. on day 1 ($F [1,3] = 36.46$, $p<0.01$) and day 5 ($F[1,3] = 16.98$, $p < 0.05$).

Effect of repeated treatment

In carrageenan-treated animals there was a significant effect of daily repeated treatment on ipsilateral GAD mRNA expression at 1h p.i. ($F[3,9] = 8.44$, $p < 0.01$); GAD mRNA expression was significantly greater on day 3 than day 5 ($p < 0.01$). There was no effect of repeated daily injection on ipsilateral or contralateral GAD 67 mRNA expression at 6 h p.i. in carrageenan-treated animals, nor in saline-treated animals at 1 h p.i. or 6 h p.i.

Lamina V

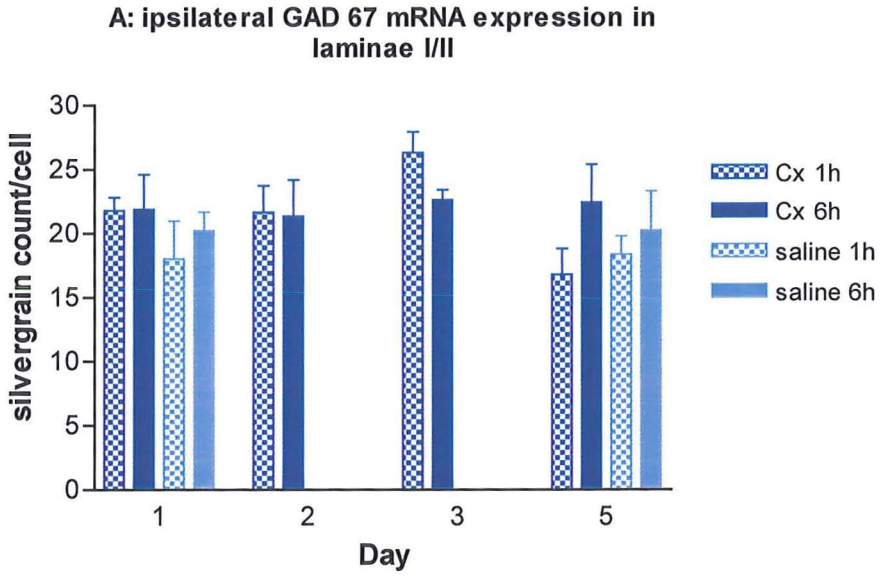
GAD 67 mRNA expression in lamina V in study 3.2 is shown in Figure 3.33.

Treatment effect

Carrageenan treatment did not alter the intensity of ipsilateral or contralateral GAD 67 mRNA expression (relative to saline treatment) at 1 h p.i. or 6 h p.i. on day 1 or day 5.

Figure 3.32: Study 3.2: GAD 67 mRNA expression in laminae I/II

Expression of GAD 67 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) laminae I/II of lumbar spinal cord measured in animals euthanased 1h and 6h following injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/ group).



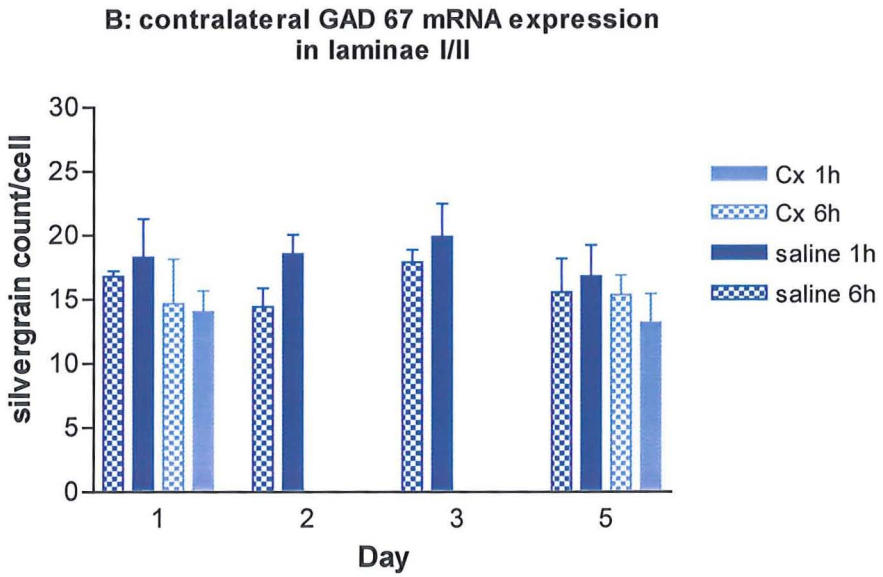
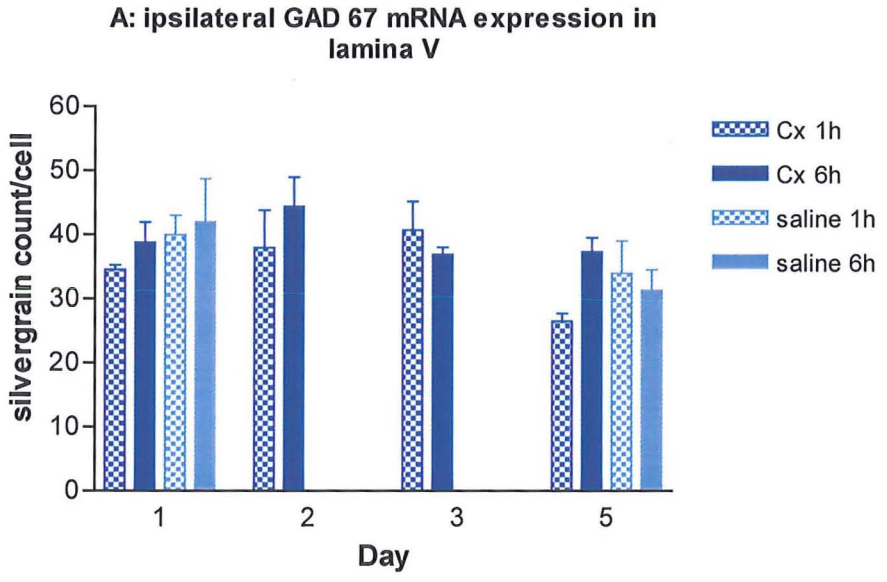
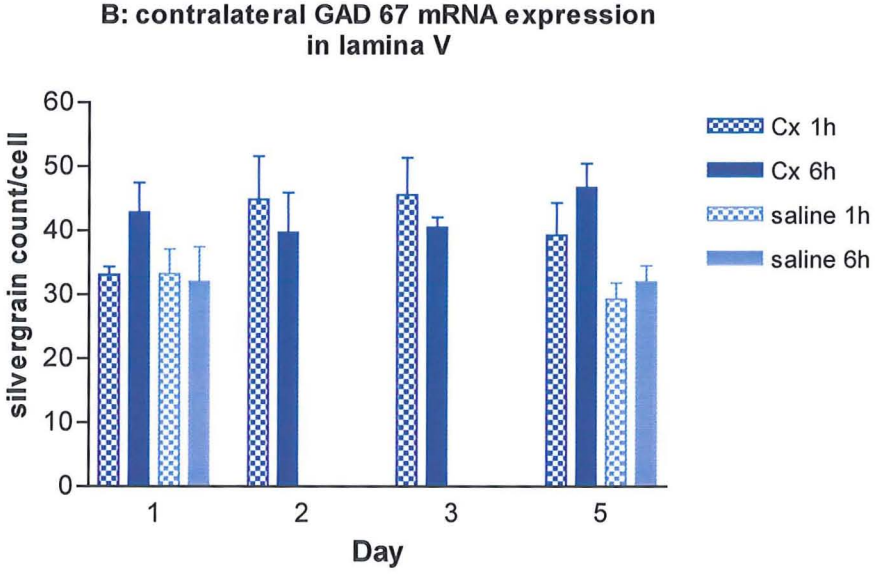


Figure 3.33 Study 3.2:GAD 67 mRNA expression in lamina V

Expression of GAD 67 mRNA, expressed as the mean ± SEM silver-grain count/ cell in ipsilateral (A) and contralateral (B) lamina V of lumbar spinal cord measured in animals euthanased 1h and 6h following injection of 50 µl of 0.1% carrageenan or 0.9% saline (n=4/ group).





Ipsilateral/contralateral effect

In carrageenan-treated animals, ipsilateral GAD mRNA expression was not significantly upregulated (relative to contralateral expression) at 1 h or 6 h p.i. on any day of the study. In saline-treated animals ipsilateral GAD mRNA expression was significantly upregulated (relative to contralateral expression) at 1 h p.i. on day 1($F[1,3] = 13.6$, $p < 0.05$) but not on day 5. Ipsilateral GAD mRNA expression was not significantly upregulated (relative to contralateral expression) at 6 h p.i. on day 1 or day 5 .

Effect of repeated treatment

Daily repeated carrageenan or saline treatment did not alter the intensity of GAD 67 mRNA expression at 1h or 6h p.i.

3.4. DISCUSSION

3.4.1. CaMKIIα

Lamina V

Minimal changes in the intensity of CaMKIIα mRNA expression in lamina V were observed in carrageenan-treated animals (relative to saline-treated animals) in studies 3.1 and 3.2, suggesting that CaMKIIα in lamina V does not contribute to the mediation of alterations in nociceptive behaviour following repeated mild inflammatory injury.

Laminae I/II

Study 3.1

0.5% (but not 0.1%) carrageenan treatment induced significant upregulation of ipsilateral CaMKIIα mRNA expression relative to saline treatment in laminae I/II. Both 0.5% and 0.1% carrageenan (but not saline) treatment induced significant ipsilateral (relative to contralateral) upregulation of CaMKIIα mRNA expression in laminae I/II. These results demonstrate that inflammatory injury (accompanied by hyperalgesia) stimulates unilateral upregulation of CaMKIIα mRNA expression in laminae I/II of the spinal dorsal horn. While 0.1% carrageenan-treated animals displayed hyperalgesic behaviour and significant upregulation of ipsilateral (relative to contralateral) CaMKIIα mRNA expression, the intensity of ipsilateral CaMKIIα expression in 0.1% carrageenan -treated animals did not differ significantly from CaMKIIα mRNA expression in saline-treated animals. Study 3.1 therefore suggests that the magnitude of upregulation of CaMKIIα mRNA expression is

directly related to the intensity of inflammatory injury and may contribute to the induction of enhanced nociceptive transmission through a centrally mediated mechanism.

Study 3.2

A similar pattern of CaMKIIα mRNA expression was observed in study 3.2, where daily repeated 0.1% carrageenan treatment induced significant ipsilateral (relative to contralateral) upregulation of CaMKIIα mRNA expression in laminae I/II at 6h p.i. on each day of the study but did not induce significant upregulation of ipsilateral CaMKIIα mRNA expression (relative to saline) on day 1 or day 5 [although contralateral CaMKIIα mRNA expression was significantly upregulated relative to saline treatment at 6 h p.i. on days 1 and 5. A significant reduction in the magnitude of ipsilateral CaMKIIα mRNA expression coincided with tachyphylaxis of hyperalgesic responses on day 3. This observation further supports the hypothesis that upregulation of spinal CaMKIIα mRNA expression may contribute to enhanced nociceptive transmission. It must be noted, however, that significant changes in contralateral CaMKIIα mRNA expression were also observed in the absence of alterations in nociceptive behaviour; contralateral CaMKIIα mRNA expression was significantly higher on day 2 than on days 3 and 5.

Involvement of CaMKIIα in the mediation of central plasticity of nociceptive transmission

As was discussed in chapter 1, CaMKIIα can modulate the activity of a multitude of intracellular agents and neurotransmitters implicated in the mediation of central nociceptive plasticity. The intensity-dependent unilateral upregulation of CaMKIIα mRNA expression in laminae I/II of the spinal dorsal horn following inflammatory injury (presumably, but not definitively accompanied by increased CaMKIIα enzyme) could thus contribute to the induction and maintenance of enhanced nociceptive processing through a modulating many different neurotransmitter pathways.

Over a rapid timescale, increased CaMKIIα activity in the superficial dorsal horn could enhance nociceptive responses through enhancing central excitatory (glutaminergic) transmission (Kolaj *et al.,* 1994; Soderling *et al.,* 1994; Pereda *et al.,* 1998). It must be noted, however, that CaMKIIα can also enhance inhibitory ('anti-nociceptive') neurotransmission in dorsal horn neurons *in-vitro*. It is possible that the intensity of neuronal stimulation and intraneuronal calcium influx determine the targets of CaMKIIα activation to subsequently drive predominantly excitatory or inhibitory transmission.

Increased CaMKIIα activity could also lead to persistent enhancement of nociceptive behaviour by increasing receptor activity (NMDA, AMPA) and activating signalling proteins (including immediate early genes) leading to the induction and maintenance of LTP of synaptic transmission in the superficial dorsal horn. As was previously discussed (chapter 1), CaMKIIα contributes to the induction and maintenance of LTP in the hippocampus (Malinow *et al.,* 1989; Fukunaga *et al,.* 1993; McGlade-McCulloch *et al.,* 1993; Miyamoto & Fukunaga, 1996) through mechanisms which have not yet been completely defined, but which include phosphorylation of NMDA and AMPA receptors and modulation of the activity of nuclear transcription factors, IEG expression and gene transcription (Lisman *et al.,* 1997). Modulation of intracellular signalling factors by protein kinase enzymes other than CaMKIIα is well recognised (Hughes & Dragunow, 1995; Bito *et al.,* 1997; Carafoli *et al.,* 1997; Lisman *et al.,* 1997; Deisseroth *et al.,* 1998).

Studies 3.1 and 3.2 investigated regulation of CaMKIIα gene expression rather than enzyme activity. Regulation of CaMKIIα mRNA expression in brain or spinal cord following natural noxious stimulation has not been reported previously; in the brain, regulation of CaMKIIα mRNA expression in the brain has been investigated predominantly using highly specific and essentially 'artificial' models of electrical stimulation (Chapter 1). It has been speculated that changes in expression and subsequent translation of CaMKIIα mRNA may critically regulate the balance of excitatory and inhibitory neurotransmission in higher centres (Liang *et al.,* 1996). It is therefore possible that modulation of CaMKIIα mRNA transcription (and its subsequent translation into the potentially active kinase enzyme) provides an additional mechanism through which CaMKIIα could regulate plasticity of nociceptive transmission in the spinal dorsal horn. The intracellular mechanisms which regulate CaMKIIα gene transcription have not yet been defined, although NMDA and NO have been shown to modulate CaMKIIα mRNA expression in hippocampal neurons (Johnston & Morris, 1995, Laabich & Cooper, 2000). NMDA and NO play key roles in central nociceptive transmission (reviewed by Milan, 1999 and in chapter 1), and therefore it is highly possible that NMDA and NO also regulate the modulation of CaMKII gene expression in response to inflammatory injury in the superficial dorsal horn

In conclusion, this study provides new insights into CaMKIIα mRNA expression activity in the spinal cord following mild inflammatory injury. CaMKII has received relatively scant attention in relation to its potentially important role in central nociceptive plasticity

in comparison with the related protein kinase enzymes PKC and PKA, which have been extensively characterised (reviewed by Millan, 1999). While upregulation of CaMKII expression was identified in the ipsilateral superficial dorsal horn in association with mild inflammatory injury in studies 4.1 and 4.2, it was not possible to localise this upregulation more precisely to excitatory or inhibitory neuronal populations. Such localisation would help to more precisely identify the role of CaMKII in the mediation of central nociceptive plasticity.

In studies 3.1 and 3.2, upregulation of CaMKIIα appeared to drive excitatory (pro) nociceptive transmission. Since CaMKII can enhance both excitatory and inhibitory central nociceptive transmission, it is possible that the degree of CaMKII upregulation and the consequences of its upregulation- potentially predominantly excitation or inhibition- are critically regulated by the intensity of inflammatory injury. For example, 'feedback' enhancement of post-synaptic glycinergic transmission by NMDA receptor activation, mediated by CaMKIIα, has been reported previously and represents an potential 'adaptive' signalling pathway which could attenuate excitatory nociceptive transmission (Xu *et al.*, 1999; 2000). Martin *et al.*(1999) recently characterised the regulation of protein kinase C expression in the spinal dorsal horn in a model of CFA inflammation, using double-labelling immunocytochemistry techniques to distinguish excitatory and inhibitory neurons in superficial laminae. In this study, upregulation of PKC immunoreactivity in the ipsilateral dorsal horn following CFA inflammation was localised to excitatory neurons, leading to the conclusion that PKC-mediated enhancement of excitatory central nociceptive transmission contributes to central sensitisation during persistent inflammation. Studies 3.1 and 3.2 clearly identify a potential role for CamKIIα in the mediation of central nociceptive plasticity following mild repetitive inflammatory stimulation, and it would be of great value to characterise the expression of CaMKIIα over a range of inflammatory stimulus intensities using techniques similar to those employed by Martin *et al.*(1999).

3.4.2. Zif/268

While previous studies (Wisden *et al.*, 1990; Herdegen *et al.*, 1991; Lanteri-Minet *et al.*, 1993; Buritova *et al.*,1995) have investigated regulation of zif/268 gene expression associated with intense inflammatory injury or noxious stimulation, current understanding of the role of zif/258 mRNA in physiological and pathophysiological central nociceptive processing leaves many important questions unanswered. Previous studies have not conclusively demonstrated a role for zif/268 mRNA upregulation in the mediation of

central sensitisation of nociceptive transmission in the spinal cord; it remains feasible that zif/268 mRNA upregulation observed in previous studies reflects a non-specific zif/268 response to intense noxious stimulation which does not directly mediate alterations in central nociceptive transmission. Several studies have reported upregulation of spinal zif/268 mRNA expression in the absence of noxious stimulation; for example, Beckman *et al* (1995) reported upregulation of zif/268 mRNA expression in brain and spinal cord 20 and 60 minutes following morphine withdrawal in morphine-dependent animals. Studies of zif/268 protein expression in the brain following noxious stimulation (Herdegen *et al.*, 1990; Lanteret-Minet *et al.*, 1993, 1994) have similarly noted that zif/268 expression is not specifically localised or upregulated in 'nociceptive' regions of the forebrain or hindbrain.

Lamina V

Minimal changes in the intensity of zif/268 mRNA expression in lamina V were observed in carrageenan-treated animals (relative to saline-treated animals) in studies 3.1 and 3.2, suggesting that zif/268 gene expression in lamina V does not contribute to the mediation of altered nociceptive behaviour following repeated mild inflammatory injury.

Laminae I/II

In study 3.2, the intensity of zif/268 mRNA expression at 1 h p.i. was not significantly different from that observed at 6h p.i. in both carrageenan and saline-treated animals, suggesting that zif/268 mRNA upregulation was either maximal at 1h p.i. and persisted for at least 6 h p.i., or that upregulation was transient and occurred between 1 h p.i. and 6 h p.i. Wisden *et al.*, (1990) reported very rapid upregulation of zif/268 expression in laminae I/II, within 15-30 minutes of noxious thermal stimulation. Lanteri-Minet *et al.*(1993) reported maximal upregulation of zif/268 mRNA within 4 hours of intraplantar CFA treatment, which was subsequently maintained for 15 days post treatment. Thus, while the time-course of zif/268 mRNA induction following inflammatory injury remains incompletely characterised, these studies suggest that zif/mRNA upregulation is rapid and maximal within 1 h of inflammatory injury.

In studies 3.1 and 3.2, no treatment effect was observed in ipsilateral or contralateral laminae I/II. No significant difference in the level of gene expression at 1 h p.i. and 6 h p.i. was observed in carrageenan or saline-treated animals. However consistent ipsilateral (relative to contralateral) upregulation of zif/68 mRNA expression was observed in both carrageenan and saline-treated animals. Weekly or daily repeated carrageenan treatment

did not alter the intensity of zif/68 mRNA expression. (although ipsilateral expression was significantly higher on day 5 than day 1 in saline-treated animals at 1 p.i.). These studies thus suggest that zif/68 mRNA upregulation in the spinal cord reflects a general neuronal response to stimulation and may contribute to the induction of central plasticity of nociceptive transmission depending on the severity of noxious stimulation.

Studies 3.1 and 3.2 used inflammatory stimuli of dramatically lower intensity than those previously employed to investigate changes in zif/268 gene and protein expression following inflammatory injury. Using immunocytochemistry techniques, Buritova *et al.* (1995) counted the number of zif/268 positively labelled cells in the lumbar superficial dorsal horn at 3 h following administration of 1-6 mg carrageenan in 150 μl saline, reporting that carrageenan induced a dose-dependent increase in the number of zif/268 mRNA positively labelled neurons in the superficial dorsal horn (at 3 h p.i.), while attenuation of inflammation by indomethacin pre-administration dose-dependently reduced the total number of zif/268 positive neurons in the superficial dorsal horn. Buritova *et al.*(1995) reported no change in the number of zif/268 positively labelled cells in the superficial dorsal horn following intraplantar saline treatment. Lanteri-Minet *et al.*(1993) reported rapid upregulation of zif/268 protein expression in the superficial dorsal horn following intraplantar CFA administration, which peaked at 4 hours p.i. (preceding maximal paw oedema by several hours). In the same study, Lanteri-Minet *et al.*(1993) observed that zif/268 expression was maintained at peak levels for 15 days following CFA treatment and was maintained at elevated levels (relative to constitutive zif/268 protein expression) for 4 weeks post treatment. It was observed that intra-articular injection of CFA induced significantly stronger and more prolonged zif/268 expression in laminae I-IV than intraplantar injection. Neither Buritova *et al.*(1993) or Lanteri-Minet *et al.*(1993) investigated the regulation of zif/268 protein expression in individual cells. While plasticity of central nociceptive processing may result from both modulation of activity in individual neurons and from recruitment or activation of additional neurons in the dorsal horn (discussed in chapter one), studies 3.1 and 3.2 focused exclusively on modulation of activity in individual neurons. The insights into the role of zif/268 in the mediation of plasticity of spinal nociceptive processing provided by these studies highlight the critical dependence of the intraneuronal molecular response to injury upon the severity of that injury.

In contrast to previous reports, in studies 3.1 and 3.2, carrageenan treatment induced hyperalgesic behaviour but did not induce significant upregulation of zif/268 gene

expression relative to saline treatment. Moreover, in both studies, upregulation of ipsilateral (relative to contralateral) zif/268 gene expression in the superficial laminae was observed in both 0.1% carrageenan and saline-treated animals. This suggests that zif/268 was upregulated in response to general neuronal stimulation, but that this upregulation was not associated with the induction of hyperalgesia, or with the plasticity of nociceptive responses observed in study 3.2. These results suggest that any potential involvement of zif/268 in the mediation of both 'physiological' central nociceptive transmission, and in plasticity of nociceptive transmission following less intense injury should be reappraised.

3.4.3. Tissue Plasminogen Activator

In study 3.1 tPA mRNA expression was identified in a very small number of cells in laminae I/II and V. A small increase in the number of tPA mRNA positive cells in laminae I/II was identified in 0.5% carrageenan-treated animals (relative to saline-treated animals), but this increase was significant only on week 1. No change in the number of tPA positive cells was observed in lamina V. It is worthy of note that the time course of tPA mRNA induction in association with inflammatory injury has not yet been characterised. It is possible that tPA is rapidly and transiently induced following inflammatory stimulation,; such transient induction would not have been detectable in study 3.2, where tPA mRNA was measured only at 6 h post-treatment. However, previous studies of tPA mRNA induction in brain tissue suggest that upregulation of tPA mRNA expression following specific stimulation is maintained for a considerable period following stimulation (Qian *et al.*,1993; Baranes *et al.*, 1998). Since strong upregulation of expression is predicted for IEG's involved in the induction central neuronal plasticity (reviewed by Morris (1997) and Herdegen & Zimmerman (1995), study 3.1 suggests that tPA expression in the dorsal horn does not contribute to the induction of central changes in nociceptive transmission following inflammatory injury.

3.4.4. Jun D

Lamina V

Minimal alteration in the intensity of jun D mRNA expression in lamina V was observed in carrageenan-treated animals (relative to saline-treated animals) in study 3.2, suggesting that jun D in lamina V does not contribute to the mediation of plasticity of nociceptive transmission associated with repeated mild inflammatory injury.

Laminae I/II

No significant alteration in the intensity of expression of ipsilateral or contralateral jun D mRNA expression in carrageenan-treated animals (relative to saline-treated animals) was observed) at 1 h or 6h p.i. on day 1 or day 5. However, significant ipsilateral (relative to contralateral) upregulation of junD mRNA was observed in both carrageenan and saline-treated animals at 1 h p.i. Daily repeated carrageenan treatment did not alter the magnitude of jun D mRNA expression in the superficial dorsal horn.

This study provides the first reported investigation of jun D gene regulation associated with mild repeated inflammatory stimulation. While the time course of jun D mRNA upregulation and subsequent protein expression in the spinal cord has not been thoroughly characterised, jun D appears to be rapidly upregulated and translated into active protein following intense noxious stimulation (Herdegen *et al.,* 1991a; 1994; Tolle *et al.*, 1994). Previous studies have shown that enhanced expression of jun D protein persists for a prolonged period following intense inflammatory injury or noxious stimulation. Herdegen *et al.*(1994) reported that ipsilateral upregulation of junD protein expression in the superficial dorsal horn persisted at maximal levels for 10 h p.i. and remained significantly greater than constitutive levels at 24 h p.i. following formalin treatment, while Lanteri-Minet *et al.,* (1993) observed elevated jun D protein expression for 7-15 days following intraplantar CFA treatment. In study 3.2, jun D mRNA expression was consistently higher at 1h p.i. than at 6h p.i. in carrageenan-treated animals, although significantly so only on day 2, suggesting that jun D mRNA upregulation was transient.

Study 3.2 provides useful new insights into the involvement of jun D in spinal nociceptive transmission. In laminae I/II, a similar magnitude of ipsilateral upregulation of jun D mRNA expression was observed in carrageenan and saline-treated animals, suggesting that the observed upregulation reflected a 'general' neuronal response to stimulation and did not contribute to alterations in nociceptive behaviour. The pattern of expression of jun D following repeated mild inflammatory stimulation is notably similar to the pattern of zif/268 discussed previously (section 3.4.2).

In conclusion, these studies highlight the necessity for thorough evaluation of IEG expression and regulation associated with different modalities and intensities of inflammatory stimulation, in order to ascertain a definitive role for these agents in both physiological nociceptive transmission and plasticity of central nociceptive transmission. While previous studies have suggested that zif/268 and jun D contribute to plasticity of central nociceptive transmission following intense injury, studies 3.1 and 3.2 suggest that

these immediate early genes are not involved in the induction of hyperalgesic behaviour or the induction of plasticity of nociceptive transmission induced by mild inflammatory stimulation.

3.4.6. CYCLO-OXYGENASE ENZYMES

3.4.6.1. COX-1

Lamina V

No significant treatment effect and no effect of repeated treatment on expression of COX-1 mRNA in lamina V were observed in studies 3.1 and 3.2, suggesting that COX-1 mediated prostaglandin synthesis in lamina V is not involved in the induction or maintenance of hyperalgesic responses following mild inflammatory injury.

Laminae I/II

In studies 3.1 and 3.2, carrageenan treatment did not alter the intensity of COX-1 mRNA expression in ipsilateral laminae I/II relative to saline treatment. A significant effect of treatment on contralateral COX-1 expression in laminae I/II was observed in study 3.1, where 0.5% carrageenan treatment induced significant upregulation of contralateral COX-1 mRNA expression relative to saline treatment. However, more detailed analysis did not identify a particular week on which this significant effect occurred. 0.1% carrageenan treatment did not alter the intensity of COX-1 gene expression relative to saline treatment in study 3.1 or study 3.2.

In study 3.1, significant ipsilateral (relative to contralateral) upregulation of COX-1 mRNA expression occurred in all treatment groups, although more detailed statistical analysis indicated no significant ipsilateral/contralateral effect on any individual week of the study. No significant ipsilateral upregulation of COX-1 mRNA expression was observed in carrageenan or saline-treated animals in study 3.2.

The ipsilateral upregulation of COX-1 gene expression observed in study 3.1 suggests that there is a central COX-1 mRNA response to peripheral stimulation. The lack of treatment effect on ipsilateral COX-1 gene expression observed in studies 3.1 and 3.2 suggests that spinal expression of COX-1 does not play an important role in the modulation of nociceptive behaviour associated with mild inflammatory injury.

These conclusions are in agreement with previous investigations of COX-1 gene regulation associated with high intensity inflammatory injury. Beiche *et al.*(1996; 1998) and

Goppelt-Struebe & Beiche(1997) assessed COX-1 mRNA induction in the rat spinal cord over 22 days following intraplantar inoculation of CFA (100 μl,) using RT-PCR techniques, and observed no change from constitutive levels of COX-1 mRNA expression over the duration of the study. A parallel study by Beiche *et al.*(1998) using western blotting techniques identified no change in COX-1 protein expression during the course of CFA-induced peripheral inflammation. In contrast to these results and of studies 3.1 and 3.2, *in-situ* hybridisation analysis of COX-1 mRNA expression in rat lumbar spinal cord by Ichitani *et al.*(*1*997) reported a complete absence of COX-1 mRNA expression in lumbar spinal cord grey matter, either constitutively or subsequent to peripheral inflammation (induced by 6mg carrageenan) or axotomy. Identification of cellular COX-1 mRNA expression cells by *in-situ* hybridisation in the present study reflects the higher sensitivity of the hybridisation method described by Wisden & Morris (1997).

COX-1 has been classically described as the 'constitutive' cyclo-oxygenase isoform, expressed in many different cell types and responsible for the production of prostanoids involved in homeostatic functions such as the regulation of vascular responses, gastric mucosal protection, and co-ordination of the actions of circulating hormones (reviewed by Rang *et al.,* 1999a). The roles of the different classes of prostaglandin molecule in central neurotransmission have not yet been characterised. While a pro-nociceptive role of prostaglandin mediated neurotransmission in the central nervous system is well established (reviewed by Dirig & Yaksh, 1998), prostaglandins may also act more generally as neuromodulatory agents within the central nervous system, having the potential to activate several different second messenger pathways through interaction with several G-protein coupled receptors (reviewed by Coleman *et al.,* 1994; Pierce *et al.,* 1995), and to activate multiple different prostanoid receptor targets (reviewed by Smith *et al.,* 1998; Rang *et al.,* 1999a). A potential 'homeostatic' role for COX-1 mediated prostaglandin synthesis in the central nervous system warrants further investigation.

3.4.6.2. COX-2

Lamina V

No significant treatment effect and no effect of repeated treatment on expression of COX-2 mRNA in lamina V were observed in studies 3.1 and 3.2, suggesting that COX-2 mediated prostaglandin synthesis in lamina V is not involved in the induction or maintenance of hyperalgesic responses following mild inflammatory injury.

Laminae I/II

Time course of COX-2 mRNA induction

Ipsilateral upregulation of COX-2 in carrageenan-treated animals was consistently recorded at 6h p.i. but not consistently at 1 h p.i., suggesting a lag period preceding COX-2 mRNA induction following inflammatory injury. This is in agreement with previous reports on the time-scale of COX enzyme induction following intense inflammatory stimulation. Hay & De Belleroche(1997) reported a two-fold increase in COX-2 mRNA expression (quantified by northern-blot analysis) at 4 h p.i. following intraplantar treatment with 2mg carrageenan. Hay *et al.*(1997) similarly reported that upregulation of COX-2 mRNA expression was maximal at 2-4h p.i. following high-dose CFA treatment. In an *in-situ* hybridisation study, Itchitani *et al.*(1997) reported that induction of COX-2 mRNA following high-dose carrageenan treatment was transient and had returned to baseline levels at 24h p.i. Using RT-PCR techniques, Beiche *et al.*(1998) reported a two-fold increase in COX-2 mRNA expression at 6 h following CFA inoculation, which had subsided at 3 days post-inoculation but was again present during the chronic phase of CFA inflammation 22 days post inoculation. Beiche *et al.*(1998) also identified COX-2 mRNA up-regulation in cervical sections of spinal cord over this period, suggestive of COX-2 mRNA beyond the region of stimulation.

Involvement of COX-2 in the mediation of central plasticity of nociceptive transmission

In study 3.1, carrageenan treatment induced significant bilateral dose-dependent upregulation of COX-2 mRNA expression (relative to saline treatment) in laminae I/II. Significant ipsilateral (relative to contralateral) upregulation of COX-2 gene expression was observed in both carrageenan and saline-treated animals. Thus there appeared to be a clear dose-dependent relationship between COX-2 gene expression in laminae I/II and hyperalgesic behaviour associated with inflammatory injury.

In study 3.2, however, no clear relationship was observed between COX-2 gene expression and the observed plasticity of nociceptive responses. Hyperalgesic behaviour observed in carrageenan-treated animals on day 5 in study 3.2 (Chapter 2, study 2.2) was not associated with upregulation of COX-2 gene expression. No consistent pattern of COX-2 mRNA upregulation occurred in study 3.2. Carrageenan treatment induced significant ipsilateral upregulation of COX-2 mRNA expression (relative to saline treatment) in laminae I/II on at 1 h p.i. and at 6h p.i. on day 1, but not on day 5. In contrast to study 3.1, no significant treatment effect on contralateral COX-2 mRNA expression was observed on day 1 or day 5. In carrageenan-treated animals, significant ipsilateral (relative to

contralateral) COX-2 mRNA upregulation was observed at 6h p.i. only on day 1, although the near-significance of upregulation observed on days 3 and 5 was likely to be a consequence of the small sample size of the study. In saline-treated animals, significant ipsilateral (relative to contralateral) COX-2 mRNA upregulation was observed at 6h p.i. on day 5 but not on day 1. In carrageenan-treated animals, significant ipsilateral (relative to contralateral) COX-2 mRNA upregulation was observed at 1h p.i. only on day 2, although the near-significance of upregulation observed on days 1 and 3 was likely to be a consequence of the small sample size of the study. In saline-treated animals, significant ipsilateral (relative to contralateral) COX-2 mRNA upregulation was observed at 1 h p.i. on day 1 but not on day 5. The intensity of COX-2 expression in carrageenan-treated animals was not significantly altered by daily repeated carrageenan treatment.

The contrasting results of studies 3.1 and 3.2 suggest that spinal COX-2 contributes to the induction of hyperalgesia associated with inflammatory injury, but its regulation in response to inflammatory stimulation is critically determined by the intensity and the frequency of that stimulation. Prostaglandins, the products of COX activity, are known to play an important role in central sensitisation. These studies suggest that induction of spinal COX-2 may play an important role in the rapid central synthesis of prostaglandins, which activate and sensitise central nociceptive neurons, contributing to the development of hyperalgesia following peripheral injury. However, COX-2 induction does not appear to play a significant role in the maintenance of enhanced nociceptive transmission, or in the induction of subsequent plasticity of nociceptive processing associated with mild repetitive inflammatory injury. It is possible that an endogenous regulatory mechanism may exist which inhibits central COX-2 induction in response to mild persistent or repeated injury. This hypothesis is supported by evidence gained from studies of the role of prostaglandins in persistent hyperalgesia induced by intraplantar CFA treatment. Hay *et al.*(1996) reported that prostaglandin levels in lumbar spinal cord measured over 7 days following CFA treatment remained elevated for the duration of the study, while COX-2 mRNA levels were only transiently increased. It must be noted that prolonged elevation of central spinal PG levels may be maintained through synthesis by non-neuronal cells, including astrocytes (Marriot *et al.,* 1990) and non-neuronal cells such as endoneurial mast cells, macrophages, neutrophils and Schwann cells (Tracy & Walker, 1995).

In non-neuronal tissues, inhibition of COX activity using non-steroidal anti-inflammatory drugs (NSAID's) induces COX-1 and COX-2 mRNA expression (Lu *et al.*, 1995). Hay and De Bellroche(1997) reported that systemic administration of a COX-2 selective

NSAID resulted in upregulation of spinal COX-2 mRNA expression. This evidence supports the existence of an feedback mechanism in which COX-2 mRNA expression is induced in response to enzyme antagonism or reduced enzyme activity. It is possible, conversely, that high spinal prostaglandin levels may provide the endogenous central regulatory mechanism which inhibits central induction of COX-2 following inflammatory injury, or central COX-2 mRNA expression may be regulated by any of the many other neurotransmitter pathways activated by inflammatory injury.

Bilateral upregulation of COX-2 following inflammatory injury

In study 3.1, significant upregulation of COX-2 mRNA was observed in both ipsilateral and contralateral laminae I/II following carrageenan treatment, although hyperalgesic behaviour was absent in the contralateral limb. There is now substantial evidence (reviewed by Koltzenburg *et al.,* 1999), to suggest that unilateral stimulation can produce bilateral effects in the somatosensory system. In addition to contralateral hyperalgesic behaviour following repeated unilateral inflammatory stimulation (Kayser *et al.,* 1998; Levine *et al.,* 1986), contralateral modulation of expression of numerous genes involved in nociceptive processing has been reported following unilateral stimulation, including substance P, CGRP, neuropeptide Y, galanin and β-endorphin (Hughes & Smith 1989; Piehl *et al.,* 1991, Zhang *et al.*, 1996). Ichitani *et al.*(1997) similarly reported strong bilateral up-regulation of COX-2 mRNA following high-dose carrageenan inflammation. The disparity between COX-2 mRNA upregulation and hyperalgesic behaviour suggests that a critical level of COX-2 and consequently prostaglandin induction is required to induce hyperalgesic behaviour; it must be noted that prostaglandins are only one of many neurotransmitters involved in the central response to peripheral inflammation.

3.4.6.3. Conclusions

These studies indicate that COX-2, but not COX-1, gene expression in laminae I/II, plays a significant role in the induction of hyperalgesia associated with inflammatory injury. Expression of cyclo-oxygenase enzymes in lamina V does not appear to contribute to the induction of hyperalgesic behaviour following inflammatory injury. Induction of COX-2 mRNA following inflammatory injury is dependent on the intensity and the frequency of inflammatory stimulation, and may be subject to endogenous inhibitory regulation.

3.4.7. ENDOGENOUS OPIOIDS-PROENKEPHALIN AND PRODYNORPHIN

3.4.7.1. Proenkephalin

Lamina V

Carrageenan treatment did not alter the magnitude of proenkephalin mRNA expression (relative to saline treatment) in lamina V, and repeated carrageenan treatment did not alter the magnitude of proenkephalin mRNA expression. In study 3.1, 0.5% carrageenan treatment induced significant ipsilateral upregulation of proenkephalin mRNA expression (relative to contralateral expression) in lamina 5, although further analysis did not indicate a particular week on which this effect occurred. This suggests no role for enkephalin-mediated mechanisms located in lamina V in the induction or maintenance of hyperalgesia associated with mild inflammatory injury.

Laminae I/II

Previous studies have identified enhanced proenkephalin expression in the superficial dorsal horn following intense inflammatory stimulation using high-dose carrageenan or CFA treatment (Iadarola *et al.*, 1988a; Draisci *et al.*, 1989; 1991; Noguchi *et al.*, 1992; Przewlocka *et al.*, 1992). Using *in-situ* hybridisation techniques, Noguchi *et al.*(1992) reported that carrageenan inflammation (4.5-6 mg in 200 μl solution) induced a two-fold increase in ipsilateral proenkephalin mRNA expression in laminae I/II. Iadarola *et al.*(1988a) reported a 50-80% increase in proenkephalin mRNA (quantified using cDNA hybridisation techniques) following intraplantar CFA treatment, which was not however followed by a significant enhancement of met-enkephalin peptide expression. Przewlocka *et al.*(1992) reported persistently enhanced ipsilateral proenkephalin mRNA expression in laminae I/II for 14 days following intraplantar CFA treatment.

The results of studies 3.1 and 3.2 are consistent with previous reports of proenkephalin gene upregulation following intense inflammatory stimulation and demonstrate, moreover, that proenkephalin mRNA is also consistently and unilaterally upregulated in response to mild inflammatory injury in an intensity-dependent fashion. In study 3.1, 0.5% (but not 0.1%) carrageenan treatment induced significant upregulation of ipsilateral proenkephalin mRNA expression (relative to saline treatment) in laminae I/II, the intensity of which was not altered by weekly repeated treatment. Ipsilateral proenkephalin mRNA expression was significantly upregulated (relative to contralateral) in carrageenan and saline-treated animals.

A similar pattern of proenkephalin mRNA expression was observed in study 3.2. Daily repeated carrageenan treatment did not induce significant upregulation of ipsilateral

proenkephalin mRNA expression (relative to saline treatment) at 1 h p.i. or 6h p.i. Ipsilateral proenkephalin mRNA expression was significantly upregulated (relative to contralateral expression) in carrageenan-treated animals at 6 h p.i. on days 2, 3 and 5 (although not on day 1) [and also at 1 h p.i. on days 3 and 5]. Ipsilateral proenkephalin mRNA expression was significantly upregulated (relative to contralateral expression) in saline-treated animals at 6 h p.i. on day 1 but not on day 5 [and also at 1h p.i. on day 1].

The induction of ipsilateral upregulation of proenkephalin mRNA expression by saline treatment in the absence of altered in nociceptive responses suggests that tonic opioid inhibition of excitatory nociceptive transmission may be enhanced in response to even mild peripheral stimulation, presumably directed towards the maintenance of nociceptive sensitivity at 'physiological baseline' up to a critical level of inflammatory insult. Due to a high constitutive level of expression of proenkephalin mRNA and met-enkephalin peptide (Majane *et al.,* 1983; Iadarola *et al.,* 1985; 1986), subtle changes in proenkephalin mRNA expression are not easily identified. The limited sensitivity of the semi-quantitative analytical employed in the present study may explain the lack of significant upregulation of ipsilateral proenkephalin mRNA expression in 0.1% carrageenan-treated animals relative to saline-treated animals in studies 3.1 and 3.2, although ipsilateral (relative to contralateral) proenkephalin mRNA upregulation was observed in both 0.1% and saline-treated animals.

While regulation of proenkephalin mRNA expression in a carrageenan model of repeated inflammatory stimulation has not been reported previously, Przewlocka *et al.*(1992) reported no change in the magnitude of ipsilateral proenkephalin mRNA upregulation over 14 days following induction of CFA-induced arthritis, a stimulus that is dramatically more intense and prolonged than that employed in the present studies. In studies 3.1 and 3.2, the magnitude of carrageenan and or saline-induced ipsilateral proenkephalin mRNA induction observed at 6 h p.i. was not significantly altered by weekly or daily repeated treatment. This suggests that the tachyphylaxis of hyperalgesic behaviour observed in study 3.2 was not mediated predominantly by a significant upregulation of opioidergic inhibitory transmission in the spinal cord.

It is worthy of note that the opioid system, while predominantly active in the spinal cord (Meagher *et al.* 1993), also enhances descending inhibition through opioid receptor stimulation in higher centers including the peri-aqueductal grey matter (Budai & Fields, 1998) and the forebrain (Grau, 1987; Meagher *et al.,* 1989; 1990). Plasticity of nociceptive behaviour following repeated inflammatory stimulation could also potentially reflect enhanced opioidergic transmission in higher centres.

3.4.7.2. Prodynorphin

Carrageenan treatment did not significantly alter the intensity of prodynorphin mRNA expression (relative to saline treatment) in laminae I/II or lamina V. The results of studies 3.1 and 3.2 contrast with previous reports of dramatic induction of prodynorphin mRNA and peptide expression following high-dose carrageenan or CFA treatment. Iadarola *et al.* (1988b) reported rapid unilateral and segmentally specific enhancement of prodynorphin mRNA expression in the dorsal horn (within 8h of treatment), followed by enhanced dynorphin A(1-8) peptide expression (from 2 days post-treatment to peak at 4 days post-treatment) associated with intense peripheral inflammation and thermal hyperalgesic behaviour induced by a variety of inflammatory agents including carrageenan and CFA. Ruda *et al.*(1988) reported enhanced expression of preprodynorphin mRNA and dynorphin peptide in laminae I/II and V/VI of the lumbar spinal dorsal horn following intraplantar CFA treatment. Nahin *et al.*(1989) reported that CFA intraplantar inflammation produced a significant increase in the actual number of neurons that expressed dynorphin in laminae I/II. Previous studies typically reported a delay of at least 24 h between the induction of prodynorphin mRNA transcription and subsequent expression of the active peptide and also typically reported that the observed expression of active peptide appeared to fall short of the magnitude of mRNA upregulation. Iadarola *et al.*(1988b) reported that dynorphin peptide levels typically began to rise 24 h after carrageenan treatment, and peaked some 72 h following CFA treatment. Two potential explanations for this disparity have been proposed (Iadarola *et al.*, 1988a,b); translation of active peptide may require substantial prior accumulation of mRNA, or diffusion of dynorphin peptide products into the extracellular environment may prevent accumulation of the active peptide at the site of production.

Controversy persists over the consequences of increased spinal dynorphin expression following inflammatory injury, with both pro- and anti-nociceptive actions attributed to the peptide. Dynorphin induces expansion of the receptive fields of lamina I projection neurons following intense inflammatory stimulation (Hylden *et al.,* 1989; 1991), and it has been speculated that dynorphin may contribute to the development of contralateral hyperalgesic behaviour following intense inflammatory injury (Millan & Colpaert 1990;1991). However, κ receptor activation has also been shown to attenuate hyperalgesia associated with inflammation (Millan *et al.,* 1985; 1988; Kayser & Guilbaud, 1991; Millan & Colpaert, 1990; 1991), while dynorphin application has been reported to produce hypoalgesia in normalgesic animals (Han & Xie, 1982; Piercey *et al.,* 1982; Przewlocki *et*

al., 1983; Spampinato & Candelletti, 1985). It is worthy of note that dynorphin administration often produces profound motor dysfunction and neurotoxicity (Hermann & Goldstein, 1985; Faden & Jacobs, 1984), so that reports of altered nociceptive behaviour following dynorphin administration should be interpreted with a degree of caution. Studies 3.1 and 3.2 identified no significant change in dynorphin gene expression following mild repetitive inflammatory injury and thus no key role for the peptide in the induction of plasticity of nociceptive behaviour associated with mild repetitive inflammatory injury.

3.7.3. *Conclusions*

The pattern of endogenous opioid gene expression induced by mild repetitive inflammatory stimulation in studies 3.1 and 3.2 differed in several respects from that which has been previously reported in association with severe acute or persistent inflammatory stimulation. Studies 3.1 and 3.2 indicate that enkephalin gene expression is consistently and dose-dependently upregulated following mild stimulation, and that the magnitude of this upregulation is not altered by repetitive stimulation. This confirms a tonic role of endogenous enkephalinergic inhibition of ascending nociceptive transmission, presumably directed towards maintenance of 'physiological' baseline central nociceptive sensitivity. In contrast, studies 3.1 and 3.2 indicate that while dynorphin may contribute to the induction and/or maintenance of central nociceptive plasticity associated with intense inflammatory injury, it does not contribute to tonic opioidergic regulation of nociceptive transmission and has no physiological antinociceptive role.

3.4.8. GAD 67

Lamina V

Carrageenan treatment did not alter the magnitude of GAD 67 gene expression (relative to saline treatment) in lamina V. Repeated carrageenan treatment did not alter the magnitude of GAD 67 gene expression in lamina V. This suggests no key role for GABA-mediated mechanisms located in lamina V in the induction or maintenance of hyperalgesia associated with mild inflammatory injury.

Laminae I/II

Previous studies have reported enhanced GABA gene expression in the superficial dorsal horn following intense inflammatory stimulation using high-dose carrageenan or CFA treatment. Castro-Lopes *et al.*(1994b) reported an ipsilateral increase in spinal GABA protein expression and in the total number of GABA-immunoreactive cells in the

superficial dorsal horn following intraplantar treatment with 6 mg carrageenan in an injection volume of 150 μl (some 120 fold greater than the dose administered in the present study). In study 3.2, carrageenan treatment did not significantly alter the intensity of GAD 67 mRNA expression (relative to saline treatment) in laminae I/II. In both carrageenan and saline-treated animals, GAD 67 mRNA expression was ipsilaterally upregulated in laminae I/II neurons at 1h p.i. (although not on day 5 in carrageenan-treated animals) but not at 6h p.i. Repeated treatment did not alter the magnitude of gene expression. There was no significant difference in the level of GAD 67 gene expression at 1h and 6h p.i., suggesting that enhancement of GAD 67 gene expression was rapid and maximal at 1 h p.i.

Saline treatment also induced ipsilateral upregulation of GAD 67 mRNA expression, in the absence of inflammation or altered nociceptive responses. This suggests that tonic GABAergic inhibitory transmission in the superficial dorsal horn is enhanced by mild peripheral stimulation.

In conclusion, study 3.2 demonstrates that even mild inflammatory stimulation induces rapid upregulation of GABA. Plasticity of nociceptive behaviour associated with daily repeated mild inflammatory injury does not appear to be mediated by upregulation of GABAergic transmission.

3.5. GENERAL CONCLUSIONS

As was discussed in chapter 1, spinally-mediated plasticity of nociceptive responses may represent both transitory, reversible, stimulus-dependent potentiation of dorsal horn neuronal responses, or persistent, phenotypic modification of dorsal horn sensory neurons such that they persistently respond in an amplified manner to a given stimulus. Consistency of baseline nociceptive responses observed in studies 3.1 and 3.2 would suggest that a fundamental phenotypic alteration in dorsal horn nociceptive neuronal responsiveness was not induced in either study. It must be recognised, however, that baseline responses were measured on each day prior to carrageenan treatment. Recognising that inflammatory injury immediately and fundamentally alters peripheral and central nociceptive transmission, it is possible, if unlikely, that long-lasting (phenotypic) alteration of inflammatory nociceptive transmission could have occurred, without simultaneous without long-lasting modulation of 'physiological' nociceptive transmission.

In-situ hybridisation analysis of gene expression was performed at 6 h p.i., since the maximal inflammatory response, accompanied by thermal and mechanical hyperalgesia

was present at this time point (Chapter 2). *In-situ* hybridisation analysis of gene expression was also performed at 1 h p.i. in study 3.2, since it was recognised that expression of several genes under investigation- most notably the immediate early genes jun D, tPA and zif/268- was transient in nature, so that analysis performed only at 6 h p.i. could fail to detect any change in the intensity of their expression. Failure to detect upregulation of immediate early gene expression in studies 3.1 and 3.2 thus does not rule out the possibility that their expression was modulated in response to inflammatory injury.

Dramatic upregulation of immediate early gene expression is a hallmark of synaptic plasticity in higher centres (Morgan & Curran, 1991, reviewed by Morris, 1997), and the absence of intensity-dependent upregulation of these IEG's would therefore suggest that LTP mediated through upregulation of zif/268, jun D or tPA did not contribute to the development of hyperalgesia in study 3.1 or 3.2. However, the transient nature of stimulus-induced IEG upregulation means that their involvement in the induction of nociceptive plasticity observed in studies 2.1 and 2.2, while unlikely, cannot be ruled out definitively.

Studies 3.1 and 3.2 investigated expression of genes which are upregulated and subsequently translated into active peptides or proteins over a wide time-scale; while immediate early genes can be upregulated in less than one hour following specific stimulation, and rapidly translated into transcription factors (Morgan & Curran, 1991), late-stage effector genes such as proenkephalin and prodynorphin, are not upregulated immediately following specific stimulation, requiring activation by up-stream intracellular mediators, and are not translated into active peptides until at least several hours following inflammatory stimulation.

The pattern of gene expression identified following mild repetitive inflammatory stimulation differed in several respects from that which has been reported previously following intense inflammatory stimulation (Herdegen & Zimmermann, 1995; Stanfa and Dickenson, 1995). It is possible that previous reports of dramatic upregulation of key genes (immediate early genes, GABA and prodynorphin) following intense inflammatory stimulation may in fact represent an 'extreme' or 'end-stage' molecular response and do not accurately represent the physiological response of the spinal cord to those levels of injury which have clear clinical relevance.

In lamina V, there was no strong evidence of altered gene expression induced by repetitive mild inflammatory injury. The role of lamina V in nociceptive processing appears to be a predominantly modulatory one in which ascending excitatory information and descending inhibitory information are integrated (reviewed in chapter 1). It is possible that the modulatory role of primary afferent fibres located in lamina V was not engaged in response the mild intensity inflammatory stimulus used in studies 3.1 and 3.2, and may require more intense or persistent noxious stimulation for its engagement in central modulation of nociceptive transmission.

In laminae I/II, both carrageenan and saline treatment induced enhanced ipsilateral (relative to contralateral) expression of all of the markers that were investigated (with the exception of prodynorphin), suggesting that those markers which were not activated specifically by noxious stimulation, nonetheless contributed to the spinal sensory response to general peripheral stimulation.

In study 3.1, hyperalgesia associated with carrageenan treatment correlated closely with significantly enhanced transcription of CaMKIIα mRNA in laminae I/II; moreover, tachyphylaxis of hyperalgesic behaviour in study 2.2 correlated with attenuation of CaMKII α upregulation in study 3.2. Since increased expression of CaMKIIα, leading to regulation of expression of a range of kinase-dependent receptors and intracellular mediators, is a hallmark of LTP induction in higher centres (reviewed by Morris, 1997), this evidence suggests that LTP-like central plasticity of nociceptive transmission in the dorsal horn could have contributed to the development of hyperalgesia observed in studies 3.1 and 3.2. As an intracellular signalling molecule that is instantaneously activated by neuronal stimulation, alterations in CaMKIIα expression could induce rapid nociceptive plasticity through modulation of the activity of many nociceptive mediators. It is worthy of note that, while predominantly associated with enhancement of glutaminergic transmission, CaMKIIα also has the potential to enhance inhibitory transmission and the factors which control the targets of CaMKIIα upregulation in dorsal horn neurons following inflammatory injury have not yet been determined.

In study 3.1, mild inflammatory injury induced consistent significant upregulation of both agents implicated in the induction of enhanced nociceptive transmission/central sensitisation (COX-2), and agents associated with the attenuation of nociceptive transmission (proenkephalin), in the superficial dorsal horn, demonstrating once again that ultimate modulation of pain sensation following inflammatory injury is determined by the

interaction of excitatory and inhibitory transmitter pathways. Evidently, the hyperalgesia observed in study 2.1 was the result of ultimate excitatory dominance, while the tachyphylaxis observed in study 2.2 reflected attenuation of excitatory dominance. Notably, enkephalin has been implicated in the induction of both LTP and LTD in higher centres, and thus could have contributed to the induction of LTD of nociceptive transmission in study 2.2.

Expression of COX-1, prodynorphin or GAD 67 mRNA did not appear to be modulated in relation to the intensity of inflammatory injury or in relation to changes in nociceptive responses. This would suggest that these mediators did not play a key role in the modulation of spinal nociceptive transmission associated with mild inflammatory injury, neither in the development of hyperalgesia (study 2.1), nor in the subsequent adaptive tachyphylaxis of hyperalgesia induced by mild repetitive inflammatory stimulation (study 2.2).

With the possible exception of CaMKIIα, changes in gene expression did not correlate closely with plasticity of nociceptive behaviour induced by daily repeated carrageenan treatment. Spinal mechanisms represent only one component of the central adaptive response to injury, and it is highly likely that that spinal mechanisms alone did not mediate the tachyphylaxis of hyperalgesia observed in study 2.2 (discussed in chapter 2).

An enormous number of candidate genes have been implicated in the induction and maintenance of synaptic plasticity in the central nervous system. Even in higher centres, where there is an infinitely greater level of understanding of the molecular basis and functional relevance of synaptic plasticity than exists at present in the spinal cord, many important issues remain unresolved (reviewed by Morris, 1997). In the hippocampus, where synaptic plasticity has been most extensively studied, experiments remain predominantly at the stage of recording gene regulation associated with plasticity, rather than directly manipulating expression of individual genes and recording the effect on neuronal function. The significance of synergistic or parallel expression of key genes in relation to the ultimate modulation of neuronal excitability is not understood. The importance of post-transcriptional processes in regulating gene expression at the mRNA level during functional plasticity is similarly poorly understood.

The selection of target genes for *in-situ* hybridisation analysis in studies 3.1 and 3.2 was hypothesis-driven, based on existing knowledge of the molecular mediators of synaptic

plasticity in higher centres. An alternative non hypothesis-driven approach to identification of genes that modulate or maintain the capacity for neuronal plasticity, using molecular screening techniques, may help to identify additional important target genes, which could then be studied in greater detail using *in-situ* hybridisation techniques to highlight the specific location of their regulation in the spinal cord. Several techniques have recently evolved to permit isolation of genes, known and unknown, which are involved in synaptic plasticity and other neuronal processes (reviewed by Befort & Costigan, 1999). These include differential display techniques, expressed sequence tags (random sequencing of clones from a cDNA library), serial analysis of gene expression (SAGE), subtractive cloning techniques, gene grids and microarrays.

Differential display uses a polymerase chain reaction (PCR)-based protocol to produce a molecular 'bar-code' of a cDNA sample. If all reaction conditions are maintained constant between control and treated animals, then it is proposed that any differences in the fingerprint are the result of differentially regulated genes (reviewed by Matz & Lukyanov, 1998). Differential display is inexpensive and relatively simple to perform, but is prone to a high false positive rate due to the fact that PCR is an amplification process.

Microarray and gene grid techniques use high-density arrays of oligonucleotides, which are generated onto silica microchips. Control and treatment RNA samples are hybridised with separate oligonucleotide microarrays, and the gene chips are then scanned to identify differential expression of specific genes. While highly sensitive, microarrays and gene grids are highly expensive and remain at present within the domain of pharmaceutical companies rather than the general research environment.

EST and SAGE techniques produce cDNA libraries for control and treated populations, which are then sequenced using an automatic sequencer. As with microarrays, these techniques are highly sensitive but highly expensive.

While these techniques may be invaluable in the identification of genes implicated in physiological and pathophysiological pain transmission, any genes identified will require thorough investigation using techniques such as *in-situ* hybridisation within models of inflammatory injury such as those used in studies 3.1 and 3.2, in order to confirm a significant role in the mechanisms that operate to produce physiological or pathophysiological pain. A combination of behavioural studies, high-sensitivity gene isolation techniques, and high-resolution molecular biology techniques such as *in-situ*

hybridisation promise to unlock the holy grail of the molecular basis of nociceptive plasticity in the future, providing thorough understanding of the molecular basis of synaptic plasticity in the dorsal horn and its contribution to physiological and pathophysiological pain transmission. This knowledge should help to identify new targets and strategies for the prevention and treatment of post-injury hyperalgesia and persistent pain states.

CHAPTER 4: CHARACTERISATION OF A METHOD FOR ORGANOTYPIC SLICE CULTURE OF SPINAL CORD

Science is magic that works

Vonnegut

4.1 INTRODUCTION

4.1.1. Principles of organotypic culture

As our knowledge about molecular mechanisms regulating central nociceptive transmission increases, we require more sensitive means of investigating and manipulating these mechanisms. A variety of culture systems have been developed to study the structure and behaviour of spinal neurons (reviewed by Morrison *et al.,* 1998). Primary neuronal cultures permit the study of either a single cell population or a heterogeneous cell population, allowing the exploration of molecular changes induced by specific stimuli, and interactions between neurons (e.g. Vartianen *et al.,* 1999; Crain *et al.,* 2000; Ma *et al.,* 1999). However, they are limited in their usefulness in the investigation of central nociceptive transmission, which is mediated through co-ordinated activity of a network of neurons located in different laminae of the dorsal horn.

Organotypic slice cultures of spinal cord provide a potentially valuable tool for the investigation of intracellular and inter-cellular processes responsible for the control and integration of neurotransmission in the spinal cord, providing an 'in-vitro' preparation of living tissue in which anatomical structure and local neuronal circuitry are preserved. Two types of system have evolved for organotypic culture of nervous tissue (reviewed by Gahwiler *et al.,* 1997), namely the 'roller-tube' technique, and the 'interface' (semi-permeable membrane) technique.

Roller-tube culture systems were first described by Hogue(1947) and subsequently characterised in detail by Gahwiler(1988). Sections of CNS tissue are stabilised in a plasma clot (Hogue, 1947) or in a collagen matrix on glass coverslips (Costero & Pomerat 1951; Crain & Peterson, 1963), which are rotated slowly to provide continuously alternating oxygenation and nutrition to cultured tissue.

An 'interface' system for organotypic culture of nervous tissue was first described by Yamamoto *et al.*(1989) using 'transwell' semiporous membranes, and by Stoppini *et*

al.(1991), using 'millicell' semiporous membranes. In interface systems cultures are continuously nourished by culture medium from below and oxygenated from above.
Tissue cultured using roller-tube or interface culture systems display slightly different properties (reviewed by Gahwiler *et al.,* 1997). Irrespective of the original thickness of the slice preparation, roller-tube cultures flatten to a greater degree than interface cultures to provide a thin near-monolayer of cells. Roller tube cultures have been used preferentially in experimental procedures such as imaging and electrophysiological recordings that demand optimum access to, and visualisation of, individual neurons. However, CNS slices cultured using roller tube techniques are embedded in plasma or collagen for the first 36-48 hours so that there is some delay before they can be used in experiments. Interface cultures are more easily prepared than roller-tube cultures and can be observed and manipulated at all stages of culturing. A novel technique for 'thin slice culture' of CNS tissue (sections up to 150 μm thick) on collagen-coated glass coverslips or culture dishes has recently been described (Parsley & Cheng, 1998), which offered the advantages of superior microscopic visualisation, enhanced tissue oxygenation and cost-effectiveness compared to previously reported methods of interface slice culture.

All organotypic culture systems share certain environmental requirements (reviewed by Gahwiler *et al.,* 1997), that include strict sterility and stable incubation conditions (standardly 37°C in an atmosphere containing 5% CO_2). Culture media used in organotypic culture of neuronal tissue typically consist of a combination of 25% serum (typically horse, donkey or foetal calf), 50% synthetic medium, such as basal medium (Eagle) or modified Dulbecco's medium and 25% balanced salt solution enriched with glucose to a final concentration of 5.6mM. Certain experiments requiring precise control of the initial composition of the medium, particularly those studies which investigate embryonic development of the CNS, utilise complex serum-free media. For example, Annis *et al.*(1990) defined a complex serum-free medium for use in the investigation of axonal connectivity and development in cerebral cortex and forebrain organotypic culture systems, reporting that precise control of the composition of the culture medium provided superior neuronal metabolism and process outgrowth.

The characteristics and viability of an organotypic culture system are critically influenced by the age (or stage of developmental) of the tissue from which the cultures are derived, and the thickness of the slice placed in culture (reviewed by Gahwiler *et al.*, 1997). Organotypic cultures of embryonic central nervous tissue have been well characterised and are reported to display superior resistance to oxidative stress relative to post-natal nervous

tissue. However, neurogenesis and neuronal migration of sensory neurons in the dorsal horn occurs throughout embryonic development and continues into the early post-natal period (reviewed by Bayer & Altman, 1995). This is of particular relevance when using organotypic culture systems to study spinal mechanisms involved in nociceptive transmission where it is important to localise the laminar arrangement of the spinal cord and ascending and descending tracts in spinal white matter. In addition, considerable ongoing cell differentiation and migration in embryonic organotypic culture systems can lead to distortion of the organotypic organisation of the cultured tissue.

The thickness of tissue sections placed in culture also critically influences their survival in culture and suitability for morphological, immunohistochemical and electrophysiological investigations (reviewed by Morrison *et al.,* 1998). Parsley *et al.,* (1998) compared the viability of 80 μm, 150 μm and 300 μm organotypic spinal cord cultures, reporting survival of 78 ± 7%, 95 ± 3% and 50 ± 7% of cells respectively after 14 days in culture. They observed that 300 μm thick sections were too thick to permit visualisation of individual cells, while considerable cell death was apparent in 80 μm sections, associated with trauma during the sectioning procedure, and concluded that 150 μm sections offered optimal visualisation of individual neurons and viability in culture.

4.1.2. Organotypic culture of spinal cord

Organotypic culture of embryonic and neonatal rat spinal cord has been described previously in both roller-tube systems (Crain & Peterson, 1963; Bunge *et al.,* 1965; Hosli & Hosli, 1987; Braschler *et al.,* 1989; Delfs *et al.,* 1989) and interface systems (Rothstein *et al.,* 1993; Rothstein & Kuncl 1995; Manabe *et al.,* 1999). In the spinal cord, organotypic culture techniques have been used to investigate a variety of neuronal processes and transmitter pathways including: cellular receptor location (Hosli & Hosli, 1987); transcription factor expression (Manabe *et al.,* 1999); neuroprotective strategies in spinal neurons (Rothstein & Kuncl, 1995) and glutamate neurotoxicity in ventral motor neurons (Rothstein *et al.,* 1993). Previously reported techniques for organotypic culture of post-natal rat spinal cord typically cultured 350-500 μm sections of spinal cord, which required fixation and further sectioning prior to further histochemical procedures.

4.1.3. Aims and Objectives

Despite its considerable potential, the technique of organotypic culture has not yet been widely used to investigate aspects of central nociceptive transmission. The objective of this study was to characterise a simple 'interface' technique for organotypic culture of post-natal spinal cord which would facilitate investigation of molecular mechanisms involved in central nociceptive transmission.

Since the superficial dorsal horn plays a dominant role in nociceptive transmission, it was of particular interest to evaluate viability and activity of neurons in this location in organotypic cultures of spinal cord.

4.2 MATERIALS AND METHODS

4.2.1. Culturing procedure

A sterile 3% solution of agar (A-6549, SIGMA, UK), was melted, set in petri dishes, and stored at 4°C to provide a stable mount against which stabilised sections of spinal cord could be sectioned.

Sprague-Dawley or Wistar rat pups were used in all experiments. In preliminary studies, spinal cord collected from embryonic rats (embryonic day 17-19) and neonatal rat pups (aged 5-14 days post-natum[PN] was sectioned, and placed in organotypic culture. However, embryonic tissue and early post-natal tissue (day 5-7 PN) was difficult to stabilise and section, and consequently, all subsequent studies were performed on neonatal rat pups aged 10-14 days PN. Rat pups were euthanased using an overdose of pentobarbitone sodium (*Sagatal*, Rhone-Merieux, 0.25ml) administered by intraperitoneal injection. Following dissection of the spinal column, the spinal cord was ejected by high-pressure hydraulic injection using a method described by Meikle *et al.* (1981). The spinal cord was immersed in ice-cold Hank's balanced salt solution (HBSS, GIBCO, 24020-091, UK) and the pia mater teased off whenever possible by fine dissection .

A 6-8mm section of lumbar spinal cord was excised, stabilised in agar and sectioned using a modification of the method described by Pickering *et al.*(1994). A sterile 2% solution of agar was prepared and melted, then poured into a custom-made mould and cooled to 35-37 °C. The excised section of spinal cord was placed in the melted agar solution, the mould was transferred to ice-cold HBSS and allowed to set. Once set, the stabilised section of spinal cord was placed against a solid 3% agar mount, fixed on the base of a vibratome, and bathed in ice-cold HBSS. With the vibratome set at slow speed and high vibration frequency, transverse slices of diameter 150-300 μm were collected, transferred to a sterile environment, and washed three times in sterile HBSS. Slices were transferred to sterile

culture wells [4 cm diameter pet-track-etched membrane cell culture inserts (Becton Dickenson, UK) placed in NUNCLON surface disposable culture wells], containing 1 ml of sterile culture medium composed of (100 ml):

49.6ml Dulbecco's modified Eagles medium (D-MEM) containing 4500mg/l D-Glucose and GlutaMAX I™, without sodium pyruvate.(61965-026, GIBCO, UK)

24.8ml HBSS

24.6 ml foetal calf serum (heat inactivated) (10108-157, GIBCO, UK)

1ml 1M penicillin/Streptomycin (15140-114, GIBCO, UK)

Cultures were incubated for up to 14 days at 35 °C in a humidified incubator supplied with 95% oxygen and 5% carbon dioxide and inspected daily using a light microscope for evidence of cell death and contamination. Culture medium was completely replaced at 36 h intervals.

4.2.2. Histochemical analyses

Histochemical procedures were performed on freshly harvested spinal cord sections and on cultured slices after 1-5 days in culture. Slices were fixed in an ice-cold solution of 4% paraformaldehyde (PFA), and washed three times (for 5 minutes) in phosphate-buffered saline (PBS) prior to NADPH-d staining and immunocytochemical analysis. All sections were mounted after processing using Histomount (HS-103, National Diagnostics, UK).

4.2.2.1 MTT Assay

The 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H tetrazolium bromide (MTT) assay was used as a simple and rapidly visualised colorimetric method of assessing cell viability, proliferation and cytotoxicity in culture systems (Leeder *et al.,* 1988; Liu *et al.,* 1997). A stock solution of 5mg/ml MTT in sterile 0.9% saline was prepared, stored at -20 °C and diluted in culture medium to a working concentration of 500μg/ml. Cultures were incubated for 2 h in MTT solution. Light microscopy was used for qualitative assessment of the intensity and localisation of MTT staining.

4.2.2.2. NADPH diaphorase assay

The reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) assay in paraformaldehyde-fixed tissue was used to identify nitric oxide synthase (NOS) expression in the central nervous system. NADPH-d staining was performed according to the method of Valtschanoff *et al.,* (1992). Sections were incubated in 0.1M PBS containing 0.25% Triton X-100 for 10 minutes then transferred to a lightproof container containing a freshly

prepared solution of 0.1 M PBS, 0.25% Triton X-100, 0.5mg/ml β-NADPH (N3886, SIGMA UK) and 0.2mg/ml nitro blue tetrazolium (NBT, 35H5043, SIGMA, UK). Sections were incubated in this solution at 37°C for 12-18 hours. Following incubation, sections were rinsed in 0.1M PBS, dehydrated in serial solutions of ethanol (70-100%) and histoclear mounted. Light microscopy was used to assess the intensity and localisation of NADPH-d staining.

4.2.2.3. Immunocytochemistry

Fixed slice cultures were incubated at room temperature in 1X PBS containing 5% Triton X-100 for 30 minutes, followed by blocking solution [1X PBS containing 15% normal goat serum (NGS)] for 60 minutes, then incubated overnight at 4°C with specific antibodies (prepared in 1XPBS with 3% NGS and 0.5% Triton X-100) against:

opioid μ-receptor (Oncogene Research, PC165L, UK), used at a dilution of 1:2000 (10μ g/ml)

calcium-calmodulin kinase IIα (CaMKIIα) (Boehringer-Mannheim 1481 703, UK), used at a dilution of 1: 2000 (10μg/ml)

methionine-enkephalin (met-enk) (Affiniti Research products EA1150, UK), used at a dilution of 1:5000 (10μg/ml)

After rinsing, cultures were incubated with 0.5% biotinylated secondary antibody (Vector, UK) in a PBS solution containing 1.5% NGS for 1 hour, rinsed three times in PBS and then incubated with ABC-conjugated horseradish-peroxidase (ABC Elite kit, Vector, UK), in accordance with manufacturer's instructions. The peroxidase was visualised using VIP peroxidase substrate kit (VIP) according to manufacturer's instructions, producing a violet reaction product 2-8 minutes after the addition of peroxidase substrate solution. Cultures were rinsed and dehydrated in serial solutions of ethanol (70-100%) and histoclear before coverslipping.

Quantitative analysis of CaMKIIα and met-enkephalin expression in the superficial dorsal horn was performed under light microscopy. Sections immunostained on day 1 and day 5 of culture (n = 3 on each day) were examined. In each section, three regions of fixed size were selected in the left and the right superficial laminae (I/II). The number of positively labelled cells in each field was counted by visual inspection. ANOVA analysis was used to compare the number of positively labelled cells.

4.3 RESULTS

4.3.1. Viability and topographical assessment of organotypically cultured spinal cord

4.3.1.1. Selection of optimal age range of subjects for collection of spinal cord

In preliminary studies, spinal cords were collected from a range of ages of embryonic and neonatal rats. Spinal cord harvested from embryonic and early neonatal rats were extremely fragile and difficult to stabilise and section. Spinal cord harvested from older rat pups aged 10-14 days was markedly more robust, was easily stabilised, and provided a good yield of anatomically intact transverse slices of spinal cord. For this reason, the majority of subsequent immunohistochemical studies were performed on organotypic cultures harvested from 10-14 day old rat pups.

4.3.1.2. Selection of optimal thickness of transverse slices for organotypic culture

The viability of cultured slices of spinal cord was qualitatively assessed using MTT and NADPH-d staining. Spinal cord slices were cultured for 5 days with minimal contamination (less than 1% of cultures were contaminated on day 5 of culture). All sections flattened (thinned) during the culture period.

150 μm sections were fragile and it was difficult to completely remove the surrounding agar prior to their transfer into culture wells. 150 μm sections were of inferior anatomical integrity when compared with thicker sections. From a total of forty 150 μm sections placed in culture, only 15 (37.5%) were suitable for further histochemical processing on day 5 of culture, as externsive cell death was evident in many slices. 150 μm sections were also more susceptible than thicker sections to disintegration during fixation and immunocytochemical analysis.

300 μm sections displayed excellent anatomical integrity when examined under light microscopy and appeared on visual inspection under light microscopy to survive well in culture: from 20 sections placed in culture, 18 (90%) were considered on visual inspection to be suitable for immunocytochemical analysis. However, poor results were obtained from immunocytochemical procedures performed on 300 μm sections: many sections failed to give positive staining, while in those sections in which positive immunostaining was observed, individual neurons could not be identified due to the thickness of the slice.

Sections cut at 200 μm displayed excellent anatomical integrity when examined under light microscopy and appeared to survive well in culture: from forty 200 μm sections

placed in culture, 36 (95%) were suitable (following visual inspection under light microscopy) for processing for immunocytochemical analysis.

4.2.2. Qualitative assessment of cell survival in 200 μm organotypic cultures

4.3.2.1. MTT staining

MTT staining of cultured 200 μm sections of spinal cord is shown in Figure 4.1. Dense positive MTT staining was observed in freshly harvested spinal cord and on days 1-5 of culture. On visual inspection of 4 sections that were MTT stained on day 5 of culture, 80-90% of each cultured section showed positive MTT staining (indicating cell viability).

4.3.2.2. NADPH-diaphorase staining

NADPH-d staining of cultured 200 μm sections of spinal cord is shown in figure 4.2. NADPH-d positive neurons were identified throughout the grey matter of the spinal cord on days 1-5 of culture. Bipolar neurons with long, branching dendrites and profuse terminal arborisation, and neurons with shorter curving dendrites, were observed in superficial laminae. Positive staining of bipolar and multipolar neurons was also observed in lamina X adjacent to the central canal. Sparse NADPH-d positive neurons were also observed in laminae III-V and in the ventral horn. On visual inspection, the pattern of expression of NOS in cultured spinal cord did not change dramatically from that observed in freshly harvested spinal cord, while the number of NADPH diaphorase-positive cells appeared to diminish over time.

4.3.2.3. Expression of CaMKIIα immunoreactivity in cultured spinal cord

CaMKIIα-IR in cultured 200 μm sections of spinal cord is shown in figures 4.3 and 4.4. CaMKIIα-IR cells were identified in the grey matter of cultured spinal cord in freshly harvested tissue and on days 1-5 of culture. CaMKIIα-IR was expressed intensely in small neurons in the superficial laminae of the dorsal horn (Figure 4.3). Positively stained bipolar and multipolar neurons were also identified in deeper laminae of the dorsal horn, particularly adjacent to the central canal (figure 4.4). On day 1, 58 ± 4.8 CaMKIIα-IR immunopositive cells were counted in each field of view in the superficial laminae, while on day 5, CaMKIIα-IR 23 ± 7.8 immunopositive cells/field of view were detected on day 5 ($F[1,8] = 86.67$, $p < 0.001$).

Figure 4.1 234

MTT staining of 200 μm transverse sections of lumbar spinal cord (10-14 days PN). Immediately post-euthanasia (A), or following 1(B) or 5 (C) days in culture, sections were incubated in MTT solution (500 μg/ml) for 2 hours.

Mitochondrial activity in live cells was indicated by a colour change from yellow to purple. Non-viable cultures remained yellow.

Dense positive staining was observed on days 1-5 of culture.

Scale bar represents 0.5mm

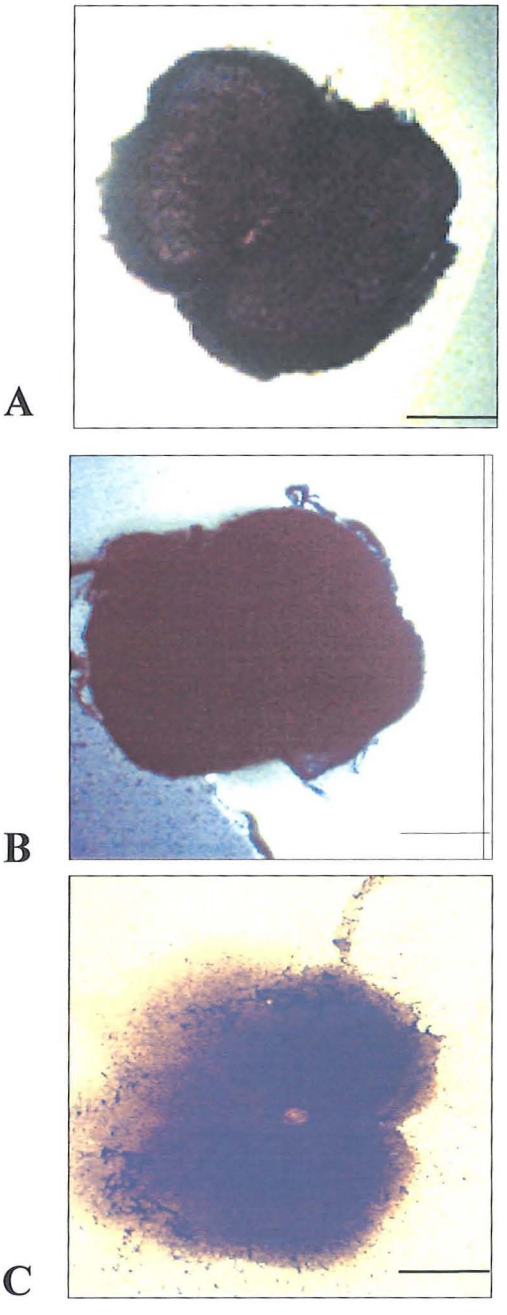
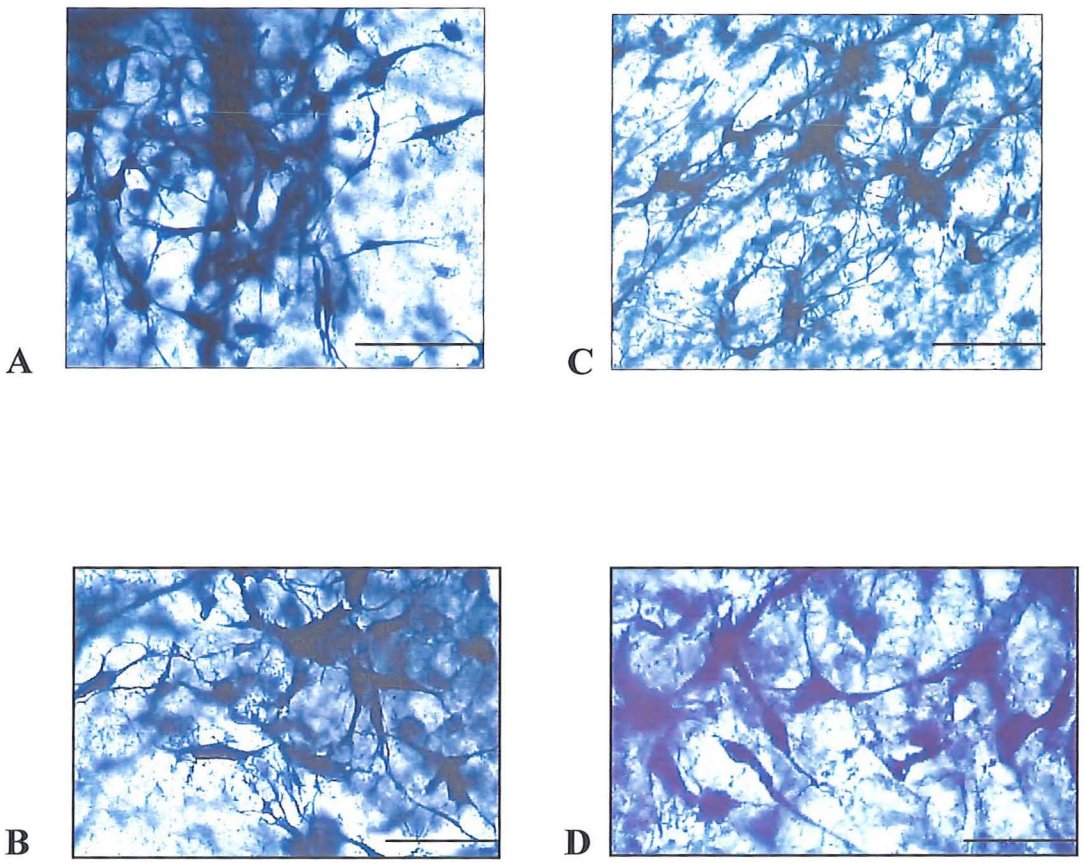


Figure 4.2 235

NADPH-d staining of 200 μm transverse sections of lumbar spinal cord (10-14 days PN), after 1 (A, B) and 5 (C, D) days in organotypic culture. NOS positive neurons were identified in both the dorsal horn (A, C) and ventral (B, D) horns. Scale bar = 50 μm



While the total number of CaMKIIα-IR cells decreased in culture, the morphology and location of CaMKIIα immunopositive cells identified on day 5 of culture appeared on visual inspection to be closely similar to that identified in CaMKIIα immunopositive cells in tissue sections prepared from freshly harvested spinal cord.

4.3.3.4. Expression of μ-opioid receptor and met-enkephalin immunoreactivity in cultured spinal cord

Expression of μOR-IR and of met-enkephalin-IR in organotypic culture of 200 μm sections of spinal cord is shown in figures 4.5 and 4.6. μOR immunoreactive cells were identified in the grey matter of cultured spinal cord on days 1-5 of culture. Expression of μO-IR was intense in the superficial dorsal horn (laminae I/II), identified in both the cell bodies of small neurons, and their extensively arborising dendrites (Figure 4.5). Scattered positively stained bipolar and multipolar neuronal cell bodies and axons were also identified in deeper laminae of the dorsal horn, particularly immediately adjacent to the central canal (Figure 4.6). On visual inspection under light microscopy, the morphology of μOR-IR positive cells identified in cultured tissue appeared to be identical to that identified in μOR-IR positive cells in tissue sections prepared from freshly harvested spinal cord.

Met-enkephalin-IR was also identified in the grey matter of cultured spinal cord on days 1-5 of culture. Met-enkephalin-IR was most intense in the superficial dorsal horn (laminae I/II), identified in both neuronal cell bodies and in their axons (Figure 4.5) but was also present in laminae IV-VII (Figure 4.6). On day 1, 31 ± 6.9 immunopositive cells were counted in each field of view in the superficial laminae, significantly greater than 19± 4.1 immunopositive cells/field of view quantified on day 5 ($F[1,8] = 13.42$, $p < 0.01$). On visual inspection under light microscopy, the morphology of met-enkephalin-IR positive cells identified in spinal cord sections on day 5 of culture appeared to be identical to that identified in met-enkephalin-IR positive cells in tissue sections prepared from freshly harvested spinal cord.

Figure 4. 3

CaMKIIα immunoreactvity(IR) in 200 μm transverse sections of lumbar spinal cord (10-14 days PN) in fresh (A) and cultured (B- D) spinal cord sections. Sections B,C and D were cultured for 24h, 3 days and 5 days respectively.

Scale bar = 250 μm

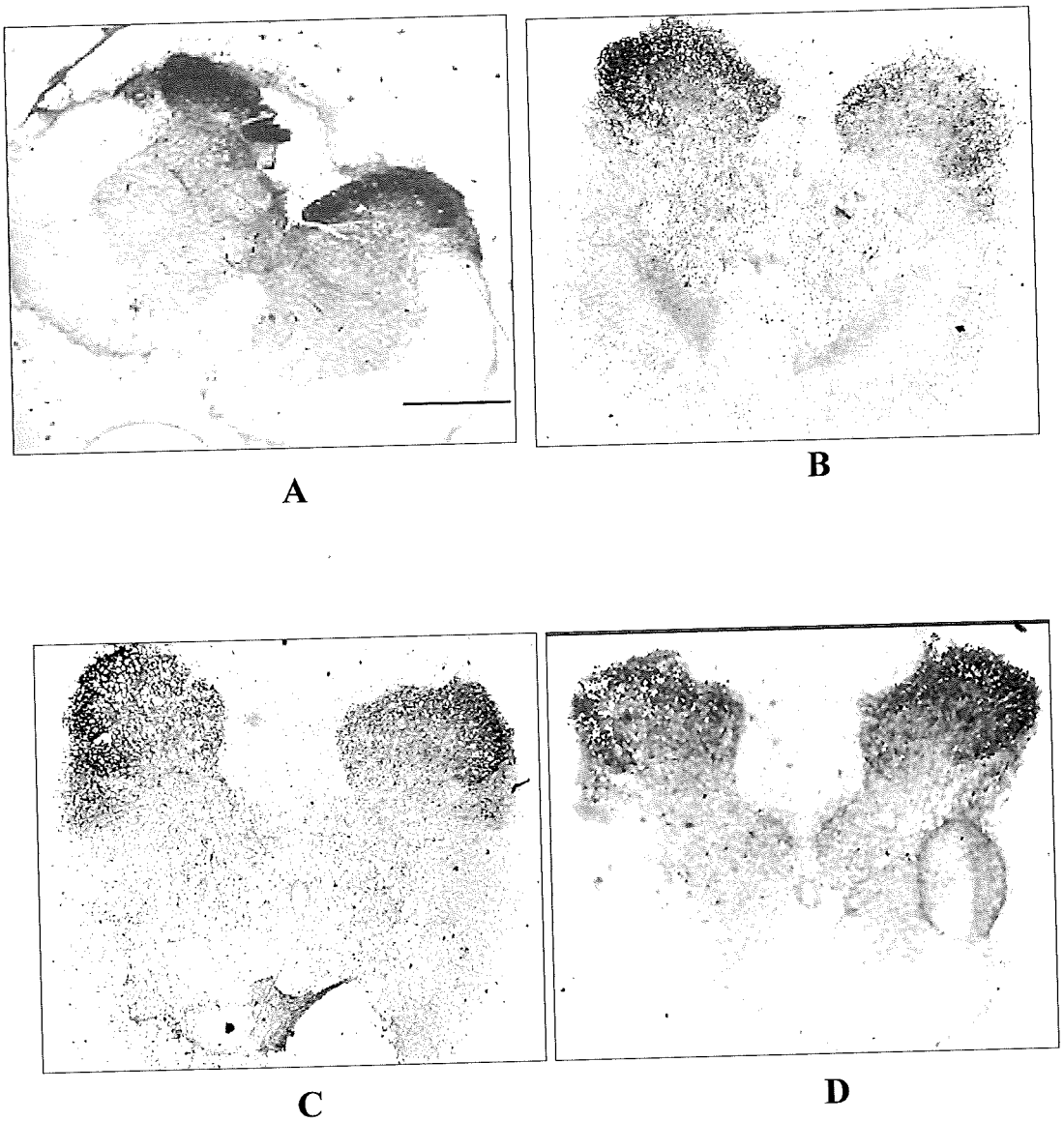


Figure 4.4 238

CaMKIIα immunoreactivity (IR) in laminae IV-VII of the dorsal horn of the spinal cord in 200 μm sections cultured for 5 days

Scale bar = 40 μm

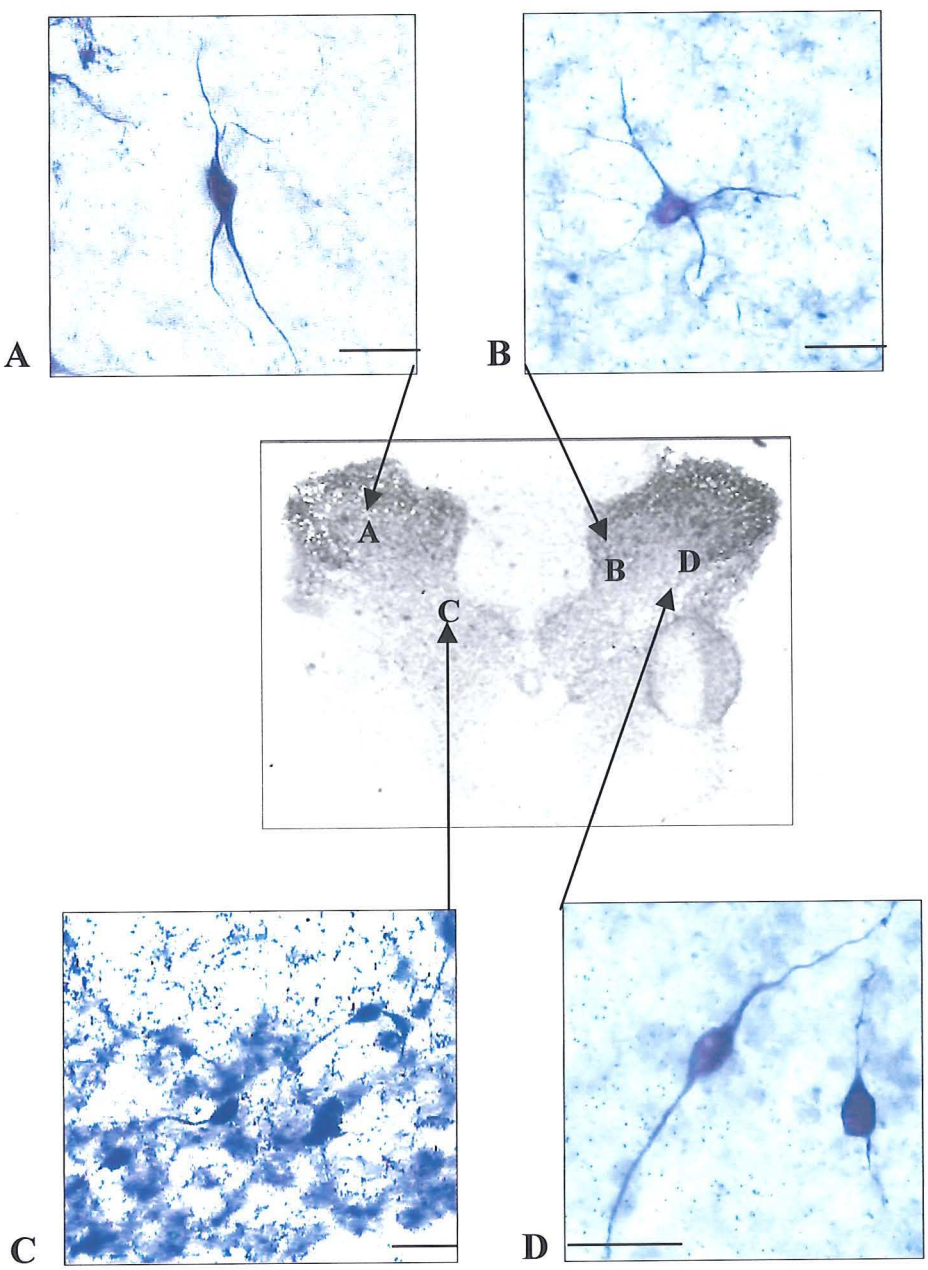


Figure 4.5

A, B: μ-opioid receptor (μ- OR) immunoreactivity (IR) in superficial laminae of the dorsal horn following 24h (A) or 3 days (B) of organotypic culture.

Scale bar = 25 μm

C, D: μ-OR –IR in lamina V (C) and lamina X (D). Scale bar = 50 μm

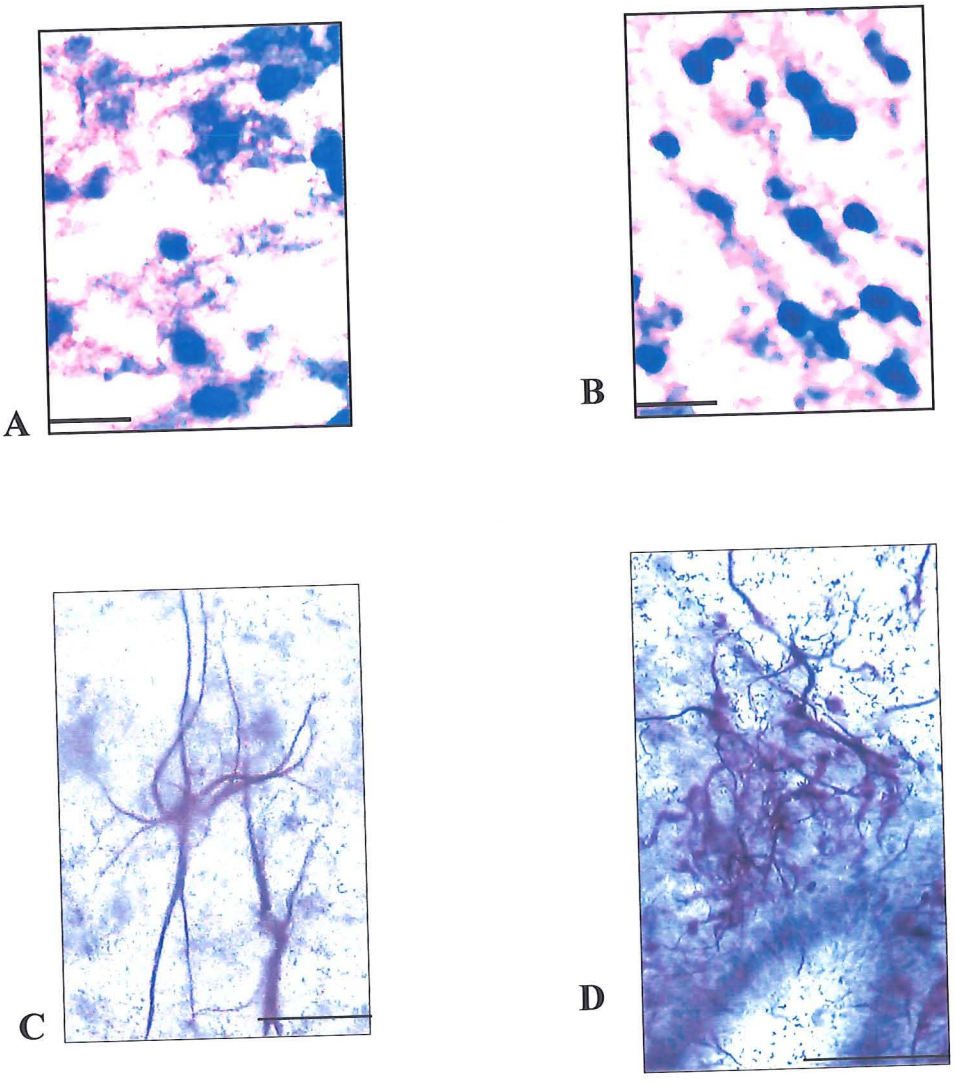
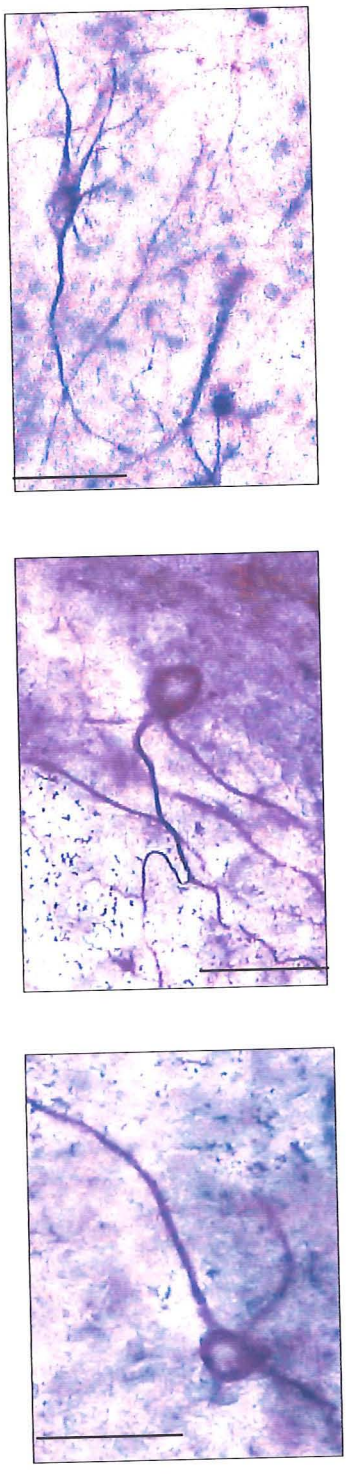


Figure 4. 6

Examples of met-enkephalin immunoreactive cells in laminae IV-VII in organotypic slice cultures. Scale bar = 50 μm



4.4. DISCUSSION

This study demonstrates that 200 μm transverse slices of postnatal spinal cord can be cultured successfully using a simple interface culture system. Histochemical and immunocytochemical assays indicated that the architecture of organotypically cultured spinal cord closely resembles that observed *in-vivo*, and, in contrast to previous methods of organotypic culture of post-natal spinal cord (Rothstein *et al.,* 1993; Manabe *et al.,*1999), the organotypic spinal cord cultures prepared in study 4 did not require further processing or sectioning prior to immunocytochemical analysis. The method described in study 4 thus provides a simple and extremely useful technique for the investigation of molecular mechanisms of spinal nociceptive transmission.

4.4.1. Age-based selection of spinal cord tissue

The selection of 10-14 day old rat pups for organotypic slice culture of spinal cord was based on a number of criteria. From a practical perspective, it was considerably more easy to harvest intact spinal cord from older rat pups than from foetal rats; preliminary studies with foetal spinal cord provided low numbers of poor-quality transverse sections of spinal cord compared with the consistent harvest of 15-20 anatomically intact sections of spinal cord obtained from each spinal cord derived from 10-14 day old rat pups.

It has been reported previously that viability of organotypic culture declines as the maturity of the tissue from which the cultures are prepared increases (reviewed by Gahwiler *et al.,* 1997). However, continued differentiation and maturation of embryonic and early neonatal spinal cord in organotypic culture is likely to disrupt the anatomical integrity of cultured slices and hinder research into molecular aspects of pain research, in which an accurate *in-vitro* representation of adult *in-vivo* spinal cord is the ideal objective. The selection of spinal cord harvested from 10-14 day old rat pups for use in organotypic culture studies was based on a compromise between these two conflicting factors. Delfs *et al.* (1989) investigated the effect of maturity on the viability of organotypic cultures of 400 μm sections of spinal cord (assessed qualitatively using phase contrast microscopy) maintained *in-vitro* for several weeks using a modified roller-tube culture technique. Delfs *et al.,* reported that organotypic cultures derived from younger animals (2-9 days old) survived better than those derived from older animals (12-16 days old); after 2 weeks in culture, 97% of cultures from 2-9 day old animals survived compared with 66% from older animals, while after 4 weeks in culture, 60% of cultures from 2-9 day old animals survived compared to only 4% from older animals. Rothstein *et al.*(1993; 1994) and Rothstein & Kuncl (1995) reported that 95% of 300-350 μm cultured sections of spinal cord harvested from 8 day old rat pups survived for at least 3 months in culture with maintenance of

excellent organotypic cellular organisation. Manabe *et al.*(1999) successfully cultured 500 μm sections of spinal cord harvested from 11 day old rat pups. Thus there was a precedent for organotypic culture of spinal cord obtained from 10-14 day old rat pups, although all previous reports cultured sections which were considerably thicker than those used in study 4.

Delfs *et al.*(1989) measured choline acetyltransferase immunoreactivity (CHAT-IR) and acetylcholinesterase (AChE) activity in organotypically-cultured sections derived from spinal cords harvested from 2-16 day old rat pups. They observed a disparity in AChE activity and CHAT-IR expression between dorsal and ventral horns in the early stages of culture which disappeared in sections cultured for longer periods. While AChE activity is present in the ventral horn at 5 days post-natum, it is does not develop in the dorsal horn until 2 weeks post-natum (Schenen, 1987). This observation demonstrates the continuation of maturation and differentiation of spinal cord harvested in the early post-natal period in organotypic culture, and highlights the advantages of culturing spinal cord tissue harvested from more mature animals when cultures are destined for use in investigation of molecular aspects of pain transmission.

4.4.2. Development of methodology for harvesting transverse sections of spinal cord

The method used in study 4 for the preparation of organotypic culture of post-natal spinal cord was adapted from a method described by Pickering *et al.,* (1) for preparation of spinal cord slices (from 7-18 day old rat pups) to be used in acute patch-clamp electrophysiological studies. In the present study, stabilisation of the spinal cord in agar prior to sectioning, and use of a vibratome to collect transverse spinal cord sections, enabled the collection of anatomically intact 150-300 μm sections suitable for organotypic culture. Although one recent study has described a similar technique, harvesting agarose-embedded foetal and postnatal (8-16 days PN) spinal cord using a vibratome, the majority of previous reports of organotypic culture of postnatal spinal cord typically harvested considerably thicker transverse sections of spinal cord (300 μm-1 mm) using a McIlwain tissue chopper. Since the thickness of the spinal cord slice critically determines its subsequent viability in culture and suitability for manipulation and use in histochemical and immunocytochemical procedures, this technique for harvesting transverse sections of 200μm or less offers distinct advantages to the McIlwain technique described previously.

4.4.3. Viability and topographical assessment of cultured spinal cord

4.4.3.1. MTT Assay

The MTT reduction assay, in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide is reduced to an insoluble purple formazan by the mitochondria of viable cells,

provides a simple and rapid colorimetric method of assessing cell viability, proliferation and cytotoxicity in culture systems (Leeder *et al.*, 1988; reviewed by Liu *et al.,* 1997) . The MTT assay has been used to assess cell survival in numerous dissociated cell culture systems, including neoplastic cells (Higashi *et al.,* 2000; Padille *et al.,* 2000), primary neuronal culture systems (Zhu *et al.,* 1997l; Blanc *et al.,* 1998; Suuronin *et al.,* 2000) and primary astrocyte culture systems (de Groot *et al.,* 1997). A strong MTT reaction was present in cultured sections of spinal cord on days 1-5 of culture, indicating a high level of cell survival in the culture system. This assay thus provided a simple and effective means of confirming culture viability prior to time-consuming selective immunocytochemical procedures.

4.4.3.2. NADPH-diaphorase staining

The reduced nicotinamide adenine dinucleotide phosphate diaphorase (NADPHd) assay in paraformaldehyde-fixed tissue has been reported previously as a useful marker of NOS expression in the central nervous system, providing highly detailed visualisation of NOS-positive neurons down to their finest processes (Dawson *et al.,* 1991; Hope *et al.*,1991; Valtschanoff *et al.,*1991; Matsumoto *et al.,* 1993).

The location and morphology of NADPH-d positive neurons in the spinal cord of the adult rat has been thoroughly described by Valtschanoff *et al.,* (1991); Saito *et al.,* (1994) and Nakamura (1997). These groups identified NADPHd activity in laminae I-VI of the dorsal horn, in the dorsal commisure, in lamina X adjacent to the central canal, and in the ventral horn. In study 4, the pattern of NADPH-d staining and the morphology of positively stained neurons in cultured spinal cord slices appeared (upon qualititative visual inspection using light microscopy) to closely resembled that described in freshly fixed tissue collected from adult rats (Valtschanoff *et al.,* 1992). While there appeared to be a gradual decline in the number of NADPH-d positive neurons over time in cultured spinal cord, the basic pattern of NADPH-d activity observed on day 5 of culture did not differ from that observed on day 1 of culture. Nitric oxide plays an important role in nociceptive transmission (Haley *et al.,* 1992; Meller *et al.,* 1992a,b) and has also been implicated in the induction and maintenance of synaptic plasticity in the central nervous system (Bohme *et al.,* 1991; O'Dell *et al.,* 1991). This assay may, therefore, subsequently provide a useful technique for *in-vitro* investigation of the role of NO in spinal nociceptive transmission and central sensitisation.

4.4.3.3. Expression of CaMKIIα, μ-opioid receptor and met-enkephalin immunoreactivity

Immunocytochemical techniques have been extensively used to investigate molecular aspects of receptor and transmitter behaviour in organotypic slice cultures of brain tissue (e.g. Dickie *et al.,* 1996; Wevers *et al.,* 1998; Gomez-Urquiho *et al.,* 1999). In organotypic cultures of spinal cord sections, immunocytochemical techniques have been used previously to evaluate choline acetyltransferase expression (Delfs *et al.,* 1989; Rothstein *et al.,* 1993; Wetts & Vaughn, 1998) and cFos expression (Manatabe *et al.,* 1999). No comprehensive immunocyctochemical assays of the expression of receptors or transmitters imlicated in nociceptive transmission in the spinal cord have been performed thus far in organotypically cultured spinal cord tissue.

Since the superficial dorsal horn plays a dominant role in nociceptive transmission, it was of particular interest to evaluate viability and activity of neurons in this location in organotypic cultures of spinal cord. CaMKIIα, μ-OR receptors, and met-enkephalin, a signal transduction agent, a neurotransmitter receptor and its ligand respectively, are all expressed in the dorsal horn of the spinal cord and contribute to the regulation of nociceptive transmission (discussed in chapters 1 and 3). Immunocytochemical analysis of CaMKIIα, met-enkephalin and μ-OR receptor expression in organotypic cultures of spinal cord provided a useful method of assessing the viability and activity of 'nociceptive' neurons in organotypic cultures of spinal cord.

CaMKIIα immunoreactivity was clearly identified in the dorsal horn of organotypically-cultured spinal cord. While concentrated in the superficial laminae of the dorsal horn, CaMKIIα-IR cells were also identified in the lateral funiculus (immediately lateral to the dorsal horn), in laminae III-VI and in laminae X adjacent to the central canal, locations which correlate closely with the location of cell bodies of several ascending nociceptive tracts including the spinothalamic tract, the spinoreticular tract and the spinomesencephalic tract (Willis *et al.,* 1995). α-CaMKII immunopositive cells were also identified in the dorsal and ventral corticospinal tracts in cultured spinal cord on days 1-5. The pattern of expression of CaMKIIα in cultured spinal cord correlates closely with that observed in freshly fixed sections derived from adult rats (Terishima *et al.,*1994). This group reported strong CaMKIIα expression in neurons in laminae I-IV, while laminae IV-X and the ventral horn were CaMKIIα immunonegative. CaMKIIα immunoreactivity was ascribed by Terishima *et al.* (1994) to three locations: presynaptic terminals, central processes of dorsal root ganglia, and dendritic processes of dorsal horn neurons.

μ-OR and met-enkephalin-immunopositive cells were identified in organotypically cultured sections of spinal cord on days 1-5 of culture, concentrated in the superficial

laminae and in lamina X adjacent to the central canal. While there was a gradual decline in the number of immunopositive cell numbers identified in the dorsal horn over time, the pattern of μ-OR and met-enkephalin immunoreactivity on day 1 of culture was closely similar to that observed on day 5 of culture, and appeared on visual inspection to closely resemble the pattern of expression in freshly harvested spinal cord tissue, fixed immediately post-euthanasia.

The pattern of expression of met-enkephalin and μ-OR in the dorsal horn of organotypic cultures of spinal cord correlates closely to that which has been reported previously in fixed spinal cord derived from adult animals. The immunohistochemical localisation of the μ-opioid receptor has been thoroughly characterised. While μ-OR's are concentrated in laminae I and II (Arvidsson *et al.,* 1995; Mansour *et al.,* 1994; 1995), they are also located in deeper laminae of the dorsal horn (Mansour *et al.,* 1994; 1995; Gouarderes *et al.,* 1985). The morphological localisation of μ-OR immunoreactivity in the dorsal horn in organotypic culture was consistent with previous reports of μ-OR in freshly fixed adult spinal cord (reviewed by Coggeshall & Carlton, 1997), where μ-OR immunoreactivity has been further localised to neuronal cell membranes, proximal dendrites and axonal fibres in the superficial dorsal horn (Arvidsson *et al.,* 1995), and predominantly to cell bodies in deeper laminae (Mansour *et al.,* 1995). Similarly, the morphological localisation of met-enkephalin expression identified in organotypic culture was consistent with previous reports of met-enkephalin expression in freshly fixed adult spinal cord (reviewed by Merchentaler *et al,* 1986), where met-enkephalin expression has been further localised to neuronal cell bodies and dendrites in the dorsal horn (Hokfelt *et al.,* 1977a; b;1979; Finley *et al.,* 1981; Merchentaler *et al.,* 1986).

4.5 CONCLUSIONS

This study describes a method of organotypic culture of post-natal spinal cord using an interface culture system which permits easy visualisation of individual neurons and easy environmental manipulation of these neurons. Such a culture system offers great potential for future *in-vitro* investigation of molecular mechanisms involved in central nociceptive transmission. There are many potential applications of this technique to numerous disciplines of pain research, including the potential for combining electrophysiological techniques and electrical stimulation, with molecular biology techniques. For example, techniques used in hippocampal slice culture systems to investigate the molecular basis of synaptic plasticity in higher centres can be applied to investigate the molecular basis of synaptic plasticity in the spinal cord, with the distinct advantage over existing techniques

that specific stimuli can be applied over a prolonged or intermittent time scale. The expression of molecular agents implicated in pathophysiological pain transmission can be studied in a sensitive system which permits the evaluation of the effects of stimulation with specific neuromodulatory agents on the function of individual spinal neurons. Organotypic slice cultures should also facilitate evaluation of time-dependent changes in spinal neuronal behaviour, such as immediate early gene expression, and facilitate more detailed evaluation of the molecular consequences of repetitive noxious (chemical or electrical) stimulation of varying intensity.

For the purposes of the present study, maintenance of spinal cord sections in culture for 5 days demonstrated the suitability of these cultures for use in investigations into molecular mechanisms of rapidly induced spinal neuroplasticity. In previous reports, organotypic cultures of thicker sections of spinal cord derived from neonatal rat pups have been successfully maintained in culture for up to 3 months (Rothstein *et al.,* 1993; 1995; Rothstein & Kuncl, 1995). It should be possible to maintain these sections of spinal cord for a prolonged period, which may be of use in studies into the molecular basis of persistent pain conditions.

CHAPTER 5: GENERAL DISCUSSION

Pain only exists in the understanding

Descartes

This study presents a new approach to the investigation of neuronal plasticity associated with tissue injury and inflammation. Wall (1999) recently speculated that classical dogma of a hard-wired, modality-dedicated relatively static pain transmission system had been superceded by the concept of pain perception as a considerably more complex 'need state' in which higher centres analyse and integrate nociceptive input, reacting in an appropriate manner through a battery of descending inhibitory and facilitatory pathways. In order to fully embrace this new concept of the 'pain system', strategies must be developed to facilitate investigation of every aspect of the central response to injury, not only those responses (predominantly excitatory) induced by severe injury.

5.1 Characterisation of nociceptive plasticity associated with mild inflammatory injury

Research into mechanisms of central nociceptive plasticity induced by inflammatory injury has classically concentrated on 'excitatory' plasticity of central nociceptive transmission, induced by induced by intense inflammatory injury resulting in extensive research into mechanisms underlying central sensitisation (reviewed by Dubner & Ruda, 1992; Yaksh, 1999). Although spinal and supraspinal mechanisms of pro-nociceptive and anti-nociceptive plasticity have been well characterised, existing knowledge about endogenous 'anti-hyperalgesic' mechanisms which the body engages to restore 'nociceptive homeostasis' has not yet been integrated into a clinically useful context and interpreted in parallel with those mechanisms which trigger pathophysiological maladaptation associated with central sensitisation and persistent hyperalgesia.

Millan (1999) speculated that the traditional emphasis on research into mechanisms underlying the induction of pathological pain sensation, rather than into endogenous mechanisms which adapt to or attenuate nociceptive transmission, reflected general frustration with attempts to develop novel analgesic or anti-hyperalgesic strategies based on the principle of engagement of endogenous antinociceptive mechanisms. Such frustration, in turn, could reflect the limitations of the high-intensity inflammatory model for facilitating evaluation of the endogenous adaptive response to injury. Improved understanding of the mechanisms underlying the engagement of endogenous

antinociceptive pathways may lead to the development of new strategies to combat persistent and other pathophysiological pain states.

The present studies demonstrate that plasticity of nociceptive responses induced by mild repetitive inflammatory challenge differs in several respects from the plasticity induced by intense acute inflammatory injury.

Mild inflammation induced by a dose of 0.25 mg carrageenan induced consistent inflammation, accompanied by thermal and mechanical hyperalgesic behaviour of similar magnitude to that induced by the doses of carrageenan (2-6 mg) standardly used to investigate nociceptive plasticity induced by inflammatory injury. This observation suggests that high-intensity models of inflammatory injury are unnecessary- and, from an animal welfare perspective, highly undesirable. Moreover, different mechanisms underlying plasticity of nociceptive responses may be induced by high intensity as opposed to low-intensity injury. High intensity injury may disable the engagement of effective endogenous adaptive mechanisms, making further investigations into physiological adaptation to injury impossible.

5.2 Investigation of molecular mediators implicated in the induction and maintenance of nociceptive plasticity

Knowledge of the molecular basis of central nociceptive plasticity is constantly evolving, reflecting the enormous number of interacting neurotransmitter and receptor systems that orchestrate the nociceptive response. *In-situ* hybridisation studies were performed to investigate the potential involvement of numerous molecular agents, previously implicated in neuronal plasticity in higher centres, in plasticity of nociceptive transmission in the spinal cord. The *in-situ* hybridisation studies presented in this thesis indicate that changes in the expression of CaMKIIα, COX-2 and enkephalin mRNA in laminae I/II of the dorsal horn may contribute to the induction and/or maintenance of central nociceptive plasticity induced by mild inflammatory injury.

As a signal transduction enzyme capable of regulating activity of many intracellular processes, CaMKIIα gene regulation clearly offers the potential to enhance or attenuate ascending nociceptive transmission, and it is highly likely that the consequences of CaMKIIα gene regulation are critically influenced by the intensity of the original stimulus. Low-dose carrageenan treatment dose-dependently induced unilateral upregulation of CaMKIIα gene expression. A significant reduction in the magnitude of ipsilateral CaMKII

α gene expression coincided with tachyphylaxis of hyperalgesic behaviour following daily repeated low-dose carrageenan treatment. Since enhanced CaMKIIα expression is a hallmark of LTP in higher centres, this evidence suggests that the development of hyperalgesia following inflammatory injury may in part reflect LTP of spinal nociceptive transmission. Since CaMKIIα has the potential to modulate both excitatory and inhibitory nociceptive transmission, further studies are clearly indicated to identify the pathways targeted by altered CaMKIIα gene expression in association with differing intensities of inflammatory injury.

Simultaneous upregulation of COX-2, an enzyme which synthesises 'excitatory' neuromodulatory prostaglandin molecules, and of proenkephalin, a classical 'antinociceptive' neurotransmitter illustrates the inherent duality of the central nociceptive response to injury, and its capacity for fine-tuned modulation of nociceptive responses in response to stimulations of differing intensity.

While a clear dose-dependent relationship between COX-2 gene expression in laminae I/II and hyperalgesic behaviour associated with inflammatory injury was evident following weekly carrageenan treatment (0.25 mg and 0.05 mg), no clear relationship was observed between COX-2 mRNA expression and the observed plasticity of nociceptive responses induced by daily low-dose (0.05 mg) carrageenan treatment. Similarly, proenkephalin mRNA was unilaterally upregulated in response to mild inflammatory injury in an intensity-dependent fashion, but no clear relationship was observed between proenkephalin mRNA expression and the observed plasticity of nociceptive responses induced by daily repeated low-dose carrageenan treatment. The involvement of COX-II (prostaglandin) and enkephalin-mediated mechanisms in nociceptive plasticity would thus appear to depend critically upon the intensity and frequency or duration of injury.

The *in-situ* hybridisation studies presented in this thesis identified a pattern of gene induction associated with mild inflammatory injury, which differed from that reported in association with high-intensity injury. Prodynorphin and immediate early gene mRNA induction associated with mild inflammatory injury was markedly lower than that previously reported following intense inflammatory injury. The magnitude of ipsilateral upregulation of tPA, junD, zif 268; COX-1; prodynorphin; and GAD 67 identified in the present study did not appear to be directly related to the intensity of inflammatory injury or to the intensity of hyperalgesic behaviour, since a similar magnitude of upregulation was observed following saline or carrageenan treatment.

With the exception of CaMKIIα, no close correlation was identified between the attenuation of hyperalgesic behaviour induced by daily repetitive mild inflammatory stimulation, and gene expression investigated using *in-situ* hybridisation studies. While it is possible that the limited time-points at which target genes were analysed could have led to failure to identify transient alterations in expression of certain genes, particularly immediate early genes, as potential mediators of the observed nociceptive plasticity, it is also highly likely that tachyphylaxis of hyperalgesia observed in following daily repeated carrageenan treatment was mediated through alternative spinal neuromodulatory pathways or indeed through supraspinal (or peripheral) mechanisms, that were beyond the scope of the present study.

In conclusion, the present study indicated that mild inflammatory injury induced changes in expression of certain molecular mediators of neuronal plasticity (most notably CaMKII α, COX-2 and potentially proenkephalin), but not of immediate early genes, GAD 67 or prodynorphin. Having identified a potential role for CaMKIIα, COX-2 and enkephalin in the mediation of central nociceptive plasticity induced by mild inflammatory injury, the contribution of these agents clearly warrants further investigation.
While the role of COX-2 in the mediation of nociceptive transmission (as distinct to its role in the generation of inflammation) has been extensively investigated in the past, associated with the development of non-steroidal anti-inflammatory drugs as analgesic agents, it is only relatively recently that the involvement of COX-2 in modulation of central nociceptive plasticity has been recognised. Numerous research strategies offer the potential for more thorough evaluation of the roles of these, and other newly identified, agents in mediating central plasticity following mild inflammatory injury, including administration of highly specific agonists/ antagonists and studies using transgenic mice (in which the effects of specific gene deletion or gene over-expression on nociceptive responses may be investigated)

Linking observations of behavioural plasticity, and plasticity of gene expression, associated with repetitive inflammatory injury

A primary aim of the research presented in this thesis was to investigate and identify similarities between central plasticity of neuronal transmission in higher centres- most notably the hippocampus- which have been heavily implicated in the development of 'memory', and central plasticity of nociceptive transmission in the spinal cord as a mechanism for the development of a 'memory of pain' (persistently enhanced pain

perception) or physiological adaptation to mild injury (persistently attenuated pain perception). Further investigation into the mechanisms underlying nociceptive plasticity induced by mild inflammatory injury may provide valuable new insights into endogenous 'anti-hyperalgesic' mechanisms. However, extensive characterisation of the mediation of endogenous 'anti-hyperalgesia' is clearly indicated.

In the first instance, the sites- spinal, supraspinal or peripheral- at which modulation of hyperalgesia induced by mild repetitive inflammatory injury occurs- must be identified. Studies on spinalised rats using a model of low-dose repetitive carrageenan model may be indicated to facilitate identification of the role of descending modulatory pathways in the induction of tachyphylaxis of hyperalgesia. Having identified these sites, and using the results of the present *in-situ* hybridisation studies as indicators of certain molecular agents implicated in the mediation of this tachyphylaxis, techniques such as differential display, microarrays and gene grid analysis may provide new insights into the pharmacological basis of adaptive nociceptive plasticity induced by mild injury, while facilitating more thorough analysis of the regulation of transiently-expressed genes (including immediate early genes) over a prolonged time span so that their involvement in the induction and maintenance of central nociceptive plasticity can be assessed more thoroughly.

Tachyphylaxis of nociceptive transmission associated with mild repetitive inflammatory injury potentially provides a behavioural correlate for LTD of central nociceptive transmission. If, as has been hypothesised (Sandkuhler, 2000), therapeutic TENS achieves pain relief in persistent pain conditions through the induction of LTD of pain transmission in primary afferent fibres and spinal projection neurons in the dorsal horn, then it is reasonable to speculate that similar endogenous mechanisms may mediate physiological 'anti-hyperalgesia' following mild inflammatory injury. Further investigation of this hypothesis is clearly indicated, potentially using electrophysiological studies to identify changes in responsiveness of neurons in the spinal cord and in higher centres associated with mild repetitive inflammatory injury.

Sherrington apocryphally described a human patient suffering from chronic pain as 'a slice preparation that could talk'; conversely, a 'living' slice preparation could offer valuable insights into mechanisms underlying conscious pain sensation, and could identify new pharmacological targets for pain control. The method for organotypic slice culture of neonatal rat spinal cord tissue described in this study provides a means of investigating the responses and interaction of individual spinal neurons following stimulation with specific

neuromodulatory agents or electrical stimuli. This technique should also facilitate analysis of the interaction of mediators involved in central nociceptive.

In conclusion, this thesis provides a new perspective on the approach to understanding plasticity of pain transmission, and a novel technique for the study of molecular processes involved in nociceptive transmission in the spinal cord. The nature of pain remains a mystery. Improved understanding of the nature of pain transmission is of universal importance. It is to be hoped that this study may have taken some steps towards elucidating 'the truth' of the mystery of pain transmission, which - if complex- is still out there...

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