ELECTROPHYSIOLOGICAL STUDIES ON THE PHARMACOLOGY OF ALTERED SPINAL NOCICEPTIVE MECHANISMS INDUCED BY INFLAMMATION IN THE RAT

by

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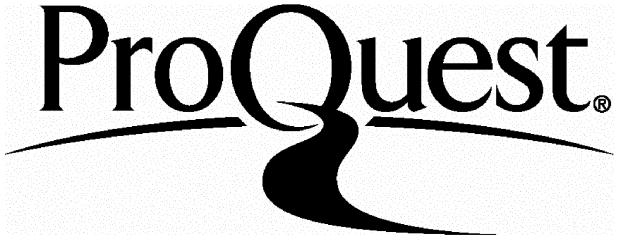

Abstract

The purpose of the *in vivo* electrophysiological experiments presented in this thesis was to investigate how the pharmacology of the spinal transmission and modulation of nociceptive messages following the development of peripheral inflammation compares with that in normal, non-inflamed animals. Recordings of convergent dorsal horn neurones were made in intact halothane anaesthetized rats, and unilateral inflammatory hyperalgesia was produced by the injection of carrageenan into the hind paw.

Alterations in spinal nociceptive transmission occurred as the inflammation developed. 58% of the neurones tested showed enhanced responses to peripheral electrical stimulation; evidence of central sensitization. In contrast, the other neurones showed decreased responses, which were linked with high levels of *N*-methyl-D-aspartate (NMDA) receptor-mediated wind-up. The role of the NMDA receptor in spinal nociceptive transmission was found to be altered post-carrageenan, with evidence for facilitated activation of the receptor.

The modulation of spinal nociceptive transmission following inflammation was studied using antinociceptive drugs, both opioid and non-opioid, to inhibit nociceptive responses. The mu opioid morphine showed a large increase in spinal potency following inflammation which was not seen with delta or kappa opioids, or the non-opioid analgesics tested. An investigation into the pharmacological systems in the spinal cord underlying this selective enhancement in the potency of morphine revealed the 'anti-opioid' peptide cholecystokinin as a factor in this phenomenon. The descending noradrenergic system, which has the potential to synergize with the antinociceptive effects of mu opioids, was not involved.

Changes in the endogenous spinal opioid systems after inflammation were also studied, revealing enhanced actions of the endogenous kappa opioid ligand dynorphin in the absence of any changes in the controls exerted by the enkephalins, the endogenous ligand at delta opioid receptors.

Complex changes in the pharmacology of spinal sensory systems occur within hours of the development of inflammation.

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CONTENTS

Publications arising from this work:

Papers

Stanfa, L.C., Sullivan, A.F. and Dickenson, A.H. (1992) Alterations in neuronal excitability and the potency of spinal mu, delta and kappa opioids after carrageenan-induced inflammation. Pain, **50**, 345-354.

Stanfa, L.C. and Dickenson, A.H. (1993) Cholecystokinin as a factor in the enhanced potency of spinal morphine following carrageenin inflammation. Br. J. Pharmacol., **108**, 967-973.

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Abstracts

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CHAPTER 1

INTRODUCTION

Over the years, much work has been carried out to elucidate both the physiology and pharmacology of the transmission and modulation of nociceptive signals arising from acute noxious stimuli. However it is equally, if not more, important from a clinical perspective to study the changes in the transmission and modulation of nociceptive messages that may occur in cases of persistent pain, since these may be more relevant to clinical problems.

Despite extensive research in the field, the analgesic management of many patients remains poor. The opioids, which have been used for thousands of years for both social and medicinal purposes to produce euphoria, analgesia and sleep, are still the first and in many cases also the last line of defence against moderate to severe pain. Although drugs such as morphine provide excellent analgesia in many cases, the incidence of side effects, the development of tolerance and the reluctance of clinicians to prescribe these powerful drugs in cases of pain associated with non-terminal conditions for fear of addiction / dependence mean that many patients still suffer pain (see Houde, 1989). In addition certain pains, particularly those arising from nerve damage, the so called "neuropathic" pains, can respond poorly to opioids even at high doses, where side effects can be a limiting factor (Arnér and Meyerson, 1988; Portenoy et al., 1990; Jadad et al., 1992). At the present time clinicians have little else to offer these patients.

Advances are being made in the treatment of pain in the form of better education about the possibilities of opioid addiction arising from medical use, patient-controlled analgesia, whereby patients are able to deliver drugs according to need rather than dosing schedule and particularly in the use of combination therapy, where the co-administration of drugs such as local anaesthetics or α_2-adrenoceptor agonists with opioids may lead to better analgesia with a lower incidence of side effects (see refs. in Dickenson and Sullivan, 1993). Another path currently being explored is the possibility that "pre-emptive" analgesia, administering analgesic drugs before surgery to prevent the development of central changes, may lead to better management of post-operative pain. The efficacy of this approach in the clinic remains controversial (McQuay, 1994).

We still know relatively little about the changes in nociceptive systems, particularly the central changes, that occur in persistent pain states. By using animals models of more prolonged pain states we can investigate the nature, and most importantly, the consequences of the changes that occur in the transmission and modulation of nociceptive messages. By further elucidating these mechanisms we may have new therapeutic targets such as preventing or reversing these changes if they are detrimental to the patient, for example leading to aberrant central processing of nociceptive information or interfering with analgesic mechanisms, or alternatively, enhancing or mimicking these changes if they are beneficial. This approach may lead to new analgesic strategies, possibly using existing licensed drugs, to treat cases of pain where conventional therapy is inadequate

The series of experiments presented in this thesis has concentrated on the changes in the pharmacology of the transmission and modulation of nociceptive messages in the spinal cord arising from the development of an inflammatory state in the periphery. The study of nociception of inflammatory origin is relevant to a variety of commonly encountered pain states such as those arising from surgery, diseases such as arthritis and aspects of the pain resulting from certain cancers. However there is a growing body of evidence resulting from both clinical and basic science studies to suggest that the underlying nature of the central changes resulting from inflammatory nociception differs from that arising from neuropathic pain. We should bear this in mind when directing treatment at reversing / enhancing changes induced by prolonged nociceptive states.

So how does the pain message get from the periphery, where the injury occurs, to the brain where it is perceived as pain by the conscious animal and what changes occur in inflammation that may affect this?

1.1. Peripheral activation of sensory fibres

Under normal conditions, the sensory fibres innervating cutaneous tissue can be broadly divided into two groups; those relaying innocuous stimuli to sensory systems in the spinal cord and those conveying noxious signals. Innocuous sensory stimuli such as touch are relayed to the dorsal horn of the spinal cord by thickly myelinated, therefore fast conducting Aβ-fibres (Burgess and Perl, 1973). The peripheral endings of these fibres innervate specialized structures which are maximally activated by low threshold, innocuous stimuli (Burgess and Perl, 1973). The peripheral endings of individual Aβ-fibres are associated with a specific receptor type, each of which responds maximally to a different quality of innocuous sensation. Thus, Aβ-fibre afferents innervating Pacinian corpuscles respond maximally to vibration, whilst rapidly adapting fibres innervating Krause receptors respond preferentially to indentation of the skin, with a further group of receptors responding preferentially to hair movements, allowing perception of the different qualities of tactile sensation (see Willis and Coggeshall, 1991).

Noxious stimuli, in non-pathological conditions, are relayed to the dorsal horn of the spinal cord, the first synapse in the relay of sensory information to the brain, by two classes of fibres, the C-fibres and some Aδ-fibres. C-fibres comprise the largest class of nociceptive fibres, the majority of which innervate nociceptors which respond well to all three types of noxious stimulation, namely thermal, mechanical and chemical (Bessou and Perl, 1969) and are hence known as C-polymodal nociceptors. These C-fibres are fine, unmyelinated fibres and therefore conduct at a much slower rate than the

thick, myelinated Aβ-fibres (Burgess and Perl, 1973). The other fibre group responsible for relaying noxious information to the spinal cord are the Aδ-fibres. These fibres are fine, myelinated fibres, conducting slightly faster than C-fibres, which innervate nociceptors responding maximally to mechanical, and in some cases thermal, stimuli in the noxious range, but responding only weakly to chemical stimuli (Burgess and Perl, 1967).

Polymodal nociceptive fibres, unlike Aβ-fibres which often reach maximum discharge rates during innocuous stimulation, respond maximally to intense noxious stimulation, showing an increasing discharge with increasing stimulus intensity (Burgess and Perl, 1973). In contrast to Aβ-fibres, the peripheral terminals of nociceptive fibres have not yet been associated with any specialized end structures, and in the case of C-fibres appear to be simply bare nerve endings which have lost their Schwann cell covering and in some cases even their basement lamella (Andres and von Düring, 1990). Although specialized end structures may exist, none have yet been visualized, leaving the mechanism by which nociceptors are activated unclear. It has been suggested that the bare nerve endings themselves act as stimulus transducers, however the means by which they might do this is unknown at the present time.

1.2. What changes occur in inflammation to affect the peripheral activation of C-fibres?

The development of inflammation in the periphery is accompanied by a change in the climate surrounding the peripheral nociceptors. Tissue damage and an influx of cells of the immune system leads to the production of an inflammatory exudate which has profound effects on the local nociceptive terminals. Under the conditions of inflammation in the periphery, nociceptors show plasticity, whereby their responsiveness to stimuli is altered. This phenomenon results from both direct activation of the nociceptors, and from sensitization of the nociceptors by inflammatory mediators, which is characterized by an increase in ongoing activity, a lowered threshold to mechanical and thermal stimuli and increased and prolonged firing to a suprathreshold stimulus (Levine et al., 1993).

Mediators known to be produced in inflammation capable of causing direct activation of nociceptors include protons (Steen et al., 1992), 5-HT (Handwerker et al., 1990) and bradykinin, which is cleaved from kininogens in the plasma following protease activation at sites of injury and produces activation of the neurones via actions at the bradykinin B_2 receptor (Dray and Perkins, 1993). The development of inflammation can also induce the expression of bradykinin B_1 receptors, although the role of these receptors is unclear. Inflammatory mediators can produce sensitization of polymodal nociceptors either by acting directly on the nociceptor, or indirectly by actions on cells other than the

nociceptors, such as sympathetic post ganglionic neurones and white blood cells, which then in turn release hyperalgesic agents which act to sensitize the polymodal nociceptors. Substances which are generated during inflammatory processes and act directly on nociceptors to produce sensitization include prostaglandins E_2 and I_2 (Pitchford and Levine, 1991), adenosine (acting at an A_2 type receptor, Taiwo and Levine, 1990) and 5-HT. The receptor subtype mediating 5-HT sensitization is a matter of some debate with evidence presented for $5HT_3$ receptors (Richardson et al., 1985; Eschalier et al., 1989), $5\text{-}HT_2$ (Rueff and Dray, 1992) and the $5HT_{1a}$ receptor (Taiwo and Levine, 1992); other receptor subtypes cannot be ruled out. In addition to the direct activation of neurones, bradykinin also sensitizes neurones to thermal and mechanical stimuli. This action of bradykinin can result from the production of prostaglandins, since the sensitizing action of bradykinin is blocked by cyclo-oxygenase inhibitors (see Dray and Perkins, 1993). There is evidence to suggest that these prostaglandins may be derived from sympathetic postganglionic neurones (Levine et al., 1986b) although this has been challenged (Koltzenburg et al., 1992). Other inflammatory mediators suggested to indirectly sensitize nociceptive neurones include interleukin-1, which stimulates the production of prostaglandins from non-neuronal cells (Ferriera et al., 1988), thereby producing an indomethacin-reversible hyperalgesia, and leukotriene B_4 which produces a hyperalgesia dependent on the presence of white blood cells (Levine et al., 1984).

Regardless of whether the final result is activation or sensitization of the nociceptor, all the mediators ultimately produce their effect through membrane ion channels. This effect may be direct, through receptor gated ion channels, or controlled indirectly through intracellular second messengers. Both of these mechanisms cause direct activation of the neurone or enhancement of the excitatory effects of other stimuli.

Nociceptive primary afferents themselves also contribute to the inflammatory process in the periphery. Once activated, C-fibres are capable of releasing neuropeptides such as substance P and CGRP at their peripheral endings in the skin following activation by electrical stimulation (White and Helme, 1985) or noxious heat (Helme et al., 1986). Substance P is the peptide most studied in this respect, and direct application of substance P to peripheral tissues has been shown to produce vasodilatation and increase vascular permeability, to attract white blood cells and increase the release of inflammatory mediators from these cells (Otsuka and Yoshioka, 1993). All these effects of substance P are pro-inflammatory and will enhance the inflammatory response. Indeed, the finding that the destruction of primary afferent nociceptors with capsaicin reduces the severity of experimental arthritis (Levine et al., 1986a) suggests that primary afferent nociceptors actively contribute to inflammation.

Thus the development of inflammation leads to the generation of mediators which

sensitize / activate nociceptors (peripheral sensitization), a mechanism that is believed to underlie the primary hyperalgesia (hyperalgesia in the area of the tissue injury) seen in inflammatory conditions. In contrast, the neural mechanisms underlying secondary hyperalgesia (hyperalgesia in areas adjacent to areas of tissue injury) are less well understood although there is much evidence to suggest that central mechanisms are involved (central sensitization) and this will be discussed later (chapter 3).

Another feature of the development of inflammation in the periphery is the finding that nociceptors which are normally unresponsive to thermal or mechanical stimuli, or silent nociceptors as they have been called, develop responsiveness to mechanical and thermal stimuli following their activation by chemical mediators generated during inflammation (see McMahon and Koltzenburg, 1990). It has been suggested that as many as 30-45% of the unmyelinated afferents in the skin may be of this type (Handwerker et al., 1991; Meyer et al., 1991). The recruitment of these fibres in inflammation will have implications for the afferent barrage reaching the spinal cord from the inflamed area, and may contribute to plastic changes in the CNS triggered by activity in nociceptive afferents.

The development of inflammation can also lead to changes in the periphery which can be regarded as anti- rather than pro-nociceptive. The most widely studied of these inflammation-induced anti-nociceptive events is the occurrence of functional opioid receptors in the periphery. Administration of opioid agonists directly into inflamed, but not non-inflamed tissue results in antinociception, an effect which can be seen within hours of the onset of inflammation (Joris et al., 1987; Hargreaves et al., 1988b; Stein et al., 1988a,b). This peripheral action of opioids in inflammatory states is now being exploited in the clinic (Khoury et al., 1990).

1.3. What happens following activation of primary afferents in the periphery?

The first synapse in the somatosensory pathway for noxious and some innocuous information is in the dorsal horn of the spinal cord and it is here that modulation of the sensory message may occur.

Nociceptive C-fibre afferents innervating peripheral cutaneous tissue enter the spinal cord via the dorsal roots. Although a proportion of unmyelinated afferents are also found in the ventral roots, it is unclear in most species whether these afferents actually enter the cord to make functional connections. Having entered the spinal cord, the C-fibres may bifurcate before traveling in the cord in a rostro-caudal direction through Lissauer's tract from which collaterals enter the grey matter to terminate in the superficial layers of the dorsal horn in laminae I and II (the substantia gelatinosa) (Willis and Coggeshall, 1991). In the rat, the rostrocaudal spread of C-fibre projections can extend

over several segments at lumbo-sacral levels (Fitzgerald, 1989). Aδ-fibres, which can also relay noxious sensory information from the periphery, follow a similar pattern, terminating either in the superficial laminae of the dorsal horn (primarily lamina I) or in the deeper layers of the dorsal horn, laminae IV-VI (Fitzgerald, 1989).

In contrast to the afferents relaying noxious information from the periphery, the majority of the large, myelinated Aβ-fibres relaying innocuous sensory information ascend via the dorsal column to terminate in the dorsal column nuclei (Brown, 1973). However, collaterals from these Aβ-fibre afferents penetrate the dorsal horn of the spinal cord where they terminate exclusively in the deeper layers of the dorsal horn, laminae III-V (Fitzgerald, 1989).

Nociceptive information transmitted to the spinal cord by C- and Aδ-fibre afferents is ultimately received by projection neurones in the dorsal horn where it is related to the higher brain centers via the ascending pain pathways (see **1.6**). However, the spinal cord is far from a simple relay station. Before nociceptive information is relayed to supraspinal structures where it is perceived as pain in the conscious animal, it is subject to amplification / modulation by both intrinsic spinal circuitry and descending influences.

Nociceptive information arriving in the dorsal horn is received by two classes of dorsal horn neurones. The first class of neurones is activated exclusively by noxious stimuli, responding only to Aδ- and or C-fibre inputs, and are known as nociceptive specific neurones. These neurones are located predominantly in lamina I of the dorsal horn and as such are able to receive mono-synaptic C- and Aδ-fibre inputs (Besson and Chaouch, 1987). However, the commonest class of dorsal horn neurone responding to noxious stimuli is the convergent, or wide dynamic range neurone. These neurones respond to innocuous cutaneous stimuli such as light touch, increasing their discharge in response to increasing intensities of thermal and mechanical stimuli in the noxious range (Le Bars et al., 1986). Unlike nociceptive specific neurones, these neurones receive inputs from Aβ-fibres as well as C- and Aδ-fibres. Convergent neurones have been recorded in the superficial zone of the dorsal horn, however they are found predominantly in laminae IV-VI of the dorsal horn, with the highest concentration in lamina V (Besson and Chaouch, 1987). This leaves the problem of how these convergent neurones located in the deeper regions of the dorsal horn receive their input from C-fibres, which terminate only in the superficial region of the dorsal horn. Although the convergent neurones can receive direct mono-synaptic inputs from Aβ-fibres and some Aδ-fibres which terminate in this deeper region of the dorsal horn, few of these convergent neurones have dendritic extensions that reach into laminae I and II (Woolf and King, 1987) where the majority of the C-fibres terminate. Consequently it is likely that nociceptor activation of these deep

convergent neurones is largely indirect, relayed via interneurones located in the intermediate laminae. This poly-synaptic pathway has implications for the modulation of the nociceptive message as it is relayed through the spinal cord.

1.4. Pharmacology of nociceptive transmission in the spinal cord

The C-fibre primary afferent transmitters are numerous and varied. Perhaps one of the most important features of C-fibres in terms of nociceptive transmission concerns the potential co-existence of a number of putative transmitters in each fibre. As many as four peptides, including substance P (SP), have been found to co-exist in small diameter dorsal root ganglion neurones in the cat (Cameron et al., 1988). Since 90% of neurones staining for substance P are also positive for glutamate (Battaglia and Rustioni, 1988), it is likely that glutamate is also found in the majority of these neurones. Glutamate and SP (De Biasi and Rustioni, 1988) and in some cases also CGRP (Merighi et al., 1991) have also been shown to co-exist in primary afferent terminals in the superficial dorsal horn. As a consequence of this, impulses arriving at the central terminals of C-fibres have the potential to evoke the release of not just one, but in some cases as many as five potential transmitters. There is evidence that glutamate and the neuropeptides are found in separate vesicles (Merighi et al., 1991), which may allow for a differential release of these substances under different nociceptive conditions. Thus, activation of the post-synaptic neurone, whether it is a projection neurone or an intrinsic dorsal horn neurone in a poly-synaptic pathway, may result not only from the release of the classical fast excitatory transmitters, the amino acids glutamate and aspartate, but may also be mediated by the barrage of peptides released from the primary afferent C-fibres. Although the role of glutamate in the transmission of nociceptive (and of course innocuous) information in the spinal cord is now well accepted, largely as a result of electrophysiological studies where agonists and antagonists at the AMPA and NMDA receptors have been employed (see introduction to chapter 4), the nature of role played by some of the primary afferent peptides in nociceptive transmission is less clear.

The most widely studied of all the C-fibre primary afferent peptides is substance P (SP), which was implicated in pain transmission long before the contribution of the excitatory amino acids was recognized. Around 20% of dorsal root ganglion neurones are positive for SP-like immunoreactivity (see refs. in Willis and Coggeshall, 1991) and neurokinin 1 (NK1) receptors, the target receptor for SP are located post-synaptically to primary afferents (Yashpal et al., 1991), around the areas where nociceptive primary afferents terminate. The release of substance P in the spinal cord (presumably originating in part from primary afferent nociceptors, although SP is also present in some intrinsic neurones and descending fibres (see Willis and Coggeshall, 1991)) is increased following

noxious but not innocuous peripheral stimulation (Yaksh et al., 1980; Duggan and Hendry, 1986; Go and Yaksh, 1987; Duggan et al., 1988). Stimulation of dorsal roots of rat spinal cord in vitro produces both an early, fast and a late, slow depolarization in dorsal horn neurones (Urban and Randic, 1984). This late, slow depolarization is mimicked by the exogenous application of SP (Henry, 1976; Murase and Randic, 1984) and blocked by substance P antagonists (Urban and Randic, 1984; De Koninck and Henry, 1991). In vivo, intrathecal administration of SP evokes behaviour attributed to pain in animals (Hylden and Wilcox, 1981; Piercey et al., 1981).

Despite this seemingly good evidence for a role of SP in pain transmission in the spinal cord, a question mark still hangs over SP as a conventional transmitter of nociception, due in part to the questioning of whether the behavioural syndrome elicited by intrathecal SP is indeed indicative of pain (Bossut et al., 1988; Frenk et al., 1988). The lack, until recently, of good neurokinin antagonists has hampered attempts to determine the nature of the role played by SP in pain transmission. Recently developed NK1 receptor antagonists such as CP-96,345 have provided further support for a role of SP in spinal nociceptive, but not non-nociceptive, transmission (e.g. De Koninck and Henry, 1991; Radhakrishnan and Henry, 1991). However, CP-96,345 and its stereoisomer are also active at calcium channels (Schmidt et al., 1992), and this property, rather than its NK1 blocking effects have been reported to be behind the antinociceptive effect of CP-96,345 against carrageenan-induced hyperalgesia and in the formalin test (Nagahisa et al., 1992). Antinociception can be produced by more recent NK1 antagonists such as RP67580 (Chapman and Dickenson, 1993) but their exact pharmacological profile remains to be determined. Consequently, care must be taken when interpreting the results of some of these antagonist studies. Regardless of whether or not SP plays an important role as a classical neurotransmitter of nociceptive information, evidence does suggest that SP also functions in a modulatory role in the spinal cord, facilitating the activation of the NMDA receptor by providing the sustained depolarization necessary for the removal of the Mg^{2+} block from the associated ion channel. In addition to the voltage dependent regulation of NMDA receptors, SP has also been shown to directly modulate NMDA receptor responses, although this effect is suppressed at physiological concentrations of glycine (Rusin et al., 1992). This modulatory role of SP is discussed further in chapter 4.

Another member of the tachykinin family of peptides present in primary afferent nociceptors is neurokinin A (NKA), which acts preferentially on neurokinin 2 receptors (see Levine et al., 1993). Although neurokinin A can be released in the spinal cord following noxious stimulation (Duggan et al., 1990), the role of this peptide in spinal nociceptive transmission has not been as extensively studied as SP. Exogenous

neurokinin A has been shown to facilitate thermal nociceptive responses of dorsal horn neurones (Fleetwood-Walker et al., 1990), but unlike substance P, the presence of NKA in the tissue can be detected long after the cessation of the stimulus, and at sites a long way removed from its presumed site of release in the substantia gelatinosa (Duggan et al., 1990). NKA may then have actions away from the site of release at the primary afferent terminals, possibly leading to effects other than direct nociceptive transmission.

One primary afferent peptide which is presumed to act as a neuromodulator rather than a neurotransmitter is calcitonin gene-related peptide (CGRP). CGRP alone produces only mild excitatory effects on dorsal horn neurones (Miletic and Tan, 1988). Perhaps more importantly, small amounts of CGRP markedly potentiate the excitatory effect of both exogenous substance P and noxious stimulation on dorsal horn neurones in vivo (Biella et al., 1991) and promote the intraspinal spreading of this peptide (Schaible et al., 1992). The use of the antibody microprobe technique has demonstrated the release of CGRP in the superficial dorsal horn following noxious stimulation (Morton and Hutchison, 1990), and whilst CGRP itself may not act as a primary afferent transmitter of nociceptive information in the dorsal horn, it is likely to contribute to this process through a potentiation of the actions of substance P, possibly by retarding the enzymatic degradation of this primary afferent transmitter (Le Greves et al., 1985).

In addition to peptides that are either directly or indirectly pro-nociceptive, C-fibre afferents also contain peptides which have been considered to be analgesic. Somatostatin, which is found in about 10% of DRG neurones (Willis and Coggeshall, 1991) has been shown to be released in the cord following noxious thermal stimulation (Kuraishi et al., 1985). Spinal administration of somatostatin has been shown to inhibit the firing of dorsal horn neurones (Randic and Miletic, 1978; Sandkuhler et al., 1990), however the lack of a specific antagonist for this substance makes the elucidation of the physiological role played by this primary afferent peptide rather difficult. Galanin, which can be co-localized with substance P and CGRP, is found in around 7% of DRG neurones (Willis and Coggeshall, 1991), and has also been reported to produce antinociceptive effects, although this tends to depend on the dose of galanin used (see Levine et al., 1993). Again, the nature of the role played by this peptide in nociceptive transmission is unclear.

Thus the final effect on the postsynaptic neurone following the activation of C-fibres depends on the interactions between a complex array of substances released from primary afferent nociceptors, each of which can contribute in a subtly different way to nociceptive transmission. A further complication arises as a result of the development of inflammation in the periphery, when complex changes occur in the levels of peptides contained in the primary afferent nociceptors. The nature and likely implications of this will be discussed in section **1.8**.

1.5. Modulation of nociceptive transmission in the spinal cord

Modulation of the transmission of nociceptive messages can occur at the level of the C-fibre terminal. The release of the primary afferent transmitters can be controlled not only by the level of activity in the primary afferent neurone itself, but also by the activity of local circuit neurones and descending fibres. The C-fibre terminals have a rich variety of receptors located on them, with many of the classes of receptor found on post-synaptic dorsal horn neurones also being found on the terminals of the C-fibre primary afferents. C-fibres terminate in the substantia gelatinosa (SG) of the dorsal horn, a region known to contain a dense network of interneurones (Willis and Coggeshall, 1991). Interneurones containing inhibitory transmitters such GABA (31% of neurones in the SG contain GABA) and less frequently enkephalin (see introduction to chapter 9) form axoaxonic synapses with the terminals of C- and Aδ-fibre primary afferents (Todd and Spike, 1993). Activation of opioid receptors on these primary afferent terminals by enkephalins has been shown to inhibit excitatory transmission in the superficial dorsal horn, an effect suggested to result from a suppression of Ca^{2+} entry into the nerve terminal (Hori et al., 1992) with transmitter release reduced as a consequence. This pre-synaptic action of enkephalin may result from a block of voltage activated Ca^{2+} channels through activation of intracellular messengers or the channels may be deactivated as a result of hyperpolarization of the terminal due to activation of K^+ channels (Hori et al., 1992). In the rat, 70% of μ-opioid receptors in the dorsal horn are located on the terminals of primary afferents (Besse et al., 1990), which suggests that the pre-synaptic action of clinically administered opioids is likely to play a major part in the spinal antinociceptive effects of these drugs.

Activation of GABA receptors located on the primary afferent terminals also leads to an inhibition of nociceptive transmission, although the mechanism by which this is produced is different from that of opioids. In contrast to the hyperpolarizations of postsynaptic neurones produced by GABA (Curtis et al., 1971), exogenous GABA has been shown to depolarize primary afferent terminals both *in vitro* (Barker and Nicholl, 1972) and *in vivo* (Curtis et al., 1977). GABA-mediated presynaptic inhibition is believed to result from this depolarization of the terminals (primary afferent depolarization or PAD) by GABA (Barker and Nicholl, 1972), which results in a reduction in the potential change produced by invading impulses with a consequent reduction in the amount of transmitter released (Eccles et al., 1963). It is unclear as to whether the ionic mechanism underlying this GABA-mediated depolarization differs from that producing hyperpolarizations postsynaptically (Willis and Coggeshall, 1991).

The presynaptic inhibition of nociceptive primary afferents by GABA has been

proposed to be one of the mechanisms underlying the segmental control of spinal nociceptive transmission resulting from the activation of large-diameter afferents, although postsynaptic effects have not been ruled out (Besson and Chaouch, 1987). This mechanism was first proposed by Melzack and Wall (1965) in the "gate control theory". This proposed that activation of Aβ-fibres can produce a segmental inhibitory control of nociceptive transmission by activating inhibitory interneurones in the substantia gelatinosa which then presynaptically inhibit nociceptive primary afferents, thereby reducing the response of spinal neurones to nociceptive stimulation. Activation of these segmental controls underlies the analgesia produced as a result of dorsal column stimulation (resulting from the antidromic activation of collaterals of Aβ-fibres in the dorsal horn), and transcutaneous nerve stimulation using high frequency, low-intensity electrical stimuli (TENS) (Besson and Chaouch, 1987).

Alpha-2 adrenoceptors have also been suggested to be present on the terminals of nociceptive primary afferents (Howe et al., 1987a), although this is a matter of some controversy (see introduction to chapter 8 for a discussion of this point). However, α_2 adrenoceptor agonists have been shown to reduce the capsaicin evoked release of the peptides CGRP and substance P from rat spinal cord, which are presumably of C-fibre origin (Takano et al., 1993). Thus, descending noradrenergic fibres, whose targets are the α_2 adrenoceptors in the spinal cord (see chapter 8), may also exert inhibitory influences on the release of transmitters from primary afferent neurones.

5-HT_1 receptors (most notably 5-HT_{1A} receptors) have also been located on unmyelinated primary afferents, and 5-HT_1 agonists have been shown to modulate the release of CGRP (but interestingly not substance P) from dorsal horn slices (Hamon et al., 1990). This suggests that descending serotonergic fibres may also inhibit nociceptive transmission at the level of the primary afferent terminal.

Activation of receptors located on C-fibre terminals by transmitters released from the network of local neurones or descending fibres terminating in the superficial dorsal horn may also have the potential to enhance the release of transmitters from primary afferent terminals, although to my knowledge this has yet to be demonstrated. Kainate receptors (Agrawal and Evans, 1986) and 5-HT_3 receptors (Hamon et al., 1990) have been shown to be located on the terminals of nociceptive primary afferents. Although the function of these two receptor types is not known, both are excitatory, therefore activation of these receptors either by transmitters released from local interneurones or descending fibres, may lead to an enhancement in the release of primary afferent transmitters. However, kainate and glutamate have been shown to depolarize C-fibres (Agrawal and Evans, 1986), which may lead to a reduction in nociceptive transmission through PAD.

Further modulation of the nociceptive transmission occurs at the level of the post-

synaptic neurones. All the inhibitory receptors that can be found on the pre-synaptic terminal of the primary afferents can be located on the post-synaptic dorsal horn neurones, so once again, interneurones and descending fibres are able to modulate nociceptive transmission. Activation of inhibitory receptors located on post-synaptic neurones leads to hyperpolarisation of the neurone by increasing a K^+ conductance (μ- and δ-opioid receptors (North et al., 1987), α_2 adrenoceptors (North and Yoshimura, 1984) and $GABA_B$ receptors (Newberry and Nicoll, 1985)) or by activating a chloride conductance ($GABA_A$ receptors, Bowery, 1982) with a resultant inhibition in the firing of the post-synaptic neurone.

In addition to these inhibitory controls on nociceptive transmission, the nociceptive response can also be enhanced by dorsal horn mechanisms. The phenomenon of wind-up, where the evoked response of dorsal horn neurones is increased despite the intensity of the stimulus remaining constant, has been observed in dorsal horn neurones following repetitive noxious stimulation (Mendell, 1966; Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990). Thus an amplification of the nociceptive response can occur within the spinal nociceptive pathway. A similar phenomenon has been described in humans following psychophysical studies (Price et al., 1977). This amplification of the nociceptive response has been shown to be dependent on activation of the NMDA receptor, since selective antagonists of this receptor block wind-up (Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990) without inhibiting the underlying response, which is mediated through non-NMDA glutamate receptors and possibly peptides (**1.4**). Wind-up, and the conditions giving rise to this phenomenon, are discussed in chapter 4.

In addition to transmitters which can directly influence nociceptive transmission in the spinal cord, dorsal horn neurones also contain neuropeptides which can modulate the actions of these transmitters. Examples of this already discussed include the ability of CGRP released from primary afferents to potentiate the actions of substance P by retarding its degradation (**1.4**) and the role played by SP in facilitating the activation of the NMDA receptor (**1.4** and chapter 4). Other spinal modulatory peptides not yet mentioned are cholecystokinin (CCK) and FLFQPQRF-NH2. Both peptides can reduce the ability of opioids to inhibit nociception, without markedly influencing transmission themselves (Tang et al., 1984b and refs. in Baber et al., 1989). A lack of antagonists for FLFQPQRF-NH2 has made investigation of the physiological role of the peptide difficult, but the role of CCK in the dorsal horn of the spinal cord is discussed further in chapter 7.

The circuitry of the dorsal horn is complex and interactive, and the battery of substances released by primary afferent nociceptors and interneurones numerous and varied. As a consequence of this, the final nociceptive message relayed to the brain from the spinal cord depends on the balance of activity in these systems.

1.6. Supraspinal nociceptive systems

The nociceptive message is relayed from the dorsal horn of the spinal cord to the brain via the spinothalamic, spinoreticular and spinocervicothalamic tracts and the post-synaptic dorsal column fibres. The spinothalamic tract is generally regarded as the most important pathway relaying noxious information from the spinal cord to higher centers. This tract terminates in several nuclei of the thalamus, including the lateral thalamus which sends projections to the primary somatosensory cortex, thought to be important for the sensory-discriminative aspects of pain. In contrast, the medial projections of the spinothalamic tract and the spinoreticular tract (which projects to the reticular formation) are thought to be concerned with the affective aspects of pain (Willis, 1989). Activation of these ascending pain pathways can in turn trigger the activation of descending controls, which feed back to modulate spinal nociceptive transmission (see below).

1.7. Descending controls

Stimulation of discrete regions of the brainstem has been shown to elicit analgesia in many species including man (Besson and Chaouch, 1987). The finding that nociceptive cells in the dorsal horn of the spinal cord are selectively inhibited by stimulation at analgesia-producing brainstem sites (Guilbaud et al., 1973), and the demonstration that this analgesic effect is prevented by lesions of the dorsolateral funiculus (Basbaum et al., 1976), demonstrated the existence of descending analgesic controls. Of the major supraspinal structures implicated in the generation of analgesia, the most widely studied are the periaqueductal grey (PAG), the rostral ventromedial medulla (RVM, which includes the nucleus raphe magnus) and the locus coeruleus, which is a major source of noradrenergic projections to the dorsal horn of the spinal cord. Activation of these regions, either by opioids or electrical stimulation leads to a selective inhibition of nociceptive dorsal horn neurones and a consequent production of analgesia (see Besson and Chaouch, 1987). The noradrenergic descending controls to the spinal cord are covered in more detail in chapter 8, and so will not be discussed further here. The periaqueductal grey sends very few direct projections to the spinal cord, but does innervate the rostral ventromedial medulla which is the major brainstem source of axons projecting to the spinal cord. Thus analgesia resulting from stimulation of the PAG is likely to result from activation of the RVM, which then projects to the spinal cord via the dorsolateral funiculus (Besson and Chaouch, 1987).

The terminals of fibres projecting from the RVM are most dense in the superficial dorsal horn (Basbaum et al., 1978), the region in which nociceptive primary afferents

terminate. Fibres innervating the spinal cord from the RVM contain a number of transmitters, some of which may co-exist. 5-HT is the transmitter most commonly associated with these projections (see refs. in Willis and Coggeshall, 1991), although some neurones projecting to the dorsal horn from this region contain GABA (Reichling and Basbaum, 1990) or enkephalin (Millhorn et al., 1987). In addition, some of the substance P (Johansson et al., 1981) and CCK (Hökfelt et al., 1988) in the dorsal horn originates from brainstem neurones. The ways in which these transmitters can modulate spinal nociceptive transmission are discussed in section **1.5**.

Despite much work in the field, the physiological function of the descending controls is not entirely clear. One explanation is that they represent a simple negative feed back system whereby nociceptive messages trigger an endogenous pain control system. However another explanation for the functional significance of these descending controls has been proposed.

Le Bars et al. (1979a,b) observed that a noxious stimulus applied to one part of the body activates descending controls which then inhibit transmission in convergent dorsal horn neurones responding to inputs from other regions of the body. These controls were termed diffuse noxious inhibitory controls or DNIC. Activation of DNIC by noxious stimuli selectively inhibits the responses of convergent dorsal horn neurones (Le Bars et al., 1979a), without affecting transmission in non-convergent neurones (1979b). Since convergent neurones are activated by innocuous as well as noxious stimuli, the basal level of activation of these neurones in the absence of noxious stimuli is likely to be relatively high. It has been proposed (Le Bars and Chitour, 1983) that in the presence of a noxious stimulus DNIC inhibits the basal activity of convergent neurones not involved in the processing of that noxious stimulus, thereby leading to a clearer perception of the nociceptive event. This involvement of DNIC in the perception of painful stimuli is supported by the finding that morphine inhibits, rather than enhancing these descending controls, thereby blurring the pain message (Le Bars et al., 1986).

1.8. Spinal changes in inflammation

Many studies have been devoted to the changes in the levels and/or release of neurotransmitters, both peptide and non-peptide, and the levels of binding sites for these transmitters in the spinal cord following the development of peripheral inflammation. The nature and consequences with regard to spinal nociceptive transmission, of these inflammation-induced changes form the basis of much of the work in this thesis, and so will be discussed in greater detail in the relevant chapters. However, a brief overview of some of the changes known to occur in the spinal cord following the development of

peripheral inflammation in a number of different animal models is given here.

One of the most widely studied of all the spinal peptides post-inflammation is substance P. Increased levels of substance P-like immunoreactivity have been shown in the dorsal root ganglia and spinal cord (particularly laminae I & II) of rats with polyarthritis of some weeks duration (see refs. in Schaible and Grubb, 1993). In one study, where SP levels were examined at several time points during the development of arthritis, the upregulation in SP levels could not be detected until some weeks after the development of the inflammatory symptoms (Marlier et al., 1991). This suggests that changes in SP levels and the development of inflammatory symptoms such as hyperalgesia are not closely correlated in this case.

However, changes in the levels of SP can occur rapidly in response to inflammatory stimuli. The number of dorsal root ganglion neurones staining for preprotachykinins has been shown to be increased within three hours of the injection of formalin into the paw (Noguchi et al., 1988) and the injection of complete Freund's adjuvant into the paw produces a detectable increase in the mRNA coding for preprotachykinin A in the dorsal root ganglia and lumbar spinal cord within 24 hours (Minami et al., 1989). This upregulation in synthesis may be a response to the enhanced spinal release of SP shown to occur in vivo within hours of the injection of kaolin and carrageenan into the knee joint of the cat (demonstrated using the antibody microprobe technique, Schaible et al., 1990) and in spinal cord slices taken from rats 3 hours after the injection of carrageenan into the paw (Garry and Hargreaves, 1992). This enhanced release of SP within the cord is likely to account for the decrease in the levels of SP-like immunoreactivity seen in the dorsal horn of rats with carrageenan inflammation of 3 hours duration (Garry and Hargreaves, 1992) and in the dorsal horn of monkeys 2-6 hours (Sluka et al., 1992), and rats 4 hours (Sluka and Westlund, 1993a), after the injection of kaolin and carrageenan into the knee joint. Interestingly, the time course of the depletion in spinal SP levels has been shown to correlate with the sensitization of dorsal horn neurones following the injection of kaolin / carrageenan into the knee (Sluka et al., 1992). In the study by Sluka and Westlund (1993a) an increase in the level of SP in the dorsal horn was seen following this initial depletion of the peptide, possibly resulting from upregulation in peptide synthesis in response to the initial enhanced release. Furthermore, the mRNA coding for the NK-1 receptor (the preferred receptor for SP, **1.4**), is increased in the dorsal horn within 6 hours of the injection of formalin, and 4 days of the injection of Freund's adjuvant, into the paw (McCarson and Krause, 1994). This may further increase the impact of an increased release of SP post-inflammation. Although the upregulation in SP levels post-inflammation may contribute to enhanced nociceptive transmission in its own right, it may also have consequences for the activation of NMDA receptors, which is discussed in chapter 4.

Spinal levels of the peptide CGRP, which is thought to be primarily of primary afferent origin (Willis and Coggeshall, 1991), have also been investigated following the development of peripheral inflammation. Levels of CGRP are increased in the dorsal root ganglia of arthritic rats 15-26 days post inoculation, although the same study failed to find any increase in the levels of CGRP in the dorsal horn (Kuraishi et al., 1989). These changes were not closely correlated with the behavioural symptoms of inflammation (Kurashi et al., 1989). In a model of more acute inflammation, an increased release of CGRP from spinal cord slices taken from animals 3 hours after the injection of carrageenan into the paw has been reported (Garry and Hargreaves, 1992). This enhanced release of the peptide is likely to be responsible for the decreased levels of CGRP found in the dorsal horn 3 hours post-carrageenan (Garry and Hargreaves, 1992). As the role played by CGRP in spinal nociceptive transmission in the normal animals is unclear (**1.4**), the consequences of an increase in spinal CGRP are open to question. However, since CGRP is able to potentiate the actions of SP by retarding its enzymatic degradation (Le Greves et al., 1985), any upregulation in the release of CGRP is likely to contribute to the enhanced actions of SP in inflammation.

Increases in the levels of SP and CGRP in DRG neurones following the development of peripheral inflammation have been linked with an upregulation in the levels of nerve growth factor (NGF) (Donnerer et al., 1992). NGF produced in peripheral tissue can bind to its receptor on sensory neurones and be retrogradely transported to the DRG (Goedert et al., 1981), where it can alter gene expression to enhance the production of SP and CGRP (Lindsay and Harmer, 1989). Within hours of the development of inflammation, the levels of NGF in the inflamed tissue are elevated (Weskamp and Otten, 1987) and an enhanced retrograde transport of NGF in the nerve innervating the tissue has been demonstrated (Donnerer et al., 1992). Donnerer et al. (1992) demonstrated that the enhanced levels of SP and CGRP in the dorsal root ganglion following the development of inflammation induced by the subcutaneous injection of complete Freund's adjuvant could be blocked by the exogenous administration of anti-NGF, suggesting NGF is responsible for the upregulation in these peptides.

The other family of spinal peptides on which attention has focused in inflammatory states is that of the endogenous opioids. Although the spinal actions of the enkephalins, which show only a modest upregulation in inflammatory states (see refs. in chapter 9), are clearly antinociceptive, both pro- (Hylden et al., 1989, 1991a; Millan and Colpaert, 1990, 1991) and anti- (Millan et al., 1985, 1987, 1988; Kayser and Guilbaud, 1991; Millan and Colpaert, 1990, 1991) nociceptive roles have been proposed for dynorphin, which shows a dramatic upregulation in inflammation (see refs. in chapter 9). The changes in the levels of opioid peptides in the spinal cord, and the consequences of these

changes are investigated in chapter 9.

In addition to changes in the levels of peptides in the spinal cord after inflammation, increases in the levels and release of excitatory amino acids such as glutamate have also been shown occur. Increased staining for glutamate in the superficial dorsal horn is seen within hours of the injection of kaolin and carrageenan into the knee joint of the monkey (Sluka et al., 1992) and rat (Sluka and Westlund, 1993a). An increase in the release of glutamate and aspartate in the dorsal horn, some of which is thought to be of interneuronal origin, has also been demonstrated following the injection of kaolin and carrageenan into the knee joint. This takes the form of a transient increase upon injection, and then a more sustained increase after several hours (Sluka and Westlund, 1992; Sorkin et al., 1992). The role of the NMDA receptor, one of the receptors on which glutamate and aspartate can act, in spinal nociceptive transmission following the development of inflammation is examined in chapter 4.

In addition to increases in the levels of excitatory non-peptide transmitters, the levels of the inhibitory transmitter GABA are increased in the dorsal horn following the development of inflammation in the periphery (Castro-Lopes et al., 1992, 1994 and see chapter 6).

The mechanism behind the upregulation in the levels of the dorsal horn transmitters is unknown, although activation of immediate early genes such as *c-fos* and *c-jun* as a result of increased neuronal activation by noxious events may underlie some of these changes. Following the development of inflammation in the periphery, a rapid (within 30 minutes (Draisci and Iadarola, 1989)) and dramatic increase in the levels of the proto-oncogene *c-fos* has been shown to occur in the dorsal horn (see refs. in Dubner and Ruda, 1992). Fos (the protein product of *c-fos*) forms a dimer with Jun (the product of *c-jun*) which is then able to bind to sequences of DNA, thereby regulating the ability of nearby genes to be transcribed into mRNA. Thus the induction of Fos by nociceptive events in the periphery can modulate the expression of genes within the cells of the dorsal horn. The genes affected by the Fos-Jun dimer, and therefore the consequences of the activation of *c-fos* and *c-jun* are not known, however the upregulation of dynorphin (Noguchi et al., 1991; Hylden et al., 1992) and enkephalin (Noguchi et al., 1992) in the dorsal horn after peripheral inflammation has been linked to the expression of Fos.

In addition to changes in intrinsic spinal systems, the development of inflammation in the periphery has been reported to increase activation of the descending controls (Cervero et al., 1991; Schaible et al., 1991b, and see chapter 8) which will also influence spinal nociceptive transmission.

All of these changes may contribute to alterations in the spinal transmission and modulation of nociceptive messages in the inflammatory state.

1.9. Suitability of convergent dorsal horn neurones for the study

The question must be addressed as to whether convergent dorsal horn neurones represent a suitable target for a study of changes in spinal nociceptive processing following the development of inflammation in the periphery. In other words, are changes in the response and modulation of these neurones in inflammatory states likely to be representative of altered spinal nociceptive processing seen in such conditions? Hence what is the evidence that convergent neurones play an important role in the transmission of nociceptive information in the normal animal?

Convergent dorsal horn neurones are known to project to higher brain centers via the ascending tracts, with this neuronal type comprising over 50% of all neurones in the spinothalamic tract (nociceptive-specific neurones comprise around 30%) (Besson and Chaouch, 1987), which is regarded as one of the most important tracts relaying sensory information to the brain (**1.6**). Convergent dorsal horn neurones also contribute to the post-synaptic dorsal column pathway, the spinoreticular and spinocervical tracts (Willis and Coggeshall, 1991). Convergent neurones show good ability to encode the intensity of a given stimulus, discharging at maximum rates to intense noxious stimulation: nociceptive-specific neurones code stimulus intensity, particularly that of thermal stimuli, poorly (Le Bars et al., 1986). This is borne out by the demonstration by Dubner et al. (1989) that the activity of convergent neurones is a better predictor of a monkey's perception of the intensity of a heat stimulus than the activity of nociceptive specific neurones. Furthermore, the rating of pain intensity by human subjects following graded levels of prolonged noxious thermal stimulation correlates closely with the sustained activity of convergent, but not nociceptive-specific, neurones recorded in unanaesthetized, spinal rats (Coghill et al., 1993b).

In addition, the discharge of convergent neurones is reduced by manipulations which result in hypoalgesia or analgesia in humans, such as systemic or intrathecal morphine administration, dorsal column stimulation and transcutaneous electrical stimulation (see refs. in Le Bars et al., 1986). In view of these observations, it seems unlikely that convergent neurones are not involved in the spinal transmission of nociceptive information and that the relay of nociceptive information in the spinal cord relies solely on nociceptive-specific neurones. However, the problem arises as to how these neurones allow the discrimination of noxious from non-noxious information, since it has been observed that innocuous stimuli given in rapid succession can evoke equal or greater levels of firing in the convergent neurones as results from stimuli at the lower end of the noxious range (Le Bars and Chitour, 1983).

One explanation that has been proposed to account for how nociceptive

information relayed by convergent neurones can be separated from the innocuous sensory information received by these neurones involves segmental convergence. It has been demonstrated in many studies (e.g. Hillman and Wall, 1969; Menétrey et al., 1977; Le Bars and Chitour, 1983; chapter 3, this study) that the peripheral excitatory receptive fields of convergent neurones often show a gradient of responsiveness, with a central area responding to both innocuous and noxious stimuli, surrounded by an area responding only to more intense, and eventually only to noxious stimuli. As a consequence of this, an innocuous stimulus applied to a given area will excite the receptive fields of fewer convergent neurones than will a more intense stimulus applied over the same area. Thus the number of convergent neurones activated by a given stimulus, as well as the frequency of firing in these neurones, may be important for a stimulus to be perceived as noxious. Indeed, there is now evidence from a psychophysical study in humans following the electrical stimulation of axons in the anterolateral quadrant of the spinal cord at differing frequencies and intensities, coupled with a metabolic imaging study in rats examining the spatial spread of activity following noxious and innocuous stimuli, that both the discharge frequency and the number of neurones activated are important for the encoding of pain (Coghill et al., 1993a).

A further system which could allow the clear differentiation of noxious from non-noxious information by convergent neurones involves the activation of diffuse noxious inhibitory controls (DNIC). As discussed in section **1.7**, noxious, but non-noxious, stimuli trigger DNIC, which then selectively inhibit the activity of convergent neurones not involved in the processing of the noxious stimulus (Le Bars et al., 1979a). This allows a contrast of the nociceptive information relayed by convergent neurones from the background barrage of information. DNIC has been demonstrated in humans, where the application of a second noxious stimulus reduces the perceived intensity of the first noxious stimulus (Talbot et al., 1987). Since animal studies have demonstrated unequivocally that DNIC reduces the responses of convergent, but not noxious-only, neurones (Le Bars et al., 1979b), the fact that DNIC can be shown to reduce the pain intensity in humans demonstrates that convergent neurones must play a major role in pain transmission.

1.10. Choice of animal model of inflammation for the project

The purpose of this study (see **1.11**) was to use in vivo electrophysiological techniques to investigate the pharmacology of the changes in the spinal transmission and modulation of nociceptive messages following the development of peripheral inflammation. To enable the study of whether phenomena such as central sensitization develop during the course of the inflammation, which may provide clues as to the central

correlates of hyperalgesia, a model must be chosen with a time scale of development such that the response of the neurones can be studied from the control period pre-inflammation through to the time of peak hyperalgesia. Thus the time scale of development of the model must be measured in hours rather than days or weeks. Furthermore, there are ethical questions to be considered when using an inflammatory model of long duration, so if it is anticipated that useful and relevant results can be obtained from a model involving a shorter time course, then this may be the model of choice. These criteria eliminate models of chronic polyarthritis, where the inflammatory changes do not reach a stable maximum until 3 weeks after the intradermal injection of Freund's complete adjuvant into the tail (Millan et al., 1986). Furthermore, polyarthritis represents a generalized disease state with changes in many organs and systems, not all of which are necessarily related to pain. Although of a much shorter duration and restricted inflammation, models of unilateral inflammatory hyperalgesia produced by the intradermal injection of complete Freund's adjuvant into the plantar surface of one paw are also unsuitable for the present study. The hyperalgesia produced in this way peaks 1-6 days post inoculation (Iadarola et al., 1988b), too long to allow for electrophysiological recordings of neurones to be performed from the onset of inflammation to the time of peak hyperalgesia.

Well characterized models of inflammatory nociception with a suitable time course include a model of acute arthritis produced by the injection of kaolin and carrageenan into the knee joint (see Schaible and Grubb, 1993), and two models of cutaneous hyperalgesia, produced by the subcutaneous injection of carrageenan (see below) or a dilute solution of formalin (see Tjølsen et al., 1992). A decision was made to study hyperalgesia of cutaneous origin, which is relevant to a variety of conditions including post-operative pain, rather than a specific study of joint inflammation, leaving the formalin test or the carrageenan model.

The formalin model varies from some of the other inflammatory models used in that following the injection of a dilute solution of formalin into the paw, the animals display tonic, bi-phasic pain related behaviour. This pain behaviour, which lasts about an hour and consists of licking and biting of the injected paw, can be visually assessed and scored and the effects of analgesic drugs on this tonic pain behaviour assessed without the need for further applied stimuli (Tjølsen et al., 1992). The peripheral injection of formalin is also used in electrophysiological studies to produce a model of acute inflammatory nociception. In this model, the dorsal horn neurones display a characteristic bi-phasic pattern of firing (Dickenson and Sullivan, 1987b and refs. in Tjølsen et al., 1992), the timing and relative magnitude of which correlates well with the pain scores obtained in behavioural studies.

However, the tonic pain related behaviour and neuronal firing associated with this model are of very short duration, terminating after one hour, despite the continuing

development of inflammation in the periphery (Tjølsen et al., 1992). In addition, the level of ongoing firing of the neurones evoked by the peripheral formalin injection complicates the investigation of the central correlates of the phenomenon of hyperalgesia which accompanies inflammatory pain states. For these reasons, the model of inflammatory hyperalgesia produced by the subcutaneous injection of carrageenan was used for the experiments presented in this thesis.

Carrageenan-induced inflammation was first described by Winter et al. in 1962, and since then has been widely used as model of inflammatory cutaneous hyperalgesia. Following the injection of 2-6mg of carrageenan into the paw, oedema rapidly develops and the animals display hyperalgesia to mechanical and thermal stimuli in the injected paw, but no behaviour indicative of spontaneous pain (Kayser and Guilbaud, 1987; Hargreaves et al., 1988a; Hylden et al., 1991b). The time course of this inflammatory model is longer than that of the formalin model, with the peak hyperalgesia in behavioural studies seen 2-4 hours after the injection of carrageenan into the paw (Kayser and Guilbaud, 1987; Hargreaves et al., 1988a; Hylden et al., 1991b). The inflammation and hyperalgesia produced by carrageenan are largely resolved within 24-72 hours.

The carrageenan model used in this study shares many of the characteristics of the more prolonged inflammatory models; for example hyperalgesia to thermal and mechanical stimuli, the induction of *c-fos* in the dorsal horn, changes in spinal peptide levels such as the dramatic upregulation in the synthesis of dynorphin (see chapter 9), and unlike the formalin model, the absence of tonic activity allows the assessment of the central correlates of hyperalgesia. in the dorsal horn neurones

1.11. Aims of this project

The purpose of the experiments presented in this thesis was to examine what the central consequences of the development of acute peripheral inflammation induced by the injection of carrageenan into the paw are on spinal systems involved in the transmission and modulation of nociception.

I have used an electrophysiological approach, recording the responses of single dorsal horn neurones to transcutaneous electrical stimulation, coupled with the direct spinal application of drugs to allow me to concentrate my study on spinal changes arising from carrageenan-induced inflammation. This approach minimizes as far as possible the influence of the peripheral changes in inflammation which may mask alterations in centrally mediated phenomena.

After an initial study of the changes in the spinal transmission of nociceptive information during the development of inflammation (chapter 3), spinal neurotransmitter

systems classically regarded to play excitatory, inhibitory or modulatory roles in nociceptive processing were examined using specific receptor agonists and antagonists to reveal if these systems were subjected to plastic changes as a result of the changing situation in the periphery. The role of excitatory amino acids acting at spinal NMDA receptors in the transmission of nociceptive information was examined using antagonists at two different sites on the NMDA receptor-channel complex (chapter 4). Attention then focused on spinal inhibitory systems, namely the activity of the endogenous opioid system (chapter 9), the spinal potency of exogenous opioids (chapter 5), the noradrenergic system (chapter 8) and the GABAergic system (chapter 6). Following the finding that the spinal potency of the mu opioid morphine is enhanced post-carrageenan, the activity of the spinal CCK system and the descending noradrenergic system were investigated since both can modulate the actions of mu opioids.

CHAPTER 2

METHODS

Abbreviations

AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
D-AP5	D-2-amino-5-phosphonovaleric acid
APS	Action potentials
CCK	Cholecystokinin
7CK	7-Chlorokynurenate
CGRP	Calcitonin gene-related peptide
CNQX	6-Cyano-7-nitroquinoxaline-2,3-dione
CNS	Central nervous system
DNIC	Diffuse noxious inhibitory controls
DRG	Dorsal root ganglion
E	A measure of wind-up (see **2.7**)
GABA	Gamma-aminobutyric acid
5HT	5-Hydroxytryptamine
I	Initial input onto the neurone (see **2.7**)
LTD	Long-term depression
LTP	Long-term potentiation
NGF	Nerve growth factor
NK1	Neurokinin 1
NKA	Neurokinin A
NMDA	*N*-methyl-D-aspartate
NO	Nitric oxide
Nor-BNI	Nor-binaltorphimine
NOS	Nitric oxide synthase
PAD	Primary afferent depolarization
PAG	Periaqueductal grey
PD	Post-discharge
PSTH	Post-stimulus-time histogram
RVM	Rostral ventromedial medulla
SG	Substantia gelatinosa
SP	Substance P
W	A measure of wind-up (see **2.7**)

In these studies, spinal nociceptive mechanisms were studied in both normal rats and in rats following the induction of peripheral inflammation produced by the injection of carrageenan into the plantar surface of one hind-paw. The technique used to study these mechanisms was the extracellular measurement of electrically evoked single unit activity of convergent dorsal horn neurones in the intact, halothane anaesthetized rat.

2.1. Preparation of the animals

2.1.1. *Animals*

Male Sprague-Dawley rats (200-250g), obtained from the University College London animal house, were used throughout these studies.

2.1.2. *Induction of anaesthesia and surgical preparation of the animal*

Induction of anaesthesia in the rat was performed in a closed perspex box using a flow of 3% halothane (Fluotec dispenser, Cyprane Ltd.) in a 33% oxygen, 66% nitrous oxide gaseous mixture. The rate of flow of oxygen and nitrous oxide was controlled by flow meters (Platon Ltd.) set to deliver 150 and 300cm^3/min respectively. Once the animal had lost consciousness, it was removed from the box and delivery of anaesthetic continued via a small glass cone placed over the animal's nose. The concentration of halothane was reduced to 2-2.25%, and surgical procedures started once the animal had lost reflex withdrawal activity to strong pinch applied to the paw.

The initial surgical procedure was the insertion of a tracheal cannula, through which anaesthesia was maintained for the remainder of the experiment. A cannula was also inserted into the jugular vein if the intravenous administration of drugs was required in the subsequent experiment. Following insertion of cannula(e), the rat was placed in a stereotaxic frame to provide stability during the electrophysiological recording. Surgical rongeurs were used to perform a laminectomy which normally extended from vertebrae L1-L3. The vertebral column was held with clamps placed rostrally and caudally to the laminectomy, and a small degree of tension applied to improve the stability of the exposed cord. If the dura mater remained after the removal of the vertebrae, this was removed to facilitate insertion of the microelectrode and the subsequent access of drugs following topical administration to the exposed surface of the cord. The pia mater was left intact. A natural flow of cerebrospinal fluid helped to keep the exposed cord moist.

Following completion of the surgical procedures, the level of halothane was reduced to 1.2-1.6% and adjusted to produce a state of complete areflexia, which was maintained for the duration of the experiment. At the end of the experiment, the rat was killed with an overdose of halothane.

2.2. Maintenance of the physiological state of the animal

During surgery and then for the duration of the experiment the core body temperature of the animal was monitored and maintained (36.5-37°C) by means of a heating blanket underneath the animal connected to a rectal thermal probe via an automatic feedback control unit (Animal Blanket Control Unit, Harvard).

The state of the peripheral circulation of the animal, i.e. the skin colour of the extremities, was assessed visually and was taken as an indication of the physiological condition of the animal. A good pink colour of the extremities of the rat was taken to indicate that the animal was well oxygenated. The rats appeared to remain in a good physiological condition throughout the experiment. In order to reduce the number of surgical procedures the animals were subjected to, the blood pressure of the animals was not measured during the experiment. However, previous studies using these anaesthetic conditions have shown that the blood pressure of the animal and the end-expiratory carbon dioxide levels remain stable throughout the experiment, even during application of noxious stimuli (Dickenson and Le Bars, 1987). The rats breathed spontaneously throughout the experiment and were therefore able to regulate their acid-base balance.

2.3. Choice of anaesthetic and the effects on dorsal horn neurones

Although techniques have been developed whereby in vivo electrophysiological recordings of dorsal horn neurones can be obtained in physiologically intact conscious cats (Collins, 1985) and sheep (Herrero et al., 1993), this is not feasible in rats at the present time. In addition, there is also the question of whether applying frankly noxious or inflammatory stimuli to conscious animals is ethical, leaving us with the choice of anaesthetizing or decerebrating / spinalizing the rats prior to the commencement of electrophysiological recording.

There are advantages and disadvantages to both decerebration / spinalization and surgical anaesthesia. In spinalized animals, following decerebration, there is no anaesthetic present to depress spinal neuronal responses or interfere pharmacologically with drug actions. However in these animals, the system of descending controls to the spinal cord (section **1.7**) is lost. Owing to the potential importance of the descending control system in animals during the development of inflammation, it was decided that studying the responses of dorsal horn neurones in the presence of an anaesthetic was preferable to spinalizing / decerebrating the animal.

The choice of anaesthetic agent to be used for this series of experiments is important since a balance must be struck between adequate anaesthesia of the animals,

able to be maintained over a duration of up to 10 hours, and yet minimal interference with the responses of dorsal horn neurones and the pharmacological systems under test.

So which anaesthetic agent is most suitable for these experiments?

Ketamine has been used as an anaesthetic agent, however this has been shown to block the channel associated with the NMDA receptor-channel complex (see Davies and Lodge, 1987), rendering it unsuitable for studies of this nature, since the NMDA receptor has been shown to play an important role in spinal nociceptive transmission, particularly in prolonged pain states (see chapter 4). Pentobarbital is another possibility, but this has been shown to prevent the development of hyperalgesia (Cleland et al., 1994), so is clearly unsuitable for the present study. Other injectable anaesthetics such as urethane and α-chloralose have also been used in electrophysiological studies such as this. However these anaesthetic agents are given as a bolus injection, with top-up injections given as required in the case of α-chloralose. As a consequence of this, the level of anaesthesia of the animal will fluctuate over the course of the experiment as a result of the distribution and elimination of these agents. Inhalational anaesthetics, which are administered in a more continuous fashion, have the advantage that the level of anaesthesia remains stable.

The inhalational anaesthetic chosen for these experiments was the volatile anaesthetic halothane delivered in a mixture of 33% oxygen / 66% nitrous oxide. The effects of varying concentrations of halothane on the responses of dorsal horn neurones have been studied in spinalized animals. Several studies in spinal cats have shown that concentrations of halothane in the therapeutic range (0.5-1.5%) depress both the spontaneous activity and the activity evoked by noxious stimulation in dorsal horn neurones, including identified wide-dynamic range neurones (Kitahata et al., 1975; Namiki et al., 1980). However, Le Bars and Chitour (1983) compared the responses of convergent dorsal horn neurones to graded noxious heat and repetitive touch in both unanaesthetized spinal rats and intact rats under halothane anaesthesia (admittedly at a slightly lower level of 0.8% but in the same mixture of 33% oxygen / 66% nitrous oxide) and found no difference in the evoked responses of the neurones between the two groups of animals. Spinalization of the rats would if anything, be expected to enhance neuronal responses slightly due to the removal of inhibitory descending controls (this effect is small, although the level of spontaneous activity increases (Le Bars et al., 1979b)) which would lead to an apparent amplification of any depressant effect of halothane on the dorsal horn neurones in the intact animals. Since Le Bars and Chitour (1983) found no difference between the neuronal responses in spinalized and anaesthetized animals, it would appear that under these anaesthetic conditions, in rats, neither halothane or nitrous oxide has a marked depressant effect on the evoked responses of convergent neurones. Recordings of dorsal horn neurones in intact conscious and halothane

anaesthetized sheep have shown that the proportions of the various neuronal types found within the dorsal horn are similar in both the anaesthetized and unanaesthetized state, although convergent neurones appear to form a larger proportion of the neurones in awake sheep (77% vs 56% in anaesthetized sheep, Herrero and Headley, 1993). In addition halothane is widely used as an anaesthetic during human surgery therefore if it is interfering in any way with the development of central hyperalgesic states then this may also happen during human surgery.

The halothane in the present study was administered in a mixture of oxygen and nitrous oxide. Although nitrous oxide has been reported to selectively depress the spontaneous activity of dorsal horn neurones in lamina V but not laminae I, IV, or VI of the cat spinal cord at a concentration of 75% (Kitahata et al., 1971), Le Bars and Chitour (1983) found no effect of 66% nitrous oxide on the evoked responses of rat dorsal horn neurones (located in laminae IV, V and VI) (see above). Thus it is probable that in the present study, the evoked responses of the dorsal horn neurones will not be markedly depressed by the presence of nitrous oxide in the anaesthetic mixture.

2.4. Electrophysiological recording of the neurones

A NeuroLog-based system (Digitimer) was used to record the responses of dorsal horn neurones. In brief, a fine parylene-coated tungsten electrode (2MΩ) was used to make single unit extracellular recordings from dorsal horn neurones. The recording system was grounded through the stereotaxic frame to the steel recording table. Electrical signals picked up by the electrode in the dorsal horn were relayed to the headstage where they formed signal A. The headstage also received an input from a clip attached to the skin of the animal which relayed background noise and formed signal B. The signals from the headstage were fed into the preamplifier switched to differential recording where signal B was subtracted from signal A to leave just the electrical activity arising from the dorsal horn. This signal was then amplified (gain = 780K) and filtered through both high and low frequency filters. The filtered output was displayed on a storage oscilloscope (1-2V / division) to allow the signal to be visualized, and also fed to an audio amp to provide an auditory representation of the signal. Neuronal spikes were differentiated from background activity for quantification purposes by the use of a window discriminator. The electrode position was adjusted to give a single unit with a signal to noise ratio of at least 4 to 1, in practice, 8 to 1 was achieved. The height of the window discriminator was adjusted such that spikes over a selected amplitude triggered a counting pulse which was relayed via the CED 1401 interface to the computer and to gated latch counters. The pulse was also displayed on the oscilloscope as a dot (BRIT brightening pulse) over the spike.

Evoked neuronal spikes were counted in relation to the latency with which they occurred after the stimulus as this allowed the conduction velocity of the primary afferent fibres activating the dorsal horn neurone to be estimated and therefore their likely fibre type determined. Thus, on commencement of a test of neuronal activity (section 2.6), the period generator simultaneously triggered the stimulating circuit (2ms wide square wave pulse (controlled by the digital width module) of adjustable amplitude (controlled by the pulse buffer)), the data capture program on the computer (MRATE software (Cambridge Electronic Design) on an IBM PC) and the delay width which controls the input to the latch counters. The latch counter was gated to count only those pulses triggered by spikes occurring between 90-800ms after the stimulus (see section 2.6). This allowed the wind-up of the neurones to be calculated (see section 2.7) by noting the incremental spike counts for the duration of the stimulation train. Trials of 16 stimuli were carried out (frequency 0.5Hz) and a system of logic gates allowed a single switch to be used to reset the spike counters, trigger the stimulation modules and halt the system after the final stimulus in the train.

The filtered neuronal signal, together with a stimulus trigger from the delay width module could also be fed into an Apple Macintosh computer via a MacLab interface where the MacLab Scope program (Analog Digital Instruments) could be used to provide a digital representation of the storage oscilloscope trace which could then be printed to provide a hard copy of the display.

2.5. Searching for neurones

Before the commencement of electrophysiological recording, the level of halothane was reduced to 1.2-1.6%. The electrode was lowered manually to the spinal cord and adjusted until it was judged (by the amplitude of the signal) that the tip of the electrode was at the surface of the dorsal horn. Once the surface of the dorsal horn was established, the microdrive was set to read zero and the electrode advanced through the dorsal horn in 10μm steps using the microdrive. As the microdrive was advanced, the ipsilateral hind paw was manually tapped and prodded to activate dorsal horn neurones with receptive fields on the toes / plantar surface of the paw. Stimulation of the paw was necessary to allow the identification of neurones within the dorsal horn during microdrive advancement since the level of spontaneous activity of dorsal horn neurones in this preparation is very low. Although there is a risk of missing neurones with a high threshold as a consequence of using a low intensity of stimulation in the initial search for the neurones, this low intensity of stimulation for search purposes was necessary to avoid evoking tissue damage / sensitization during the application of repetitive stimuli.

Once a potential neurone was identified by an amplitude which was clearly above the background noise, the electrode was carefully moved to the position in which the amplitude of the neuronal signal was greatest. The recording was judged to be of a single neurone if the amplitude of all the spikes was uniform. In some cases, two neurones could be identified as having spikes with an amplitude greater than background in which case the window discriminator was adjusted so that only the neurone with spikes of greater amplitude was counted. This was visually monitored on the oscilloscope throughout the experiment by checking for the presence of a dot over the spikes being counted. The level of the window was adjusted as necessary to maintain this separation. The depth of the neurone within the dorsal horn was taken from the microdrive reading.

2.6. Characterizing the neuronal responses and method of stimulation

Once an individual neurone had been separated on an amplitude basis from the background noise, the peripheral receptive field of the neurone was identified by prodding and pinching the skin of the plantar surface of the foot. Some neurones responded only to touch / brush of their receptive field with no increase in firing rate when a noxious stimulus such as pinch was applied. If these neurones were subsequently tested by electrical stimulation of their receptive field (see below) they were found to receive only an Aβ-fibre input. These neurones were not studied further. Only convergent neurones, which receive both C- and Aβ-fibre inputs, were used for this study.

Once the peripheral receptive field of the neurone had been identified by tapping and pinching the skin of the foot, two fine steel stimulating needles were inserted just under the skin in the centre of the receptive field, usually on two adjacent toes. This allowed transcutaneous electrical stimulation to be used as the test stimulus for the rest of the experiment. This method of electrical stimulation has been shown to produce similar responses to those induced by direct nerve stimulation (Menétrey et al., 1977) but avoids the possibility of complications such as nerve damage which might occur with direct nerve stimulation, arising from the need to isolate and expose the nerve thereby changing its local environment. Electrical stimulation at both noxious and non-noxious intensities, rather than natural stimulation of the afferents, was chosen as the test stimulus for the experiment since this provides a constant, reproducible stimulus which is non-tissue damaging. The non-damaging nature of the stimulus is important since the receptive field of the neurone was stimulated periodically for up to 8 hours. In addition, electrical stimulation activates the neurones directly thus bypassing the nociceptors which may become sensitized to natural stimuli by inflammatory mediators in the carrageenan animals, complicating the interpretation of the results. Furthermore, the transduction of

natural stimuli such as heat or pressure through the tissue may be altered by the oedema produced by the inflammation, thereby altering the magnitude of the stimulus reaching the nociceptors. The transduction of electrical stimuli through inflamed tissue would not be compromised however, since the stimulator used is a constant current device, compensating for changes in tissue resistance in inflammation.

Once the stimulating needles had been inserted, the preparation was left to settle for 10-15 minutes before the threshold current required for activation of the C- and then the Aβ-fibres was determined. The C- and Aβ-fibre evoked responses of the dorsal horn neurone could be differentiated on the basis of both threshold and latency. Low intensity stimulation of the peripheral receptive field activates only Aβ-fibres, which are fast conducting and therefore evoke action potentials in the dorsal horn neurones with a short post-stimulus latency. Activation of C-fibres requires higher intensity stimulation and the slower conduction velocity of these fibres results in the action potentials evoked in dorsal horn neurones by C-fibre activity being of a measurably longer post-stimulus latency than those evoked by Aβ-fibres. The threshold of the Aβ-fibres was taken as the current necessary to consistently evoke an action potential in the dorsal horn neurone within 20msec of the stimulus (determined on the basis of the conduction velocity of Aβ-fibres). The pulse triggered by this action potential was displayed on the computer on a post-stimulus-time histogram (PSTH) and could be heard via the audio amp. The threshold current for activation of C-fibres was taken as the current required to consistently evoke an action potential in the dorsal horn neurone between 90-300msec after the stimulus (determined on the basis of the conduction velocity of C-fibres).

Once the threshold currents required for Aβ- and C-fibre activation for each particular neurone had been established, the response of the neurone to a trial of 16 stimuli given at either 3x the C- or 3x the Aβ-fibre threshold was determined. The cumulative response of the neurone to the 16 stimuli was displayed on the computer as a post-stimulus-time histogram (PSTH) of the number of action potentials evoked against post-stimulus latency. From this, the number of action potentials resulting from Aβ-fibre activity (action potentials evoked within 20msec), Aδ-fibre activity (20-90msec) and C-fibre activity (90-300msec)* could be quantified. The post-discharge of the neurone (action potentials occurring 300-800msec after the stimulus), which is thought to represent activity generated within the dorsal horn as a result of high intensity stimulation rather than activity evoked by slowly conducting fibres, was also quantified from the PSTH.

Tests consisting of trials of 16 stimuli at 3x the C-fibre and then at 3x the Aβ-fibre threshold were conducted every 10 minutes during the experiment.

* the 'C-Fibre evoked response'

2.7. Calculation of the wind-up of dorsal horn neurones

In addition to the total number of C-fibre evoked action potentials per trial of 16 stimuli, a measure of the degree of the C-fibre evoked wind-up of the neurone, which is defined as the increasing response of a neurone to a constant intensity stimulus, could also be calculated from the number of spikes of C-fibre latency or longer evoked per stimulus displayed on the gated latch counters.

For the purposes of looking at the effect of drugs on the wind-up of a neurone, i.e. changes in the wind-up of an individual neurone, as in chapters 4 and 5, the wind-up of a neurone was expressed as E. This is illustrated in figure **2.1**.

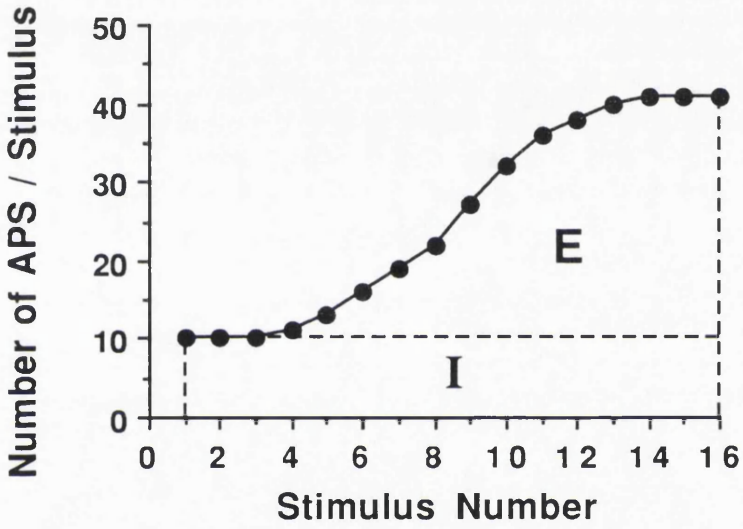


Figure 2.1. The response of a neurone to a train of stimuli can be divided into two components I, which results from the steady response of the neurone, and E, which can be thought of as the excess action potentials (APS) generated as a result of wind-up mechanisms.

E was calculated as the number of excess spikes generated as a result of wind-up mechanisms. E = actual number of C-fibre evoked spikes in response to 16 stimuli - I,* which is the number of C-fibre evoked spikes expected if no wind-up occurred, i.e. the C-fibre evoked response to the first stimulus x 16. Therefore a reduction in E for an individual neurone signifies a reduction in the amount of wind-up exhibited by that neurone and vice versa.

Whilst E, the number of excess spikes due to wind-up, is suitable for studying changes in the wind-up of an individual neurone, for example resulting from the actions of drugs, E is not a suitable measure of wind-up for comparisons of the degree of wind-up between neurones. E alone tells us nothing about the degree to which a neurone

* I = initial input onto neurone (response to 1st stimulus) x 16

winds-up unless we know the overall magnitude of the neuronal response. For example, a value of E of 150 may represent a large degree of wind-up for a neurones whose total C-fibre evoked response is only 300 spikes, but may constitute a relatively constant response with very little wind-up in a neurone with a C-fibre evoked response in the order of 900 spikes. Thus for comparisons between neurones, a different measure of wind-up, W, was used.

So, as a measure of the degree to which a neurone "winds up" for the purposes of comparing the degree of wind-up between neurones in a population, as in chapter 3, W was calculated. W is expressed as a ratio of how many times greater the actual evoked response to the 16 stimuli is than the response that would be expected if no wind-up occurred and the neurone responded steadily to the 16 stimuli. Thus W = actual number of C-fibre evoked spikes in response to 16 stimuli / number of C-fibre evoked spikes expected if no wind-up occurred, i.e. the C-fibre evoked response to the first stimulus x 16. Thus in neurones with little wind-up, W is close to 1, whilst neurones displaying a large degree of wind-up have a larger value of W.

2.8. Induction of peripheral inflammation

Inflammation was induced in the periphery by the injection of 100µl of 2% λ-carrageenan in saline (2mg / paw) into the ipsilateral (or in a few cases the contralateral) hind paw of the rat. Injections were made into the plantar surface of the paw in the centre of the receptive field of the neurone once three stable control trials had been obtained. Following the injection of carrageenan, the electrically-evoked response of the neurone was tested at 10 minute intervals for periods up to 3 hours post injection. Only one injection of carrageenan was given per animal. As a control for the effects of the injection trauma, 100µl of physiological saline alone were injected into the ipsilateral hind paw of 8 animals and the evoked responses of the neurones followed in the same way as in the carrageenan animals.

Changes in paw volume as a consequence of the inflammation were not assessed. In some animals however, the degree of swelling of the paw induced by the carrageenan injection was gauged by measuring the diameter of the inflamed paw 3 hours after the injection of carrageenan and comparing this to the diameter of the contralateral paw at the same time point.

* Dose-response curves for the actions of drugs on the evoked responses of dorsal horn neurones were constructed following cumulative drug administration of up to 3 doses per neurone at 40-60 minute intervals, with ideally a 10-fold increase in dose between each administration. As a consequence of this cumulative dosing, drug effects in the carrageenan animals were studied between 3 and 5.5 hours after the injection of carrageenan into the paw. However for clarity these responses were grouped and referred to as drug effects 3 hours post-carrageenan.

2.9. Administration of drugs

Drugs were administered following three stable control trials (less than 10% variation in the C-fibre evoked response). Most of the drugs in this study were administered directly onto the exposed surface of the cord, which is equivalent to an intrathecal injection. Drugs applied directly to the cord were administered using a Hamilton microsyringe in a volume of 50µl which was sufficient to cover the exposed cord and form a small pool in the "well" formed by the laminectomy.

Drugs administered by the subcutaneous route were given by an injection into the scruff of the neck in a volume of 1ml/kg. Drugs given intravenously were administered via a jugular cannula in a volume of 0.8ml/kg. Vehicle controls were carried out as appropriate.*

2.10. Analysis of results and statistics

The electrically evoked neuronal responses for the three tests immediately prior to drug administration were averaged and used as controls for the subsequent drug effects. When drugs were administered following 1 or 3 hours of carrageenan inflammation, the 3 trials immediately prior to the drug administration were used as controls. Mean maximal drug effects were expressed as a percentage of control, percentage inhibition, or in some cases where the drug produced both increases and decreases in the neuronal response, as a percentage change from control (i.e. 160% of control and 40% of control both represent a change from control of 60%) ± standard error of the mean (S.E.M.).

Drug effects were tested for significance (compared with control responses) using the paired Student's t-test (parametric) or Wilcoxon signed rank test (non-parametric) on the original spike count data. Significant differences between the effects of drug combinations were tested using Student's t-test or the Alternate Welch t-test (for unequal S.D.s) (parametric), the Mann-Whitney test (non-parametric), or two factor analysis of variance (ANOVA) followed by Newman-Keuls Student's test as appropriate. Statistical analysis of the data was carried out with the aid of a Macintosh computer and InStat (GraphPad) or Super ANOVA (Abacus Concepts Inc.) software. A p value less than 0.05 was regarded as significant.

For the purposes of comparing dose-response curves obtained in normal animals and following carrageenan inflammation, the dose-response curves were fitted to sigmoid curves using the InPlot curve fitting program (GraphPad), and ED_{50} values and their 95% confidence intervals obtained from these fitted curves. Overlapping confidence intervals were taken to indicate that the ED_{50} values were not significantly different.

In order to establish whether two measured variables were related, linear correlation was performed. The Pearson (parametric) or Spearman (non-parametric) correlation was used as appropriate, with the correlation coefficient, r, and associated p value calculated using InStat (GraphPad).

2.11. Drugs

The drugs used in this study and their sources are listed below. All drugs were dissolved in physiological saline (0.9% NaCl) unless otherwise stated.
Atipamezole (Farmos Group Ltd., Finland); (±)-Baclofen (Research Biochemicals Incorporated, U.S.A); λ-Carrageenan (Sigma, U.K.); 7 Chlorokynurenate (buffered to pH 6, Tocris Neuramin, U.K.); Dexmedetomidine (Farmos Group Ltd., Finland); DSTBULET (Tyr-D-Ser(Otbu)-Gly-Phe-Leu-Thr, a gift from Prof. B.P. Roques, Université René Descartes, Paris); Idazoxan HCl (Sigma, U.K.); Kelatorphan (a gift from Prof. B.P. Roques, Université René Descartes, Paris); L-365,260 (3R(+)-N-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)-N^1-(3-methyl-phenyl) urea) (in 10% tween / alcohol, a gift from Merck Sharp and Dohme); (+)-MK-801 hydrogen maleate (Dizocilpine) (Research Biochemicals Incorporated, U.S.A); Morphine sulphate (Thornton and Ross, U.K.); Naloxone hydrochloride (Sigma, U.K.); Nor-Binaltorphimine (nor-BNI) (a gift from Institute de Recherche Jouveinal, France); Sulphated cholecystokinin-8 (CCK) (Sigma, U.K.); U69593 ((5α.7α,8β)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro [4.5]dec-8-yl)-benzeneacetamide) (Sigma, U.K.).

CHAPTER 3

CONVERGENT DORSAL HORN NEURONES: CHARACTERISTICS AND THEIR RESPONSE TO THE DEVELOPMENT OF CARRAGEENAN-INDUCED INFLAMMATION

3.1. Introduction

Convergent dorsal horn neurones play an important role in the spinal transmission of nociceptive information (see **1.9**). Many electrophysiological studies have monitored the responses of these neurones in order to investigate the spinal mechanisms involved in the transmission and modulation of nociceptive information. These studies frequently utilize acute noxious stimuli such as transcutaneous electrical stimulation or brief natural stimuli such as noxious pressure or heat applied to normal tissue. However, it is of equal, if not greater, importance to understand the spinal transmission and modulation of nociception arising from pathological conditions such as inflammation. Despite this need to study animal models more related to clinically important pain states, there have been relatively few electrophysiological studies performed which have characterized the changes that occur in spinal nociceptive mechanisms during inflammation (Menétrey and Besson, 1982; Calvino et al., 1987a,b; Schaible et al., 1987; Hylden et al., 1989; Neugebauer and Schaible, 1990; Dougherty et al., 1992b; Grubb et al., 1993).

The aim of this chapter is to investigate how spinal nociceptive transmission alters during the development of inflammation in the periphery. The hyperalgesia produced by the injection of carrageenan into the paw, the inflammatory model chosen for this study (**1.10**), has been shown in behavioural studies to peak 2-4 hours following inoculation (Kayser and Guilbaud, 1987; Hargreaves et al., 1988a; Hylden et al., 1991b). This time course allows continuous recording from single dorsal horn neurones for the period over which the inflammation / hyperalgesia develops, allowing inflammation evoked alterations in their discharge to be observed directly, thereby avoiding the need to compare the responses of populations of neurones recorded in normal animals with those recorded in animals with peripheral inflammation. Thus sampling problems are minimized.

Rather than using the response of the neurones to natural stimuli to determine changes in the transmission and modulation of nociceptive information in the inflammatory state, transcutaneous electrical stimulation was used. The advantages of using electrical rather than natural stimulation, outlined in section **2.6**, are that this stimulus, although noxious, is non-tissue damaging and activates the afferents directly thereby avoiding complicating factors such as sensitization of peripheral nociceptors to natural stimuli in the inflammatory state. Whilst we need to understand the changes in peripheral nociceptive systems that occur following the development of inflammation, it is important to be able to separate inflammation-induced alterations in spinal nociceptive systems from the influence of these peripheral changes. The direct activation of primary afferents by electrical stimulation in this study allowed me to concentrate on centrally mediated changes in nociceptive responses which may occur as a result of inflammation.

3.2. Results

3.2.1. The responses of convergent dorsal horn neurones to natural stimuli in normal animals

Although electrical stimulation was used as the test stimulus in the experimental studies, the dorsal horn neurones were initially characterized on the basis of their responses to natural uncontrolled stimuli. The neurones used in this study responded to both innocuous stimuli such as light touch and prod, and noxious stimuli such as pinch and hence can be termed convergent or wide dynamic range neurones. An example of the response of one of these convergent neurones to a variety of natural stimuli applied to its receptive field is shown in figure **3.1**. The neurones responded to pinch with firing which was sustained over the period of the stimulus (generally 10 seconds), showing little adaptation over time, with the neuronal activity often outlasting the stimulus, dying down over a period of tens of seconds. The response to prod, which was of lower frequency than that seen with pinch, did not extend beyond the cessation of the stimulus.

The peripheral receptive fields of the convergent neurones displayed a graded sensitivity to the various intensities of mechanical stimuli. The area over which brush could evoke firing was small, seldom extending beyond one toe or foot pad. This area was surrounded by a larger area, which typically extended over 2 toes and possibly 1 or 2 footpads, in which prod was able to evoke firing. The receptive field in which pinch would evoke firing was generally larger still, covering up to 3 toes. An example of the differing sensitivity of the peripheral receptive field of a neurone is shown in figure **3.1.**

A number of neurones responding strongly to innocuous stimuli such as touch, but which did not give a sustained response to pinch were also encountered whilst searching. If these neurones were subsequently tested with electrical stimulation, they were found to have only a short latency response corresponding to Aβ-fibre conduction velocities, and so were not used for further study.

On rare occasions "noxious-only" neurones were found. These neurones gave no response to brush, touch or persistent prodding of their receptive field, responding only when an area of the receptive field was pinched with forceps. Noxious-only neurones were encountered infrequently in this preparation, probably due in part to the fact that the search procedure used relied initially on non-noxious stimuli such as prod, moving on to noxious stimuli such as pinch only when a responsive neurone had been isolated. As so few noxious-only neurones were found, no attempt was made to study these neurones to judge if the pharmacology associated with these neurones differed in any way from that seen with the convergent neurones studied.

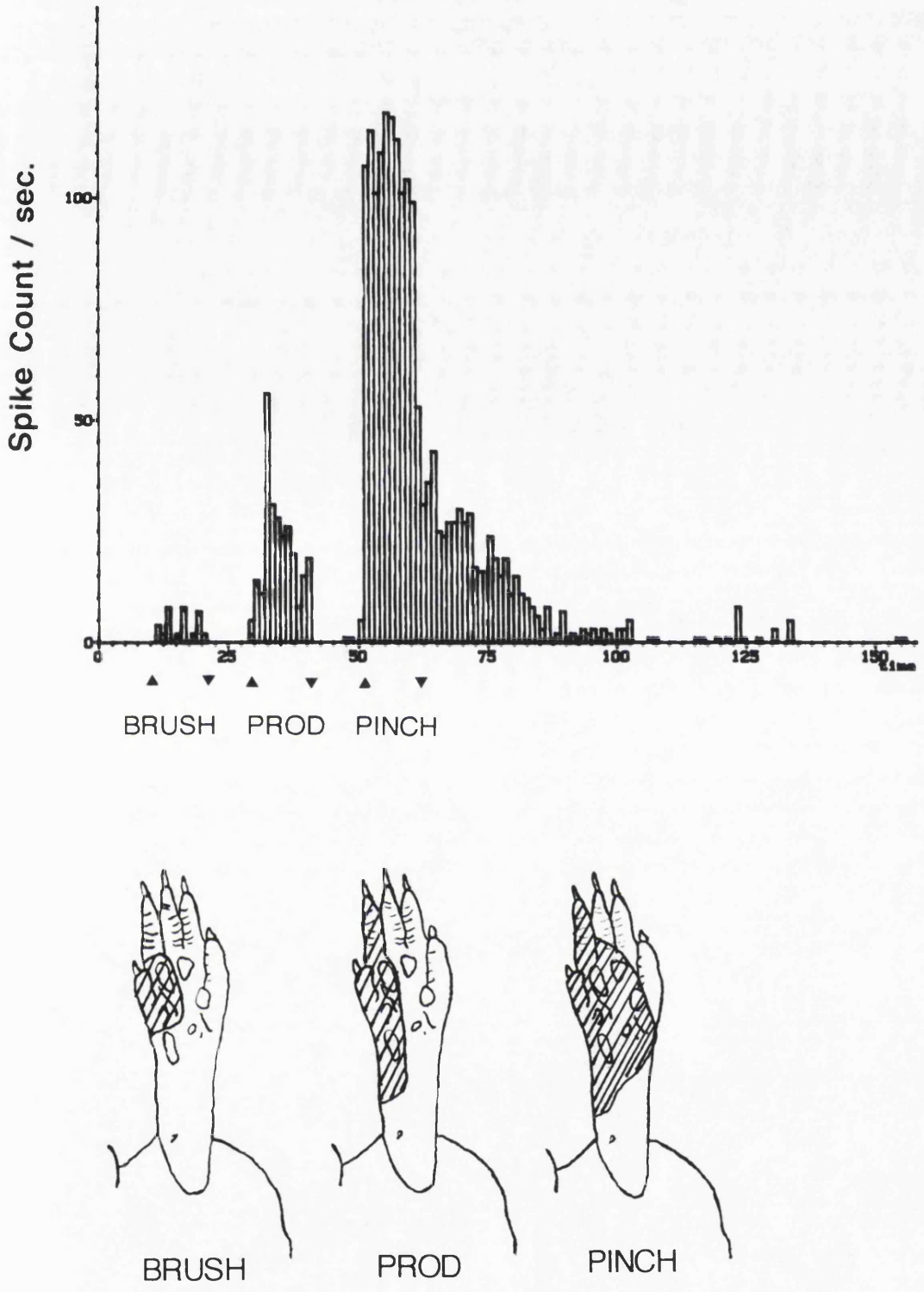


Figure 3.1. Rate recording of the response of a typical convergent dorsal horn neurone to natural stimulation of its peripheral receptive field located on the ipsilateral hind paw. The graded sensitivity of the receptive field to the different stimulus intensities is shown below. Note the continued firing of the neurone following cessation of the pinch stimulus, whilst the firing elicited by brush and prod of the receptive field did not outlast the stimulus. All stimuli were applied for a period of 10 seconds.

3.2.2. Response of dorsal horn neurones in normal animals to electrical stimulation of their receptive field

The convergent dorsal horn neurones used in this study were located mainly in the deep dorsal horn (500-1000μm, mean = 774 ± 9μm, n=433) or in a few cases in the superficial dorsal horn (0-250μm, mean = 197 ± 22μm, n=23). Antidromic stimulation of the ascending pathways was not performed and thus the dorsal horn neurones used in these studies cannot be confirmed as being projection neurones. However, the neurones recorded in this study were located in the regions of the cord corresponding to the zones where the neurones of the spinoreticular and spinothalamic tracts originate (Besson and Chaouch, 1987) and as such are likely to include a large proportion of projection neurones.

Neurones encountered within the band presumed to include the substantia gelatinosa (250-400μm below the dorsal surface of the cord, Magnuson and Dickenson, 1991) were not studied since many of these neurones are inhibitory interneurones and show differing pharmacology from projection neurones (Woolf and Fitzgerald, 1981; Sastry and Goh, 1983; Magnuson and Dickenson, 1991).

Transcutaneous electrical stimulation applied via two needles inserted into the peripheral receptive field of the neurone resulted in firing of the dorsal horn neurone. Low intensity stimulation (0.01-0.2mA) evoked only a response with a short post-stimulus latency, which was recorded in the cord 4-20msec after the stimulus. This short latency low threshold response was attributed to activation of the fast conducting Aβ-fibres (latency band corresponds to conduction velocities of 6-30 m/sec). The mean threshold current required to evoke an Aβ-fibre mediated response in dorsal horn neurones was 0.12 ± 0.006mA, with 76.7 ± 2.2 spikes being evoked following a trial of 16 stimuli given at three times the Aβ-fibre threshold (n=225).

Increasing the stimulus strength (0.1-2.5mA) evoked an additional longer latency response in the dorsal horn neurones (recorded at latencies of 90-300msec post-stimulus) attributed to activation of slower conducting C-fibres. This latency band corresponds to conduction velocities of 0.4-1.25m/sec which is consistent with the conduction velocities of C-fibres recorded from the rat hind limb by Lynn and Carpenter (1982), which ranged from 0.49-0.89m/sec. The mean threshold current required to evoke a C-fibre mediated response was 1.42 ± 0.02 mA (n=456), with an average of 302.9 ± 7.4 spikes of C-fibre latency evoked per trial of 16 stimuli given at 3 times C-fibre threshold. Aδ-fibre evoked responses could also be quantified (post-stimulus latency 20-90msec). Figure **3.2** shows an example of the response of a neurone to a single stimulus of C-fibre intensity.

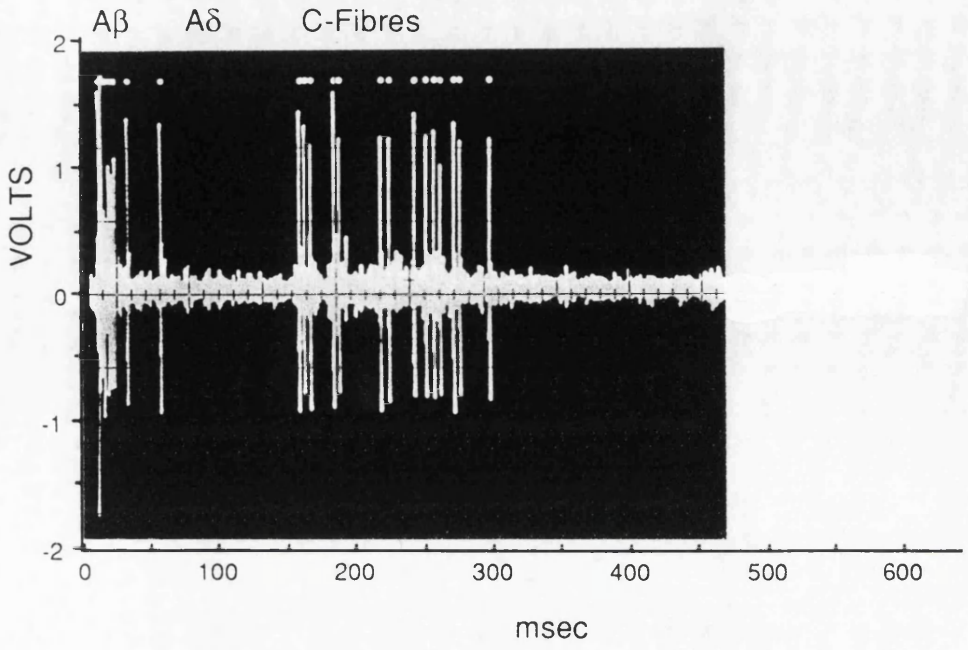


Figure 3.2. Single sweep oscilloscope trace of the response of a convergent dorsal horn neurone to a single stimulus given at 3x C-fibre threshold. The response of the neurone evoked by Aβ-, Aδ-, and C-fibre activity can be separated on the basis of post-stimulus latency. The recording was obtained from a single neurone with spikes of constant amplitude (as seen on a storage oscilloscope).

Following determination of the C- and Aβ-fibre thresholds for each particular neurone, trials of 16 stimuli were given at 3 x the C- and then 3 x the Aβ-fibre threshold and post-stimulus histograms (PSTH) of the number of spikes evoked against post-stimulus latency constructed (see section **2.6**).

The majority of the convergent dorsal horn neurones in this study responded to the repeated electrical stimulation at C-fibre intensity with firing that continued beyond the latency associated with C-fibre conduction velocities. This activity was termed post discharge and is likely to result from activity generated within the dorsal horn as a consequence of wind-up mechanisms (see **3.2.3.**) and not produced by very slowly conducting afferents. The mean post-discharge of the neurones in this study was 170.5 ± 8.3 spikes per trial (n=456). Figure **3.3** shows examples of PSTH s of a neurone with (bottom) and without (top) a post discharge following a train of 16 stimuli at 3x their C-fibre threshold.

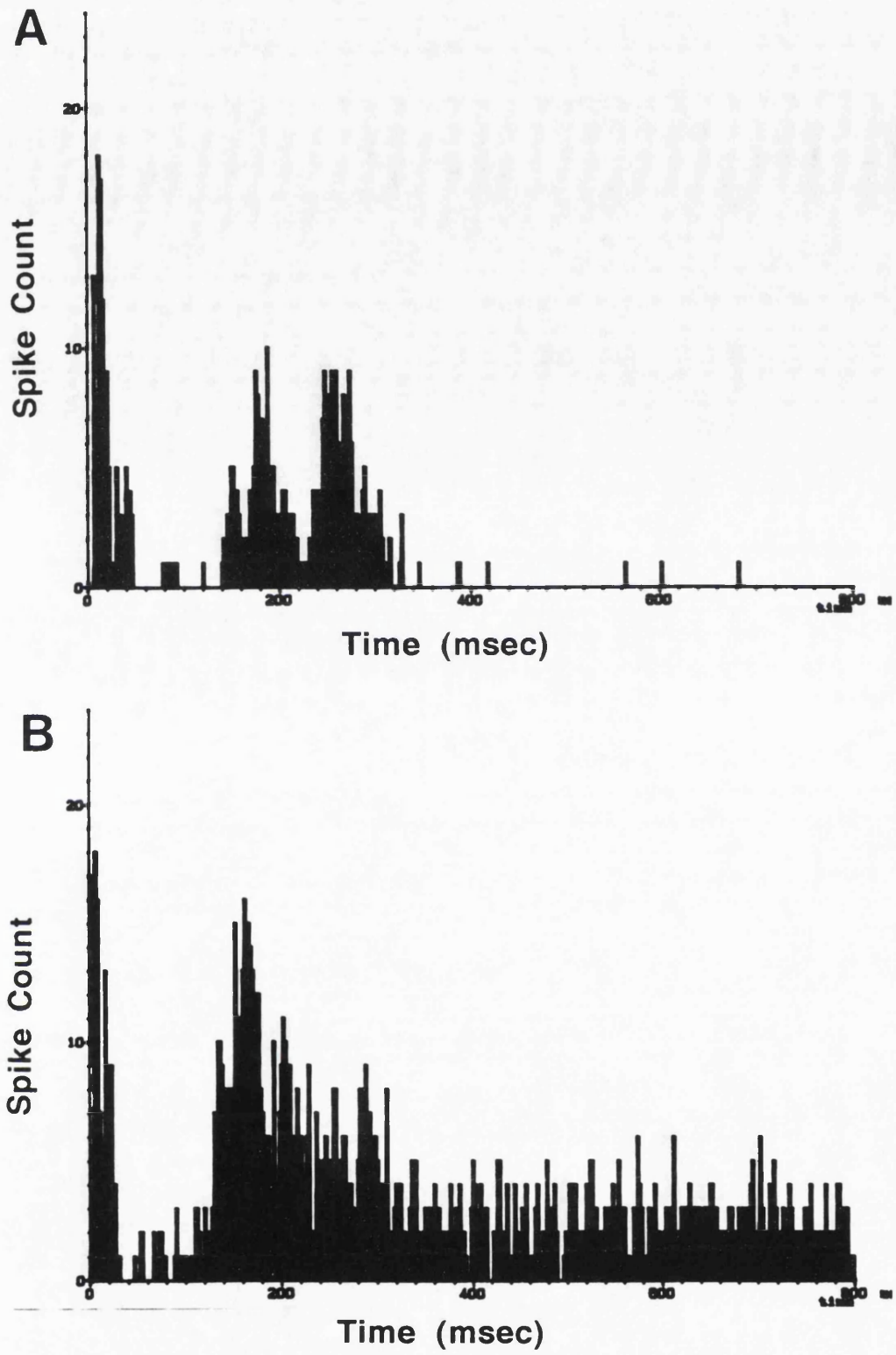


Figure 3.3. Post-stimulus histograms constructed from the responses of two convergent dorsal horn neurones following a train of 16 stimuli given at 3x the C-fibre threshold of the individual neurones. A) PSTH constructed from the responses of a neurone with no post discharge. B) PSTH from a neurone displaying post-discharge which is seen as continued firing after the post-stimulus latency band associated with C-fibre conduction velocities (>300ms).

3.2.3. Wind-up of dorsal horn neurones following electrical stimulation in normal animals

Neurones displaying a post-discharge following repetitive stimulation (0.5Hz) at C-fibre intensities were found to exhibit the phenomenon of wind-up when the C-fibre response evoked by each individual stimulus in the train was examined. Wind-up is defined as an increasing neuronal response to a repetitive stimulus delivered at a constant intensity (Mendell, 1966). The stimulating conditions used in the present study evoked wind-up (to a varying degree) in the majority of the neurones in this preparation.

A small proportion of the neurones studied displayed no wind-up, with each of the 16 stimuli in the train evoking a roughly equal number of C-fibre responses. An example of the response exhibited by such a neurone is shown in figure **3.4A**. A perhaps more typical neuronal response to repetitive C-fibre intensity stimulation is shown in figures **3.4B** and **3.5**. With neurones exhibiting wind-up, only the response of the neurone to the first few stimuli is constant. After this, the evoked response becomes greater with each successive stimulus, eventually reaching a plateau around the 12^{th}-14^{th} stimulus.

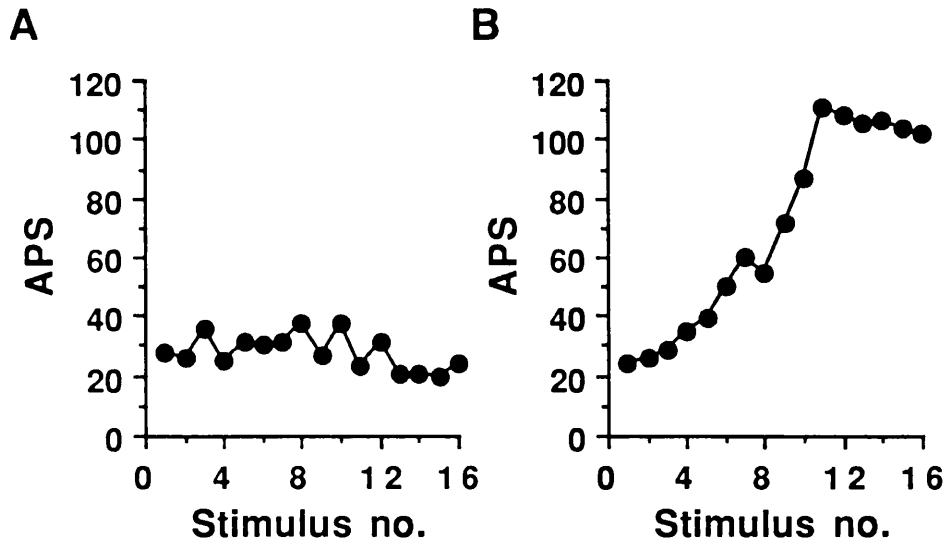
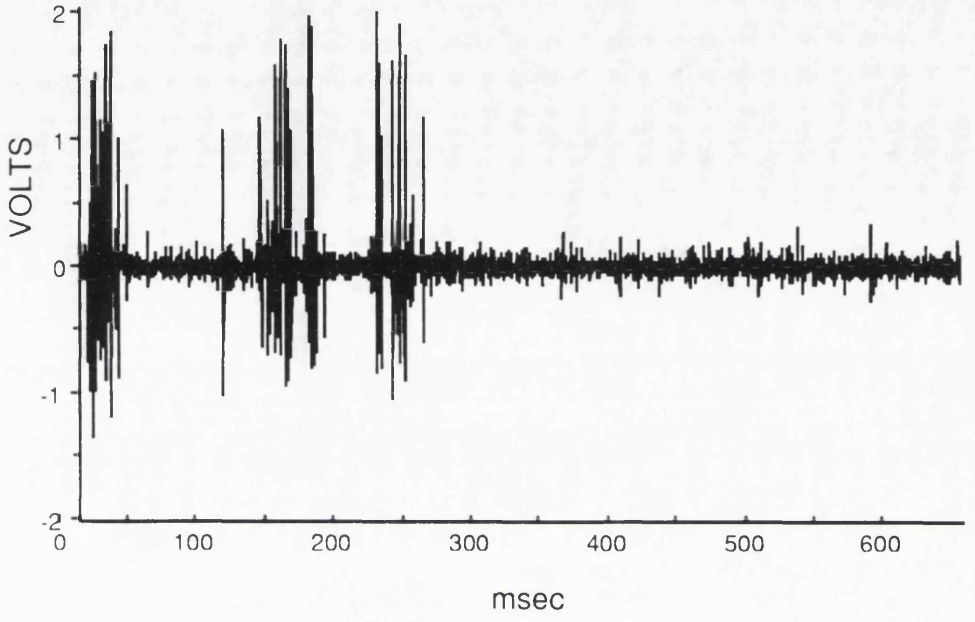


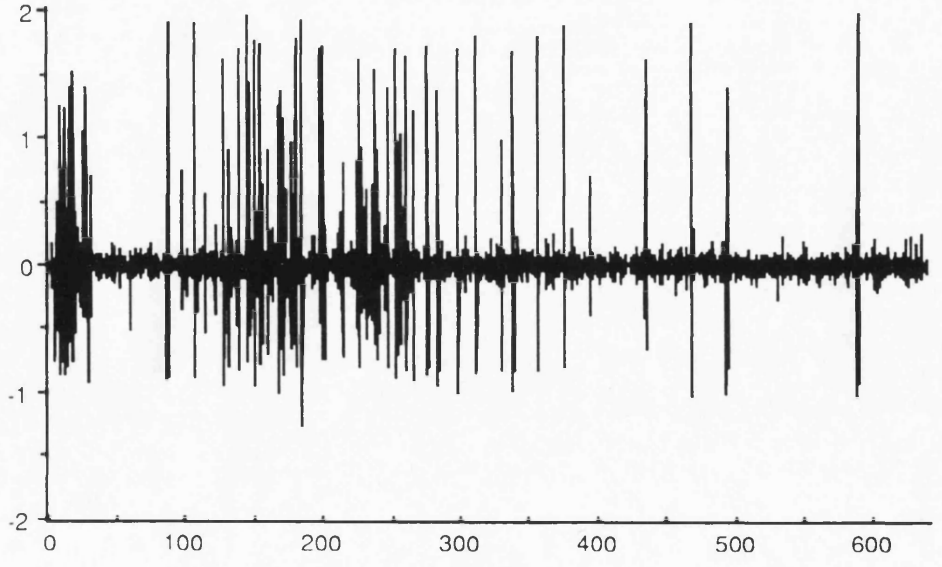
Figure 3.4. Examples of the response of individual dorsal horn neurones to a train of 16 stimuli given at 3x their C-fibre threshold. Plots of the number of action potentials (APS) evoked per stimulus are given against stimulus number. Neurone A displays no wind-up (W=1.00), giving a relatively constant response to each stimulus in the train. In contrast, neurone B exhibits the phenomenon of wind-up (W=2.91) and does not respond to the constant intensity stimulation with a steady response. Each stimulus in the train evokes an increasing number of action potentials with the response only plateauing around the 12^{th} stimulus. Oscilloscope traces of the response of neurone B to the 1^{st}, 8^{th} and 16^{th} stimuli in the train are shown in figure 3.5.

Figure 3.5.

Response of neurone to 1st stimulus in the train.



Response of neurone to 8th stimulus in the train.



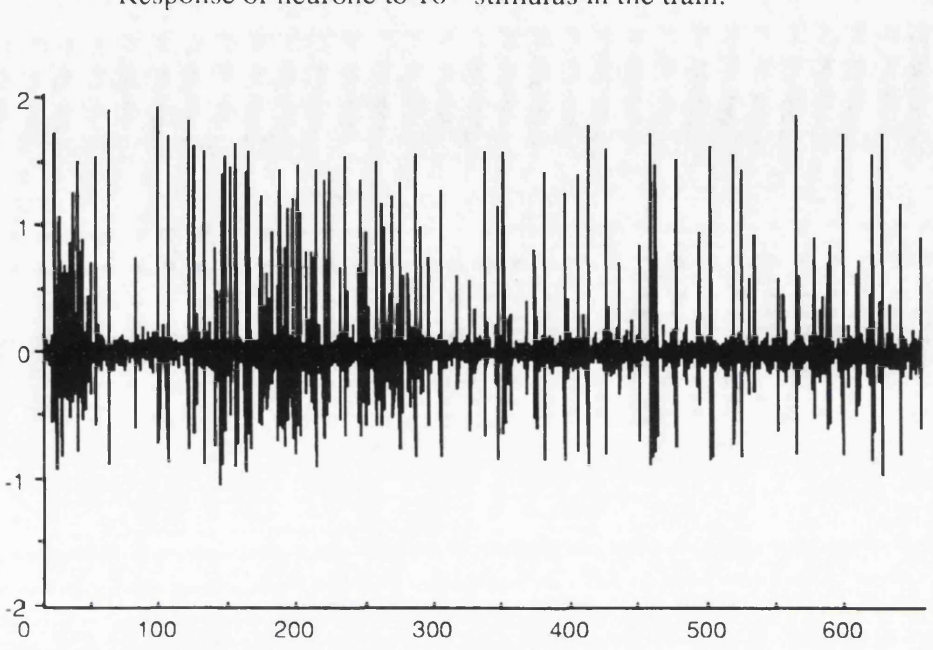


*Figure 3.5. Single sweep oscilloscope traces of the response of a convergent dorsal horn neurone to the 1st, 8th and 16th stimuli in a train. The first stimulus in the train elicits no post-discharge. However by the 8th stimulus, a greater number of C-fibre evoked action potentials (90-300ms) together with a small degree of post discharge (>300ms) are evoked. The final stimulus in the train evokes a yet larger C-fibre response in the convergent neurone together with a considerable post-discharge. Thus although the stimulus intensity remains constant throughout the train, dorsal horn circuitry is capable of amplifying the response of the central neurones, which is manifest as the phenomenon of wind-up.**

The degree of wind-up exhibited by the neurones varied within the population, although there was no significant correlation between the depth of the neurone within the deep dorsal horn (500-1000μm, which corresponds roughly to lamina IV-VI/VII) and the degree of wind-up (W) it exhibited (Spearman $r = 0.17$, $p = 0.09$, $n = 433$). In addition, there was no relationship between the degree of wind-up of the neurone and the magnitude of the total C-fibre evoked response (Spearman $r = 0.06$, $p = 0.51$, $n = 433$).

* ***The recording was made from a single neurone with spikes of constant amplitude (as seen on a storage oscilloscope, see fig. 3.2); however, the trace shown here is a digital representation and so the spike amplitude does not appear to be uniform.***

3.2.4. Peripheral signs of inflammation following the injection of carrageenan

Following the subcutaneous injection of 100μl of λ-carrageenan into the plantar surface of the hind paw, erythema and oedema of the paw were observed. These signs were first obvious within 30 minutes of the injection of carrageenan. The oedema continued to develop over the course of the experiment with the increase in paw volume appearing maximal around 4 hours after the injection. In a few experiments, a comparison of the diameters of the injected and non-injected paws was made 3 hours after the injection of carrageenan. The diameter of the inflamed paw was approximately 25% greater than that of the non-injected paw at this time point (30.0 ± 1.0mm vs 37.2 ± 0.6mm for the inflamed paw (n=6)).

3.2.5. Response of convergent dorsal horn neurones to the development of carrageenan inflammation

Although many of the neurones studied fired for a brief period (usually 1-2 minutes) following the insertion of the syringe needle and subsequent injection of carrageenan into their peripheral receptive field, after this initial period, in the absence of stimulation of their receptive field, the dorsal horn neurones displayed no spontaneous activity.

3.2.6. Response of convergent dorsal horn neurones to electrical stimulation following the injection of saline into the peripheral receptive field

To ensure that neither the injection procedure itself or the repeated electrical stimulation of the peripheral receptive field over a period of some hours altered the evoked responses of the dorsal horn neurones, 0.1ml of saline were injected into the peripheral receptive field, and the electrically evoked response of the neurones followed for three hours in the same way as for carrageenan.

There was no significant change in the electrically evoked C-fibre mediated responses of the dorsal horn neurones three hours after the injection of saline into the paw (C-fibre evoked response 103.6 ± 3.5% of control at three hours, range 87-118% of control; mean maximal change from control, 7.9 ± 2.2%, n=8). The Aβ-fibre evoked responses of the neurones were similarly unaffected following the injection of saline into the receptive field (Aβ-fibre responses 101.1 ± 5.4% of control, range 80-122%). There

was more variability in the post-discharge of the neurones following the injection of saline, although the post-discharge of the neurones was not altered significantly from control (PD 110.3 ± 7.6% of control, range 70-127%; mean change from control 19.1 ± 3.9%, n=8) (figure **3.6** and figs. **3.8, 3.9**). Thus any changes seen in the electrically evoked neuronal responses following the injection of carrageenan into the hind paw are most likely to be a direct result of the inflammatory process rather than, for example, the repetitive stimulation of the peripheral receptive field.

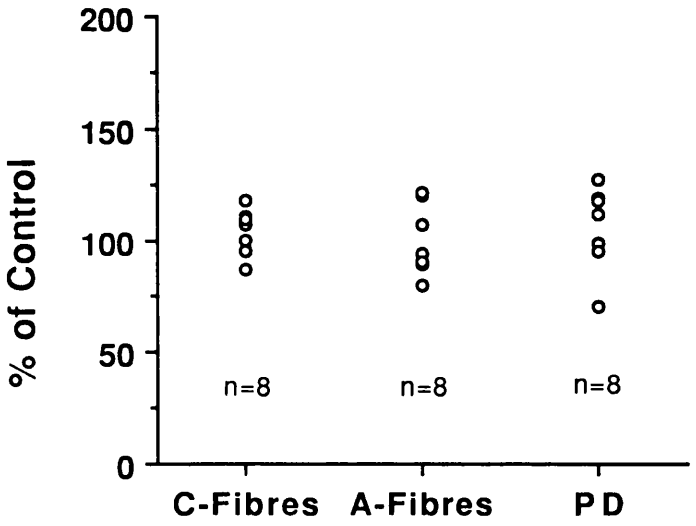


Figure 3.6. Electrically evoked C- and Aβ-fibre responses and the post-discharge (PD) of individual dorsal horn neurones three hours after the injection of 100μl of saline into their peripheral receptive field. Neuronal responses are expressed as a percentage of their pre-saline control value. n=8.

3.2.7. Response of dorsal horn neurones to electrical stimulation of their peripheral receptive field following the injection of carrageenan

The response of a sample of 119 convergent dorsal horn neurones to electrical stimulation of their peripheral receptive field (at 10 minute intervals) was followed throughout the 3 hours after the injection of carrageenan into the ipsilateral hind paw. The electrically-evoked responses of these neurones were altered as the inflammation developed. However, this did not take the form of a uniform enhancement in neuronal responses as might be expected in view of the hyperalgesia seen in behavioural studies. The inflammation-induced changes in the evoked responses of individual dorsal horn neurones are shown in figure **3.7**.

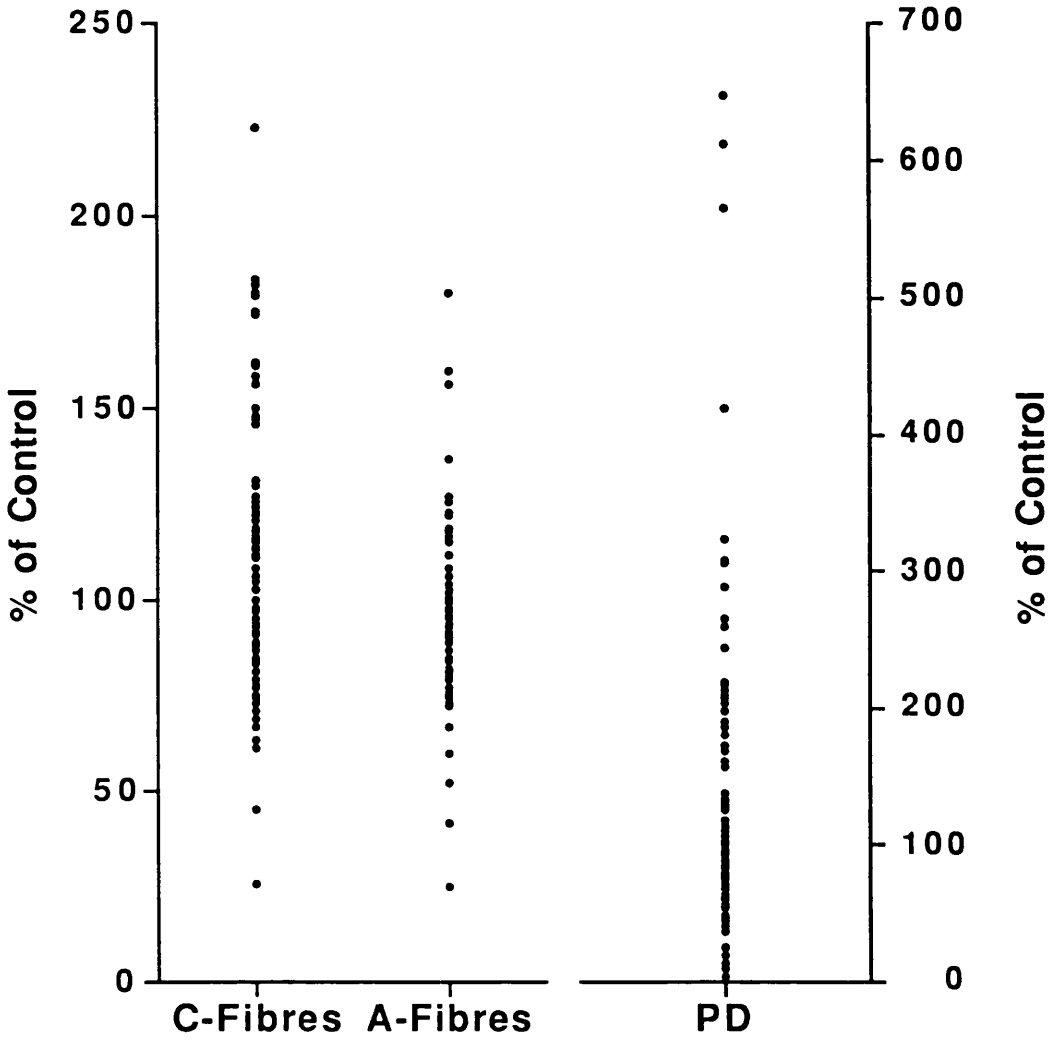


Figure 3.7. Changes in the electrically evoked C- and Aβ-fibre responses and the post-discharge (PD) of individual dorsal horn neurones three hours after the injection of carrageenan into their peripheral receptive field. Neuronal responses are expressed as a percentage of their pre-carrageenan control value. Note the differing scale of the vertical axis for the PD. n=90-119 neurones.

Figure **3.7** shows that the C-fibre evoked response of just over half of these dorsal horn neurones (69 neurones, 58%) increased to a mean of 133.1 ± 3.5% of control (n=69) (range 103-223% of control) three hours after the injection of carrageenan. In contrast, the C-fibre evoked response of the remaining 50 neurones (42%) paradoxically decreased following the development of inflammation, being reduced to a mean of 79.9 ± 1.9% of control (n=50) (range 98-26% of control). When the C-fibre evoked responses of all the neurones 3 hours post-carrageenan are taken together, the net result of the carrageenan inflammation is a slight increase in the C-fibre evoked response of the neuronal population to 110.8 ± 3.3% of control (n=119) (figure **3.8**). However this increase does not represent a significant change from pre-carrageenan control values (two-tailed p-value = 0.091, Wilcoxon signed rank test).

When the magnitude of the change in the C-fibre response post-carrageenan, irrespective of whether the response had increased or decreased, was compared to that produced by the peripheral injection of saline alone (figure **3.9**), the magnitude of the changes in the neuronal response produced by carrageenan (mean change from control, irrespective of direction of change 27.6 ± 2.3%) were significantly greater than those resulting from saline alone (7.9 ± 2.2%) (one-tailed p-value = 0.0019, Mann-Whitney test). Thus the development of carrageenan-induced inflammation resulted in significant, although complex changes in the C-fibre evoked responses of dorsal horn neurones.

The post-discharge (PD) of the neurones showed a similar bidirectional change post-carrageenan, with 58% of the neurones showing an increased response (mean 216.1 ± 17.2% of control, range 103-646% of control) whilst the remaining 42% of the neurones displayed a decreased response post-carrageenan (mean 60.2 ± 4.1% of control, range 4-97% of control) (figure **3.7**). The magnitude of the change in the post-discharge of the neurones three hours post-carrageenan was significantly greater than that produced by the injection of saline alone (mean change from control evoked by carrageenan = 82.5 ± 10.7% (n=90) vs 19.1 ± 3.9% produced by saline (n=8), one-tailed p-value = 0.0022, Mann-Whitney test) (figure **3.9**).

In contrast to the overall change in the C-fibre evoked response post-carrageenan, the change in the post-discharge of the neurones was significant and produced a net increase in the post-discharge of the neuronal population to 150.6 ± 12.9% of control (one-tailed p-value = 0.013, Wilcoxon signed rank test, n=90) (figure **3.8**).

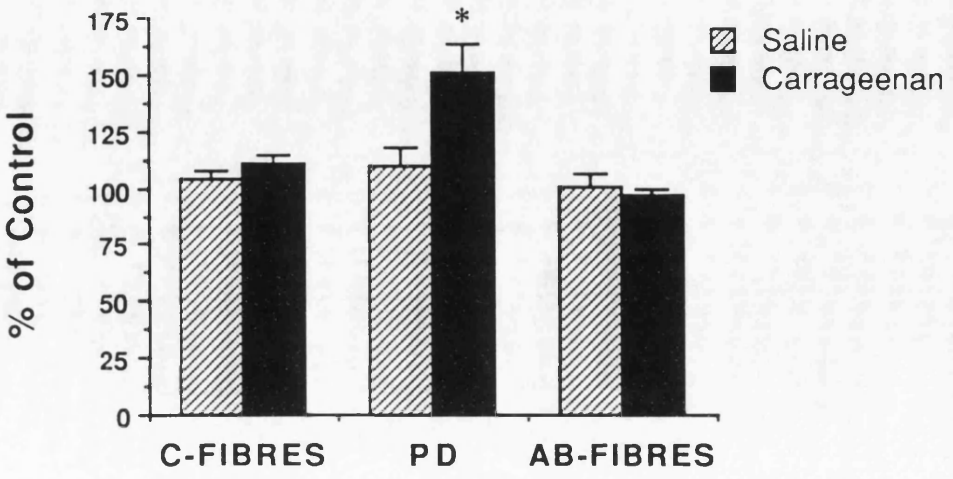


*Figure 3.8. Mean C- and Aβ-fibre evoked response and post-discharge (PD) of the neuronal population three hours after the injection of saline (shaded columns) or carrageenan (filled columns) into the peripheral receptive field. Neuronal responses are expressed as a percentage of their pre-injection control value. n=8 (saline) or 90-119 (carrageenan). * p≤0.05 compared with pre-injection control.*

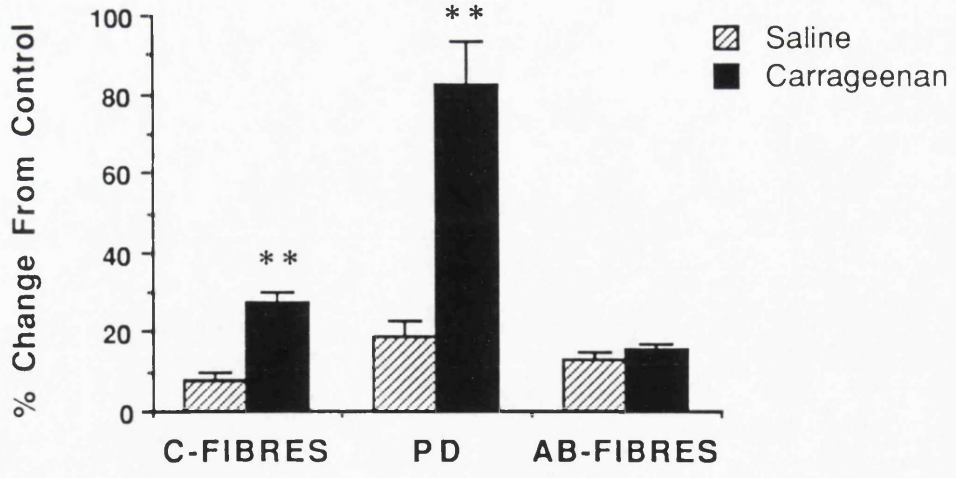


*Figure 3.9. Magnitude of change in the C- and Aβ-fibre evoked response and the post-discharge (PD) of the neurones produced by saline (shaded columns) or carrageenan (filled columns). Post-injection responses are expressed as a percentage change from control, regardless of direction. n=8 (saline) or 90-119 (carrageenan). ** p≤0.01 compared with saline alone.*

There was less change in the Aβ-fibre evoked responses of the neurones post-carrageenan. As with the C-fibre evoked response and post-discharge, a proportion of the neurones showed an enhanced Aβ-fibre evoked response following the development of inflammation (39/103 (38%), mean 117.5 ± 3.0% of control, range 101-180% of control), whilst the others showed a decreased response to the constant intensity stimulation (64/103 (62%), mean 84.3 ± 1.7% of control, range 25-98% of control) (figure **3.7**). When the responses of all the neurones were considered together, there was no net effect of the peripheral injection of carrageenan on the Aβ-fibre evoked responses of dorsal horn neurones (mean 96.9 ± 2.2% of control, n=103) (figure **3.8**). In contrast to the C-fibre evoked responses, the magnitude of the change in the Aβ-fibre evoked response was not significantly different to that produced by saline alone (mean change in the Aβ-fibre evoked response produced by carrageenan =15.8 ± 1.3% (n=103) vs 12.9 ± 2.4% for saline alone (n=8), $p>0.05$) (figure **3.9**).

The complex changes in the C-fibre evoked response of the neurones following the injection of carrageenan were not related to the depth of the neurone within the dorsal horn. Of the 119 neurones studied throughout the three hours following the injection of carrageenan, 110 were located in the deep dorsal horn of the spinal cord (500-1000μm, mean depth 772.6 ± 15.7μm). There was no correlation between the depth of the neurone in the dorsal horn and whether it showed an increased or decreased response post-carrageenan (Spearman correlation, $r = -0.028$, $p=0.77$). The responses post-carrageenan of the nine neurones located in the superficial dorsal horn (0-250μm, mean depth 193.8 ± 37.7μm) did not appear to differ from those of neurones located in the deep dorsal horn, however the number of neurones involved was too small to allow a comprehensive study to be performed.

3.2.8. Thresholds for electrical activation of the neurones post-carrageenan

The changes in the electrically evoked C- and Aβ-fibre evoked responses of the dorsal horn neurones following the development of carrageenan inflammation are unlikely to result from alterations in the threshold current required for electrical activation of the neurones. In some neurones, the threshold current required for C-fibre activation was re-established 3 hours after the carrageenan injection and these currents were not significantly different from their control values; 0.86 ± 0.19mA vs 0.88 ± 0.22mA respectively (n=10). There was no indication of a lowered threshold in neurones with an enhanced response 3 hours after the injection of carrageenan and vice versa. In addition, there was no significant correlation between the control C-fibre threshold of a neurone and the response of the neurone three hours after the injection of carrageenan (figure **3.10**) (Spearman correlation r = 0.1358, two-tailed p-value = 0.15, n=114).

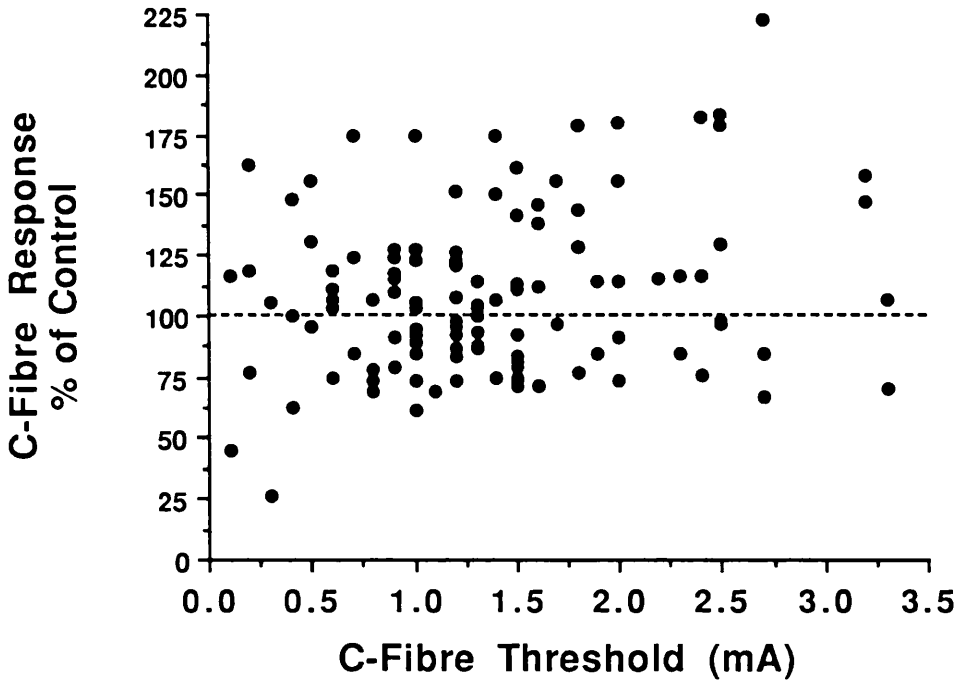


Figure 3.10. The changes in the electrically evoked C-fibre responses of the neurones following the injection of carrageenan into the paw (vertical axis) were not related to the C-fibre thresholds of the neurones prior to the injection (horizontal axis). Correlation coefficient, r = 0.14, p>0.1; n=114.

3.2.9. Relationship between the neuronal response post-carrageenan and wind-up

Although at first sight the changes in the electrically evoked C-fibre mediated responses of dorsal horn neurones three hours after the injection of carrageenan appear random, the magnitude and direction of the change in the C-fibre evoked response was found to be highly significantly correlated with the degree of wind-up of the neurones (W) pre-carrageenan (Spearman correlation, r = -0.52, two-tailed p-value <0.0001, n=119) (figure **3.11**). The correlation between the degree of wind-up of the neurones prior to the injection of carrageenan and the altered neuronal response post-carrageenan was inverse.

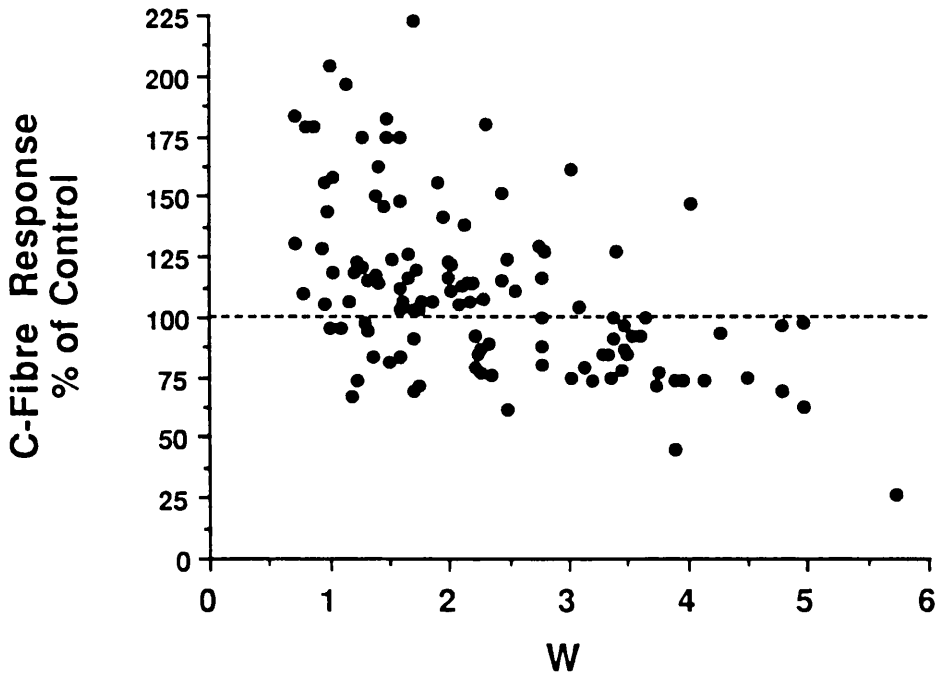


Figure 3.11. Relationship between the direction and magnitude of the change in the C-fibre evoked response of the neurones 3 hours after the injection of carrageenan into the paw and the initial degree of wind-up of the neurone, W. Neurones with little wind-up in the control period (low value of W) tended to display enhanced C-fibre evoked responses post-carrageenan, whilst the responses of those neurones exhibiting a large degree of wind-up initially (high W) tended to be reduced.
Correlation coefficient, r = -0.52, p<0.0001; n=119.

Thus as figure **3.11** shows, those cells with a large degree of wind-up in the control period (high value of W) subsequently exhibited a decreased response to the constant intensity C-fibre stimulation after carrageenan, whilst those with little wind-up initially (low W) tended to display an increased response as the inflammation developed.

This inverse correlation was also seen between the changes in the post-discharge of the neurones following the development of inflammation and the degree of wind-up (W) pre-carrageenan (Spearman correlation, r = -0.58, p<0.0001) (figure **3.12**).

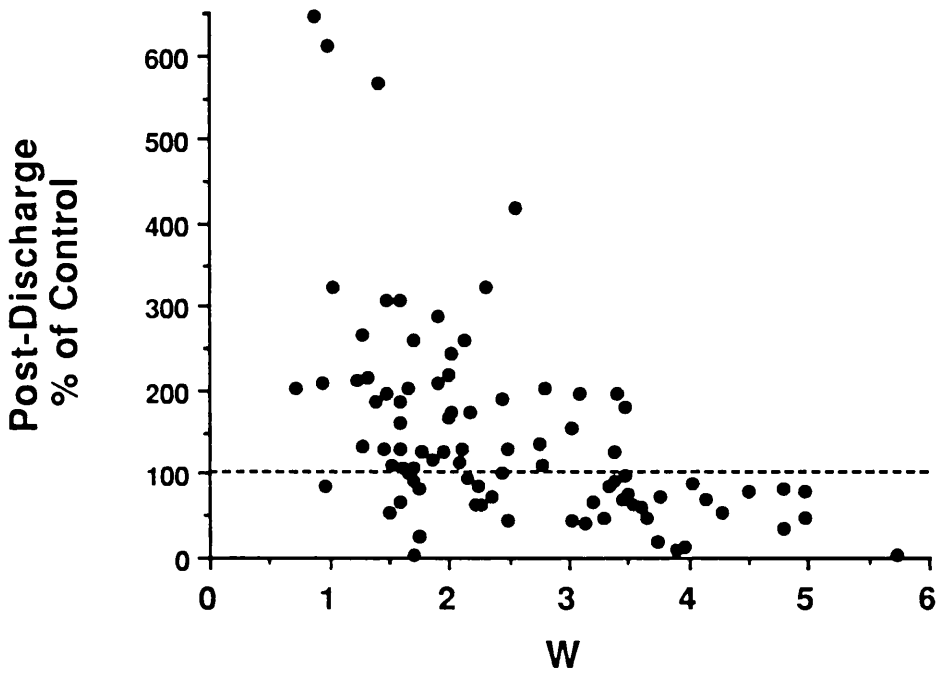


Figure 3.12. The direction and magnitude of the changes in the post-discharge of the neurones post-carrageenan are significantly correlated with the degree of wind-up exhibited by the neurone prior to the injection of carrageenan. Correlation coefficient, r = -0.58, p<0.0001; n=90.

The increased neuronal responses following carrageenan were due both to an enhanced input onto the cells and also to the cells exhibiting a greater degree of wind-up as the inflammation developed. Neurones with a reduced response post-carrageenan tended to have less wind-up than in the control period and a smaller input. Some individual examples of this are shown in figure **3.13**.

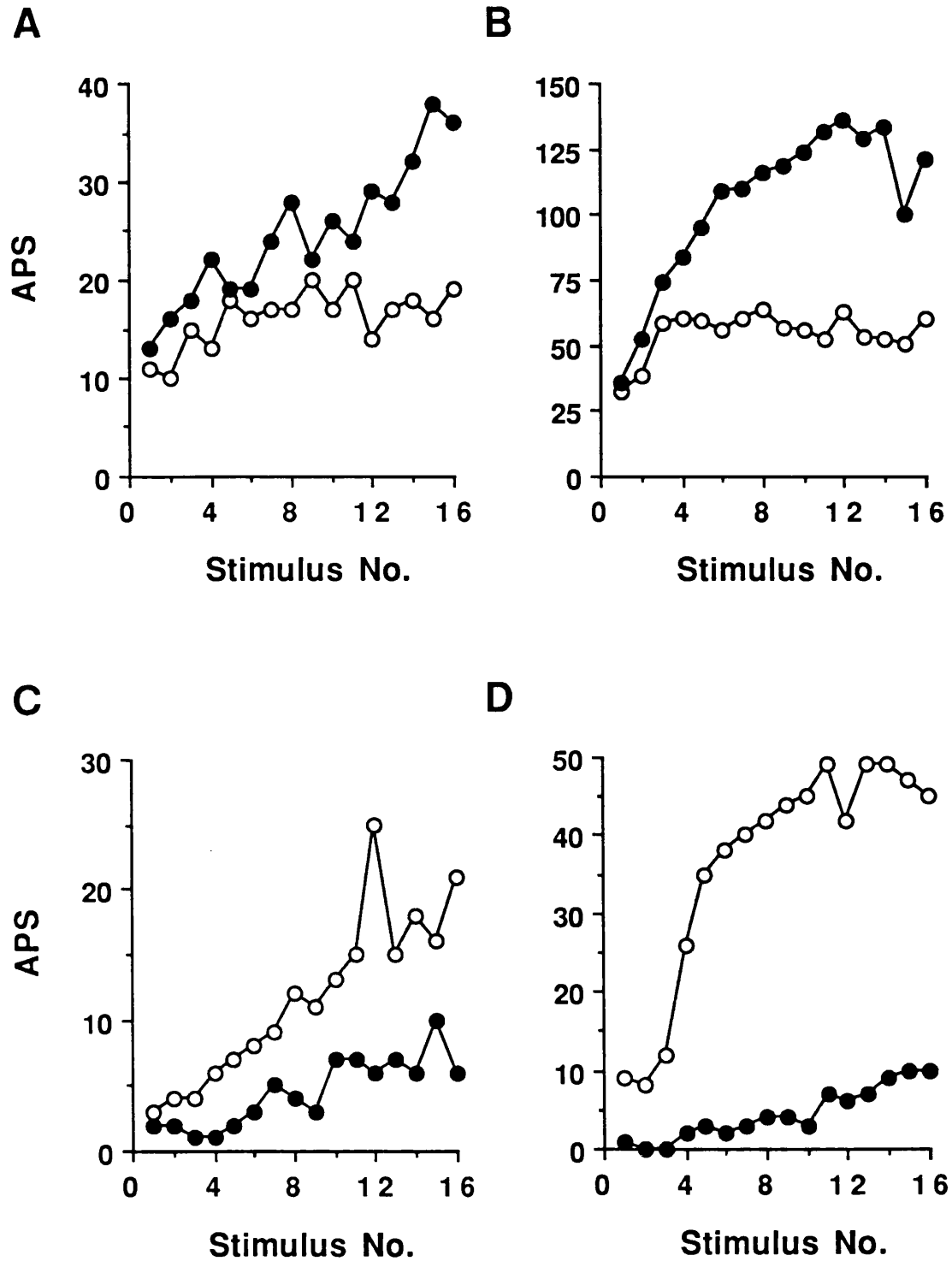


Figure 3.13. Examples of wind-up graphs of neurones before (-o-) and 3 hrs after (-●-) carrageenan. Plots of the number of actions potentials evoked / stimulus (APS) are given against stimulus no.. Neurones A & B exhibited little wind-up during the control period (W=1.5 & 1.6) and displayed enhanced responses as the inflammation developed. By contrast, cells C & D had a large degree of wind-up in the control period (W=3.9 & 6.9); this was reduced as the inflammation developed. Note the different scales on the y-axis.

Both the changes in the input (I) and the changes in the wind-up of individual neurones (E) were strongly correlated with the changes in the C-fibre evoked response post-carrageenan (Spearman r = 0.7324 (I) and 0.8064 (E) respectively, $p<0.0001$) (figure **3.14**).

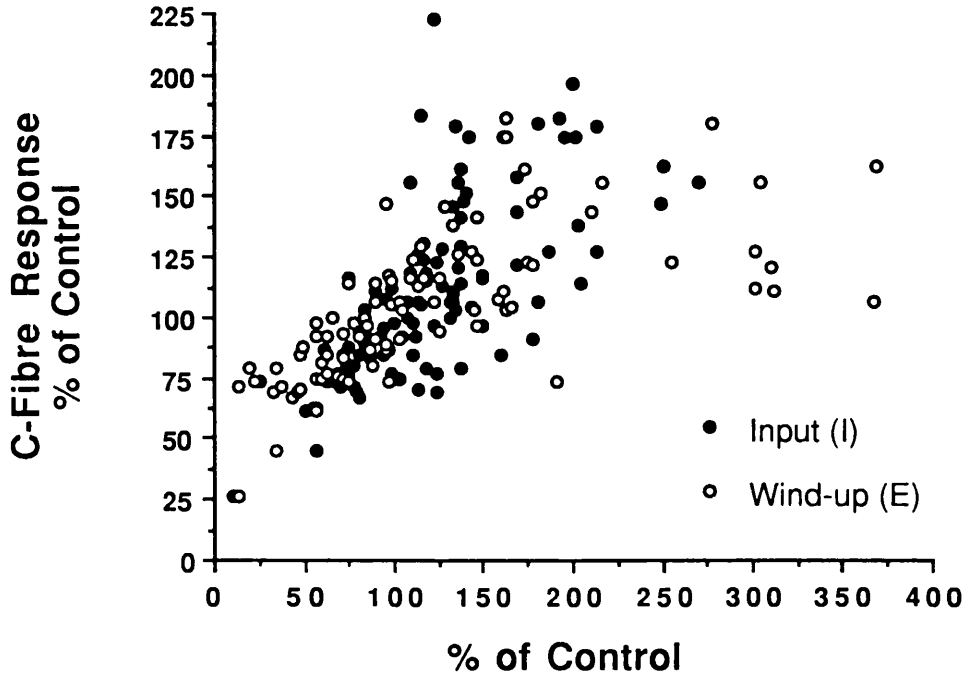


Figure 3.14. The change in the C-fibre evoked response of the dorsal horn neurones post-carrageenan results from changes in both the initial input onto the neurones (I) and in the wind-up of the individual neurones (E). The changes in both parameters are highly correlated with the changes in the total C-fibre evoked response. The correlation coefficient (r) between the altered C-fibre evoked responses and the changes in input (I) is 0.73 and for wind-up (E) it is 0.81; p<0.0001 for both.

The alterations in the Aβ-fibre evoked responses of the same cells post-carrageenan were also inversely correlated with the degree of C-fibre evoked wind-up in the control period (Spearman r = -0.20, p=0.041) (figure **3.15**), although this correlation was much weaker than that seen between the altered C-fibre evoked response post-carrageenan and wind-up.

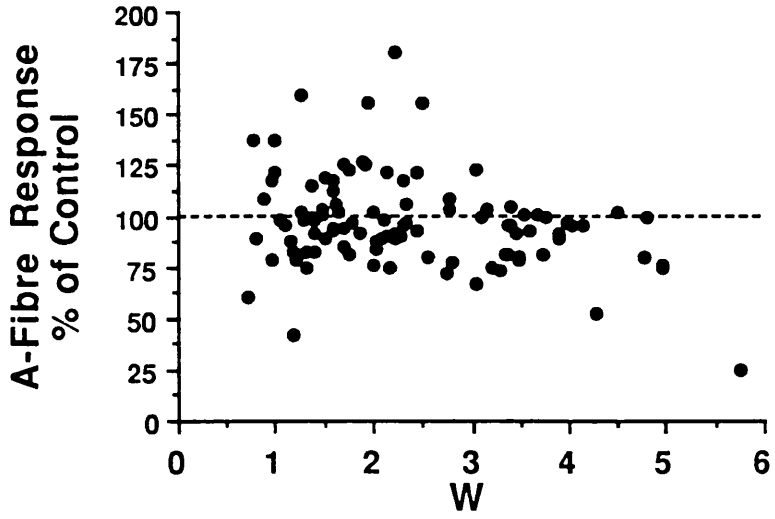


Figure 3.15. The changes in the Aβ-fibre evoked responses of the neurones post-carrageenan show a significant inverse correlation with the degree of C-fibre mediated wind-up (W) in the control period. Correlation coefficient, r = -0.20, p=0.04; n=103.

There was no significant correlation between the size of the C-fibre evoked response of a neurone during the control period and the subsequent magnitude and direction of change in the C-fibre evoked response of the neurone following the development of inflammation (Spearman r = -0.17, p=0.063, n=114) (figure **3.16**).

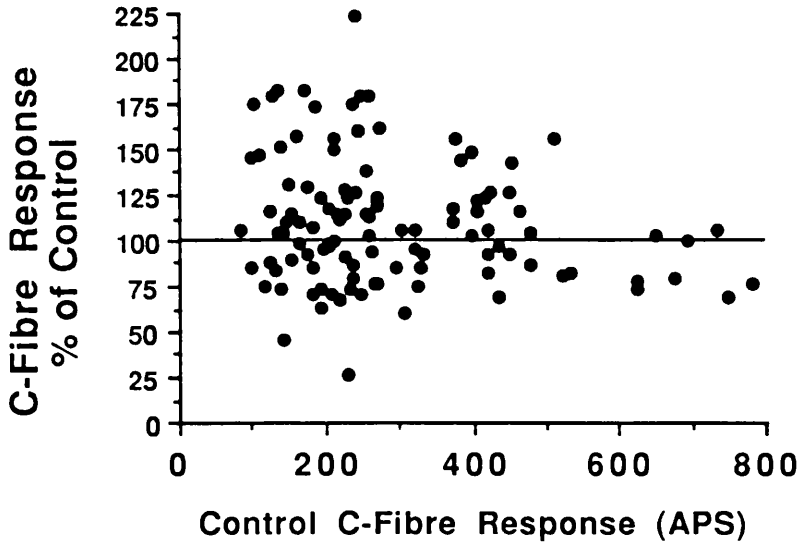


Figure 3.16. Lack of correlation between changes in the C-fibre evoked responses post-carrageenan (vertical axis) and the control C-fibre response (number of action potentials evoked (APS)). Correlation coefficient, r = -0.17, p>0.05, n=114).

3.3. Discussion

The results presented in this chapter show that complex changes occur in the electrically evoked C-fibre mediated responses of convergent dorsal horn neurones following the development of inflammation in the periphery. Three hours after the injection of carrageenan into the ipsilateral hind paw, the time corresponding to peak hyperalgesia in behavioural studies using this inflammatory agent (Kayser and Guilbaud, 1987; Hargreaves et al., 1988a; Hylden et al., 1991b), C-fibre evoked responses of convergent dorsal horn neurones to constant intensity electrical stimulation were either facilitated or inhibited compared with their control responses.

Transcutaneous electrical stimulation, used in the present study, by-passes the peripheral nociceptors to activate the neurones directly thus sensitization of the peripheral nociceptors by inflammatory mediators is unlikely to influence the response to electrical stimulation. This is supported by the demonstration that the electrical threshold for activation of the neurones is unaltered by the development of carrageenan inflammation. The altered response of these neurones to constant intensity electrical stimulation following the development of inflammation in the absence of any changes in the electrical threshold of the neurones strongly suggests that these changes are likely to result primarily from central rather than peripheral mechanisms.

The suggestion that there is a strong centrally mediated component to the alterations in the electrically evoked C-fibre response is further supported by the finding that the direction and magnitude of the changes in the C-fibre evoked response post-carrageenan are strongly correlated with the initial degree of wind-up of the dorsal horn neurones, a centrally mediated phenomenon. The dependence of the carrageenan-induced changes in the C-fibre evoked response on the initial wind-up of the neurones points to an involvement of the NMDA receptor which has been shown to be important in the manifestation of C-fibre evoked wind-up in these dorsal horn neurones (Mendell, 1966; Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990). Although Aβ-fibre stimulation is not capable of inducing wind-up in dorsal horn neurones (Dickenson and Sullivan, 1987a) the relatively weak alterations in the Aβ-fibre evoked responses were also correlated, although not as strongly, with the initial wind-up of the neurones. This is not entirely unexpected since wind-up mechanisms (initiated by C-fibre stimulation) are known to increase both the C-and Aβ-fibre evoked responses of deep dorsal horn neurones (Schouenborg and Sjölund, 1983). Thus Aβ-fibre evoked responses arriving on a convergent dorsal horn neurone rendered hyperexcitable as a result of C-fibre evoked wind-up mechanisms are liable to be altered in a similar way to those evoked by C-fibres.

The potential involvement of the NMDA receptor in the altered neuronal responses

post-carrageenan, as suggested by the strong correlation between the changes in the evoked neuronal responses post-carrageenan and the initial wind-up of the neurones (an NMDA receptor mediated event), is not surprising. The phenomenon of central hypersensitivity, the increased excitability of spinal systems, was first demonstrated by Woolf (1983) and subsequently shown to be dependent on the activation of NMDA receptors (Woolf and Thompson, 1991). In addition, it has been shown that the NMDA receptor is important in the response of neurones to another inflammatory stimulus, formalin, which by contrast to carrageenan causes ongoing activity in the neurones (Haley et al., 1990; Dickenson and Aydar, 1991).

Since the NMDA receptor is excitatory, the increases in the evoked responses of some neurones after carrageenan are predictable, particularly in view of the hyperalgesia seen in behavioural studies. However, approximately half the neurones displayed reduced responses to electrical stimulation following the development of inflammation. Furthermore, these were the neurones which exhibited the most wind-up prior to carrageenan. At first sight it may seem paradoxical that the NMDA receptor may be involved in decreased neuronal responses. However there is evidence to suggest that NMDA receptor activation can result in the activation of antinociceptive as well as nociceptive mechanisms. Raigordsky and Urca (1987) demonstrated both analgesia and behaviour indicative of nociception following the administration of NMDA into the intrathecal space, with the analgesia but not the hyperalgesia being reversed by naloxone, suggesting the involvement of the endogenous opioids. Since this initial study, several further studies have reported findings indicative of antinociception as well as nociception resulting from NMDA receptor activation. Furthermore, several of these studies show that the antinociception is associated with high as opposed to low levels of NMDA receptor activation.

In an electrophysiological study, Dougherty and Willis (1991a) examined the response of primate spinothalamic tract neurones following the iontophoretic administration of NMDA alone and NMDA co-administered with substance P. The co-administration of SP with NMDA, which would be expected to further enhance the activation of the NMDA receptor by providing the sustained depolarization necessary to remove the Mg^{2+} block of the NMDA receptor channel (see **1.4**), led to the enhancement of the response to NMDA in 14/21 neurones. However, the response of the remaining 7 neurones to NMDA was reduced following co-administration with SP. In addition, the response of a proportion of the neurones to brush and pinch of their peripheral receptive field was reduced during the combined application of NMDA and SP. A similar proportion of neurones were found to display reduced responses following the combined administration of NMDA and SP when this study was extended to cover non-NMDA

ligands (Dougherty et al., 1993) although in this instance, no accompanying reductions were found in the responses to mechanical stimuli.

In the formalin response, another model of inflammatory hyperalgesia, the intrathecal administration of NMDA alone was found to decrease nociceptive behaviour in the second phase of the formalin test (Mjellem-Joly et al., 1992). Thus in the second phase of the formalin response where there is already a high level of NMDA receptor activation (Haley et al., 1990; Dickenson and Aydar, 1991; Coderre and Melzack, 1992), akin to neurones displaying a large degree of wind-up in response to electrical stimulation in the present study, further activation of the NMDA receptor, in this case by exogenous NMDA itself, decreased nociceptive responses. However in the same study, the intrathecal co-administration of NMDA with substance P significantly increased and prolonged the response to formalin (Mjellem-Joly et al., 1992).

Recently Kolhekar et al. (1993) showed that whilst low doses of intrathecal NMDA produced facilitations of the tail flick reflex in rats, the highest dose tested inhibited the tail flick reflex. The inhibition of the tail flick reflex by intrathecal NMDA was shown to be due to activation of descending inhibitory controls (Kolhekar et al., 1993). This demonstrates that high levels of NMDA receptor activation can lead to the recruitment of inhibitory systems, which are of sufficient magnitude to produce analgesia.

Of particular relevance to this chapter, is the study by Dougherty et al. (1992b) in which the responses of spinothalamic tract neurones to the iontophoretic administration excitatory amino acids were followed during the development of acute arthritis in monkeys. Whilst the response of the neurones to glutamate and quisqualic acid was progressively enhanced as the arthritis developed over a period of 3-4 hours, the response to iontophoretically administered NMDA was progressively reduced. Furthermore, these decreases in response were most evident with the highest doses of NMDA used (Dougherty et al., 1992b). Although this is not a direct example of NMDA receptor activation leading to an inhibition of neuronal responses, it demonstrates that the excitatory consequences of NMDA receptor activation can be reduced following the development of inflammation.

These studies illustrate that activation of NMDA receptors can, under certain conditions, lead to a reduction in spinal nociceptive transmission. This process may have parallels with homosynaptic long-term depression (LTD) in the hippocampus, in which NMDA receptors play a pivotal role in reducing synaptic efficacy (Bear and Malenka, 1994).

To my knowledge, only three other studies have been performed in which the evoked responses of dorsal horn neurones have been monitored during the development of peripheral inflammation (Schaible et al., 1987; Neugebauer and Schaible, 1990;

Dougherty et al., 1992b), all of which have employed models of acute arthritis induced by the injection of kaolin and carrageenan into the knee joint. Two of these studies, performed in cats, (Schaible et al., 1987; Neugebauer and Schaible, 1990) reported that the neuronal responses evoked by flexion of the knee joint or pressure across the joint were progressively enhanced in all neurones tested as the inflammation developed (up to 6 hours). However, 2/10 neurones tested did show a reduced response to brush during this time (Neugebauer and Schaible, 1990). In the study of Dougherty et al. (1992b) in monkeys, 4/12 neurones showed a reduced response to brush, whilst 6/12 showed an enhanced response as the arthritis developed. In contrast to the two studies in the cat however, 3/12 neurones also showed decreased responses to flexion of the joint as the arthritis developed (Dougherty et al., 1992b). Thus although inflammation-induced reductions in evoked neuronal responses have been reported in previous studies, the predominant change reported is an enhancement in neuronal responses, with the emphasis of these studies placed on this increased excitability post-inflammation.

Although one possibility for the apparent discrepancy between the results presented in this chapter, where around 50% of the neurones displayed reduced responses as the inflammation developed, and the studies discussed above (Schaible et al., 1987; Neugebauer and Schaible, 1990; Dougherty et al., 1992b), which report predominantly enhanced responses following inflammation, is the different inflammatory model (and species) used, other possibilities exist. The acute arthritis studies have concentrated mainly on the responses of the spinal neurones to natural mechanical stimuli which activate the neurones via the peripheral nociceptors. The nociceptors are known to become sensitized following inflammation (see refs in **1.2**) thus the C-fibre barrage reaching the spinal cord in response to natural stimuli is likely to be enhanced, which could mask some of the central changes. In addition, the intensity of these natural stimuli compared with the intensity of the electrical stimulation used in this chapter is not known. The natural stimulation used may not evoke wind-up mechanisms to the same degree as the electrical stimulation used in the present study, and consequently may not produce the inhibitions of evoked responses associated with high levels of wind-up seen in this study.

One study has followed the response of dorsal horn neurones to electrical stimulation during the development of acute arthritis in the cat's knee and in this study the electrically evoked responses of the neurones were progressively enhanced as the inflammation developed (Neugebauer and Schaible, 1990). However, the intensity of the electrical stimulation used was described as "sufficient to elicit reproducible small responses of the cell" and "usually sufficient to stimulate small myelinated axons" suggesting that the intensity of stimulation used was much lower than that employed in this chapter (3x that required to consistently evoke responses with C-fibre latencies). Thus in the study of Neugebauer and Schaible (1990) it is likely that the intensity of

stimulation used evoked little or no wind-up. I have shown in this chapter that neurones in which little or no wind-up is evoked tend to display an enhanced response post-inflammation which fits with the results of Neugebauer and Schaible (1990).

A further difference between the study presented in this chapter and that of Neugebauer and Schaible (1990) is that the cats used by Neugebauer and Schaible (1990) were spinalized, whereas the rats used in the present study were intact. The descending control system, which is absent in the spinalized cats, may play some role in the reduced neuronal responses seen in the present study post-carrageenan since Kolhekar et al. (1993) have shown that inhibitory descending controls play an important part in the NMDA receptor-mediated reductions in neuronal responses.

3.3.1. What mechanisms could underlie the altered neuronal responses post-carrageenan?

The strong correlation between the direction and magnitude of the neuronal changes post-carrageenan and the degree of wind-up of the neurone prior to this event, in the absence of a correlation between these neuronal changes and either the C-fibre threshold or the size of the C-fibre response of the neurone, strongly suggests a specific involvement of the NMDA receptor in these changes as opposed to just the excitability of the neurone.

Following the development of carrageenan inflammation, the responses of many convergent dorsal horn neurones to C-fibre intensity electrical stimulation are increased, indicating the development of central sensitization. This enhanced response is partly due to these neurones exhibiting a greater degree of wind-up post-carrageenan. Wind-up is mediated through the activation of NMDA receptors by glutamate or aspartate following the removal of the voltage-dependent Mg^{2+} block of the associated ion channel. The depolarization of the neurone necessary to relieve the Mg^{2+} block of the NMDA receptor channel and thus allow wind-up to occur is provided by activation of non-NMDA or more likely peptide receptors such as those for substance P (see chapter 4). Thus an increase in the levels of glutamate and particularly peptides released in the dorsal horn following inflammation would facilitate the activation of the NMDA receptor and could explain the enhanced neuronal responses seen. Several studies using models of acute or polyarthritis have demonstrated that following the development of inflammation an increase occurs in the intraspinal release of the excitatory amino acids glutamate and aspartate (Sluka and Westlund, 1992; Sorkin et al., 1992) and substance P (Oku et al, 1987; Schaible et al., 1990) following mechanical stimulation of the inflamed area. However, these increases in amino acid and peptide release in the cord may simply reflect increased activation of the

primary afferent fibres as a result of sensitization of the primary afferent nociceptors and consequently would not occur in the present study where electrical activation of the afferents bypasses the sensitized nociceptors.

There is evidence to suggest that this increased release does not simply reflect increased afferent activation as a result of peripheral sensitization. Increases in the levels of staining for SP and CGRP in the dorsal root ganglia (see refs. in Schaible and Grubb, 1993) and for glutamate in the superficial dorsal horn (Sluka et al., 1992; Sluka and Westlund, 1993a) have been found following the development of inflammation which may mean that a given stimulus is able to evoke a greater release of transmitter from the afferent terminals. However alterations in staining for the primary afferent transmitters are not indicative of the actual release of the substance in question and the time course of the changes does not always correlate with the hyperalgesia (see **1.8**). More convincing evidence to support the notion that a given stimulus can evoke a greater intraspinal release of peptides following inflammation, independently of peripheral sensitization of the nociceptors, is provided by the studies of Nanayama et al. (1989) and Garry and Hargreaves (1992). Nanayama et al. (1989) demonstrated that the capsaicin evoked release of CGRP from spinal dorsal horn slices is enhanced in adjuvant arthritic rats, and of more relevance to the present study, Garry and Hargreaves (1992) demonstrated an increase in both the spontaneous and capsaicin evoked release of SP and CGRP from spinal dorsal horn slices taken from rats following 3 hours of carrageenan inflammation. This increase in the spinal release of the peptide CGRP following carrageenan inflammation may further enhance the actions of the increased levels of SP in inflammation since CGRP retards the degradation of SP (Le Greves et al., 1985). The increase in the spontaneous release of substance P may also be important as this could increase the membrane potential of the dorsal horn neurones (without reaching the threshold for firing) thereby facilitating the removal of the voltage dependent Mg^{2+} block. This would allow activation of the NMDA receptor earlier in the stimulus train and hence a greater degree of wind-up. Evidence is presented in chapter 4 to suggest that this early activation of the NMDA receptor is occurring. Thus in the present study, an increase in the electrically evoked release of SP and CGRP and possibly glutamate and aspartate following the development of carrageenan inflammation could explain the enhancement in the neuronal responses.

However, if the enhanced neuronal responses following carrageenan inflammation were simply due to an enhanced release of primary afferent transmitters, then why do the changes in neuronal response correlate so well with the degree of NMDA receptor mediated wind-up of the neurones? One explanation is that activation of spinal NMDA receptors is producing the enhancement in transmitter release. Activation of post-synaptic

NMDA receptors can lead to the generation of nitric oxide (NO) which is potentially capable of diffusing back to the pre-synaptic terminal to increase transmitter release (see Meller and Gebhart, 1993). The intrathecal administration of NMDA has been shown to produce an L-NAME sensitive release of glutamate in the spinal cord (Sorkin, 1993). Additionally, NMDA receptor antagonists can block the increased release of excitatory amino acids seen following the development of acute arthritis in the rat (Sluka and Westlund, 1993b). Furthermore, the nitric oxide synthase inhibitor L-NAME has been shown to reduce the second phase of the formalin response (Haley et al., 1992; Malmberg and Yaksh, 1993), and to reverse the thermal (but not the mechanical) hyperalgesia produced by the peripheral injection of carrageenan (Meller et al., 1994). Thus NMDA receptor mediated generation of NO following the development of carrageenan inflammation could enhance transmitter release and thereby enhance neuronal responses. Although this scheme could provide the link between the degree of wind-up, and hence NMDA receptor activation, of a neurone and the neuronal changes seen post-carrageenan, the correlation seen in this study appears to be the opposite to that which would be expected if this mechanism involving NMDA receptor mediated NO production were the link. In other words, neurones with a large degree of NMDA receptor activation would be expected to produce a greater amount of NO which in would turn lead to a greater release of transmitter and thereby a greater enhancement in neuronal response, which is the opposite to that found. So although NO production may still be involved in the altered neuronal responses, possibly through some of its other actions such as the activation of protein kinases (Meller and Gebhart, 1993) it seems unlikely that an NMDA / NO mediated enhancement of transmitter release is responsible for the altered neuronal responses post-carrageenan.

Although increased transmitter release could account for the enhanced neuronal responses post-carrageenan, how would this relate to the reduced neuronal responses seen in nearly half the neurones post-carrageenan? Other mechanisms must be considered. One possibility which could underlie both the enhanced and reduced neuronal responses seen post-carrageenan involves the peptide dynorphin. Marked increases in the levels of preprodynorphin in the dorsal horn of the spinal cord have been shown to occur within 4 hours of the injection of carrageenan into the paw (Draisci and Iadarola, 1989). Furthermore, exogenous dynorphin applied to the spinal cord has been shown to produce both facilitations and inhibitions of the evoked response of dorsal horn neurones (Knox and Dickenson, 1987). Increases in dynorphin in the spinal cord have also been linked to the expansion of the receptive fields of the neurones seen in inflammation, which may contribute to the phenomenon of hyperalgesia (Hylden et al., 1991a). Some of the actions of dynorphin appear to be non-opioid in nature and have been reported to be linked to the NMDA receptor (Caudle and Isaac, 1988), providing the

Another factor which could account for the bidirectional changes in the evoked neuronal responses of the dorsal horn neurones following the development of carrageenan inflammation is that the convergent dorsal horn studied form a heterogeneous population. It is possible that different populations of convergent dorsal horn neurones respond differently to the inflammatory stimulus. Whilst this possibility cannot be ruled out, the continuous nature of the distribution of altered neuronal responses post-carrageenan (fig. **3.7 & 3.11**) suggests that distinct neuronal populations are not an explanation for the bidirectional changes in the evoked neuronal responses.

necessary link between the neuronal responses and wind-up. This possibility is examined further in chapter 9.

The neurones displaying a reduced response post-carrageenan tended to display a large degree of wind-up in the control period. It is possible that when an already high degree of activation of NMDA receptors is enhanced further by the development of inflammation, this excessive activation of the receptor may lead to functional modulation of the NMDA receptor-channel complex through phosphorylation / dephosphorylation mechanisms to downregulate its actions. This would lead to a decrease in the NMDA receptor-mediated component of the C-fibre evoked response and therefore a decrease in the magnitude of the evoked response. A decrease in the response to ionophoretically administered NMDA has been observed in animals with acute arthritis (Dougherty et al., 1992b). The reduction in the neuronal response evoked by NMDA in these animals was greatest with the highest doses of NMDA (Dougherty et al., 1992b) which could imply that a downregulation mechanism such as this is operating.

Alternatively, the decreases in neuronal response may result from high levels of NMDA receptor activation triggering inhibitory systems. Activation of descending inhibitory controls has already been implicated in NMDA receptor mediated antinociception (Kolhekar et al., 1993). It is possible that the recruitment of intrinsic spinal inhibitory systems may also play a role. In normal rats, NMDA receptor antagonists have been shown to facilitate the responses of neurones located in the substantia gelatinosa of the dorsal horn which has been suggested to result from the disinhibition of inhibitory interneurones (Dickenson and Sullivan, 1990). Hence NMDA receptors appear to be involved in the control of inhibitory systems in the dorsal horn. An increase in glutamic acid decarboxylase (Nahin and Hylden, 1991) and in the levels of GABA itself (Castro-Lopes et al., 1992, 1994) have been demonstrated in the spinal cord following the development of unilateral inflammation of the hind-limb. This increase in GABA may be responsible for some of the inhibitions of the C-fibre evoked responses seen post-carrageenan, possibly through increased activation of GABA interneurones.

Having established that an inhibition of the responses of some neurones occurs as a result of inflammation in the periphery, and that these inhibitions may involve the NMDA receptor system, what might the purpose of these inhibitions be? Neurones with little wind-up in the control period showed increased responses following the development of inflammation in the periphery, partly due to an increase in the degree of wind-up exhibited. By contrast, neurones exhibiting a high level of wind-up in the control period showed a reduced C-fibre response with less wind-up post-carrageenan. Excessive activation of the NMDA receptor has been implicated in cell damage / death (Olney, 1990). Thus it may be the case that high levels of activation of the NMDA

receptor (i.e. high levels of wind-up), enhanced by the development of inflammation, results in the recruitment of inhibitory systems (possibly including descending systems) as an autoinhibitory mechanism to avoid cell damage. However, this remains to be seen.

3.3.2. How do the changes seen in the neuronal responses post-carrageenan relate to the hyperalgesia seen in behavioural studies?

The problem arises as to how the bi-directional changes in the electrically evoked neuronal responses of convergent dorsal horn neurones seen following the development of carrageenan inflammation relate to the hyperalgesia to mechanical and thermal stimuli seen in behavioural studies following the peripheral injection of this inflammatory agent. There are several possibilities which may be considered.

1. *The presence of the anaesthetic.* Under the conditions of full surgical anaesthesia used in this study, the response of the dorsal horn neurones to the developing carrageenan inflammation may be suppressed and not represent those of dorsal horn neurones in awake animals. Although this cannot be ruled out, this seems unlikely since Le Bars and Chitour (1983) found the responses of convergent dorsal horn neurones to a number of natural stimuli to be the same in both non-anaesthetized spinal rats and intact halothane anaesthetized rats suggesting that halothane anaesthesia does not markedly alter neuronal responses. Additionally, patients undergoing operations under full surgical anaesthesia experience pain and hyperalgesia from the moment they regain consciousness, indicating that the hyperalgesic state is most likely to have been set up under anaesthesia.

2. *The method of stimulation used.* The use of electrical stimulation in this study bypasses the peripheral nociceptors which are known to become sensitized by inflammatory mediators such as bradykinin (see **1.2**). Thus much of the hyperalgesia seen behaviourally may be a result of the sensitization of these nociceptors leading to an increased afferent barrage reaching the cord. However, there is clearly also a central component as demonstrated with this and other studies (Woolf, 1983; Hylden et al., 1989; Neubauer and Schaible, 1990).

3. *The expansion of peripheral receptive fields following inflammation.* Several studies have demonstrated that an expansion of the peripheral receptive fields of dorsal horn neurones occurs following the development of inflammation (Hylden et al., 1989; Grubb et al., 1993). As a consequence of this, a noxious mechanical or thermal stimulus applied to the inflamed area would lead to the activation of a greater number of dorsal horn neurones than if the same stimulus was applied to non-inflamed tissue. This spatial

summation may be interpreted by higher order neurones as the phenomenon of hyperalgesia.

4. *Post-discharge of the neurones.* The electrically evoked C-fibre responses of the dorsal horn neurones were altered in a bi-directional fashion following the development of carrageenan inflammation such that only a small increase occurred in the net C-fibre evoked response of the population. Although this complex pattern of changes may be interpreted as hyperalgesia, the C-fibre generated post-discharge of the neurones may be of more importance in this respect. The post-discharge of the neurones displayed the same bi-directional changes seen in the C-fibre evoked response after carrageenan however in contrast to the C-fibre evoked response, a net increase occurred in the neuronal post-discharge evoked by the C-fibre intensity stimulation. Thus it may be this increase in the C-fibre / wind-up generated post-discharge that is interpreted as the phenomenon of hyperalgesia.

5. *Silent nociceptors.* The occurrence of so called 'silent nociceptors' has been reported in normal animals (see McMahon and Koltzenburg, 1990). These nociceptors are unresponsive to natural stimuli in the normal animal, but become responsive in the presence of inflammation following sensitization by inflammatory mediators (see **1.2**). The additional input to the spinal cord resulting from the activation of these 'silent' nociceptors by mechanical and thermal stimuli in the inflammatory state may contribute to the hyperalgesia. The use of electrical stimulation, which by-passes the nociceptors, will activate the afferents arising from these silent nociceptors irrespective of the presence of inflammatory mediators. Thus the novel contribution of these silent nociceptors to the afferent barrage resulting from natural stimulation in the presence of inflammation will be masked.

6. *The responses of neurones other than convergent dorsal horn neurones are critical in the hyperalgesic response.* This study was designed to follow the evoked responses of convergent dorsal horn neurones during the development of peripheral inflammation. Convergent dorsal horn neurones have been proposed to play an important role in the spinal transmission of nociceptive information in the normal animal (see section **1.9**). However the selection of these neurones may not represent the true picture of the transmission of nociceptive information in the dorsal horn following carrageenan inflammation. The neurones selected for this study received inputs from both C- and Aβ-fibres: neurones receiving only Aβ-fibre inputs were discarded and not studied further. In addition, no nociceptive specific neurones were studied. It is possible that it is the

responses of these neurones which are important in determining hyperalgesia. Whilst it is very unlikely that the neurones appearing to receive only Aβ-fibre inputs play any role in nociceptive transmission in the normal animal, this may not be the case after inflammation. It is possible that these neurones may then develop an active C-fibre input subsequent to the development of inflammation due to the strengthening of previously subthreshold central synaptic connections.

Although possibilities such as this cannot be ruled out, on a number of occasions neurones were lost during the three hours following the injection of carrageenan into the paw. In these cases, a search was conducted for another neurone on the ipsilateral side of the cord in order to study the actions of drugs in animals with peripheral inflammation. These neurones, which were encountered between 1 and 3 hours after the injection of carrageenan, did not appear to differ in any way, either in terms of their response to electrical stimulation, or their response to drugs, from that of neurones in the inflammatory state which were characterized as convergent neurones before the injection of carrageenan. Thus it would appear unlikely that a "new" population of convergent neurones appears following the development of inflammation.

7. In addition to the mechanisms discussed in points 2, 3, 4 & 5 which are likely to be important in the overall spinal events determining hyperalgesia, a further possibility exists. Studies presented in chapter 4 using NMDA antagonists suggest a plausible mechanism whereby the relative involvement of the NMDA receptor in the input and wind-up of the cells, minor and critical respectively, in normal animals, radically alters after inflammation. This may be interpreted in such a way as to explain centrally generated hyperalgesia.

Although the changes in the evoked neuronal responses following the development of inflammation appear complicated, the correlation between the direction and degree of these changes and the degree of wind-up exhibited by the neurone pre-carrageenan suggests that these changes are not random. It may be the case that all neurones show a tendency towards enhanced responses post-carrageenan (central sensitization) but in neurones where this enhancement in response leads to excessive activation of the NMDA receptor, inhibitory systems are recruited, either local inhibitory neurones or descending controls, which balances out the excitations, in extreme cases tipping the balance of the system such that inhibitions predominate. Reducing the stimulus strength or frequency in the present study of neuronal responses post-carrageenan (both of which would reduce NMDA receptor activation) could provide clues as to whether the level of NMDA receptor activation is indeed critical in determining the neuronal response post-carrageenan.

CHAPTER 4

EFFECTS OF NMDA RECEPTOR ANTAGONISTS ON THE EVOKED RESPONSES OF CONVERGENT DORSAL HORN NEURONES IN NORMAL AND CARRAGEENAN ANIMALS

4.1. Introduction

Since Curtis et al. (1959) first reported that iontophoretic administration of L-glutamate produces a marked excitation of spinal neurones, evidence has accumulated to support a role of excitatory amino acids in both nociceptive and non-nociceptive transmission in the spinal cord.

The excitatory amino acids, glutamate and aspartate, are found in a large proportion of dorsal root ganglion neurones, both large and small, and in both large- and small-diameter primary sensory neurones (Wanaka et al., 1987; Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988; Maxwell et al., 1990; Westlund et al., 1989, 1990; Merighi et al., 1991), often coexisting with peptides such as substance P and CGRP in the small diameter fibres (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988; Merighi et al., 1991). In addition, many intrinsic and projection neurones within the dorsal horn are immunoreactive for excitatory amino acids (Miller et al., 1988). The release of both aspartate and glutamate within the dorsal horn has been demonstrated following mechanical or chemical stimulation of the tissue and following both high- and low-intensity electrical stimulation of peripheral nerves (Skilling et al., 1988; Kangrga and Randic, 1991; Sorkin et al., 1992). Thus the excitatory amino acids are not associated with any one particular fibre type or response modality.

Attention has therefore focussed on the various receptors for the excitatory amino acids to see if these are associated with specific sensory pathways. The receptor targets for the excitatory amino acids in the dorsal horn include both ionotropic and metabotropic receptors. At the present time, little is known about the possible role of metabotropic glutamate receptors in sensory transmission in the spinal cord. However there is much evidence to implicate ionotropic receptors, both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors (primarily α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors), in excitatory transmission in the spinal cord (Davies and Watkins, 1983; Peet et al., 1983; Schouenborg and Sjölund, 1986; King et al., 1988; Morris, 1989; Gerber and Randic, 1989; Jeftinija, 1989). Although non-NMDA receptors play a role in both nociceptive and non-nociceptive transmission in the dorsal horn as demonstrated by the ability of antagonists of non-NMDA receptors, such as the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), to reduce responses of dorsal horn neurones to both noxious and non-noxious stimuli (Dougherty et al., 1992a; Neugebauer et al., 1993a), NMDA receptors are primarily associated with nociceptive transmission in the dorsal horn (Dickenson and Sullivan, 1990; Dougherty et al., 1992a; Neugebauer et al., 1993a), although some reports of involvement of NMDA receptors in non-nociceptive transmission exist (e.g. Dougherty and Willis, 1991b).

NMDA receptors in the spinal cord are well placed to modulate nociceptive transmission, being concentrated in the superficial laminae of the dorsal horn (Monaghan and Cotman, 1985; Mitchell and Anderson, 1991), the region in which nociceptive C- and Aδ-fibres terminate (Besson and Chaouch, 1987). They are not however, thought to be located on the presynaptic terminals of these fibres (Agrawal and Evans, 1986). Spinal administration of NMDA enhances the response of dorsal horn neurones to peripheral noxious stimuli (Aanonsen et al., 1990; Sher and Mitchell, 1990a; Dougherty and Willis, 1991a) and in behavioural studies, produces behaviour thought to be indicative of nociception such as caudally directed biting and scratching and a decrease in nociceptive thresholds (Cahusac et al., 1984; Aanonsen and Wilcox, 1987; Raigorodsky and Urca, 1987). Consistent with these studies, antagonists at the NMDA receptor-channel complex reduce nociceptive neuronal activity evoked by suprathreshold noxious stimuli (Headley et al., 1987; Dickenson and Sullivan, 1990; Dougherty et al., 1992a; Neugebauer et al., 1993a) and have been shown to depress aversive responses to nociceptive skin heating and paw pressure in conscious rats (Cahusac et al., 1984).

However, the majority of behavioural studies using NMDA receptor antagonists failed to find effects on nociceptive reflexes in acute nociceptive tests in normal animals (Yaksh, 1989; Davar et al., 1991; Ren et al., 1992a,b; Yamamoto and Yaksh, 1992), whilst electrophysiological studies have found NMDA receptor antagonists to be most effective against the C-fibre evoked wind-up and consequent post discharge of dorsal horn neurones, which are only evoked following high frequency repetitive stimulation (Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990). At resting membrane potentials, the NMDA receptor channel is blocked in a voltage-dependent manner by Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984), which must be removed by sustained depolarization of the neurone mediated through the activation of receptors other than NMDA receptors before ions can traverse the NMDA receptor channel and the consequences of activation of this receptor occur. The presence of this resting Mg^{2+} block of the NMDA receptor channel, together with the variable ability of NMDA receptor antagonists to inhibit acute nociceptive responses in normal animals suggests that it is likely that the NMDA receptor participates in the transmission of acute noxious stimuli only when the intensity and / or duration of the stimuli are sufficient to allow the NMDA receptor complex to become active. Whilst there is evidence to show that NMDA receptors play a role in the response to acute nociceptive stimuli in normal animals, it is the role played by this receptor in nociception arising from pathological conditions, particularly the development of hyperalgesia / central hypersensitivity that has attracted the most attention.

Repetitive activation of small diameter primary afferents leads to activity dependent

changes in the excitability of neurones in the spinal cord. These changes can take the form of wind-up, the progressive increase in the number of action potentials elicited by constant intensity repetitive C-fibre stimulation, first described by Mendell in 1966, or a prolonged increase in the excitability of spinal neurones following a brief, high intensity C-fibre input, known as central sensitization (Woolf, 1983). Activation of the NMDA receptor has been shown to underlie both these phenomena (Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990; Woolf and Thompson, 1991). The nature of these C-fibre evoked, NMDA receptor-dependent phenomena described above is such that similar mechanisms of amplification / prolongation of neuronal responses have been proposed to underlie much of the altered nociceptive processing seen in protracted pain states. Indeed, antagonists at the NMDA receptor have been shown to reduce the second phase of the formalin response in both electrophysiological (Haley et al., 1990) and behavioural studies (Coderre and Melzack, 1992), reverse the hyperexcitability of dorsal horn neurones resulting from ischaemia of the tail (Sher and Mitchell, 1990b), to prevent or reverse the development of thermal hyperalgesia in a rat model of neuropathic pain (Davar et al., 1991; Yamamoto and Yaksh, 1992) and to reduce the thermal and mechanical hyperalgesia resulting from the development of carrageenan inflammation (Ren et al., 1992a,b; Ren and Dubner, 1993; Yamamoto et al., 1993), suggesting a role for the NMDA receptor in the aberrant nociceptive processing in all these pain states. Despite this interest, much remains to be resolved regarding the role of the NMDA receptor in the induction and maintenance of the hyperalgesic state.

In the previous chapter (chapter 3), it was shown that the alterations in the neuronal responses seen in the three hours after the peripheral injection of carrageenan were highly correlated with the degree of wind-up exhibited by the neurone, an NMDA receptor-mediated event (Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990). Studies in the rat using a model of acute arthritis produced by the injection of kaolin and carrageenan into the knee joint, which produces an inflammation with a similar time course to the carrageenan model used in the present study, have demonstrated a transient increase in the release of both glutamate and aspartate in the dorsal horn upon injection of the inflammatory agents into the joint and then a more sustained increase in release 3.5 hours after the injection (Sluka and Westlund, 1992). This increased release of excitatory amino acids in the cord during the development of peripheral inflammation, together with the correlation between the altered neuronal responses post-carrageenan and the NMDA receptor-mediated wind-up of the neurones, suggests that the NMDA receptor may play an enhanced role in the spinal transmission of evoked neuronal responses in the carrageenan animals. The findings of behavioural studies showing that NMDA receptor antagonists are effective at prolonging the paw withdrawal latencies of the inflamed but not

the non-inflamed paw in animals with carrageenan inflammation supports this enhanced role (Ren et al., 1992a,b; Ren and Dubner, 1993; Yamamoto et al., 1993).

In view of the potential importance of NMDA receptors in spinal nociceptive events during the development of carrageenan-induced inflammation, the role played by this receptor in spinal nociceptive transmission was investigated. The NMDA receptor-channel complex has a number of sites at which agents can act to block the activation of the receptor, namely the glutamate binding site (which is blocked by D-2-amino-5-phosphonovaleric acid (D-AP5)); the binding site for the co-agonist glycine (blocked by 7-chlorokynurenate and HA 966) and a binding site within the channel itself which is blocked in a non-competitive fashion by ketamine and MK-801 (Lodge and Johnson, 1991). Antagonists at two of these sites, MK-801 (dizocilpine) and 7-chlorokynurenate (7CK), were used to investigate the role of the NMDA receptor in nociceptive transmission in the spinal cord three hours after the injection of carrageenan into the hind paw of the rat compared with that in normal animals.

4.2. Results

4.2.1. Effects of intrathecal MK-801 on the electrically evoked responses of dorsal horn neurones in normal animals

The effects of four doses (0.5, 5, 50 and 200μg) of MK-801, applied cumulatively to the surface of the spinal cord at intervals of 40 minutes, were tested on the electrically evoked responses of 7 dorsal horn neurones located in the deep dorsal horn (500-1000μm). One of the neurones tested, although located in the same region of the cord as the other 6, and not differing from the others in terms of its response pattern to electrical stimulation, showed a facilitated response to electrical stimulation following all doses of MK-801 and was therefore excluded from the results.

The C-fibre evoked responses of the neurones were relatively insensitive to MK-801, with only the highest doses tested (50 and 200μg) producing a significant inhibition of the C-fibre evoked response (paired Student's t-test on raw spike-count data, one-tailed p-value <0.001, n=6). The effects of MK-801 on the C-fibre evoked response showed little dose dependence, with 0.5, 5 and 50μg of MK-801 producing roughly equal effects (16.2 ± 8.6% inhibition with 0.5μg (n=5), 18.0 ± 4.0% inhibition with 50μg (n=6)), which then increased to 47.0 ± 8.2% with the highest dose of 200μg (n=6) (figure **4.1**). The Aβ-fibre evoked response of the neurones was also inhibited by MK-801, with 0.5, 50 and 200μg of MK-801 producing a significant inhibition of the response (0.001<p<0.01). As with the inhibition of the C-fibre evoked response, the effect was small, with the highest dose (200μg) only producing an inhibition of 23.0 ± 4.5% (n=5) (figure **4.1**).

The most pronounced effect of intrathecal MK-801 in these animals was on the wind-up of the neurones (E) and on the post discharge (which is generated as a result of wind-up mechanisms). Figure **4.2** shows that as with the C-fibre evoked response, the inhibitory effects of MK-801 on the wind-up and post-discharge of the neurones showed little dose dependence, reaching an early plateau of around 40% inhibition of wind-up (E) with 0.5, 5 and 50μg of MK-801, which then showed a further increase with 200μg of MK-801 to 58.3 ± 9.5% (n=6). All the doses of MK-801 produced a significant inhibition of the wind-up of the neurones (E) (0.005<p<0.05), with the exception of 5μg of MK-801 which just failed to reach significance (0.05<p<0.10). The inhibition of the post-discharge (PD) of the neurones by MK-801, although closely related to the inhibition of wind-up seen, was more variable. As a consequence of this variability, only the inhibition of the post-discharge by 0.5 and 200μg of MK-801 proved to be significant

(p= 0.04 and 0.0009 respectively), although the inhibitions of the post-discharge by 5 and 50μg of MK-801 were close to reaching significance ($0.05<p<0.08$).

In contrast to the effects of MK-801 on the wind-up and post-discharge of the neurones, the initial input onto the neurones (I) was unaffected by all but the highest dose of MK-801 (inhibition of I of 7.3 ± 8.2% (n=6) with 50μg of MK-801, rising to 64.2 ± 7.9% with 200μg, (n=6)) (see figure **4.2**). The top dose of MK-801 used (200μg) is high in comparison with those used in behavioural studies, where motor effects are seen following the intrathecal administration of 32μg (Ren et al., 1992a). The lack of effect of 0.5-50μg of MK-801 on the initial input onto the neurones indicates at most a minor involvement of the NMDA receptor in the transmission of the steady input onto the neurones in normal animals.

Stimulus-response graphs showing typical examples of the response of individual neurones to a train of stimuli illustrating the inhibition of the wind-up of the neurones by MK-801 in the absence of inhibitions of the steady neuronal input can be seen in figure **4.10**.

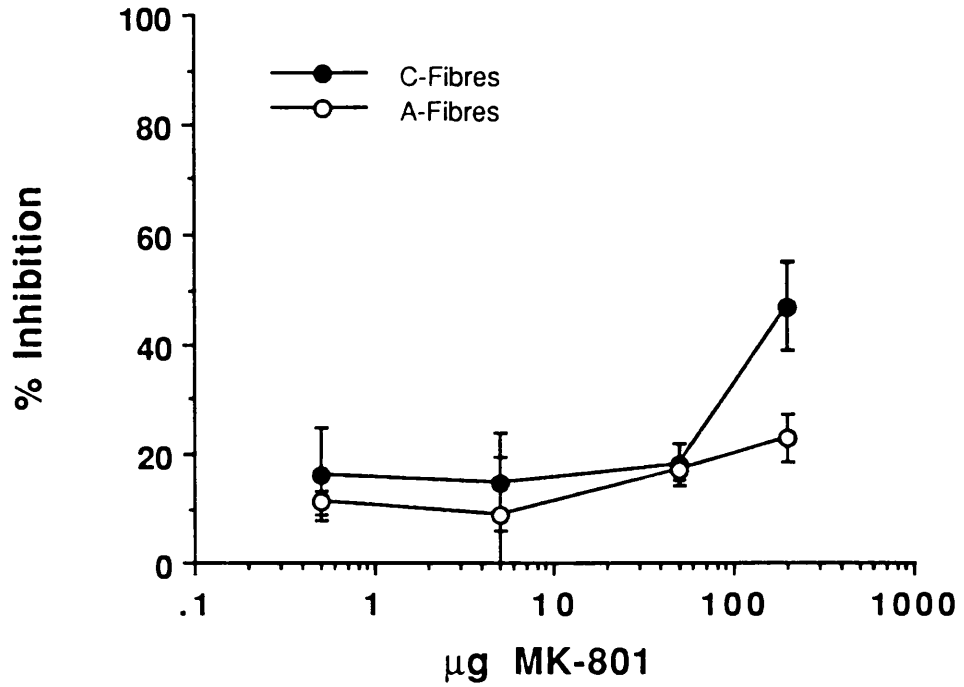


Figure 4.1. Inhibition of the C- and A-fibre evoked response of dorsal horn neurones by intrathecal MK-801 in normal animals. n=5-6 neurones per dose.

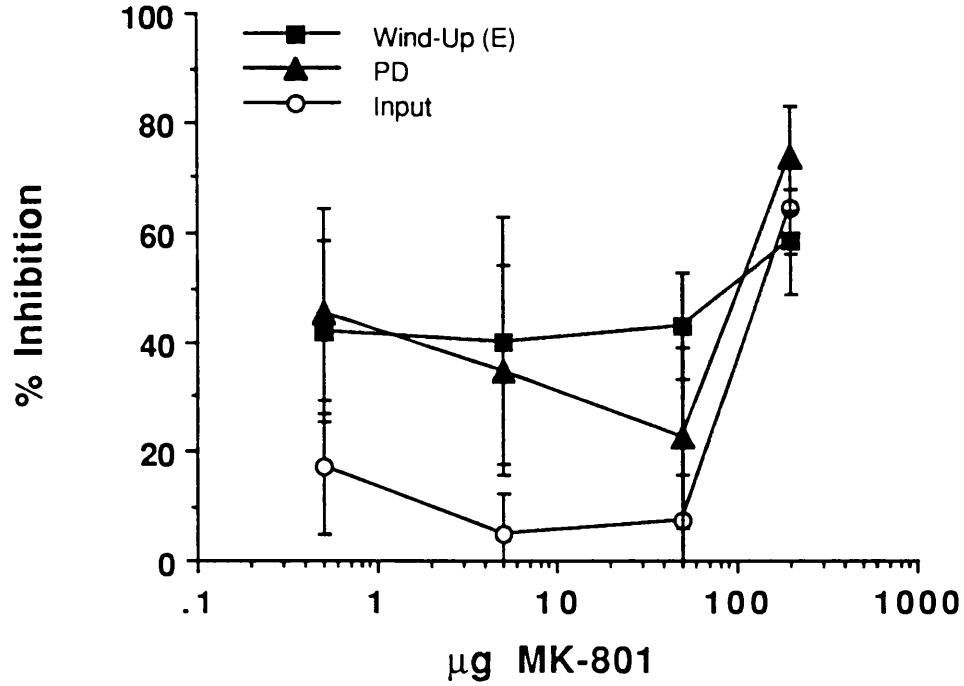


Figure 4.2. Inhibition of the wind-up (E), post-discharge (PD) and initial input (I) of dorsal horn neurones by intrathecal MK-801 in normal animals. n=5-6 neurones / dose.

4.2.2. Effects of intrathecal MK-801 on the electrically evoked responses of dorsal horn neurones following carrageenan inflammation

The effects of the same four doses of MK-801 (0.5, 5, 50 and 200μg) on the electrically evoked activity of dorsal horn neurones were tested three hours after the injection of carrageenan into the ipsilateral hind paw. The effects of MK-801 were examined on the evoked responses of 9 neurones located in the deep dorsal horn (500-1000μm). As in normal animals, one of these nine neurones, despite having the same general location and characteristics as the other neurones, was facilitated by all doses of MK-801 and is excluded from the analysis.

Figure **4.3** shows that increasing doses of intrathecal MK-801 produced a dose dependent inhibition of the C-fibre evoked response of the neurones, although this effect was not large, with only 50 and 200μg of MK-801 producing a significant inhibition of the response (50.3 ± 7.0% inhibition with 200μg, p=0.005, paired Student's t-test on original spike counts, n=6). The Aβ-fibre evoked responses of the neurones were not significantly inhibited by any dose of MK-801 in these animals, with 200μg of MK-801 producing a maximum inhibition of the Aβ-fibre evoked response of 8.3 ± 13.1% (n=6) (figure **4.3**).

As figure **4.4** shows, intrathecal MK-801 produced a dose dependent inhibition of the post-discharge of the neurones when applied three hours after the onset of carrageenan inflammation, which was significant following 50 and 200μg (inhibitions of the PD of 33.6 ± 9.8% (n=7) and 70.5 ± 13.5% (n=6) respectively, $p<0.05$). In contrast to the post-discharge, the wind-up of the neurones (E) was not significantly inhibited by any of the doses of MK-801 tested in the carrageenan animals (although 200μg of MK-801 produced an inhibition of 40.5 ± 16.7%, $p>0.05$) (figure **4.4**).

The greatest effect of MK-801 in the carrageenan animals was seen on the steady neuronal input onto the neurones (I), which was dose-dependently reduced by intrathecal MK-801 (figure **4.4**). This inhibition of I was only significant with the higher doses tested, with 50 and 200μg of MK-801 producing inhibitions of 41.8 ± 11.8%, p=0.02 (n=7) and 74.8 ± 6.1%, p=0.003 (n=6) respectively.

Stimulus-response graphs showing typical examples of the response of individual neurones in the carrageenan animals to a train of stimuli, illustrating the inability of MK-801 to overcome the wind-up of the neurones in these animals whilst reducing the steady neuronal input, can be seen in figure **4.10**.

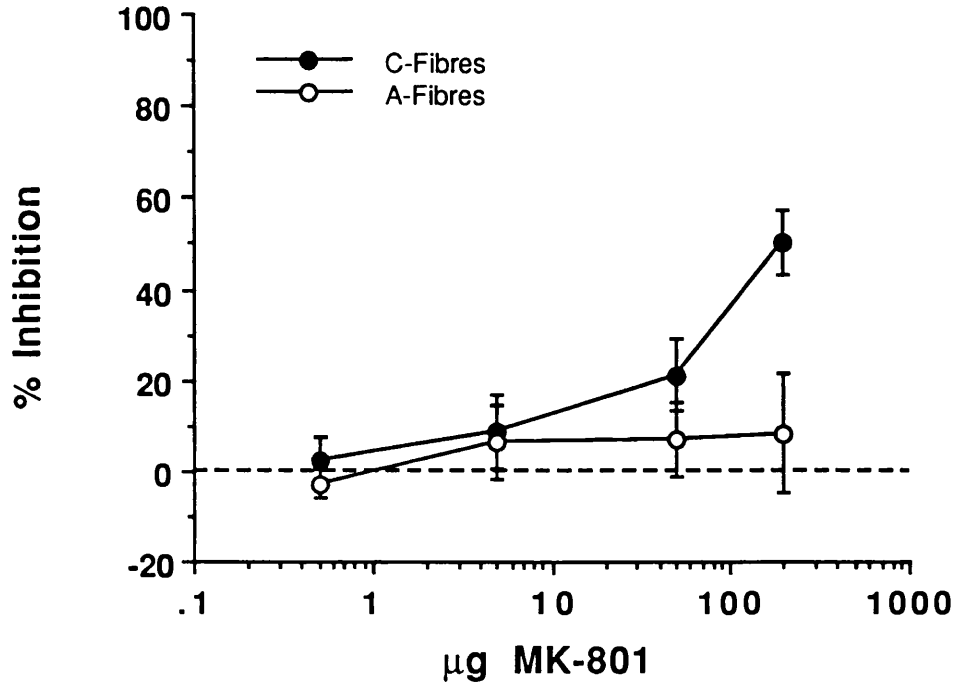


Figure 4.3. Inhibition of the C- and A-fibre evoked responses of dorsal horn neurones by intrathecal MK-801 three hours after the injection of carrageenan into the paw. n=6-8 neurones per dose.

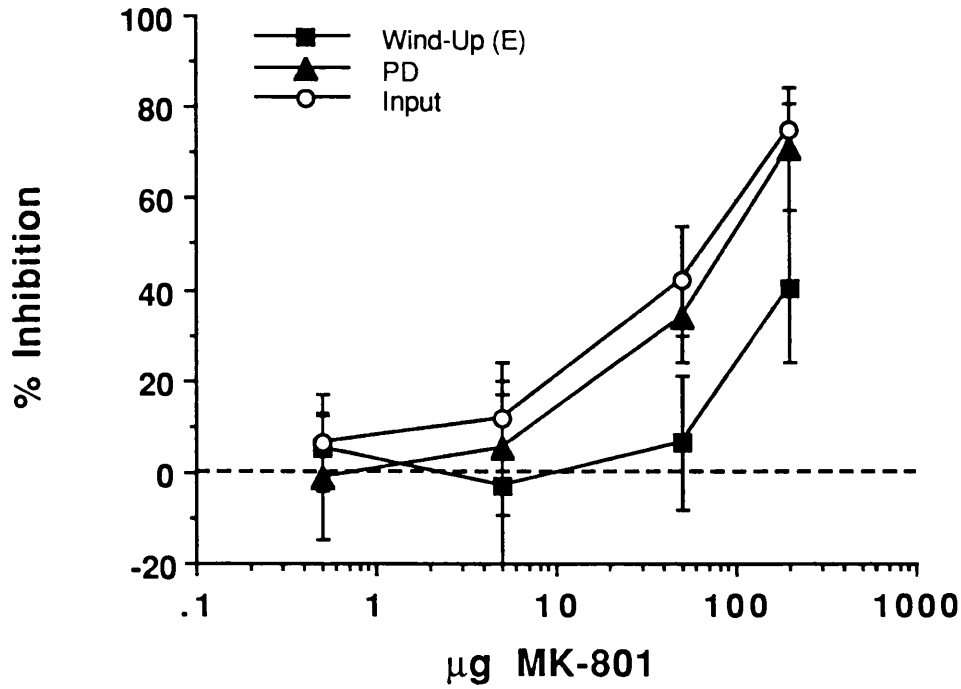


Figure 4.4. Inhibition of the wind-up (E), post-discharge (PD) and initial input (I) of the neurones by intrathecal MK-801 three hours after the injection of carrageenan into the paw. n=6-8 neurones per dose.

4.2.3. Comparison of the effects of intrathecal MK-801 on the evoked neuronal responses in normal and carrageenan animals

All 4 doses of intrathecal MK-801 tested produced similar inhibitions of the C-fibre evoked response (i.e. the responses in the defined C-fibre latency band, attributed to the direct transmission of afferent C-fibre information) in both normal and carrageenan animals (figure **4.5**). Although the inhibition of the C-fibre evoked response by MK-801 in the carrageenan animals showed greater dose dependency, with the lowest dose appearing less effective at inhibiting the C-fibre evoked response than in normal animals, no significant differences were found between the groups.

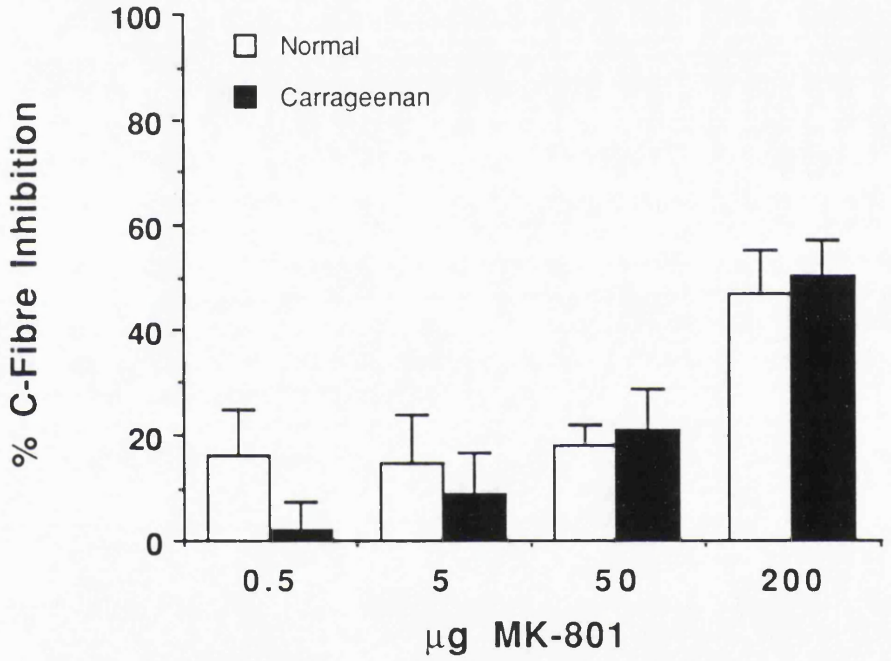


Figure 4.5. Comparison of the inhibition of the C-fibre evoked response of dorsal horn neurones by intrathecal MK-801 in normal animals and 3 hours after the injection of carrageenan into the paw. No significant differences were found between the groups. n=5-8 neurones per group.

The inhibition of the Aβ-fibre evoked response by MK-801, although small, did appear to be marginally reduced in the carrageenan animals compared with that seen in normal animals, however this was only significant with the dose of 0.5µg ($p=0.005$) (figure **4.6**).

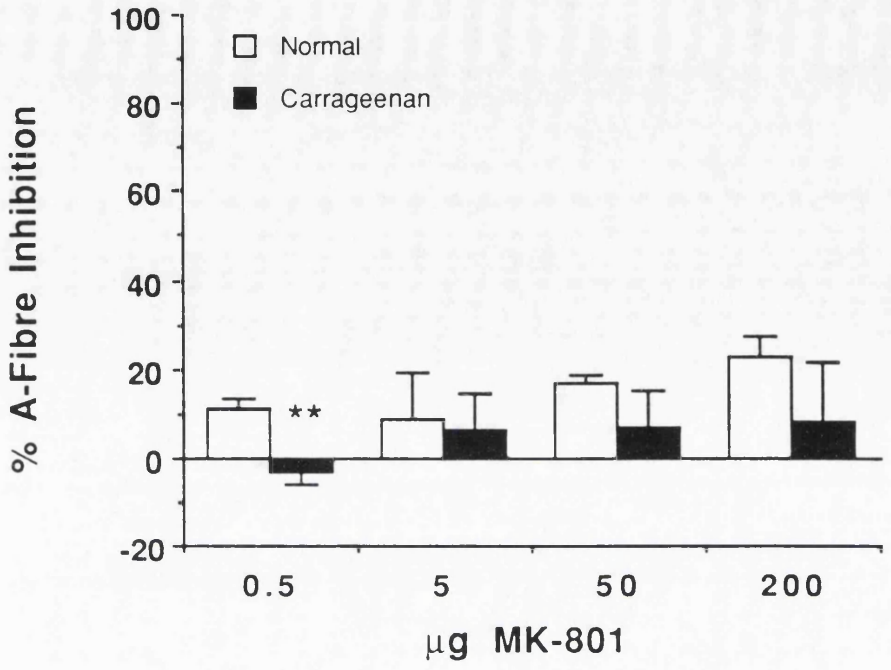


*Figure 4.6. Comparison of the inhibition of the Aβ-fibre evoked response of dorsal horn neurones by intrathecal MK-801 in normal animals and 3 hours after the injection of carrageenan into the paw. n=5-8 neurones per group. ** p<0.01 compared with normal animals.*

In contrast to the effects of MK-801 on the neuronal response evoked in the defined C-fibre latency band, which were unaltered by the development of inflammation (see figure **4.5**), when the augmented C-fibre-generated activity was considered in terms of the wind-up and post-discharge of the neurones, and the response of the neurone to the initial stimulus in the train (I) considered, differences were seen between the two groups of animals.

Following the development of carrageenan inflammation the response of the neurone to the first stimulus I, considered as the neuronal response prior to augmentation by wind-up mechanisms, appeared to be more sensitive to the channel blocker in the carrageenan animals, although this difference was only marked with the dose of 50µg (one-tailed p-value = 0.02, Student's t-test, figure **4.7**).

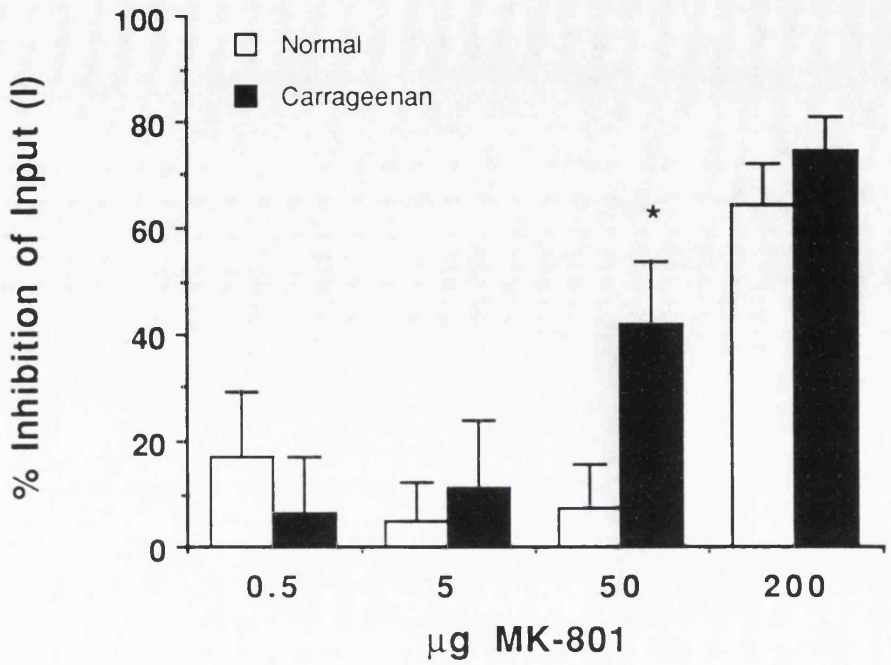


*Figure 4.7. Comparison of the inhibition of the input (I) onto the neurones by intrathecal MK-801 in normal and carrageenan animals. n=5-8 neurones per group. * p<0.05 compared with normal animals.*

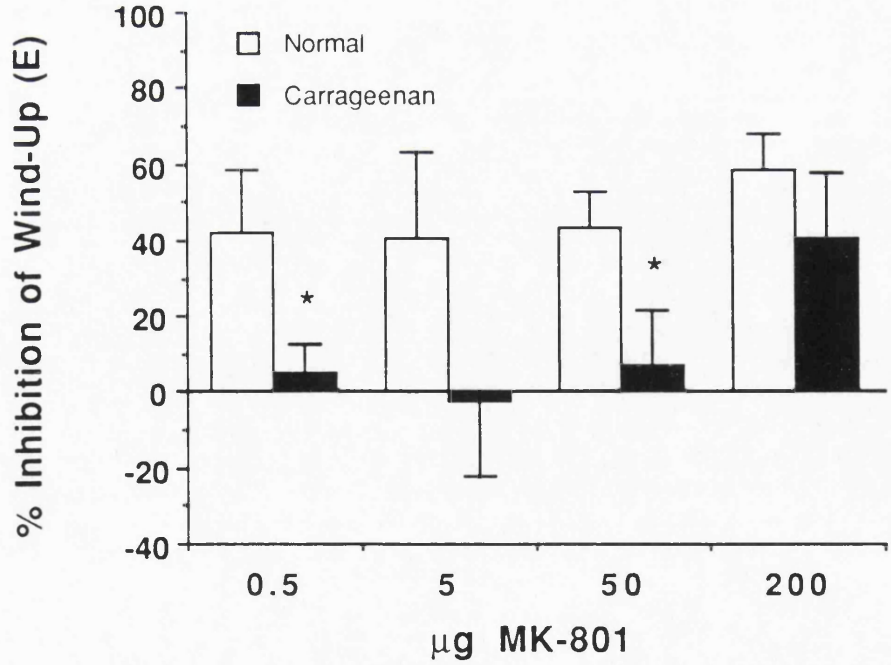


*Figure 4.8. Comparison of the inhibition of the wind-up of the neurones (E) by intrathecal MK-801 in normal and carrageenan animals. n=5-8 neurones per group. *p<0.05 compared with normal animals.*

The greatest difference in the actions of MK-801 on the neuronal responses in the two groups of animals was seen on the wind-up of the neurones (E) (figure **4.8**). Whilst in normal animals MK-801 was able to produce a marked, although not dose-dependent, inhibition of the wind-up (E) of the neurones, this was not the case after three hours of carrageenan-induced inflammation. In the carrageenan animals, the same doses of MK-801 were no longer able to produce any effect on the wind-up of the neurones, with the exception of the highest dose (200μg) which produced an inhibition similar to that seen in normal animals (figure **4.8**). Likewise, the post-discharge of the neurones in the carrageenan animals was less sensitive to inhibition by the low doses of MK-801 (figure **4.9**), although this difference was lost as the dose was increased.

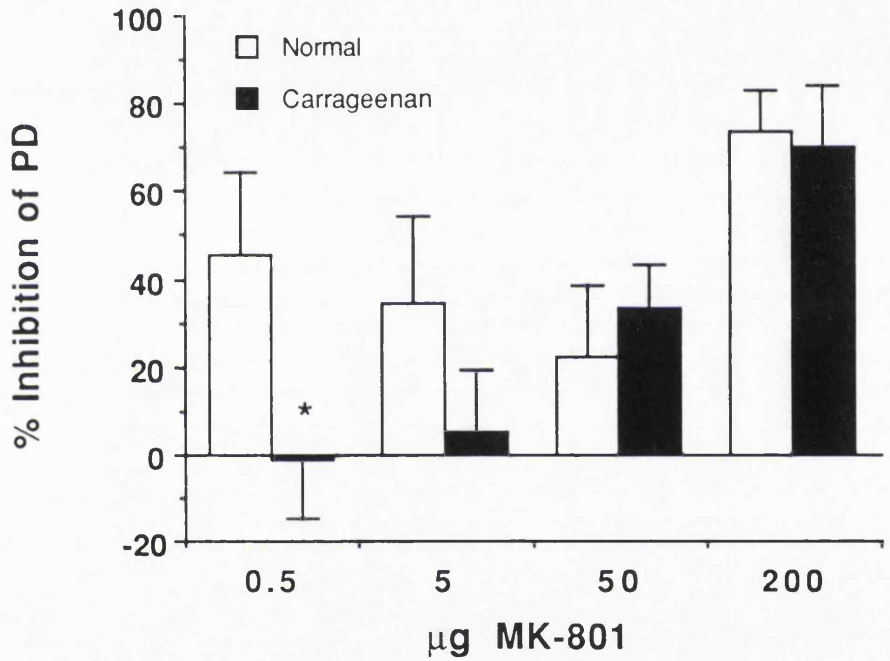


*Figure 4.9. Comparison of the inhibition of the post-discharge of the neurones (PD) by intrathecal MK-801 in normal animals and three hours after the injection of carrageenan into the paw. n=5-8 neurones per group. *p<0.05 compared with normal animals.*

Thus following three hours of carrageenan inflammation, a change has occurred in the ability of MK-801 to inhibit the various components of the C-fibre generated neuronal response. These can be summed up as follows:-

1. A reduction in the sensitivity of the wind-up of the neurones to MK-801 in the carrageenan animals,
2. A modest, although significant, enhancement in the sensitivity of the initial C-fibre input to MK-801 following carrageenan inflammation.

The differing profile of action of MK-801 in the carrageenan animals compared with that seen in normal animals is illustrated in the individual stimulus-response graphs shown in figure **4.10**. From the typical examples shown, it can be seen that following carrageenan inflammation, MK-801 produces some reduction in the initial input onto the neurones but despite this, the neurones still wind-up with the same slope as pre-MK-801. In normal animals, the wind-up of the neurone is clearly inhibited by MK-801 whilst the steady neuronal response continues unabated (figure **4.10**).

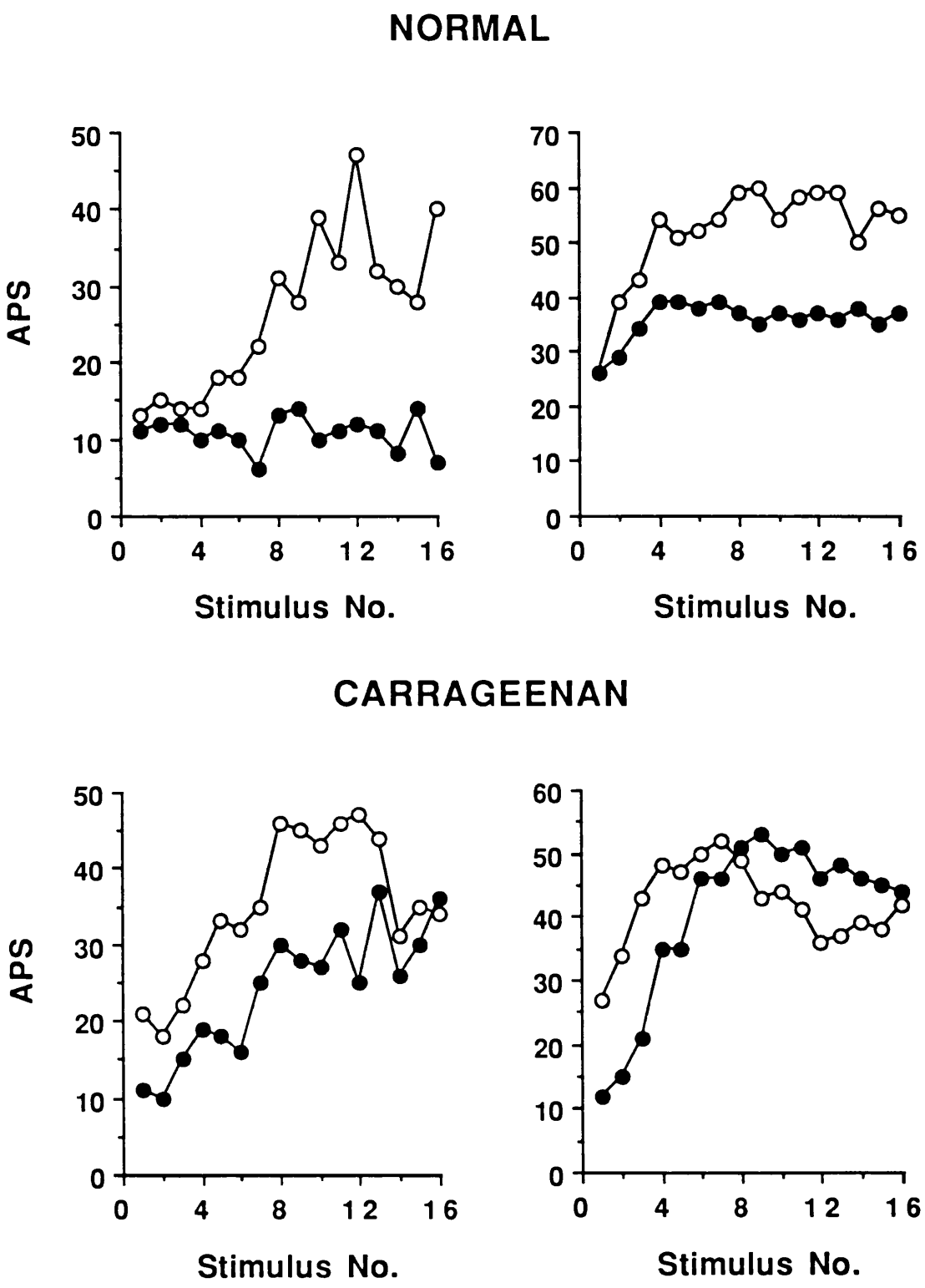


Figure 4.10. Stimulus-response graphs for individual dorsal horn neurones recorded in normal (top) and carrageenan (bottom) animals showing the wind-up of the neurones alone (-o-) and following the intrathecal administration of 50μg of MK-801 (-●-). Plots of the number of action potentials evoked (APS) are given against stimulus number. Note the attenuation of the wind-up by MK-801 in the absence of a reduction in input in normal animals, whilst post-carrageenan, it is the input that is reduced by MK-801.

4.2.4. Is there a relationship between the direction of change in the neuronal response post-carrageenan and the subsequent effects of MK-801?

In chapter 3 it was shown that the direction of change in the electrically evoked neuronal response post-carrageenan was correlated to the prior degree of wind-up of the neurone. Since wind-up is an NMDA receptor mediated event (Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990) activation of the NMDA receptor is likely to be involved in the altered neuronal responses post-carrageenan. Consequently it could be envisaged that the effect of MK-801 on the evoked response of a neurone post-carrageenan might be related to whether the neurone displayed an enhanced or reduced response following the injection of carrageenan into the paw.

Unfortunately, the number of neurones tested with MK-801 post-carrageenan for which the response of the neurone pre-carrageenan, and therefore direction and degree of change in the neuronal response was known was small (7/9 of the neurones). In addition, of the 7 neurones whose pre-carrageenan responses were known, 6 of these neurones displayed enhanced C-fibre evoked responses post-carrageenan allowing little chance to examine the effects of MK-801 on neurones displaying reduced neuronal responses post-carrageenan.

From the limited data available, there were no strong indications of any link between the direction of change in the C-fibre evoked neuronal response and the subsequent effect of MK-801. Whilst the neurone displaying a reduced C-fibre evoked response post-carrageenan was moderately facilitated by the low doses of MK-801, another neurone displaying an enhanced response post-carrageenan was also facilitated by 0.5 and 5μg of MK-801. More convincing evidence for a role of the NMDA receptor in the altered neuronal responses post-carrageenan was obtained by considering the changes in the neuronal input and the wind-up of the neurones separately, although the limited number of neurones and the biased sample still presented problems.

Six of the neurones subsequently tested with MK-801 displayed an enhanced input post-carrageenan. Although there was no significant correlation, those neurones showing the greatest facilitation in input post-carrageenan subsequently tended to show the greatest inhibition of their input by MK-801 (figure **4.11**). This suggests that activation of NMDA receptors is involved in this enhanced response.

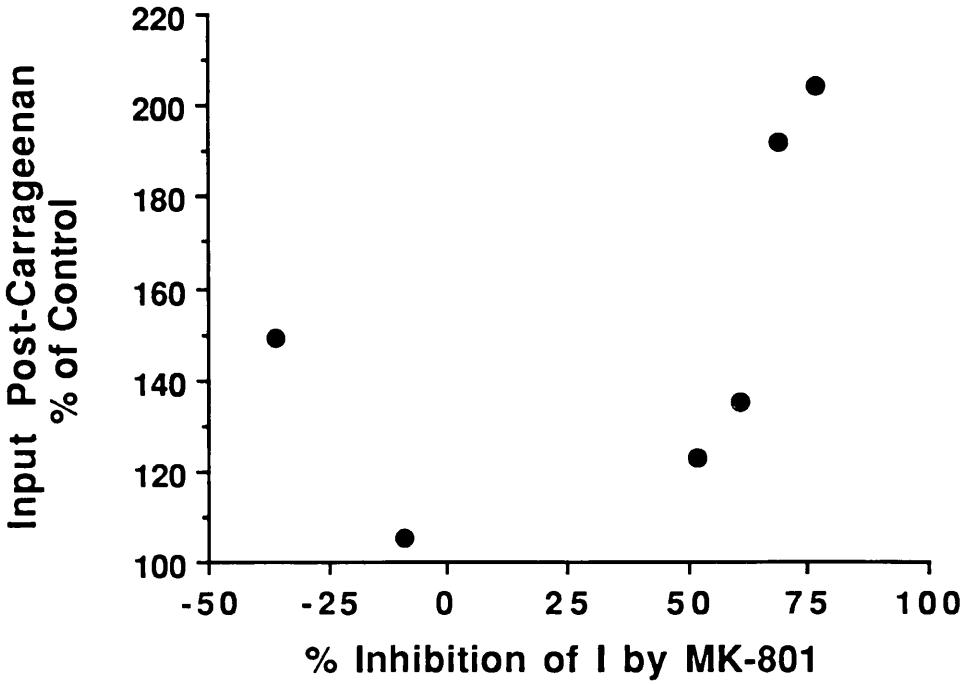


Figure 4.11. Relationship between the increase in the input onto a neurone (I) post-carrageenan and the subsequent inhibition of that input by 50μg of MK-801. Note that MK-801 produced the greatest reduction in input in neurones which showed the greatest enhancement in I post-carrageenan.

The wind-up of the neurones subsequently tested with MK-801 showed more variability post-carrageenan. Of the 7 neurones whose post-carrageenan responses were known, 4 showed an enhanced wind-up whilst the other 3 had a reduced wind-up after three hours of inflammation. In 5 of the neurones, the effects of 50μg of MK-801 were opposite to those produced by the inflammation, i.e. MK-801 enhanced the wind-up of those neurones where wind-up was reduced post-carrageenan, and reduced the wind-up of the neurones where the inflammation enhanced wind-up. The magnitude of the effect produced by MK-801 did not appear to reflect the magnitude of the change in response post-carrageenan. In the other two neurones, MK-801 produced effects in the same direction as those produced by the development of inflammation. Thus the NMDA receptor may be playing a role in both the enhanced and reduced neuronal responses post-carrageenan (also see chapter 3), although the number of neurones involved in this study is too small to allow firm conclusions to be drawn.

4.2.5. Effects of intrathecal 7-Chlorokynurenate on the electrically evoked responses of dorsal horn neurones in carrageenan animals: comparison with normal animals

The effects of two intrathecal doses (1 and 50μg) of 7-chlorokynurenate (7CK), an antagonist at the glycine site of the NMDA receptor-channel complex, were tested on the electrically evoked responses of 6 dorsal horn neurones three hours after the injection of carrageenan into the ipsilateral hind paw. In order to reduce the number of animals used, the data obtained with 7CK in the carrageenan animals in the present study has been compared with that obtained in normal animals in a previous study in this laboratory by Dickenson and Aydar (Dickenson and Aydar, 1991). The experimental conditions, doses and routes of administration of the drug were identical between the two studies.

In the carrageenan animals, all of the neurones tested were inhibited to some degree by 7CK, with none of the neurones showing any consistent signs of facilitation following the administration of 7CK. The lower dose of 7CK tested (1μg, n=5) failed to produce a significant inhibition of any of the components of the electrically evoked neuronal response measured (figures **4.12**, **4.14**, **4.15**). However, when the dose of 7CK was increased to 50μg, significant inhibitions of the electrically evoked neuronal responses were observed. Figure **4.12** shows that the most marked effect of this dose of 7CK in the carrageenan animals was on the input of the neurones (93.3 ± 3.1% inhibition, n=6), whilst the wind-up of the neurones (E) was almost unaffected by this dose (35.7 ± 12.0% inhibition, $p>0.05$, n=6). This insensitivity of the wind-up of the neurones to antagonism by 7CK and the dramatic reduction in the input onto the neurones produced by 7CK in carrageenan animals contrasts with the almost total abolition of wind-up and insensitivity of the neuronal input which has been shown previously in this laboratory to occur with this dose of 7CK in normal animals (Dickenson and Aydar, 1991, data reproduced in figures **4.12**, **4.14**, **4.15** for comparison). In the study of Dickenson and Aydar (1991), performed in this laboratory using the same techniques, 50μg of 7CK produced an inhibition of the wind-up of the neurones in normal animals of 70 ± 10% (n=4) which is significantly greater than that produced in the present study following three hours of carrageenan inflammation (one-tailed p-value = 0.04, Student's t-test, figure **4.12**).

Conversely, the inhibition of the input by 50μg of 7CK in the carrageenan animals is significantly greater than that seen in the study of Dickenson and Aydar (1991) in normal animals (93 ± 3% vs 12 ± 22% in normal animals, $p=0.001$, figure **4.12**).

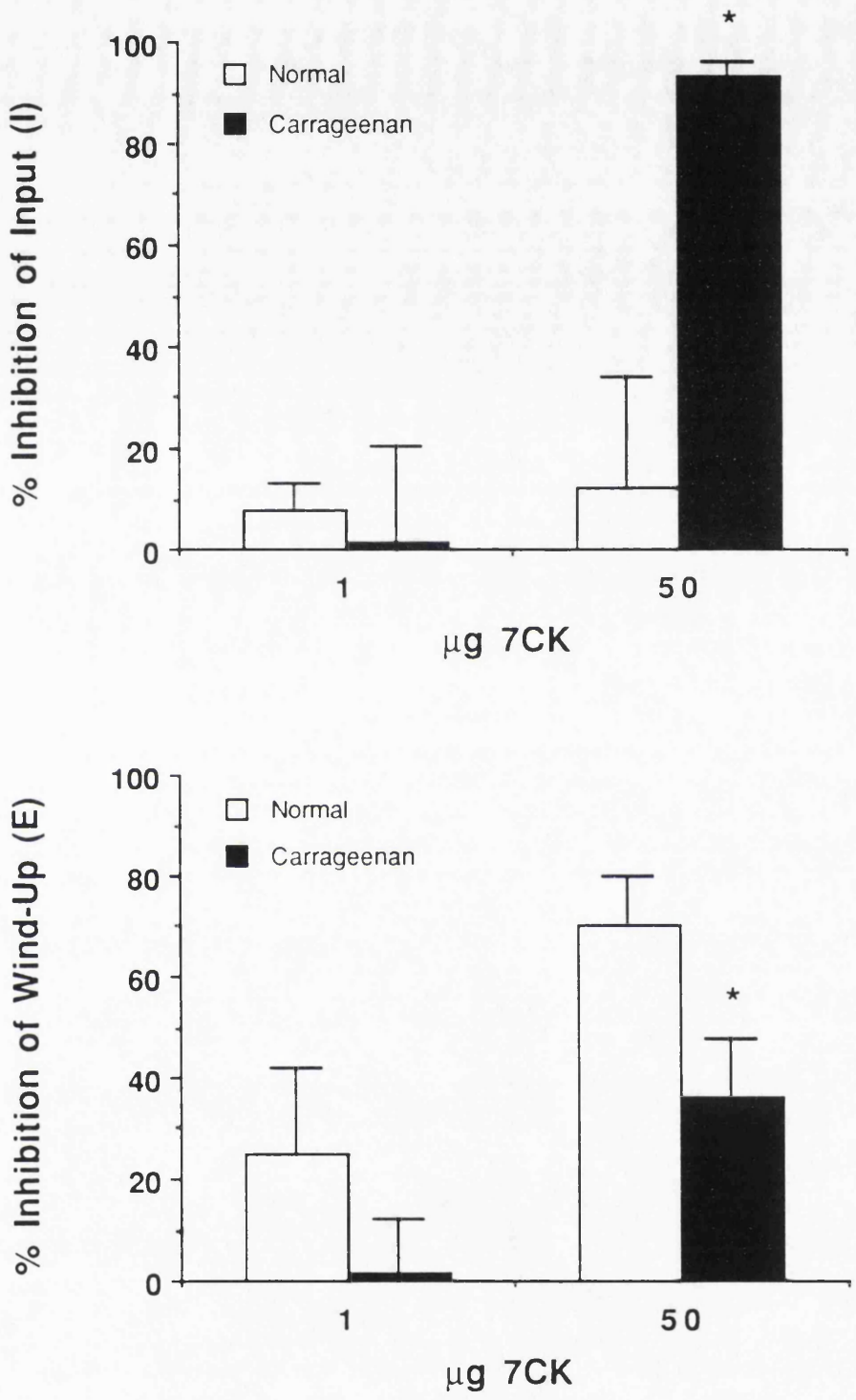


*Figure 4.12. Comparison of the inhibition of the input (I) (top) and wind-up (E) (bottom) of the neurones by intrathecal MK-801 in normal animals (data from Dickenson and Aydar, 1991) and 3 hours post-carrageenan. Note the relative sensitivity of the input and insensitivity of the wind-up in the carrageenan animals to 7CK. n=4-6 neurones per group. *p<0.05 compared with normal animals.*

Figure **4.13** shows examples of stimulus-response graphs illustrating the effects of 7CK on the wind-up of individual neurones following three hours of carrageenan inflammation. An example of the effect of 7CK on the wind-up of a neurone recorded in a normal animal (recorded during the present study and so not included in results of Dickenson and Aydar, 1991) is shown alongside the wind-up curves for the effects of 7CK in carrageenan animals for comparison (figure **4.13**).

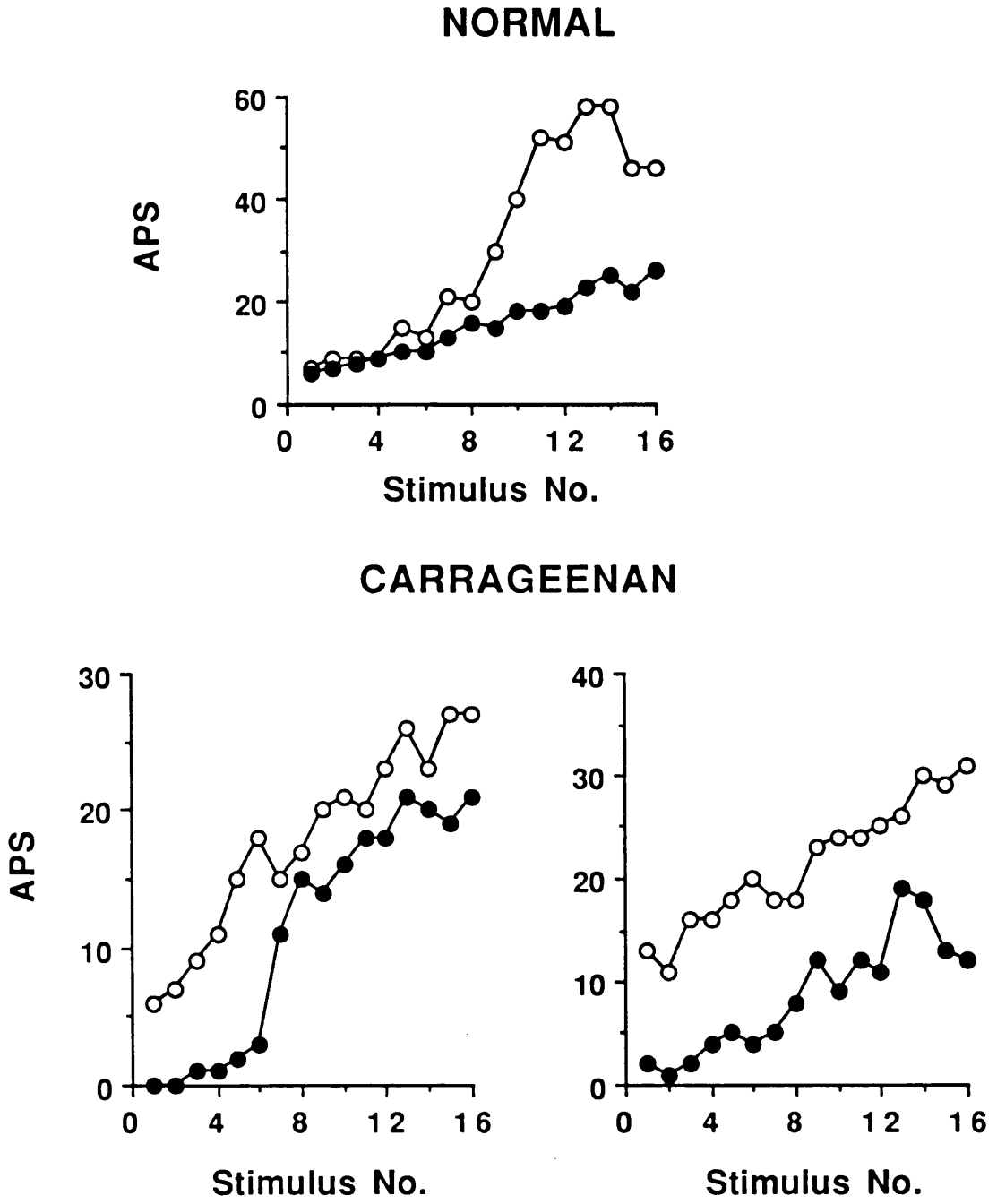


Figure 4.13. Stimulus-response graphs for individual neurones recorded in normal (top) and carrageenan (bottom) animals showing the wind-up of the neurones alone (-o-) and following the intrathecal administration of 50μg of 7CK (-●-).

Although the wind-up of the neurones post-carrageenan was only moderately reduced following 50μg of 7CK (inhibition of 35.7 ± 12.0%, figure **4.12**), the post-discharge of the neurones, which usually follows closely changes in wind-up, showed an inhibition in the carrageenan animals equal to that seen in normal animals (61.0 ± 13.2% in carrageenan animals (n=6) vs 61 ± 19% in normal animals with 50μg of 7CK (Dickenson and Aydar, 1991, n=4), figure **4.14**).

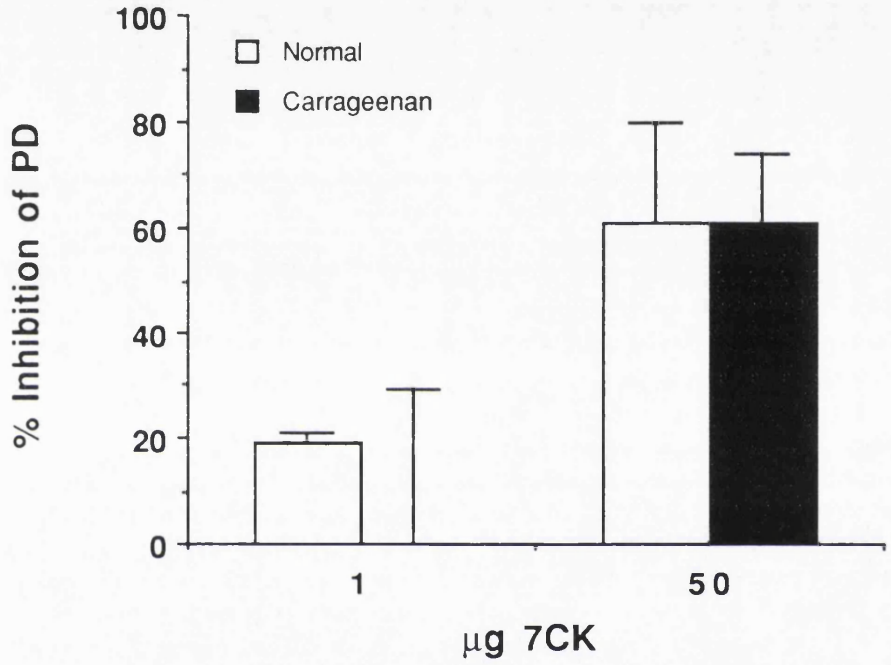


Figure 4.14. Comparison of the inhibition of the post-discharge of the neurones (PD) by intrathecal 7CK in normal animals (data from Dickenson and Aydar, 1991) and 3 hours after the injection of carrageenan into the paw. No difference was found between the two groups of animals. n=4-6 neurones per group.

In addition, the C-fibre evoked response (i.e. the responses in the defined C-fibre latency band, attributed to the direct transmission of afferent C-fibre information) was inhibited to a greater extent by 50μg of 7CK in the carrageenan animals (69.5 ± 5.6%, n=6) than in normal animals (Dickenson and Aydar, 1991; 17 ± 18%, n=4, one-tailed p-value = 0.01, Student's t-test, figure **4.15**).

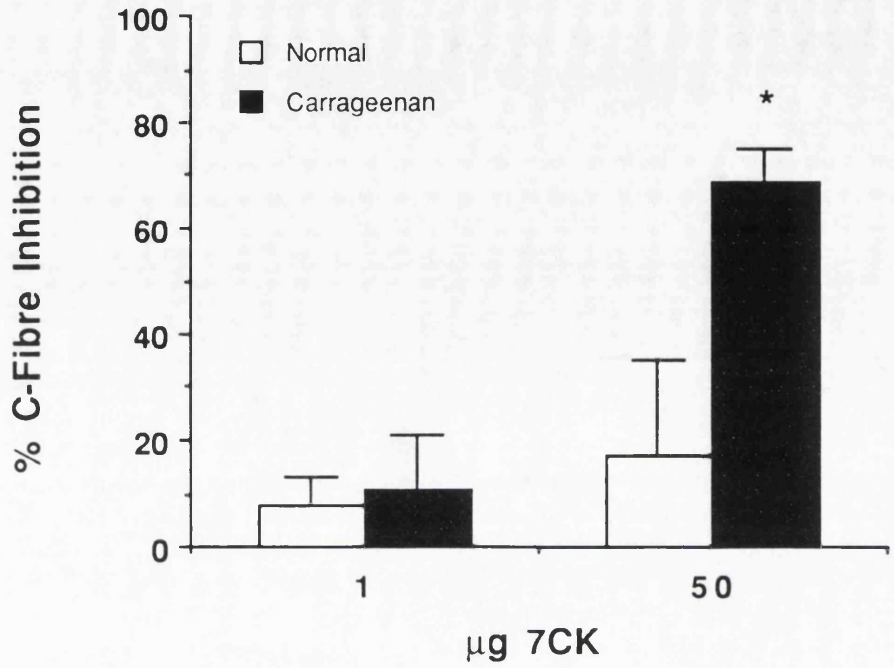


*Figure 4.15. Comparison of the inhibition of the C-fibre evoked response by intrathecal 7CK in normal animals (data from Dickenson and Aydar, 1991) and 3 hours after the injection of carrageenan into the paw. n=4-6 neurones per group. *p<0.05 compared with normal animals.*

Interestingly, 50µg of 7CK produced an inhibition of the Aβ-fibre evoked response of 40.1 ± 5.5% in the carrageenan animals (n=6, p<0.01), which is in contrast to the modest inhibitions of the Aβ-fibre evoked response produced in these animals by MK-801 (see figure **4.6**).

The reduced sensitivity of wind-up and the increased sensitivity of the neuronal input to the effects of the glycine site antagonist 7CK in carrageenan animals (this chapter) compared with that seen in normal animals (Dickenson and Aydar, 1991) is comparable to changes reported in this chapter for the spinal actions of the NMDA receptor-channel blocker MK-801 post-carrageenan (**4.2.3.**).

4.3. Discussion

The suggestion of the involvement of NMDA receptors in the alterations in the electrically evoked neuronal responses seen following the development of carrageenan inflammation (chapter 3), and the demonstration that a number of diverse nociceptive conditions appear to be mediated, at least in part, through activation of NMDA receptors (see introduction) led to the investigation of the role of the NMDA receptor in spinal nociceptive processing following carrageenan inflammation. The results presented in this chapter demonstrate that within three hours of the development of carrageenan-induced inflammation in the periphery, the role played by the NMDA receptor in spinal nociceptive processing, as revealed by the NMDA receptor antagonists MK-801 and 7CK, is altered compared with that seen in normal animals. This altered role of the NMDA receptor post-carrageenan is manifest in two ways:-

1. An increased participation of NMDA receptors in the early response of a neurone to a train of noxious stimuli compared with normal animals, with the receptor now appearing to be active during the initial C-fibre input onto the cell instead of needing a sustained non-NMDA receptor drive to allow it to become activated.
2. A reduced sensitivity of the wind-up of the neurones to NMDA receptor antagonists following the development of inflammation in the periphery.

These findings are discussed below.

4.3.1. Involvement of the NMDA receptor in the initial input onto the neurones following carrageenan inflammation.

In normal animals, the initial response of a dorsal horn neurone to a train of stimuli delivered at intensities sufficient to activate C-fibres, is unaffected by NMDA receptor antagonists (this chapter; Davies and Lodge, 1987; Dickenson and Sullivan, 1987a, 1990; Dickenson and Aydar, 1991). This insensitivity of the initial input onto a neurone to NMDA receptor antagonists can be explained by the voltage-dependent Mg^{2+} block of the channel associated with the NMDA receptor. At resting membrane potentials the NMDA receptor-channel is blocked by Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984). Before Na^{+} and Ca^{2+} influx through the NMDA receptor channel can occur, the Mg^{2+} must be expelled from the channel by a sustained depolarization of the neurone produced by transmitters acting at receptors other than NMDA receptors. The temporal summation of the slow excitatory postsynaptic potentials produced by peptides such as substance P (Henry, 1976; Murase and Randic, 1984) is believed to play a major part in overcoming the Mg^{2+} block of the NMDA receptor channel allowing the NMDA receptor to participate

in the neuronal response. There is evidence from both a release study carried out in slices of rat ventral spinal cord (Iverfeldt et al., 1989) and an *in vivo* study employing a NK1 receptor antagonist against the responses of dorsal horn neurones (De Koninck and Henry, 1991), that substance P is preferentially released by repetitive rather than brief noxious stimuli. This potentially delayed release of SP, and the need for cumulative depolarization of the neurone can explain why the first few stimuli in a train are usually unable (insufficient) to overcome the Mg^{2+} block of the NMDA receptor channel and thus the NMDA receptor is able to make at most a small contribution to the neuronal response to these initial noxious stimuli. As the stimulation continues, the temporal summation of the slow synaptic potentials resulting from the actions of peptides such as substance P overcomes the Mg^{2+} block of the NMDA receptor channel allowing the NMDA receptor to participate in the neuronal response, hence the sensitivity of the responses evoked by later stimuli in the train to NMDA receptor antagonists.

However, following three hours of carrageenan inflammation, both the channel blocker MK-801 and the glycine site antagonist 7CK dose-dependently reduced the initial input onto the neurones, thereby revealing an increased contribution of the NMDA receptor to the neuronal response at this early stage.

The electrophysiological evidence presented in this chapter for a greater contribution of NMDA receptor-mediated events to the neuronal response to the initial noxious stimuli following the development of inflammation mirrors the findings of behavioural studies in animals with unilateral carrageenan-induced inflammation. Behavioural studies in these animals have shown that NMDA receptor antagonists reduce the hyperalgesia to noxious heat or pressure associated with the development of inflammation in the injected paw whilst having no effect on the reflex withdrawals to these acute noxious stimuli applied to the non-inflamed paw (Ren et al., 1992a,b; Ren and Dubner, 1993; Yamamoto et al., 1993). The procedures employed in reflex withdrawal tests in behavioural studies, such as the paw withdrawal tests used in the carrageenan animals, are such that the animal withdraws from the stimulus (heat or pressure) as soon as it perceives it as noxious. Thus under these conditions, the stimulus delivered to the animal represents a brief, probably low intensity noxious stimulus for which the correlate in this electrophysiological study is most likely to be the response of the neurone to the initial stimuli in the train.

Thus in a normal animal, for the reasons given above, the response of a neurone to the initial stimuli in the train is relatively unaffected by NMDA receptor antagonists. Equally, the brief noxious stimuli used to elicit reflex withdrawals in behavioural studies are unlikely to be sufficient to lead to activation of the NMDA receptor, hence the lack of effect of NMDA receptor antagonists in these acute nociceptive tests in normal animals.

However following the development of inflammation in the periphery, the reflex withdrawal response of the animal to acute noxious thermal and mechanical stimuli applied to the inflamed tissue, like the response of the dorsal horn neurones to the initial stimuli in the train, is reduced by NMDA receptor antagonists.

4.3.2. What changes might underlie this novel involvement of the NMDA receptor in the initial input onto the neurones following carrageenan inflammation?

The earlier involvement of the NMDA receptor in the neuronal response to noxious stimuli following the development of carrageenan inflammation demonstrated in this electrophysiological study and probably underlying the reductions in behavioural hyperalgesia by NMDA receptor antagonists, suggests that there has been a reduction in the degree of non-NMDA receptor activation required to enable the NMDA receptor to participate in the neuronal response. A number of factors could contribute to the involvement of the NMDA receptor in the neuronal input following carrageenan inflammation.

One possibility is that following the development of carrageenan inflammation, there is an increase in the release of glutamate within the spinal cord which, by way of enhanced activation of non-NMDA excitatory amino acid receptors, leads to the earlier removal of the Mg^{2+} block of the NMDA receptor channel. However, although an increase in the release of glutamate and aspartate in the cord has been reported within hours of the injection of kaolin / carrageenan into the knee joint of rats (Sluka and Westlund, 1992), there must be some doubt as to whether enhanced AMPA receptor-mediated depolarizations could contribute to the removal of the Mg^{2+} block given the transient nature of the current activated by the AMPA receptor. Wind-up, and by analogy, removal of the Mg^{2+} block of the NMDA receptor in this case, only occurs when the duration of the postsynaptic depolarizations exceeds the interstimulus interval resulting in a summation of the potential (Thompson et al., 1990). Given the 2-second interval between stimuli in the present study, AMPA-mediated depolarizations are very unlikely to summate under these conditions, consequently alternative explanations must be sought.

Glutamate is only one of the transmitters found in small diameter primary afferents (see section **1.4**). Substance P is found in a number of small diameter primary afferent neurones (see Willis and Coggeshall, 1991), frequently coexisting with glutamate (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988; Merighi et al., 1991). The long slow depolarizations produced by peptides such as SP (Henry, 1976; Murase and

Randic, 1984), which are capable of temporal summation, are ideal for removing the Mg^{2+} block of the NMDA receptor channel. In support of this, it has been shown that the co-administration of small doses of SP with exogenously administered NMDA augments the facilitatory actions of NMDA on the responses of dorsal horn neurones (Dougherty and Willis, 1991a), presumably by aiding the removal of the voltage-dependent Mg^{2+} block. Thus, an increase in the evoked release of SP, possibly at an earlier stage in the stimulus train, occurring within the first hours after the injection of carrageenan into the paw would facilitate the expulsion of the Mg^{2+} from the NMDA receptor channel thus allowing it to participate in the neuronal response at an earlier stage. In addition to this voltage dependent regulation of NMDA receptors by SP, SP has also been reported to directly modulate NMDA receptor currents (Rusin et al., 1992), although this effect is suppressed at physiological concentrations of glycine.

Increases in both the synthesis (Minami et al., 1989) and levels (see Schaible and Grubb, 1993) of SP in the dorsal root ganglia and spinal cord have been shown to occur in the days and weeks after the induction of unilateral adjuvant-induced inflammation or more generalized polyarthritis. Consistent with this is the demonstration that, when examined in polyarthritic rats of 2-3 weeks duration, both the spontaneous and evoked release of SP in the dorsal horn of the spinal cord is enhanced (Oku et al., 1987). However, it is the spinal release of this peptide within the first few hours following the inflammatory stimulus that is critical to the present discussion.

One study, performed in monkeys, has examined the dorsal horn content of SP within the first hours of the injection of kaolin and carrageenan into the knee joint and found the tissue levels of SP to be reduced at this time (Sluka et al., 1992). This decrease in the levels of SP in the dorsal horn probably represents an early depletion of the peptide as a consequence of enhanced release, since antibody microprobes have been used to demonstrate an increased release of SP in the dorsal horn within 6 hours of the injection of kaolin and carrageenan into the knee joint of cats (Schaible et al., 1990). However, of even greater relevance to the present study is the finding that both the spontaneous and capsaicin-evoked release of substance P is enhanced in spinal cord slices taken from rats three hours after the injection of carrageenan into the paw (Garry and Hargreaves, 1992).

Thus an increased release of SP within the cord does occur following the development of peripheral inflammation, which should facilitate the removal of the Mg^{2+} block from the NMDA receptor channel and therefore may explain the novel NMDA receptor-mediated component in the neuronal input post-carrageenan. In addition to the enhancement in the evoked release of SP, the potential increase in the spontaneous release of SP within the cord post-carrageenan may be sufficient to cause a slight depolarization of the resting membrane potential of the postsynaptic neurones such that when stimulus evoked release of transmitters occurs from the primary afferents, much less non-NMDA

receptor mediated depolarization is needed to overcome the Mg^{2+} block of the channel. However to my knowledge no studies have specifically investigated whether alterations occur in the resting membrane potential of dorsal horn neurones during the development of inflammation. An additional factor which may influence the actions of SP post-carrageenan is the enhanced release of calcitonin gene-related peptide (CGRP) in the cord following carrageenan inflammation (Garry and Hargreaves, 1992). Whilst CGRP can produce a slow-onset, long-lasting excitation of dorsal horn neurones (Miletic and Tan, 1988) which could itself contribute to the cumulative depolarization needed to remove the Mg^{2+} block of the NMDA receptor channel, CGRP can also interact with SP. It has been shown *in vivo* that concentrations of CGRP that alone have little effect on neuronal responses, markedly potentiate the excitatory effects of SP (Biella et al., 1991). The mechanism behind this potentiation by CGRP is unknown, however CGRP has been shown to retard the breakdown of SP (Le Greves et al., 1985) which would prolong its active life and therefore its actions. In addition, CGRP has been shown to promote the intraspinal spreading of SP (Schaible et al., 1992). Consequently, the observed increase in the release of CGRP post-carrageenan may also enhance the levels of SP in the cord and hence SP-mediated depolarizations, further contributing to the relief of the Mg^{2+} block of the NMDA receptor channel.

A further possibility is that a conformational change occurs in the spinal NMDA receptor-channel complex, triggered by the development of inflammation in the periphery, such that removal of the Mg^{2+} block is made easier. Activation of protein kinase C (PKC) has been shown to reduce the Mg^{2+} block of the NMDA receptor channel (Chen and Huang, 1992). Thus if an increase in PKC levels occurs as a result of increases in neuronal activity following inflammation this may bring about a reduction in the Mg^{2+} block of the NMDA receptor channel. Consequently less of a non-NMDA receptor drive would be required to relieve the block, allowing earlier participation of the NMDA receptor in the neuronal response. Indeed, the involvement of PKC in persistent nociception has recently been proposed on the basis that the intrathecal administration of H-7, which inhibits PKC, reduces the nociceptive behaviour elicited by the subcutaneous injection of formalin (Coderre, 1992).

All of the above possibilities may contribute to the enhanced sensitivity of the neuronal input to NMDA receptor antagonists following carrageenan inflammation.

4.3.2. Insensitivity of the electrically evoked wind-up of dorsal horn neurones to NMDA receptor antagonists following carrageenan inflammation

Following repetitive electrical stimulation of the peripheral receptive field at intensities sufficient to activate C-fibres, the majority of neurones in this preparation exhibit the phenomenon of wind-up. In normal animals, the manifestation of this wind-up can be reduced by more than 50% by antagonists acting at a number of sites on the NMDA receptor-channel complex including the glutamate binding site (Dickenson and Sullivan, 1987a, 1990), the channel itself (Davies and Lodge, 1987; this chapter) and the glycine modulatory site (Dickenson and Aydar, 1991). However, three hours after the onset of carrageenan inflammation, the ability of the NMDA receptor antagonists MK-801 and 7CK to reduce the wind-up of the neurones was markedly diminished.

The differing effects of MK-801 on the Aβ-fibre evoked response in the normal animals and post-carrageenan are likely to be related to the ability of MK-801 to block the wind-up of the neurones in the two groups of animals. Whilst it has been shown that stimulation of Aβ-fibres under the experimental conditions used in the present study is unable to produce wind-up (Dickenson and Sullivan, 1987a), Aβ-fibre impulses arriving on a convergent neurone which has been 'wound-up' by prior C-fibre stimulation are likely to be amplified as a result of this (Schouenborg and Sjölund, 1983). In normal animals, MK-801 reduces the wind-up of the convergent dorsal horn neurones and thereby the C-fibre mediated amplification of the Aβ-fibre evoked responses. Hence the modest reduction in the Aβ-fibre evoked responses of dorsal horn neurones produced by MK-801 in these animals. However, in the carrageenan animals, MK-801 is unable to block the C-fibre mediated wind-up of the convergent neurones and as a consequence, the Aβ-fibre evoked responses of the neurones are no longer affected by the drug.

The reduced influence of MK-801 and 7CK on the neuronal wind-up following the development of inflammation is unlikely to result from an increase in the degree of wind-up occurring post-carrageenan such that an escalation of the doses of the NMDA receptor antagonists is required to cope with the enhanced responses. Although the wind-up of the neurones following carrageenan was on average 129 ± 11% of the value seen in the control period (chapter 3), subsequent to the development of carrageenan inflammation, the NMDA receptor antagonists were unable to inhibit the wind-up even of those neurones not displaying an enhanced wind-up or C-fibre evoked response post-carrageenan. In addition, the actions of the channel blocker MK-801 are use dependent (Wong et al., 1986) thus increased neuronal activity post-carrageenan would be expected

to aid rather than hinder the actions of this drug. Other mechanisms must therefore be considered.

One possibility is that the characteristics of the NMDA receptor-channel complex are altered by events accompanying the the development of carrageenan inflammation in such a way that the ability of MK-801 and 7CK to inhibit NMDA receptor activation is reduced. There are conditions which could occur in the spinal cord following the development of inflammation which could lead to the reduction in the sensitivity of wind-up to NMDA receptor antagonists, at least in terms of the actions of MK-801.

Increases in the levels of glutamate and glycine have been reported to decrease the affinity of MK-801 binding in the presence of physiological levels of Mg^{2+} (von Euler and Liu, 1993). An increased release of glutamate (but not glycine) in the dorsal horn of the spinal cord has been described three hours after the induction of inflammation produced by the injection of kaolin and carrageenan into the knee joint of rats (Sluka and Westlund, 1992). It is possible that this increase in the levels of glutamate in the spinal cord in inflammation is sufficient to reduce the binding of MK-801 to such an extent that its ability to inhibit wind-up is compromised.

Another event which could diminish the effectiveness of MK-801 following the development of carrageenan inflammation is an increase in the generation of free arachidonic acid in the spinal cord. One of the consequences of the activation of NMDA receptors is the release of arachidonic acid by phospholipase A_2 as a result of the elevation in intracellular calcium (Dumuis et al., 1988; Lazarewicz et al., 1990). Arachidonic acid potentiates the current though the NMDA receptor by increasing the opening probability of the channel (Miller et al., 1992). However, arachidonic acid also makes the NMDA current more transient (Miller et al., 1992). Since MK-801 is only able to enter the NMDA receptor channel when the channel is open (Foster and Wong, 1987; Huettner and Bean, 1988), a decrease in open time would be expected to reduce the chance of MK-801 entering the channel and hence blocking it, a phenomenon which might not be entirely compensated for by the increased opening probability of the channel. However, neither of these scenarios account for the parallel reduction in the sensitivity of the neuronal wind-up post-carrageenan to 7CK, which acts as an antagonist at the glycine site on the NMDA receptor, or why the input onto the neurones should become sensitive to NMDA receptor antagonists if the effectiveness of these antagonists is reduced. It is therefore unlikely that the mechanisms proposed above underlie the reduction in the sensitivity of wind-up to NMDA receptor antagonists in the carrageenan animals.

Another possibility for this phenomenon is that following three hours of carrageenan inflammation, wind-up in the dorsal horn of the spinal cord is now mediated by systems other than glutamate / aspartate acting at NMDA receptors. NMDA receptor-mediated wind-up could be diminished following inflammation if the Mg^{2+} block of the

NMDA receptor channel was reduced under these conditions such that the NMDA receptor could be near maximally activated from the start (hence the involvement of this receptor in the initial input following carrageenan), leaving little scope for sequential removal of the Mg^{2+} block and hence conventional wind-up. This could result from the increased release of SP post-carrageenan (Garry and Hargreaves, 1992) or from more complex changes resulting in a reduction in the Mg^{2+} block of the NMDA receptor channel such as that which can result from increased activation of protein kinase C (Chen and Huang, 1992). NMDA receptor antagonists would then reverse a component of the neuronal response resulting from activation of the NMDA receptor, although this may now be a constant rather than a continually increasing proportion of the response. Although the shape of the wind-up curves obtained following three hours of carrageenan inflammation do not appear to differ noticeably in their shape (i.e. the rate of increase in the response and the plateauing of this effect in many cases) from those obtained in normal animals, which would tend to imply that the underlying mechanisms are not that different, in view of the relative insensitivity of the wind-up to NMDA receptor antagonists post-carrageenan, alternative mechanisms must be considered.

So what other mechanisms could result in an increasing neuronal response per stimulus following repetitive C-fibre intensity stimulation? NMDA receptor antagonists are not always capable of producing a complete inhibition of wind-up (e.g. the effects of MK-801 on wind-up in normal animals appear to reach a plateau of around 50% in this chapter), although how much of this relates to the efficacy of the drug and how much is due to the contribution of other mechanisms is not clear. The summation of the slow depolarizations produced by peptides such as substance P, as well as relieving the Mg^{2+} block of the NMDA receptor channel, may themselves contribute to the increasing neuronal response with each stimulus in the train. The limited, steady wind-up exhibited by some superficial neurones in this preparation is not sensitive to NMDA receptor antagonists in normal animals (J. Haley, personal communication), indicating another mechanism at work, possibly involving only the cumulative peptide-mediated depolarizations. The stimulus-evoked release of SP is increased following three hours of carrageenan inflammation (Garry and Hargreaves, 1992) which would allow the cumulative peptide-induced depolarizations to play a greater role in wind-up in the carrageenan animals.

A further possibility for the production of wind-up in dorsal horn neurones involves the generation of nitric oxide (NO). Intense staining for nitric oxide synthase (NOS) is seen in neurones in laminae I-IV of the spinal cord (Saito et al., 1994). Activation of NMDA receptors, which share the same general location in the cord as NOS (Monaghan and Cotman, 1985; Mitchell and Anderson, 1991; Saito et al., 1994), leads to

an influx of Ca^{2+} into the neurones which can in turn activate a calmodulin sensitive site on NO synthase leading to the generation of NO (see Meller and Gebhart, 1993). NO produced in this way may be able to diffuse from its site of production in the post-synaptic neurones to various sites, possibly including pre-synaptic terminals where NO can increase transmitter release (see Meller and Gebhart, 1993). Inhibitors of nitric oxide synthase have been shown to be effective against both formalin-induced nociception (Moore et al., 1991; Haley et al., 1992; Malmberg and Yaksh, 1993) and carrageenan-induced thermal hyperalgesia (Meller et al., 1994). Both nociceptive models involve activation of the NMDA receptor (Haley et al., 1990; Coderre and Melzack, 1992; Ren et al., 1992a,b).

If NO generation can occur in the spinal cord following acute electrical stimulation of the afferents, then activation of postsynaptic NMDA receptors may generate NO which could then feed back to enhance the evoked release of primary afferent transmitters. The increased levels of transmitter released would further enhance the excitability of the postsynaptic neurone through the greater activation of both non-NMDA receptors and NMDA receptors (which would generate more NO) and hence a positive feed-back cycle is generated which could be seen as wind-up. In normal animals, if the generation of NO is contributing to wind-up, and there is evidence to suggest that this might be the case, at least in motor neurones (Semos and Headley, 1993), then blocking NMDA receptors would effectively prevent the whole process since NMDA receptor activation is necessary for NO production. However, following the development of inflammation, it is conceivable that receptors other than the NMDA receptor may be able to trigger the generation of NO (such as metabotropic glutamate receptors for example, which can also raise $[Ca^{2+}]_i$, through phospholipase C-mediated production of IP_3 (Miller, 1991)) such that blockade of NMDA receptors is now ineffective against NO production and NO-mediated wind-up. This has not yet been demonstrated in any other system to my knowledge.

Alternatively, drawing parallels with long-term potentiation (LTP) in the hippocampus, NMDA receptors may be important in the generation of altered spinal nociceptive processing, as in the induction of LTP, but once established, other mechanisms take over and NMDA receptor antagonism is ineffective (Collingridge and Bliss, 1987). Long-term potentiation of primary afferent neurotransmission has also been demonstrated in spinal cord slices following high frequency stimulation of the dorsal root afferents (Randic et al., 1993). As in the hippocampus, the induction of LTP of the AMPA receptor-mediated response can be blocked by the NMDA receptor antagonist APV, but once LTP is established, NMDA receptor antagonists are without effect (Randic et al., 1993). However, LTP of NMDA receptor-mediated responses can be blocked by

APV after induction (Randic et al., 1993). How, if at all, this relates to the insensitivity of wind-up to NMDA receptor antagonists post-carrageenan is unclear.

Again drawing parallels with hippocampal events, where potentiation requires not only NMDA receptor activation but a concurrent turning off of GABA-mediated inhibitions (Bliss and Collingridge, 1993), it can be noted that NOS and GABA often colocalize in dorsal horn neurones (Valtschanoff et al., 1992; Spike et al., 1993). As NO has been proposed to be involved in generating the wind-up of the neurones, part of this may be mediated through a reduction in GABA inhibitions, akin to the situation in the hippocampus. Whilst it is hard to see how activation of NOS in GABAergic neurones following afferent barrage could switch off GABA-mediated inhibitions in this situation, this colocalization could be the source of the inhibitions that seem to be evoked in the cord by high levels of NMDA receptor activity post-carrageenan (chapter 3).

Recent evidence has shown that a rapid downregulation occurs in the levels of mRNA coding for the NMDA R1 receptor in the lumbar spinal cords of rats following the injection of complete Freund's adjuvant into the paw (Kus et al., 1994). Although this decrease in the levels of mRNA can be detected 7.5 hours post-injection, the corresponding decrease in MK-801 binding is not seen until 3 days post-injection (Kus et al., 1994). Whilst it appears from this study (Kus et al., 1994) that there is a downregulation in the number (or affinity) of NMDA receptors to compensate for the enhanced activity of this receptor in inflammation, the time course of these changes is such that it is not likely to contribute to the the changes in the role of the NMDA receptor detected in the present study three hours after the injection of carrageenan into the paw.

The relative insensitivity of the neuronal wind-up following the development of inflammation might initially seem at odds with the results of behavioural studies observing the effects of NMDA receptor antagonists on hyperalgesia resulting from carrageenan-induced inflammation, where they have been found to be highly effective (Ren et al., 1992a,b; Ren and Dubner, 1993; Yamamoto et al., 1993). However, the brief noxious stimuli used to evaluate hyperalgesia in these reflex behavioural studies are unlikely to lead to large scale activation of wind-up mechanisms within the cord. Consequently the novel sensitivity of the input onto the neurones to NMDA receptor antagonists seen in the present electrophysiological study can explain the actions of NMDA receptor antagonists against inflammatory hyperalgesia in behavioural studies. Wind-up mediated amplification of the responses is unlikely to be generated in behavioural studies and hence the insensitivity of this amplified response to NMDA receptor antagonists in inflammation is unlikely to be encountered. In addition, the effects of NMDA receptor antagonists on motor events within the cord may contribute to the analgesic results in behavioural studies.

A limited number of other electrophysiological studies have also been carried out

in which the actions of NMDA receptor antagonists have been examined on the evoked responses of dorsal horn neurones following the development of peripheral inflammation (Schaible et al., 1991a; Ren et al., 1992a; Neugebauer et al., 1993b). The studies of Schaible et al. (1991a) and Neugebauer et al. (1993b) used models of acute arthritis in the knee joint of the cat and rat respectively, and found the response evoked in dorsal horn neurones by flexion of the inflamed joint or noxious pressure across the joint to be markedly reduced by ketamine and AP5. In contrast, in a model of unilateral inflammation produced by the intraplantar injection of complete Freund's adjuvant, Ren et al. (1992a) found the responses of dorsal horn neurones evoked by noxious pinch of the inflamed paw were only inhibited by MK-801 in 36% of the neurones, with the rest showing facilitations (24%) or mixed effects following MK-801. These latter results are very much in line with those I describe in this thesis. One possibility for the differences between the ability of NMDA antagonists to inhibit the evoked responses in these studies may be the method of stimulation used. The development of inflammation leads to the expansion of the peripheral receptive fields of dorsal horn neurones (Menétrey and Besson, 1982; Calvino et al., 1987a; Hylden et al., 1989; Ren et al., 1992a; Grubb et al., 1993; Neugebauer et al., 1993b). This expansion is reversed by NMDA receptor antagonists (Ren et al., 1992a; Neugebauer et al., 1993b). In the present study, and in the study of Ren et al. (1992a), the noxious stimuli were applied to a localized area in the centre of the receptive field of the neurone, whereas in the animals with acute arthritis of the knee joint (Schaible et al., 1991a; Neugebauer et al., 1993b) the stimulus (flexion of the knee joint or mechanical compression of the knee joint) affected a wide area. Consequently, during the development of acute arthritis, stimulation of the knee joint will activate an increasing number of inputs onto the dorsal horn neurone under study due the expansion of peripheral receptive fields. NMDA receptor antagonists restore the receptive field to the control size, hence reducing the number of active inputs onto the neurone, thereby reducing the evoked response. With the application of discrete stimuli however (Ren et al., 1992a; present study), the expansion of the receptive field during the development of inflammation, although still occurring, will have little impact on the response of a neurone evoked by these localized stimuli since the expanded portion of the receptive field is likely to be outside the area being stimulated. Consequently, the smaller, sometimes mixed effects of NMDA receptor antagonists in models where discrete stimuli have been used may result from the neuronal responses studied being independent of expansions in receptive fields.

CHAPTER 5

ALTERED SPINAL POTENCY OF MU, DELTA AND KAPPA OPIOIDS FOLLOWING CARRAGEENAN-INDUCED INFLAMMATION

5.1. Introduction

The analgesic properties of opioid drugs are widely exploited in the clinic. Many studies, both behavioural and electrophysiological, have investigated the antinociceptive actions of opioids against acute nociceptive responses. However, perhaps of more relevance to clinical situations are the actions of opioids in more persistent / prolonged pain states such as those with an inflammatory basis, arising from peripheral pathology or as a result of operative procedures.

A number of behavioural studies have investigated the antinociceptive effects of opioids in models of inflammatory nociception including models of generalized arthritis (Kayser and Guilbaud, 1983; Neil et al., 1986; Millan et al., 1987), and those producing more localized, unilateral inflammation induced by the intraplantar injection of Freund's complete adjuvant (Stein et al., 1988b) or carrageenan (Joris et al., 1990; Kayser et al., 1991; Hylden et al., 1991b), and have found that the antinociceptive potency of opioids in these animals is much greater than that seen against acute noxious stimuli in normal animals. However, with the exception of Hylden et al. (1991b), these studies have used systemic administration of the opioids. In inflammatory conditions, administration of opioids directly into the inflamed but not non-inflamed tissue produces a naloxone-reversible analgesia (Joris et al., 1987; Hargreaves et al., 1988b; Stein et al., 1988a, 1989). Consequently, opioids administered systemically in inflammatory states have the potential to act at supraspinal, spinal, and also peripheral sites, making the exact site or sites at which inflammation induced changes are occurring hard to localize. Studies have shown that at least part of the enhanced potency of systemically administered opioids seen in inflammation results from novel actions of the drugs at sites in the inflamed peripheral tissue (Stein et al., 1988b; Kayser et al., 1991).

What about the central sites at which opioids act to produce analgesia, the brain stem and the spinal cord? Do changes occur in these nociceptive systems following the development of peripheral inflammation which affect the ability of opioids to produce analgesia? This chapter sets out to investigate whether the potency of opioids acting at one of these sites, the spinal cord, is altered following the development of peripheral inflammation.

The transmission and processing of nociceptive information in the dorsal horn of the spinal cord is dynamic, with great capacity for change, allowing it to adapt to changes in nociceptive input produced by trauma or disease. In the previous chapters I have shown how plasticity in spinal nociceptive mechanisms, namely complex alterations in the electrically evoked neuronal response (chapter 3) and changes in the role played by the NMDA receptor in spinal nociceptive transmission (chapter 4), can result within three

hours of the onset of peripheral inflammation induced by the injection of carrageenan into the paw. Therefore, it is conceivable that the development of inflammation in the periphery may induce changes in spinal nociceptive systems which may alter the ability of opioids acting at sites in the spinal cord to modulate nociceptive transmission. Indeed, Hylden et al. (1991b) have shown in a behavioural study that the potency of spinal opioids is enhanced following carrageenan inflammation.

The electrophysiological study presented in this chapter examines the sensitivity of the C-fibre evoked responses of dorsal horn neurones to intrathecal opioids three hours after the onset of carrageenan inflammation (the time corresponding to peak hyperalgesia in behavioural studies (Kayser and Guilbaud, 1987; Hargreaves et al., 1988a; Hylden et al., 1991b)) compared with that in normal, non-inflamed animals. Electrically evoked activation of peripheral primary afferent fibres, via needles inserted into the peripheral receptive field, rather than activation by mechanical or thermal stimuli, was used to evoke dorsal horn neuronal responses to circumvent changes in baseline responses to natural stimuli which can accompany the inflammatory state. These shifts in baseline responses (i.e. lowered nociceptive thresholds to heat and pressure) can complicate interpretation of shifts in dose response curves. In addition, the presence of oedema and the increased blood flow in the inflamed region can potentially alter the intensity of thermal or mechanical stimuli reaching the afferents, further complicating comparisons between drug effects in normal animals and animals with carrageenan-induced inflammation. The use of constant current electrical stimulation of the afferents in this study overcomes this problem (see **2.6**). No change in the threshold current required for the activation of C-fibres occurs following the development of inflammation (see **3.2.8.**) thus drug-induced reductions in nociceptive responses are measured against the same starting point.

The ability of intrathecal mu, delta and kappa opioid agonists to inhibit the electrically evoked nociceptive responses of convergent dorsal horn neurones was tested following three hours of carrageenan inflammation, with morphine, DSTBULET and U69593 being chosen as typical agonists for these receptor subtypes respectively. Changes in the spinal potency of these opioid agonists as a result of the development of inflammation in the periphery was assessed.

5.2. Results

The convergent cells used in the study were located in both the superficial (mean depth 266μm, n=5), and deep (mean depth 748μm, n=92) dorsal horn. No difference could be determined between the two groups of cells either in terms of their response after carrageenan or the effects of opioids so the results are pooled for the whole population.

5.2.1. Morphine dose-response curve in normal animals

Intrathecal morphine in the dose range 0.1-50μg was tested on the C- and Aβ-fibre evoked responses of 26 dorsal horn neurones in normal animals. Doses of morphine of 0.5μg and above produced a dose dependent inhibition of the C-fibre evoked responses of the neurones, with the highest dose tested (50μg) giving a maximum inhibition of 90.0 ± 4.1% (n=9) (figure **5.1**). It should be noted that there is no relationship between the degree of inhibition produced by a given dose of morphine and the magnitude of the pre-morphine C-fibre evoked response. The Aβ-fibre evoked responses of the neurones were inhibited to a lesser extent than those evoked by C-fibres, with a maximum inhibition of 28.2 ± 9.1% (n=9) produced by 50μg (figure **5.1**). In contrast to the effects of the higher doses of morphine, the lowest dose of morphine tested (0.1μg) produced a significant facilitation of the C-fibre evoked response of 26.2 ± 8.5% (paired Student's t-test, one-tailed p-value = 0.005; n=5) (figure **5.1**), whereas only dose dependent inhibitions of the C-fibre evoked response were observed as the dose was increased.

The dose-response curve obtained for the inhibition of the C-fibre evoked response was fitted to a sigmoid curve using the curve fitting program InPlot (GraphPAD) in order to determine the ED_{50} value with 95% confidence limits. An ED_{50} of 5.07 μg (3.98 - 6.44μg) for the inhibition of the C-fibre evoked response by morphine was obtained from the fitted curve.

The ability of intrathecal morphine to inhibit the initial input onto the neurones (I) and the wind-up of the neurones (E) was also studied. In agreement with a previous study in this laboratory (Dickenson and Sullivan, 1986), the initial input onto the neurones showed a greater sensitivity to morphine than the wind-up. For example, the ED_{50} dose of 5μg of morphine produced an inhibition of I of 80.3 ± 11.3% compared with 41.8 ± 19.9% for the wind-up (E) (n=6). A stimulus-response graph illustrating the effects of 5μg of morphine on an individual neurone is shown in figure **5.2**.

Intrathecal naloxone (5μg) administered after 25 or 50μg of morphine reversed the inhibition of C-fibre evoked responses to 101.8 ± 6.2 % of control within 30 minutes.

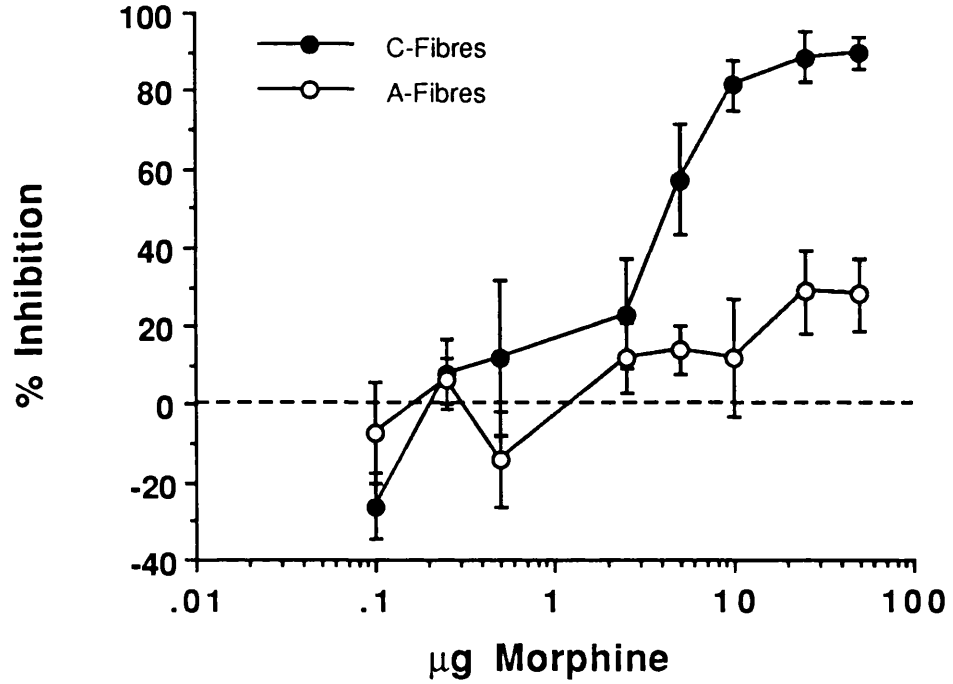


Figure 5.1. Dose-response curve for the inhibition of the electrically evoked C- and Aβ-fibre evoked responses of dorsal horn neurones by intrathecal morphine in normal animals. ED_{50} for the inhibition of the C-fibre evoked response by morphine = 5.07 µg (3.98 - 6.44µg). n=5-9 neurones per dose.

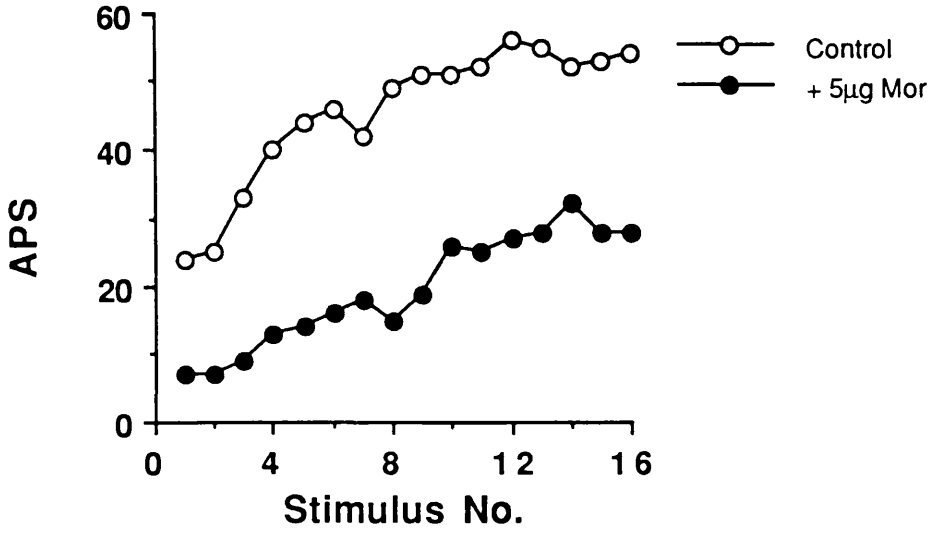


Figure 5.2. Stimulus-response graph showing the effect of 5µg of morphine on the wind-up of an individual dorsal horn neurone. The number of action potentials evoked (APS) are given against stimulus number. Note that despite the marked reduction in input in the presence of morphine, the neurone continues to wind-up almost as before.

5.2.2. Dose-response curve for intrathecal morphine following 3 hours of carrageenan inflammation

Intrathecal morphine in the dose range 0.001-10μg, administered 3 hours after the injection of carrageenan into the ipsilateral hind paw, was tested on the C-and Aβ-fibre evoked responses of 14 dorsal horn neurones. Morphine produced dose dependent inhibitions of the C-fibre evoked response, with the highest dose tested (10μg) giving a maximum inhibition of 83.0 ± 4.7 % (n=7) (figure **5.3**). Aβ-fibres were also inhibited by morphine but to a lesser extent than the C-fibres, with a maximum inhibition of 35.8 ± 9.7% (n=9) produced by 2.5μg of morphine (figure **5.3**). Despite the administration of a range of doses of morphine at the lower end of the dose-response curve, no facilitations of the C-fibre evoked response were seen, which is in contrast to the findings obtained in normal animals. The ED_{50} dose obtained from the fitted curve for the inhibition of the C-fibre evoked response by morphine in these animals was 0.28μg (0.15 - 0.57μg).

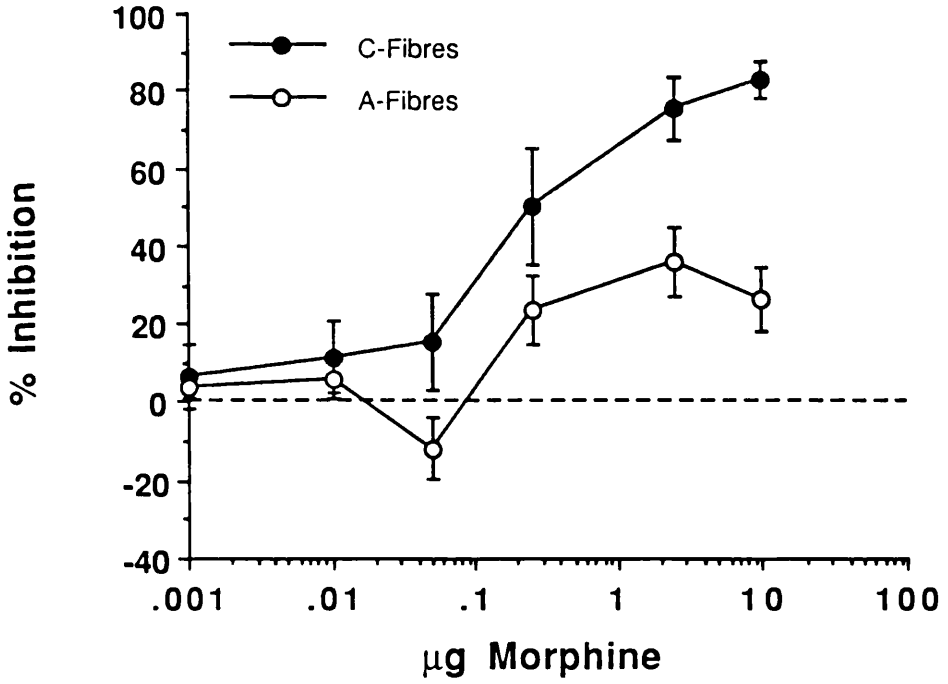


Figure 5.3. Dose-response curve for the inhibition of the electrically evoked C- and Aβ-fibre evoked responses of dorsal horn neurones by intrathecal morphine administered 3 hours after the injection of carrageenan into the ipsilateral hind paw. ED_{50} for the inhibition of the C-fibre evoked response by morphine = 0.28 μg (0.15 - 0.57μg). n=4-9 neurones per dose.

Intrathecal application of 1μg of naloxone administered after 2.5 or 10μg of morphine reversed the inhibition of C-fibre evoked responses to 68.7 ± 7.0 % of control within 30 minutes.

As in normal animals, the magnitude of the post-carrageenan neuronal responses had no influence on the effects of the opioid. For example, the correlation coefficient between degree of inhibition and magnitude of pre-drug C-fibre response for 0.25μg of morphine was 0.04, (p=0.92; n=8). In addition, the degree of inhibition produced by morphine was independent of whether a cell showed an increased or decreased response post-carrageenan.

5.2.3. Dose-response curve for intrathecal morphine following 1 hour of carrageenan inflammation

Intrathecal morphine, administered one hour after the induction of carrageenan inflammation, was tested on the evoked responses of 18 neurones. In these animals morphine produced a dose dependent inhibition of the C-fibre evoked responses, with a maximum inhibition of 100 ± 0 % produced by 10μg of morphine (n=4) (figure **5.4**), and an ED_{50} of 0.79μg (0.26 - 2.37μg). The facilitations of the C-fibre evoked responses seen with low doses of intrathecal morphine in non-inflamed animals did not occur at any of the doses tested following 1 hour of carrageenan induced inflammation (figure **5.4**).

5.2.4. Comparison of the effects of intrathecal morphine in normal and carrageenan animals

The ED_{50} obtained for the inhibition of the C-fibre evoked activity by intrathecal morphine in animals following 3 hours of carrageenan inflammation was 0.28μg (0.15 - 0.57μg). When this dose-response curve obtained in carrageenan animals is compared with that obtained in normal, non-inflamed, animals (ED_{50} = 5.07μg (3.98 - 6.44μg)) it can be seen that the dose-response curve for inhibition of the C-fibre evoked response by intrathecal morphine 3 hours after the onset of carrageenan inflammation is shifted to the left, although this shift is not parallel (figure **5.4**). When the morphine dose-response curve obtained in animals following just one hour of carrageenan inflammation (ED_{50} = 0.79μg (0.26-2.37μg)) is compared with that obtained in normal and 3 hour-carrageenan animals, it can be seen that the leftward shift in the dose response curve for morphine is already similar to that obtained following three hours of inflammation (figure **5.4**).

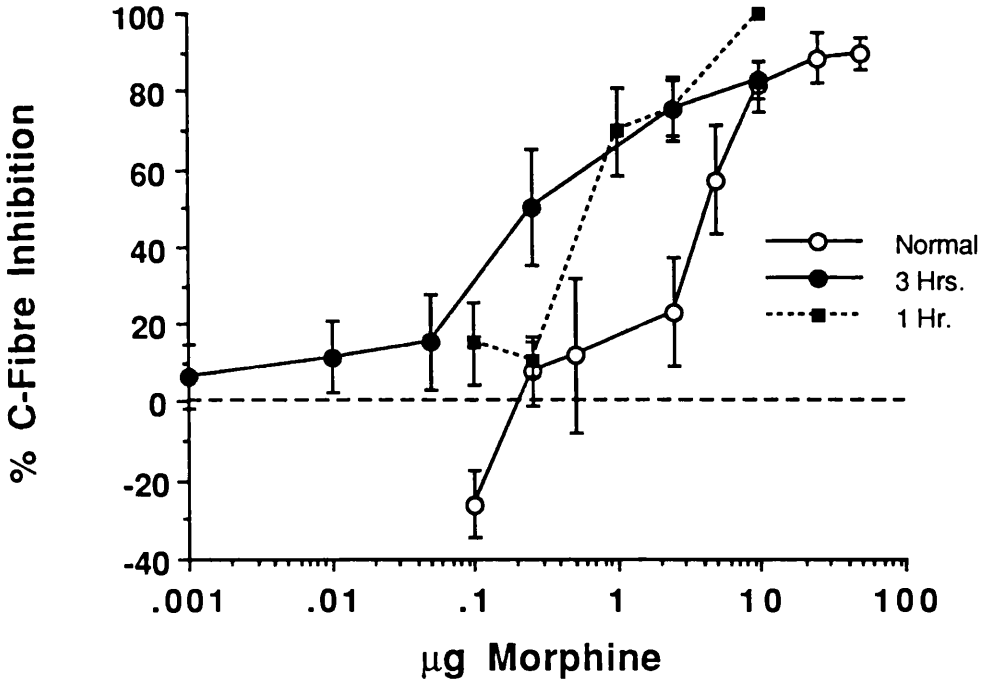


Figure 5.4. Comparison of the dose-response curves obtained for the inhibition of the C-fibre evoked responses by intrathecal morphine in normal animals, and one (1 Hr.) and three (3 Hrs.) hours after the injection of carrageenan into the ipsilateral hind paw. Note the rapid leftward shift (within one hour) in the dose-response curve for intrathecal morphine following the development of inflammation which results in the ED_{50} dose of morphine being reduced 18-fold within 3 hours of the carrageenan injection. Note the absence of morphine induced facilitations of the response in the carrageenan animals.

These results show that spinal morphine is substantially more potent (18-fold shift in the ED_{50} dose at 3 hours) against nociceptive responses in animals with carrageenan induced inflammation compared with normal animals. Furthermore, this inflammation-induced enhancement in the spinal potency of morphine occurs very rapidly, with a significant reduction in the ED_{50} dose occurring within one hour of the injection of carrageenan.

Morphine also appeared to be more potent against the Aβ-fibre evoked responses of the neurones in the carrageenan animals (figure **5.5**) although due to the relatively small inhibitions of the Aβ-fibre evoked response produced by morphine, no attempt was made to quantify the extent of the potency shift.

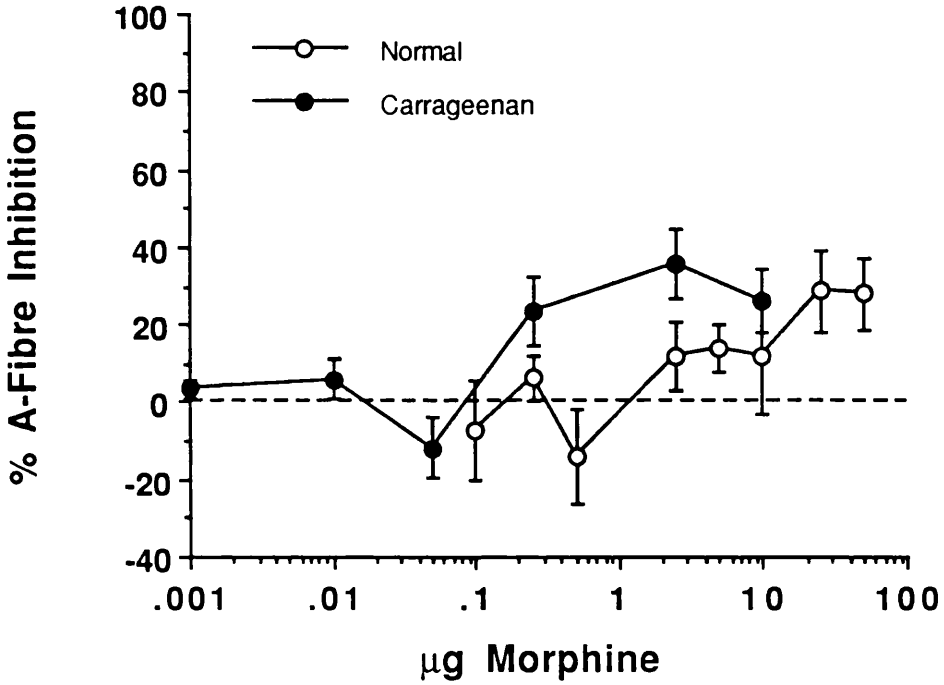


Figure 5.5. Comparison of the dose-response curves obtained for the inhibition of the Aβ-fibre evoked response by intrathecal morphine in normal animals and in animals three hours after the injection of carrageenan into the ipsilateral hind paw.

Although morphine was significantly more potent against C-fibre evoked neuronal responses following the development of carrageenan inflammation, the profile of its actions was not altered: in the carrageenan animals, the input onto the neurones still showed a greater sensitivity to the effects of morphine than the neuronal wind-up. This is illustrated in figure **5.6** using doses of morphine which produce an equivalent inhibition of the C-fibre evoked response in the two groups of animals.

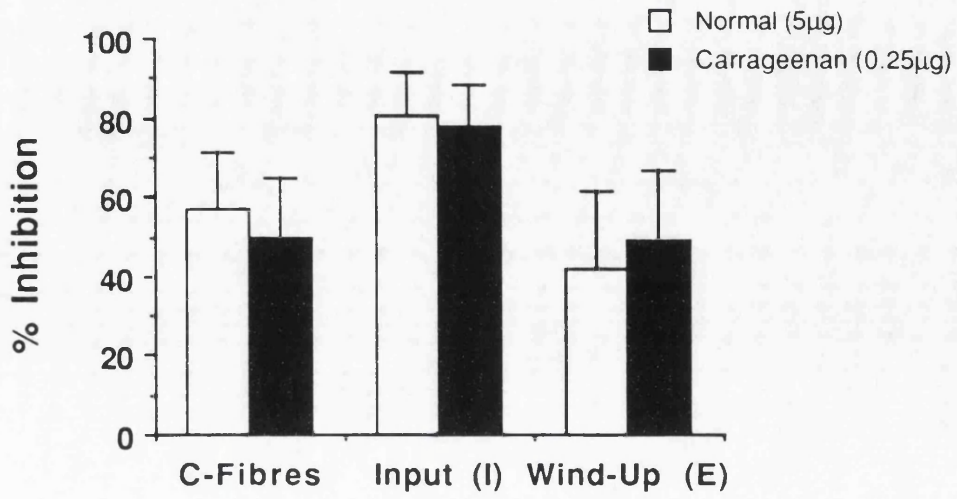


Figure 5.6. Inhibition of the C-fibre evoked response, and the initial input onto the neurones (I) and wind-up of the neurones (E) by intrathecal morphine in normal animals and three hours after the injection of carrageenan into the paw. Doses of morphine producing an equivalent inhibition of the C-fibre evoked response in normal and carrageenan animals (5µg and 0.25µg respectively) also produce an equivalent inhibition of the input and wind-up of the neurones. n=6-8 neurones per group.

To exclude the possibility that the trauma of the carrageenan injection or the repeated electrical stimulation over a period of three hours are factors in the enhancement in the potency of morphine seen in the carrageenan animals, 5µg of morphine were applied to the cord 3 hours after the injection of 100µl saline into the plantar region of the paw. The C-fibre inhibition produced by this dose under these conditions (43 ± 14%; n=4) was not significantly different from that produced in the normal non-injected animals (57 ± 14%; n=5), indicating that it is the development of peripheral inflammation that induces the increased sensitivity to spinal morphine.

To conclusively demonstrate that the enhancement in morphine potency seen was due to alterations in spinal mechanisms, systemic effects of these intrathecal doses had to be discounted. Systemic administration of morphine at doses up to 1mg/kg had no significant inhibitory effect on the evoked neuronal responses in carrageenan animals (12 ± 13% inhibition of the C-fibre evoked response by 1mg/kg morphine i.v. ; n=4), thereby ruling out an action of morphine at peripheral or supraspinal sites as a factor in the enhanced effects of spinal morphine. Interestingly the systemic administration of 10µg/kg of morphine i.v. in the carrageenan animals produced a slight facilitation of the C-fibre evoked response to 124 ± 8.5% of control (n=4), a phenomenon not seen following spinal administration in the carrageenan animals.

5.2.5. Morphine dose-response curve following contralateral carrageenan injection

In order to determine whether the changes in the antinociceptive potency of spinal morphine seen after the injection of carrageenan into the ipsilateral hind paw extend to neurones receiving input from the non-injected paw, the potency of spinal morphine was examined following the injection of carrageenan into the contralateral hind paw. Three doses of morphine (0.25, 1 and 5μg) which lie at the lower end of the dose-response range in normal animals (figure **5.1**), but which produce inhibitions of 50% or more following injection of carrageenan into the ipsilateral hind paw (figure **5.3**) were tested on the evoked responses of 6 dorsal horn neurones 3 hours after the injection of carrageenan into the contralateral hind paw. The three doses of morphine tested following the contralateral injection of carrageenan produced inhibitions of the C-fibre evoked response identical to those produced by the same doses in normal, non-injected animals (figure **5.7**). Therefore the shift in the antinociceptive potency of spinal morphine observed after unilateral carrageenan inflammation appears to be localized to dorsal horn neurones ipsilateral to the inflamed region.

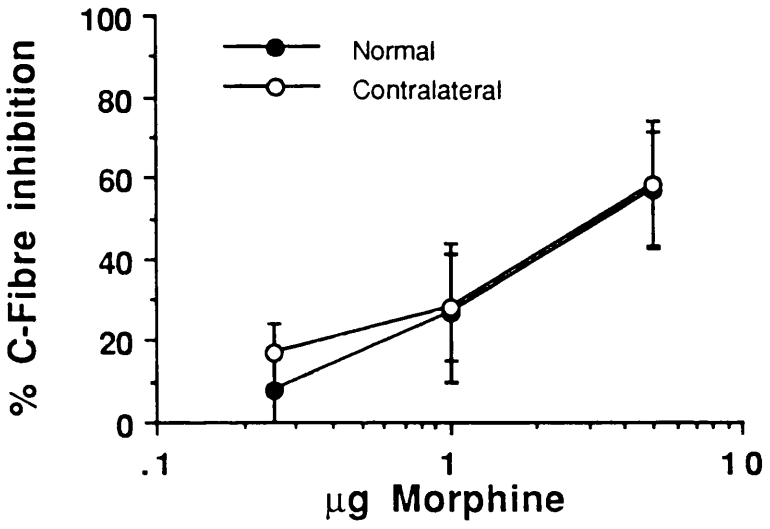


Figure 5.7. Dose-response curves for the inhibition of the C-fibre evoked response of dorsal horn neurones by intrathecal morphine in normal animals and in animals 3 hours after the injection of carrageenan into the contralateral paw. n=5-7 neurones per dose.

5.2.6. U69593 dose-response curve in carrageenan animals

Doses of U69593, the kappa opioid receptor agonist, in the range 0.5-500μg applied to the surface of the cord were tested on a total of 13 neurones 3 hours after the injection of carrageenan into the ipsilateral hind paw. The lowest two doses applied had no effect on the C-fibre evoked response, causing neither excitation or inhibition. The higher doses produced a dose dependent inhibition of the C-fibre evoked response, giving an ED_{50} of 83.0μg (75.7 - 91.0μg) and a maximum inhibition of 93.6 ± 2.0% with a dose of 250 μg (n=5) (figure **5.8**). The Aβ-fibre evoked activity of the neurones was also inhibited by U69593, but to a much lesser extent than the C-fibres, with the highest dose only producing an inhibition of 29.4 ± 19.0% (n=5) (figure **5.8**).

Intrathecal naloxone (250μg) administered intrathecally after 250 or 500μg of U69593 produced a reversal to 80.6 ± 9.6 % of the C-fibre control within 20 minutes.

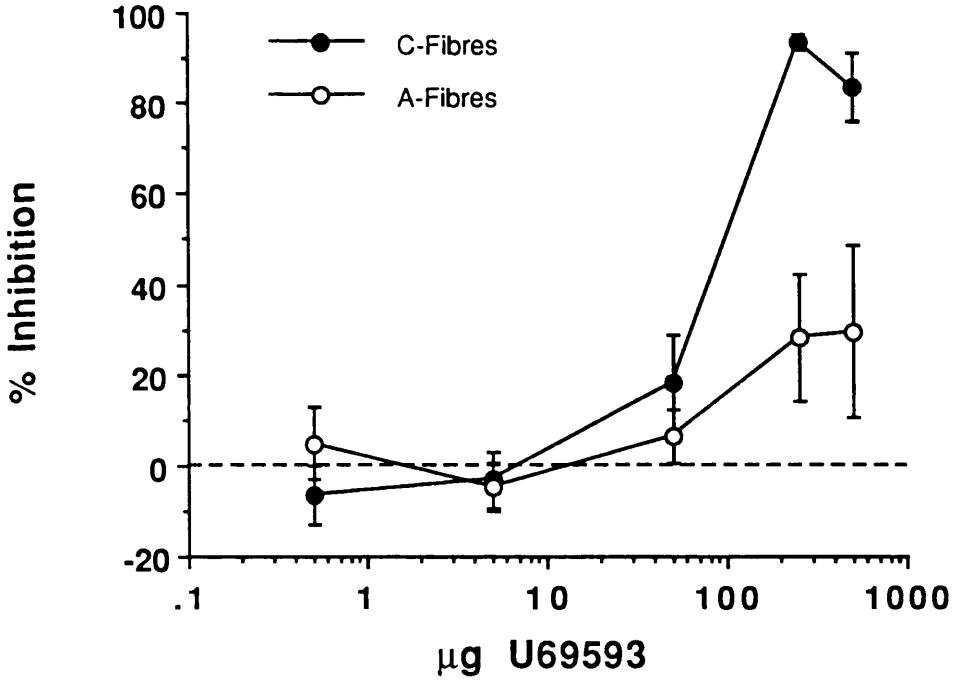


Figure 5.8. Dose-response curve for the inhibition of the C- and Aβ-fibre evoked responses of dorsal horn neurones by intrathecal U69593 three hours after the injection of carrageenan into the ipsilateral hind paw. ED_{50} for the inhibition of the C-fibre evoked response by U69593 = 83.0μg (75.7 - 91.0μg). n=5-8 neurones per dose.

5.2.7. Comparison of the effects of intrathecal U69593 in normal and carrageenan animals

The dose response curve for morphine in normal animals obtained in the present study is almost identical to that obtained in a previous study in this laboratory examining the antinociceptive effects of intrathecal morphine in this model (Dickenson and Sullivan, 1986, ED_{50} estimated at 5.5μg; ED_{50}=5.07μg this study). Thus, in order to reduce the number of animals used, a dose response curve for U69593 in normal animals, obtained in this laboratory by Sullivan and Dickenson (1991) under the same experimental conditions was used as a control for the effects of U69593 in the carrageenan animals.

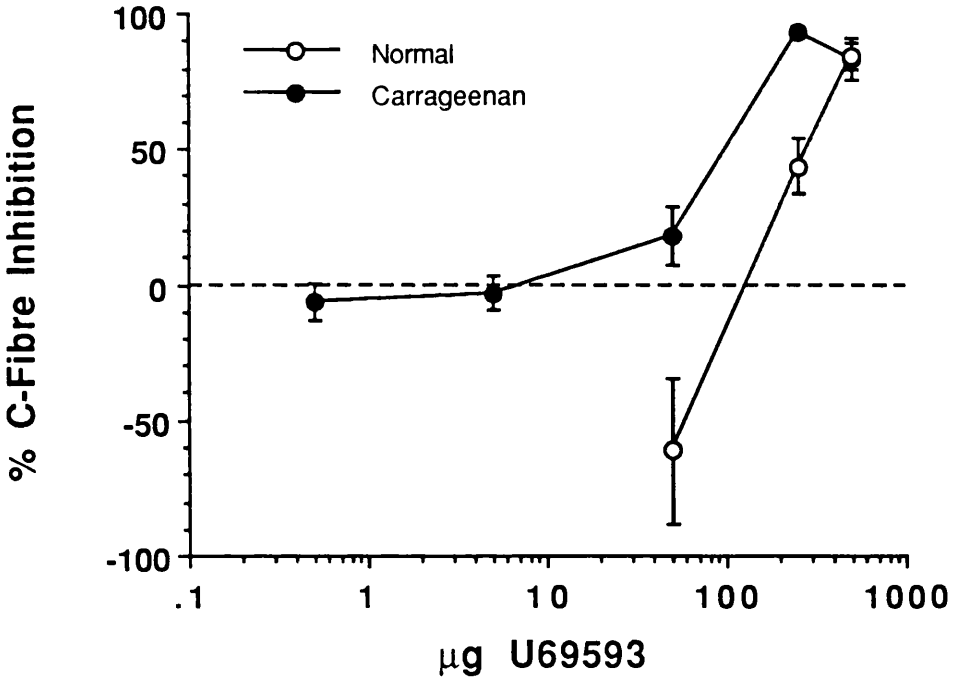


Figure 5.9. Comparison of the dose-response curves obtained for the inhibition of the C-fibre evoked responses by intrathecal U69593 in normal animals (data from Sullivan and Dickenson, 1991) and in animals three hours after the injection of carrageenan into the ipsilateral hind paw. Whilst facilitations of the C-fibre evoked response predominate with the lowest dose of U69593 in normal animals, the effects of U69593 are purely inhibitory following the development of inflammation. The ED_{50} dose of U69593 is approximately 3-fold lower in the carrageenan animals compared with normals.

When the dose response curve for the inhibition of the C-fibre evoked response by U69593 in the carrageenan animals is compared with that obtained in normal animals (data from Sullivan and Dickenson, 1991) (figure **5.9**), the most obvious difference is the lack of facilitation that has been reported by many studies to occur with low doses of U69593 and other kappa agonists (Knox and Dickenson, 1987; Sullivan and Dickenson, 1991; Hylden et al., 1991a). The dose response curve obtained in carrageenan animals lies slightly to the left of the upper part of that produced under normal conditions (ED_{50} estimated as 250μg, Sullivan and Dickenson, 1991), indicating a 3-fold increase in potency under these conditions of inflammation (figure **5.9**).

There did not appear to be any marked difference in the effect of U69593 on Aβ-fibre evoked responses in normal animals, which do not show facilitations with low doses of U69593 (Sullivan and Dickenson, 1991), and on these responses in carrageenan animals (figure **5.10**).

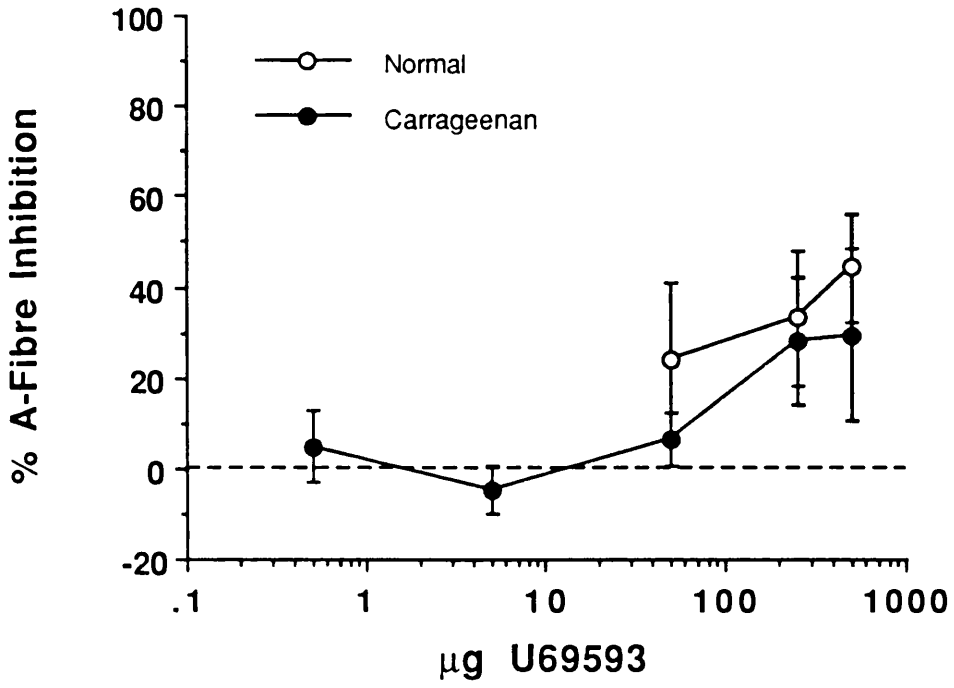


Figure 5.10. Comparison of the dose-response curves obtained for the inhibition of the Aβ-fibre evoked response by intrathecal U69593 in normal animals (data from Sullivan and Dickenson, 1991) and in animals three hours after the injection of carrageenan into the ipsilateral hind paw. U69593 appears to be equally potent at inhibiting the Aβ-fibre evoked response of the neurones in both groups of animals.

5.2.8. DSTBULET dose-response curve in carrageenan animals

DSTBULET, the delta opioid agonist, was tested on a total of 12 neurones 3 hours post-carrageenan. Intrathecal DSTBULET dose dependently inhibited the C-fibre evoked activity of the neurones, with the highest dose tested (100 μg) giving a complete inhibition of 100 ± 0% (n=4) (figure **5.11**). The ED_{50} dose determined from the fitted dose response curve for the inhibition of the C-fibre evoked response by DSTBULET was 4.31μg (2.68 - 6.94μg). The Aβ-fibre evoked activity was also inhibited, but to a much lesser extent than that evoked by C-fibres, with the highest dose tested only giving an inhibition of 33.3 ± 5.7 % (n=4) (figure **5.11**).

10μg of naloxone produced a reversal of the C-fibre response to 89 ± 9% of control within 30 minutes.

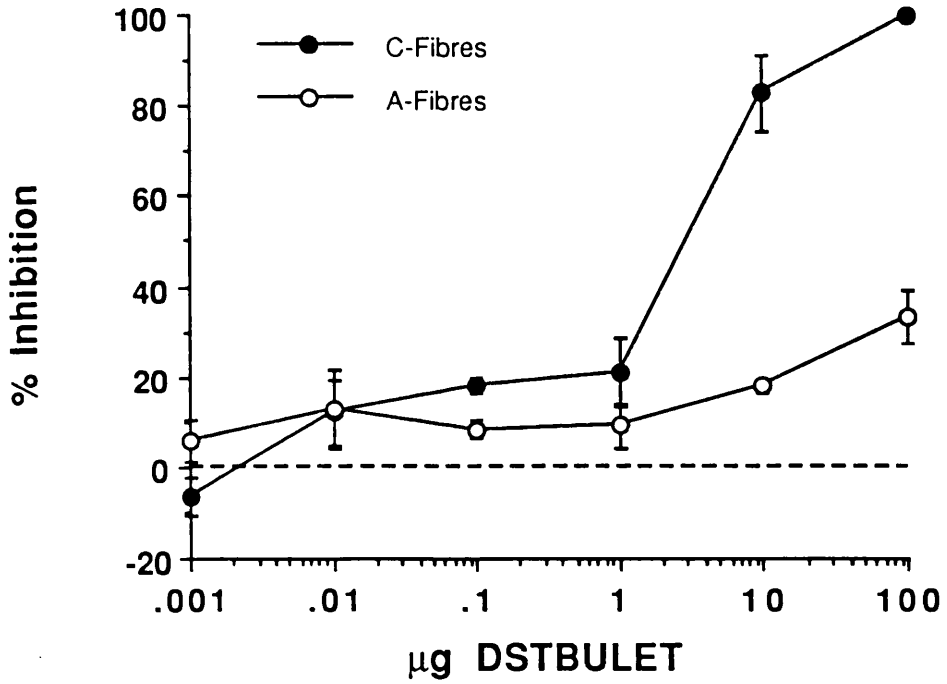


Figure 5.11. Dose-response curve for the inhibition of the C- and Aβ-fibre evoked responses of dorsal horn neurones by intrathecal DSTBULET three hours after the injection of carrageenan into the ipsilateral hind paw. ED_{50} for the inhibition of the C-fibre evoked response by DSTBULET = 4.31μg (2.68 - 6.94μg). n=4-5 neurones per dose.

5.2.9. Comparison of the effects of intrathecal DSTBULET in normal and carrageenan animals

When the dose response curve obtained for DSTBULET in the carrageenan animals is compared to one obtained previously in this laboratory in normal animals (data obtained from Sullivan et al., 1989 to reduce to number of animals used), it can be seen that there is a parallel shift to the left in the C-fibre dose response curve for DSTBULET following 3 hours of carrageenan-induced inflammation (figure **5.12**). When the ED_{50} dose for DSTBULET in the carrageenan animals (4.31μg (2.68 - 6.94μg)) is compared to that obtained in normal animals (calculated as 14.6μg, (10.0 - 21.4μg), data from Sullivan et al., 1989), it can be seen that in the presence of carrageenan inflammation, DSTBULET is approximately 3 times more potent (figure **5.12**). The inhibition of the Aβ-fibre evoked response by DSTBULET was not affected by the inflammation.

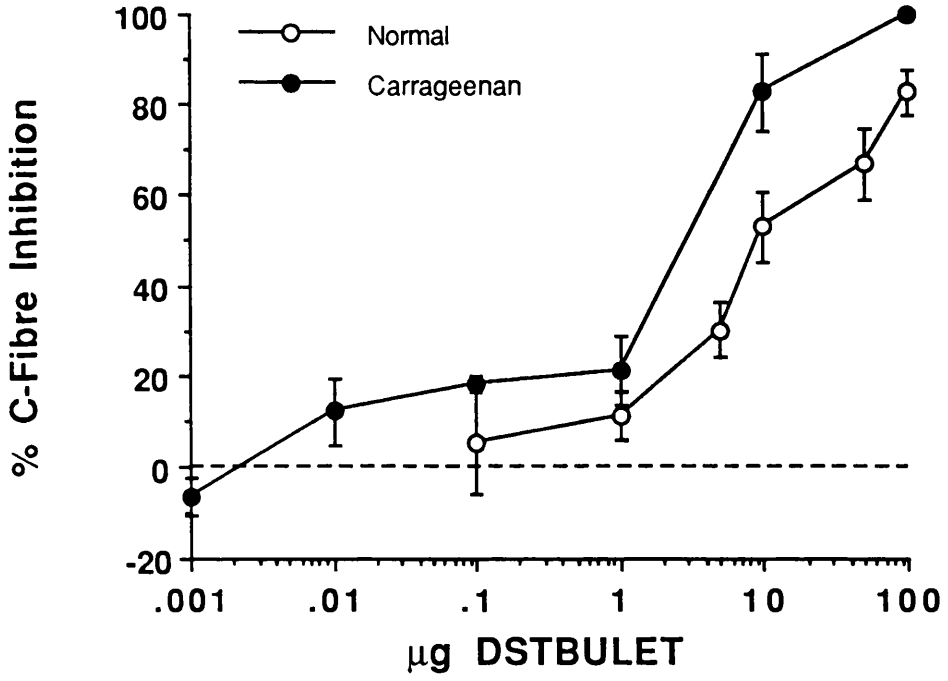


Figure 5.12. Comparison of the dose-response curves obtained for the inhibition of the C-fibre evoked responses by intrathecal DSTBULET in normal animals (data from Sullivan et al., 1989) and three hours after the injection of carrageenan into the ipsilateral hind paw. There is a parallel leftward shift in the dose response curve for DSTBULET in the carrageenan animals, resulting in a 3-fold reduction in the ED_{50} dose of DSTBULET in these animals. Note that DSTBULET did not facilitate the C-fibre response in either the normal or carrageenan animals.

5.3. Discussion

In a number of animal models of inflammation, producing either a generalized inflammatory state such as arthritis or those involving more acute localized inflammation such as the carrageenan model, behavioural studies have demonstrated an enhanced antinociceptive action of systemically administered opioids against noxious stimuli applied to the inflamed tissue (Kayser and Guilbaud, 1983; Neil et al., 1986; Millan et al., 1987; Stein et al., 1988b; Joris et al., 1990; Kayser et al., 1991). At least part of the enhanced antinociceptive potency of systemically administered opioids in inflammation has been attributed to a novel action at sites in the periphery (Stein et al., 1988b; Kayser et al., 1991), since naloxone administered directly into the inflamed paw is able to antagonize the actions of systemically administered opioids, whereas the same dose of naloxone given systemically is without effect (Stein et al., 1988b; Kayser et al., 1991). However, until recently, nothing was known about the ability of inflammation to induce changes at the other sites at which these systemically administered opioids act, the brain stem and spinal cord, which may also contribute to alterations in opioid potency.

The results of the electrophysiological study presented here, along with the behavioural study of Hylden et al. (1991b), demonstrate that the antinociceptive potency of opioids acting at spinal sites is also enhanced following the development of inflammation in the periphery.

At the time corresponding to peak hyperalgesia demonstrated in behavioural studies, which occurs just three hours after the injection of carrageenan into the hind paw (Kayser and Guilbaud, 1987; Hargreaves et al., 1988a; Hylden et al., 1991b), morphine, DSTBULET and U69593 (selective agonists of the μ, δ and κ opioid receptors respectively) administered directly onto the spinal cord, were more potent against the electrically evoked C-fibre activity of dorsal horn neurones in animals with carrageenan inflammation than in normal animals. The direction and degree of change in the C-fibre evoked response in the three hours following the injection of carrageenan into the paw (see chapter 3) did not bear any relation to the effects of the subsequently administered opioids. Thus the changes in opioid effectiveness after carrageenan are independent of the changes in neuronal responses and so are not simply a consequence of the altered magnitude of the neuronal responses.

Although the spinal antinociceptive potency of all three opioids tested in this study was enhanced in the carrageenan animals compared to normals, the mu opioid morphine showed a far greater increase in potency than DSTBULET or U69593 which showed only relatively modest increases in potency (see table **5.1**).

Table 5.1. Spinal potency of opioids in normal and carrageenan animals.

	ED_{50} (95% confidence limits)		Potency Shift
Drug	**Normal**	**3 hrs Post-Carrageenan**	
Morphine (μ)	5.07 (3.98 - 6.44) μg	0.28 (0.15 - 0.57) μg	18
DSTBULET (δ)	14.6 (10.0 - 21.4) μg*	4.31 (2.68 - 6.94) μg	3
U69593 (κ)	Estimated at 250 μg**	83.0 (75.7 - 91.0) μg	3

* Data from Sullivan et al. (1989) ** Data from Sullivan and Dickenson (1991).

This non-uniform enhancement in the potency of opioids acting at the different opioid receptors was also observed in the behavioural study of Hylden et al. (1991b). In this study, a greater increase was seen in the spinal potency of the mu agonist DAMGO than for the mixed mu and delta agonist DADL and the delta agonist DPDPE, although difficulties in determining the ED_{50} for DPDPE in non-inflamed animals, and the the altered nociceptive threshold as a result of the inflammation made comparisons difficult. Thus the enhancement in opioid potency in inflammation, at least at spinal sites, cannot simply be regarded as a general increase: specific changes must be occurring which preferentially lead to the greater enhancement in the potency of mu rather than delta or kappa opioid agonists. Interestingly, agonists at all three opioid receptors produce an antinociceptive effect when administered into inflamed tissue (Stein et al., 1989) and studies to date have not revealed any differences between the three receptor subtypes with regard to their ability to produce analgesia in this state.

In light of these findings, it is perhaps not surprising that where behavioural studies have used systemic administration of opioids, which will have actions at sites in the spinal cord amongst others, to examine opioid potency in inflammatory models, they have consistently demonstrated an enhancement in the potency of mu agonists such as morphine and DAMGO (Kayser and Guilbaud, 1983; Neil et al., 1986; Millan et al., 1987; Joris et al., 1990; Kayser et al., 1991). In contrast, although studied less frequently, studies have been unable to demonstrate significantly enhanced effects of delta agonists (Neil et al., 1986), whilst the results of studies with kappa agonists are conflicting, showing both enhanced (Neil et al., 1986; Stein et al., 1988b) and diminished antinociceptive effects (Millan et al., 1987) of kappa agonists in inflammation.

5.3.1. What changes may underlie this shift in the spinal potency of opioids in inflammation?

Three important factors to consider when addressing this problem are:-

i) The speed at which the enhancement in potency occurs.

The enhancement in the spinal potency of the exogenously administered opioids occurred within three hours of the injection of carrageenan into the paw, and when the potency of the mu opioid agonist morphine was tested just one hour after the injection of carrageenan, a substantial shift in spinal potency had already occurred.

ii) The localization of the enhancement in potency to those dorsal horn neurones receiving input from the inflamed paw.

The potency of morphine was unaltered (compared to normal animals) three hours after the injection of carrageenan into the contralateral hind paw, indicating that the changes leading to the enhancement in the spinal potency of morphine are not bilateral.

iii) The preferential enhancement in the spinal potency of the mu opioid agonist morphine

Although the potency of all the opioids tested was enhanced to an extent, the mu opioid morphine showed a 6-fold greater increase in spinal potency compared with the delta and kappa opioids tested.

The finding that the potency of agonists acting at the three opioid receptor classes is not uniformly enhanced following carrageenan inflammation suggests that specific changes in spinal systems are being induced by the development of inflammation in the periphery. Generalized changes in nociceptive systems such as altered transmitter release, levels of activity in the primary afferents etc. are unlikely to underlie the changes in the ability of the opioids to inhibit nociceptive transmission since this would be expected to produce more or less equal changes in the potency of all three agonists tested. The lack of correlation between the change in the neuronal response induced by carrageenan and the subsequent ability of the opioids to inhibit this neuronal response also argues against the alterations in nociceptive transmission being directly responsible for the changes in opioid potency.

What about changes in the opioid receptors themselves, either in terms of number or affinity? The rapid onset of the enhancement in opioid potency (within one hour of the injection of carrageenan in the case of morphine) argues against an upregulation in the

number of spinal opioid receptors resulting from increased synthesis. Increased axonal transport of opioid receptors to the periphery, and so presumably also to the central terminals of these primary afferents, has been shown to occur following the intraplantar injection of Freund's adjuvant (Hassan et al., 1993). However, this process is relatively slow and does not lead to a significant increase in opioid binding sites in the paw until 3-4 days after injection, too slow to explain the enhanced potency of spinal morphine even taking into an account the shorter distance between the ganglion and central terminal. Indeed, the majority of binding studies in animals with inflammation show no change in mu, delta or kappa opioid receptor number or affinity in the spinal cord even after several weeks of inflammation (Cesselin et al., 1980; Iadarola et al., 1988a; Ruda et al., 1988; Delay-Goyet et al., 1989). A few studies have reported complex changes in opioid receptor binding in the spinal cords of arthritic rats (Millan et al., 1986; Besse et al., 1992). The study by Millan et al. (1986), conducted three weeks after the onset of adjuvant-induced polyarthritis, used the "universal" opioid ligand diprenorphine and demonstrated a relative decrease in the proportion of κ- as compared to μ-opioid receptors in the spinal cord, although no modification in the total number of opioid binding sites was seen. The recent study by Besse et al. (1992) used selective ligands for the three opioid receptor subtypes to examine modifications in opioid receptor binding 2-6 weeks after the onset of monoarthritis. Complex changes in opioid receptor binding were found, including an increase in the specific binding of the mu agonist DAMGO and the delta agonist DPDPE at two weeks, the earliest time point tested. It is possible that this increase in mu binding, which appeared to be greater than that observed with the delta ligand, may in fact be present much earlier in the inflammatory process and hence potentially explain the increase in morphine potency. However, even if these changes could occur in the time scale of the present study, the increase in binding seen in the study of Besse et al. (1992) was bilateral, with no difference found between the ipsilateral and contralateral sides of the cord. It is therefore difficult to envisage how this could explain the unilateral increase in opioid potency seen in the present study following carrageenan.

Binding studies only look at the number of sites at which opioids are bound and are unable to tell us whether these in fact represent functionally coupled receptors. Therefore, it is conceivable that whilst it appears that no increase in the number opioid receptor binding sites occurs during inflammation, an increase in the proportion of functionally coupled opioid receptors may be triggered by spinal events in inflammation leading to an increased ability of opioids to inhibit nociceptive transmission. One way of testing this hypothesis would be to look at the ability of opioids to stimulate GTP hydrolysis in membranes prepared from the spinal cords of normal rats and those taken from rats with a peripheral inflammation. Koski and Klee (1981) showed that opioids

inhibit adenylate cyclase by stimulating GTP hydrolysis and this technique could be used to examine the ability of opioids to evoke post-receptor events following inflammation. Any enhancement in GTP hydrolysis in membranes prepared from rats with peripheral inflammation, in the absence of changes in opioid receptor binding, would suggest (in this isolated system) that the proportion of functionally coupled receptors is altered following the development of inflammation.

Both the central (this chapter) and the peripheral (Hargreaves et al., 1988b; Joris et al., 1990) changes in opioid sensitivity have been shown to occur within 2 hours of the onset of carrageenan-induced inflammation. Hence it appears that both the peripheral and central changes develop in parallel so is there a common mechanism?

One possibility is that the opioid receptors present on the peripheral C-fibre terminals in normal animals are covered by the perineurium, rendering them inaccessible to opioid agonists and thus non-functioning in normal animals. In inflammation the conditions surrounding the nerve fibre terminal change, particularly the pH of the environment, and this may lead to altered drug access possibly as a result of the opening of tight junctions in the perineurium allowing the opioid agonists to reach and therefore activate the receptors (C. Stein, personal communication). This scenario would not occur at the central terminals of the C-fibres therefore there must be another explanation for the enhancement in the potency of spinal opioids, most likely resulting from pharmacological means rather than anatomical changes.

There are two transmitter systems in the spinal cord which could lead to the enhancement in spinal opioid potency observed, based on their ability to preferentially affect the operation of the mu opioid receptor.

The first is based on the ability of the endogenous spinal peptides, cholecystokinin and FLFQPQRFamide to reduce spinal mu (Faris et al., 1983; Tang et al., 1984b; Wiesenfeld-Hallin and Duranti, 1987; Barbaz et al., 1989; Magnuson et al., 1990; Kellstein et al., 1991) but not delta (Magnuson et al., 1990; Wang et al., 1990) or kappa opioid actions (Barbaz et al., 1989, but see Wang et al., 1990). A selective increase in mu opioid potency could then be predicted if there was a depletion or a reduced release of these "anti-opioid" peptides in the inflammatory state. Changes of this nature could happen in the short timescale of the altered opioid potency. The possibility that CCK is a factor in the enhanced spinal potency of morphine following carrageenan inflammation is investigated in chapter 7. The lack of any antagonist of FLFQPQRFamide made investigation of the role of this peptide in inflammation impossible. Some authors have used antibodies to this peptide to investigate the actions of endogenous FLFQPQRFamide (Tang et al., 1984b) but there must be some doubt as to the ability of these to penetrate the spinal cord after intrathecal application.

The other possible mechanism is that the development of carrageenan inflammation

leads to an increase in the activity descending noradrenergic systems. Alpha$_2$-adrenoceptor agonists such as clonidine, dexmedetomidine and noradrenaline potentiate the antinociceptive effects of mu agonists such as morphine (Yaksh and Reddy, 1981; Hylden and Wilcox, 1983; Sullivan et al., 1987; Wilcox, et al. 1987; Loomis, et al. 1988; Ossipov et al., 1990; Plummer et al., 1992; Sullivan et al., 1992a) but not those of delta (Sullivan et al., 1992a, but see Omote, et al. 1991; Roerig, et al. 1992) or kappa (Gordon et al., 1992) opioid agonists. The involvement of noradrenergic mechanisms in the enhanced effects of spinal opioids has recently been proposed on the basis of the use of carrageenan in behavioural studies (Hylden et al., 1991b). Although the finding that the enhancement in morphine potency is restricted to the inflamed side would appear to make alteration in descending controls, which would affect both sides of the cord equally, unlikely, the possibility that the descending noradrenergic system do play a role in the enhancement in the potency of morphine after carrageenan inflammation is investigated in chapter 8.

5.3.2. Absence of low dose opioid facilitations after carrageenan inflammation

Although the predominant action of opioids is an inhibition of nociception, the spinal administration of low doses of mu and kappa opioids in normals animals leads to the facilitation of nociceptive transmission (Dickenson and Sullivan, 1986; Sullivan and Dickenson, 1991; Wiesenfeld-Hallin et al., 1991; this chapter). Interestingly, in the carrageenan animals both morphine and the kappa agonist U69593 were purely inhibitory over the range of doses studied.

The mechanism behind these excitatory effects produced by low doses of mu and kappa opioids in normal animals is unclear. One interpretation of these effects is based on a disinhibition mechanism involving presynaptic inhibition of inhibitory interneurones. Although this mechanism could explain the facilitations of the C-fibre evoked response of convergent dorsal horn neurones seen in normal animals in this model, dose-dependent dual excitatory and inhibitory effects of opioids have been seen in isolated dorsal root ganglion neurones where this disinhibition mechanism could not exist (Shen and Crain, 1989). It has been proposed (Crain and Shen, 1990), and recently demonstrated (Cruciani et al., 1993) that opioid receptors can be linked to both inhibitory and excitatory G proteins, leading to either increased or decreased K^+ (μ and δ) or Ca^{2+} (κ) conductance. The excitatory effect of low doses of opioids could then result from an increased release of excitatory transmitters from the presynaptic terminal, an effect that has been reported to be responsible for the facilitation of the spinal flexor reflex by low

doses of intrathecal morphine (Wiesenfeld-Hallin et al., 1991). Whatever the mechanism behind the excitatory effects of opioids, all studies, whether in vitro (Shen and Crain, 1989) or in vivo (Dickenson and Sullivan, 1986; Wiesenfeld-Hallin et al., 1991; this chapter), show these excitatory effects to be linked with low doses of opioid agonists, with inhibitory effects predominating as the dose is increased. The trigger for the switch between excitatory and inhibitory effects of opioids is unknown although it may be the case that at higher doses of opioids, both the excitatory and inhibitory effects occur, with the inhibitions masking the excitations.

Whatever the mechanism of the low dose facilitations, changes are induced by the development of carrageenan inflammation such that these facilitatory events no longer occur following spinal opioid administration. Paradoxical hyperalgesia following low doses of morphine has been reported in arthritic animals (Kayser et al., 1987; Kayser and Guilbaud, 1990). However this effect occurred following systemic administration of morphine and was only seen using the centrally integrated nociceptive test (vocalization threshold) and not when using the spinally co-ordinated paw withdrawal test (Kayser and Guilbaud, 1990). Thus it is likely that this hyperalgesic effect of low doses of systemic morphine results from actions at supraspinal, rather than spinal sites. In support of the facilitatory effects of opioids in animals with inflammation being mediated at non-spinal sites is the finding in this study that whilst spinal opioids did not produce facilitations in the carrageenan animals, a dose of 10μg/kg of morphine given i.v. did appear to produce a facilitation of the C-fibre evoked response in these animals.

5.3.3. What triggers the changes in spinal opioid potency?

The trigger for the central changes which lead to the enhancement in opioid potency is not obvious. In some other models of inflammatory nociception such as the formalin model, the dorsal horn neurones develop ongoing activity (Dickenson and Sullivan, 1987b), providing an obvious candidate for the driving force behind changes in central nociceptive processing. The acute effect of carrageenan injection on neuronal activity is only a brief period of firing of the cells and then the slower development of either increases of decreases in the evoked cell responses (chapter 3). No ongoing activity develops either in the convergent dorsal horn neurones (chapter 3) or in the primary afferents (Heapy et al., 1987). Thus, unless subthreshold depolarizations are responsible, another explanation must be sought.

One possibility for the driving force behind the central changes in opioid potency involves the retrograde transport of nerve growth factor (NGF) from the inflamed tissue (see section **1.8**). An enhanced retrograde transport of NGF within the sciatic nerve has

been reported within 24 hours of the injection of Freund's adjuvant into the hind paw, and this has been linked with an increased synthesis of neuropeptides within the dorsal root ganglia (Donnerer et al., 1992). This study demonstrated that the calcitonin gene-related peptide content of the dorsal root ganglia is increased as early as 12 hours (the earliest time point tested) after the injection of Freund's adjuvant in the paw, with substance P levels increasing within days. The inflammation-induced increases in both peptides were blocked by the prior administration of anti-NGF (Donnerer et al., 1992).

Thus it is conceivable that an increase in the peptide content of primary afferent neurones in inflammation, stimulated by the uptake and retrograde transport of NGF from the inflamed tissue, may lead to an increase in the release of these peptides at the central terminals. This may in turn trigger changes in spinal nociceptive systems leading to enhanced opioid actions. The question is whether the time scale of the enhancement in opioid potency would allow for these changes to occur since following the development of inflammation in the periphery, NGF would have to be retrogradely transported to the ganglion, peptide synthesis increased, and then the increased peptide content transported to the central terminals. It seems highly unlikely that all this could occur within the hour following the injection of carrageenan, after which the changes in opioid potency are evident.

Although the increased retrograde transport of NGF following the development of inflammation may not be fast enough to produce alterations in primary afferent peptide levels, Garry and Hargreaves (1992) have demonstrated an increase in both the spontaneous and capsaicin evoked release of SP and CGRP from spinal dorsal horn slices taken from rats following 3 hours of carrageenan inflammation. Thus although the mechanism behind these changes is not known, this enhanced peptide release in inflammation may trigger central changes. In chapter 3 complex changes in the electrically-evoked responses of dorsal horn neurones following the injection of carrageenan were described, whilst chapter 4 discusses the changes in the role of the NMDA receptor in spinal nociceptive processing in the carrageenan animals. Events of this nature could be involved in initiating the changes in the systems underlying the enhancement in spinal opioid potency. As will be seen in chapter 7, a reduction in the spinal levels of the anti-opioid peptide cholecystokinin seems to be an important factor in the observed selective increase in the spinal potency of morphine in the carrageenan animals. It is not known whether this change result from altered afferent peptide release, spinal intrinsic mechanisms (e.g. NMDA receptors) or the other factors discussed here. Thus, at the present time, the link between the development of inflammation in the periphery and the changes in spinal nociceptive systems remains unclear.

CHAPTER 6

SPINAL ANTINOCICEPTIVE POTENCY OF THE $GABA_B$ AGONIST BACLOFEN IN NORMAL AND CARRAGEENAN ANIMALS

6.1. Introduction

In the previous chapter, it was shown that the antinociceptive potency of agonists acting on one of the inhibitory systems in the spinal cord, the opioid system, is enhanced in animals with carrageenan-induced inflammation. It was therefore of interest to see if the potency of an agonist acting on another inhibitory system in the spinal cord, the GABAergic system, is similarly altered in inflammation.

The GABAergic system in the spinal cord is well sited to modulate nociceptive transmission. Studies in the rat using antisera to glutamate decarboxylase (GAD, the rate limiting enzyme in the biosynthesis of GABA) which is localized in the terminals of GABAergic neurones (Hunt et al., 1981), and more recently antisera against GABA itself (Magoul et al., 1987; Todd and McKenzie, 1989; Todd and Sullivan, 1990) have shown that the highest concentrations of these immunoreactive cells are found in the superficial part of the dorsal horn (laminae I-III), with lower levels present in the deeper laminae. In the superficial laminae, between 28-46% of the neurones show GABA-like immunoreactivity (Todd and Sullivan, 1990). The profile of many of these GABAergic neurones is that of islet cells (Todd and McKenzie, 1989) which are considered to be inhibitory interneurones by virtue of the morphology of their axonic processes (Gobel, 1978). GABAergic neurones make synaptic contacts both pre and post-synaptic to primary afferent neurones, with many axo-axonic contacts found between GABAergic neurones and terminals of primary afferent origin (Barber et al., 1978; Magoul et al., 1987).

GABA receptors can be divided into $GABA_A$ and $GABA_B$ subtypes, with $GABA_A$ receptors being linked to chloride channels (Bowery, 1982), whilst activation of $GABA_B$ receptors reduces calcium currents (Désarmenien et al., 1984) and increases K^+ conductance (Newberry and Nicoll, 1985). Although $GABA_A$ receptors are found in large numbers in the spinal cord, including the terminals of C- and Aδ-fibres (Désarmenien et al., 1984), the difficulty in separating the analgesic effects of agonists acting at this receptor from effects on motor function (Hammond and Drower, 1984) has led to attention being focussed on the ability of $GABA_B$, rather than $GABA_A$, agonists to produce analgesia at the level of the spinal cord.

$GABA_B$ receptors are located throughout the grey matter of the spinal cord, both pre- (50%) and post-synaptic to primary afferent terminals (Price et al., 1984, 1987). Price et al. (1987) demonstrated that a greater reduction in $GABA_B$ binding in lamina IV occurs following dorsal rhizotomy than following capsaicin treatment, suggesting $GABA_B$ receptors are located on the terminals of large diameter Aβ-fibres as well as on C- and Aδ-fibres (Désarmenien et al., 1984; Price et al., 1984, 1987). In contrast to

$GABA_A$ agonists, $GABA_B$ agonists have been shown to produce analgesia in behavioural studies following intrathecal administration in rats (Wilson and Yaksh, 1978; Hammond and Drower, 1984; Sawynok and Dickson, 1985; Aran and Hammond, 1991), cats (Wilson and Yaksh, 1978) and monkeys (Yaksh and Reddy, 1981) at doses that do not produce motor impairment. In addition, the intrathecal (Dickenson et al., 1985) or intravenous (Piercey and Hollister, 1979; Henry, 1982; Dickenson et al., 1985) administration of the $GABA_B$ agonist baclofen inhibits the nociceptive responses of dorsal horn neurones in electrophysiological studies. Thus the location and function of the $GABA_B$ receptor to some extent, acts as a comparator for inflammation-induced changes in mu opioid systems.

Despite this clear evidence for antinociceptive actions of the $GABA_B$ agonist baclofen across a range of animals species, analgesia in humans following baclofen administration is less clear. Baclofen has been shown to reduce pain in patients with spasticity, however this may be a result of muscle relaxation rather than an analgesic effect of baclofen in its own right (Pinto et al., 1972). Thus humans may show differences from a range of other animal species with regard to baclofen analgesia.

This chapter examines the spinal potency of the $GABA_B$ agonist baclofen in normal animals and compares this with the potency of the drug three hours after the injection of carrageenan into the hind paw in order to assess whether the potency of this drug is altered following the development of peripheral inflammation.

6.2. Results

6.2.1. Effects of intrathecal baclofen in normal animals

The effects of L-baclofen, the active isomer, (0.01-5μg), applied intrathecally, on the C- and Aβ-fibre evoked response of dorsal horn neurones were tested in normal, non-inflamed animals. A slight, non-significant facilitation of the C-fibre evoked response of the neurones was seen with the lowest dose tested (0.01μg) which then turned to a dose dependent inhibition as the dose was increased (figure **6.1**). The highest dose of baclofen tested (5μg) produced a maximum inhibition of the C-fibre evoked response of 75.6 ± 10.9% (n=8). The Aβ-fibre evoked response of the neurones was inhibited to a lesser extent, with a maximum inhibition of 28.6 ± 8.2% being produced by 5μg (n=7).

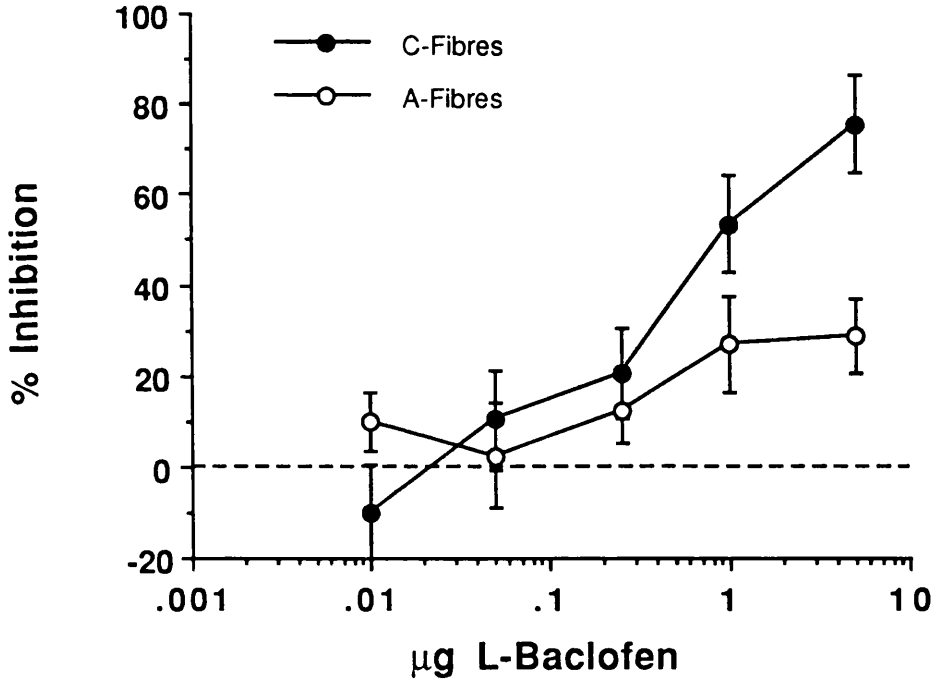


Figure 6.1. Dose-response curve for the inhibition of the C- and Aβ-fibre evoked response of dorsal horn neurones by intrathecal L-baclofen in normal animals. n=7-8 neurones per dose.

Due to the lack of a definite plateau at the top of the dose-response curve, it was not appropriate to determine the ED_{50} value using the GraphPad curve-fitting program. However, the dose of baclofen required to produce a 50% inhibition of the C-fibre

evoked response in normal animals was estimated from the graph to be 0.9μg.

D-Baclofen, the inactive isomer of baclofen was also tested on the responses of the dorsal horn neurones to check that the effects of L-baclofen were due to a specific interaction with $GABA_B$ receptors. 30μg of D-baclofen, a dose 6 times higher than the dose of L-baclofen which produced a 75% inhibition of the C-fibre evoked response, had no effect on the C-fibre evoked response (96.5 ± 3.0% of control, n=8).

No attempt was made to reverse the neuronal inhibitions produced by L-baclofen with a $GABA_B$ antagonist.

6.2.2. Effects of intrathecal baclofen in carrageenan animals

L-Baclofen (0.01-2.5μg) produced dose dependent inhibitions of the C- and Aβ-fibre evoked responses of dorsal horn neurones when applied to the spinal cord three hours after the injection of carrageenan into the ipsilateral hind-paw. As in normal animals, the inhibition of the C-fibre evoked response of the neurones was greater than that observed with the Aβ-fibre evoked response, with the highest dose of baclofen tested in these animals (2.5μg) producing an inhibition of 69.8 ± 10.2% (n=8) of the C-fibre response compared with 20.0 ± 15.8% of the Aβ-fibre response (figure **6.2**).

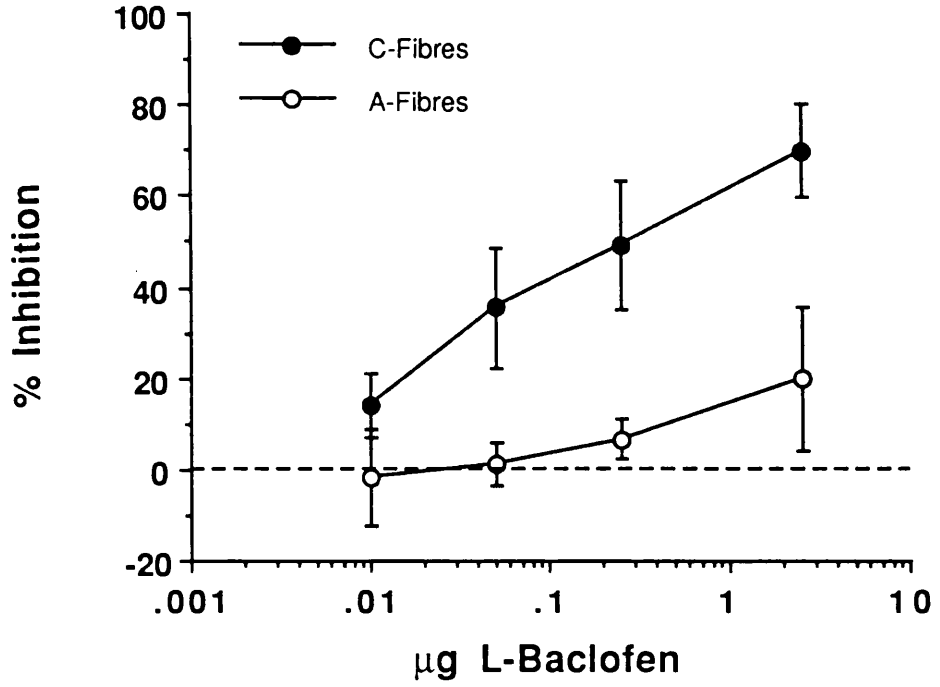


Figure 6.2. Dose-response curve for the inhibition of the C- and Aβ-fibre evoked response of dorsal horn neurones by intrathecal L-baclofen 3 hours after the injection of carrageenan into the ipsilateral hind paw. n=7-8 neurones per dose.

Due to the shape of the dose-response curve for baclofen in the carrageenan animals, it was not appropriate to perform a curve fit to obtain an ED_{50} value. Thus an estimation of the dose of L-baclofen required to produce a 50% inhibition of the C-fibre response was made from the graph (figure **6.2**). In the carrageenan animals, this dose was estimated as 0.3μg.

6.2.3. Comparison of the effects of intrathecal baclofen in normal and carrageenan animals

When the estimated doses required to produce a 50% inhibition of the C-fibre evoked response in normal (0.9μg) and carrageenan (0.3μg) animals are compared, it can be seen that intrathecal baclofen is 3-fold more potent in the carrageenan animals. Although the dose-response curve for baclofen in carrageenan animals lies to the left of that in normal animals, the shift in the curve is not parallel (figure **6.3**), with the curve becoming flattened in the carrageenan animals.

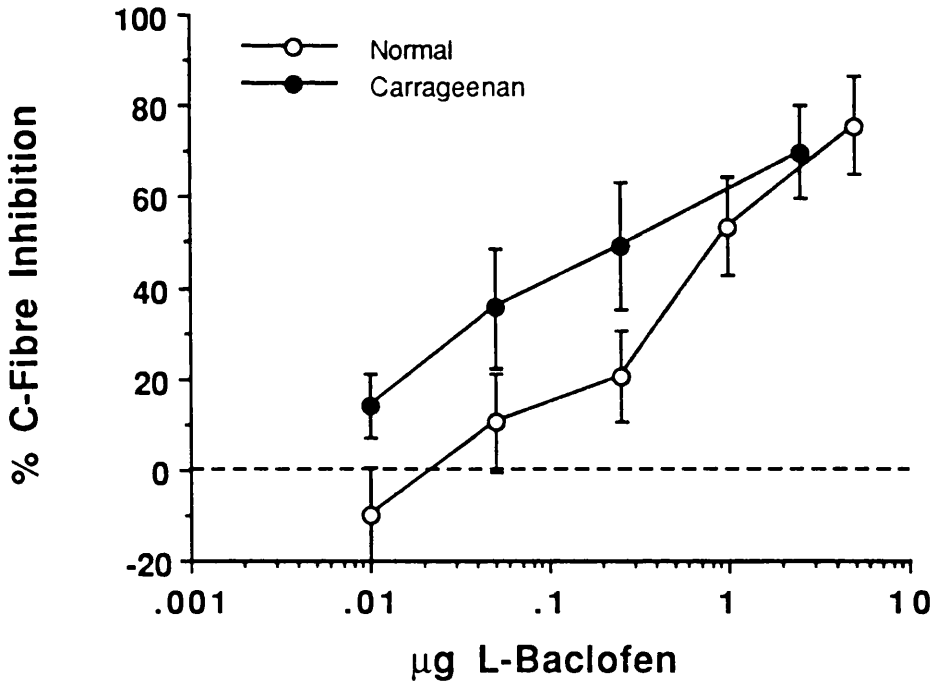


Figure 6.3. Comparison of the dose-response curves obtained for the inhibition of C-fibre evoked responses by i.t. baclofen in normal animals and 3 hours post- carrageenan. There is a non-parallel leftward shift in the dose response curve for baclofen in the carrageenan animals, resulting in a 3-fold reduction in the dose of baclofen required to produce a 50% inhibition of the C-fibre evoked response in these animals.

6.3. Discussion

The spinal potency of the $GABA_B$ agonist baclofen in animals with carrageenan-induced inflammation is enhanced compared with that seen in normal animals. Thus as well as the enhanced opioid potency seen in the carrageenan animals, an increase in the potency of an agonist acting at another inhibitory system within the cord, the GABAergic system, is enhanced, albeit modestly, following the development of carrageenan inflammation. The 3-fold enhancement seen in the spinal potency of baclofen in the carrageenan animals contrasts with the dramatic 18-fold enhancement in the spinal potency of the μ-opioid morphine in these animals (chapter 5). This again suggests that a very specific change is occurring in spinal systems which interact preferentially with the operation of the μ-opioid receptor. The 3-fold shift seen in the spinal potency of baclofen and the δ-and κ-opioids (chapter 5) suggests that there is probably another mechanism at work, possibly this time involving a more general but unknown mechanism. Interestingly, the non-parallel nature of the shift in the dose-response curve for the inhibition of the C-fibre evoked neuronal response by baclofen post-carrageenan is very similar to that seen in the dose-response curves for both morphine (chapter 5) and the alpha-2 adrenoceptor agonist dexmedetomidine (chapter 8) in the carrageenan animals.

Does the role of GABA in the spinal cord change in protracted pain states?

Studies have demonstrated an increase in the levels of glutamic acid decarboxylase (Nahin and Hylden, 1991) and in the levels of GABA itself in the spinal cord following the development of unilateral inflammation induced by Freund's adjuvant (Castro-Lopes et al., 1992) or carrageenan (Castro-Lopes et al., 1994). This upregulation in the levels of GABA in the spinal cord following the development of carrageenan inflammation, although not significant until 4 days post-injection, (Castro-Lopes et al., 1994), combined with the enhancement in potency (as shown in the present study) may lead to an enhanced GABAergic control of spinal nociceptive mechanisms, possibly to help counter the increased nociception associated with this pain state.

It was suggested in chapter 3 that increased activation of the NMDA receptor post-carrageenan may lead to the recruitment of inhibitory systems to help counter the consequences of NMDA receptor activation. One such inhibitory system which could be involved is the GABAergic system within the cord, particularly in view of the apparent upregulation in this system following carrageenan inflammation. Studies with a selective $GABA_B$ antagonist in normal and carrageenan animals would help to reveal the actions of the endogenous GABAergic system in the control of spinal nociceptive mechanisms in these animals, although as yet the status of available $GABA_B$ antagonists remains unclear.

CHAPTER 7

CHOLECYSTOKININ AS A FACTOR IN THE ENHANCED POTENCY OF MORPHINE FOLLOWING CARRAGEENAN INFLAMMATION

7.1. Introduction

The peptide cholecystokinin (CCK) was first isolated from porcine intestine (Mutt and Jorpes, 1968) but it was not until the mid-1970's that Vanderhaeghen and colleagues first described CCK-like immunoreactivity in the brain (Vanderhaeghen et al., 1975). Although widely distributed within the CNS, the function of CCK in the brain and spinal cord remains relatively unclear. CCK has been suggested to act as a neuromodulator, or even a neurotransmitter since it is able to excite neurones in many regions of the brain (Dodd and Kelly, 1981; Chiodo and Bunney, 1983; Denavit-Saubié et al., 1985; Hommer et al., 1985; Boden and Hill, 1988; Bohme et al., 1988) and in the dorsal horn of the spinal cord (Jeftinija et al., 1981a; Willetts et al., 1985). Cholecystokinin is known to play a role in satiety, anxiety, and to interact with dopaminergic mechanisms in the brain (see refs. in Lindefors et al., 1993). However it is the role played by CCK in nociception, via the ability of the peptide to negatively modulate the antinociceptive effects of opioids that forms the basis for the studies in this chapter.

The predominant form of cholecystokinin found in the mammalian central nervous system is the sulphated octapeptide (CCK-8) (Dockray, 1976; Rehfeld, 1978) and there is considerable evidence that this fragment may play an important role in pain transmission by modulating CNS opioid mechanisms. This was first proposed following the demonstration that small doses of CCK suppress β-endorphin-induced analgesia in the rat hot-plate test (Itoh et al., 1982) and reduce morphine analgesia in the rat tail flick test (Faris et al., 1983). In addition, the weak CCK receptor antagonist proglumide was shown to enhance opioid analgesia (Watkins et al., 1984). Thus a potential physiological role for CCK in the modulation / control of opioid induced antinociception in the normal animal was established.

It appears that modifications leading to an increase in the functional activity of CCK systems may be responsible for the diminished antinociceptive potency of opioids seen in certain conditions: for example, CCK has been proposed to be a factor in the development of opioid tolerance based on the evidence that CCK receptor antagonists can either reverse or prevent morphine tolerance (Watkins et al., 1984; Tang et al., 1984a; Panerai et al., 1987; Dourish et al., 1988, 1990; Xu et al., 1992). More recently, an upregulation in CCK levels has been implicated in another condition where there is reduced sensitivity to the antinociceptive effects of morphine, this time in an animal model of neuropathic pain (Xu et al., 1993, and see discussion).

So what about situations where there is an *enhanced* sensitivity to opioids, such as that seen after inflammation? Features of the enhancement in the antinociceptive potency of spinal opioids following carrageenan-induced inflammation suggest that

alterations in the functional activity of CCK may also underlie this phenomenon, although this time resulting from a decrease rather than an increase in activity. There is good evidence from studies using exogenous CCK itself and CCK receptor antagonists that in the normal animal, physiological levels of CCK can interfere with the actions of μ-opioid agonists such as morphine at the level of the spinal cord (Tang et al., 1984a; Watkins et al., 1984, 1985; Wiesenfeld-Hallin and Duranti, 1987; Magnuson et al., 1990; Kellstein et al., 1991; Zhou et al., 1993). Although not so extensively studied, this negative modulation of opioid actions by CCK does not appear to extend to opioids acting at δ- (Magnuson et al., 1990; Wang et al., 1990) or κ- (Barbaz et al., 1989, but see Wang et al., 1990) opioid receptors. This same pattern of selectivity was seen with the potency of spinal opioids following carrageenan inflammation. In both the electrophysiological study presented in chapter 5, and to a more modest extent in the behavioural study of Hylden et al. (1991b) in animals with carrageenan inflammation, spinal mu opioid agonists showed a far greater enhancement in antinociceptive potency than either delta or kappa selective opioid agonists.

The questions addressed in this chapter are

i) what is the physiological role of spinal CCK in normal animals?

ii) is this role different in the inflammatory state?

iii) do reductions in the activity of spinal CCK systems lead to the enhancement in the potency of spinal morphine seen following carrageenan inflammation?

The majority of the CCK receptors in the brain of rodents, primates and man are of the CCK_B receptor type (Hill & Woodruff, 1990). However in the spinal cord, species differences occur with regard to predominant CCK receptor type found. In primates and man, the predominant spinal CCK receptor is the CCK_A receptor (Hill et al., 1988; Hill and Woodruff, 1990; Ghilardi et al., 1992). In rats however, the spinal cholecystokinin receptor is the CCK_B receptor (Hill et al., 1988; Hill and Woodruff, 1990; Ghilardi et al., 1992). Interestingly, in both the rat and primate, the pre-synaptically located CCK receptors are CCK_B receptors (Ghilardi et al., 1992). The differences in spinal CCK receptor types between rodents and primates may then be less obvious than suggested by some early accounts (see Baber et al., 1989).

Thus in the studies presented in this chapter, sulphated CCK-8 and the selective CCK_B receptor antagonist L-365,260 (Dourish et al., 1990) were used to investigate whether physiological levels of CCK exert a tonic inhibition of the antinociceptive effect of intrathecal morphine in normal (control) animals and whether the same is true in animals following the development of carrageenan-induced inflammation.

In addition, the CCK_B receptor antagonist L-365,260 was used to investigate whether the release of CCK, which is capable of exciting neurones in the spinal cord (Jeftinija et al., 1981a; Willetts et al., 1985; Magnuson et al., 1990), is responsible for the facilitations of the evoked response seen with low doses of morphine in normal animals but absent in animals with carrageenan induced inflammation (chapter 5).

7.2. Results

The convergent neurones used in this study were located in the deeper laminae of the dorsal horn (500-1000μm, mean depth = 738 ± 31μm (n=45)), and formed a similar population to those used to investigate the potency of morphine in normal animals and in animals following the development of carrageenan inflammation (chapter 5).

7.2.1. Lack of effect of the CCK_B receptor antagonist L-365,260 on spinal nociceptive transmission in normal animals

The selective CCK_B receptor antagonist L-365,260 used in the present study has been shown previously to have a bell-shaped dose-response curve (Dourish et al., 1990). The maximum effective dose of L-365,260, given subcutaneously in rats, was shown by Dourish et al. (1990) to be 0.2mg/kg, thus this dose was chosen for the present studies.

The subcutaneous administration of 0.2mg/kg of L-365,260 alone in normal animals (n=4) produced a modest inhibition (14.0 ± 3.2%, n=4) of the C-fibre evoked response 10 minutes post-injection. This inhibition was transient, with the C-fibre evoked response returning to control levels by 20 minutes, and remaining there for the rest of the 90 minute time course over which the response of the neurone was followed (figure **7.1**). No effect was seen on the Aβ-fibre evoked response. In some animals, the effects of administration of the vehicle alone were examined, and this was also found to be without effect on the evoked neuronal responses (n=4, figure **7.1**).

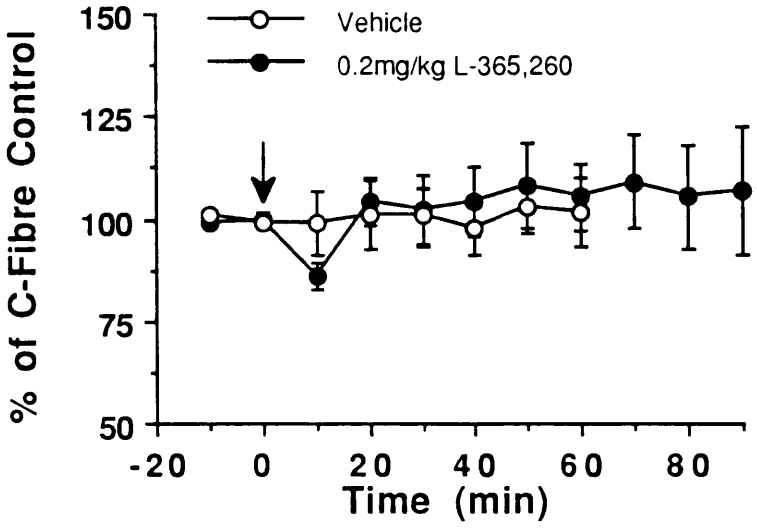


Figure 7.1. Lack of effect of the subcutaneous administration of 0.2mg/kg L-365,260 or vehicle alone (injected at t=0) on the C-fibre evoked responses of dorsal horn neurones in normal animals. n=4 neurones per group.

7.2.2. Effect of the CCK_B antagonist L-365,260 on the antinociceptive potency of intrathecal morphine in normal animals

The intrathecal administration of 0.25 and 10μg of morphine in normal animals produced inhibitions of the C-fibre evoked response of dorsal horn neurones of 7.9 ± 9.1% (n=7) and 81.3 ± 6.5% (n=6) respectively (chapter 5 and figure **7.2**). When these two doses of intrathecal morphine were administered following the subcutaneous injection of 0.2mg/kg of L-365,260 (10 minute pre-treatment), the antinociceptive potency of both doses was significantly enhanced. This produced a leftward shift in the dose response curve with the degree of inhibition of the C-fibre evoked response produced by 0.25μg of morphine being enhanced to 34.7 ± 9.3% (Student's t-test, 1-tailed p-value = 0.033; n=6) and that produced by 10μg to 99.4 ± 0.6% (Alternate Welch t-test, 1-tailed p-value = 0.020; n=5) (figure **7.2**).

Intrathecal naloxone (5μg) reversed the morphine induced inhibitions of the C-fibre evoked response to 84.3 ± 12.1% of control values within 20 minutes.

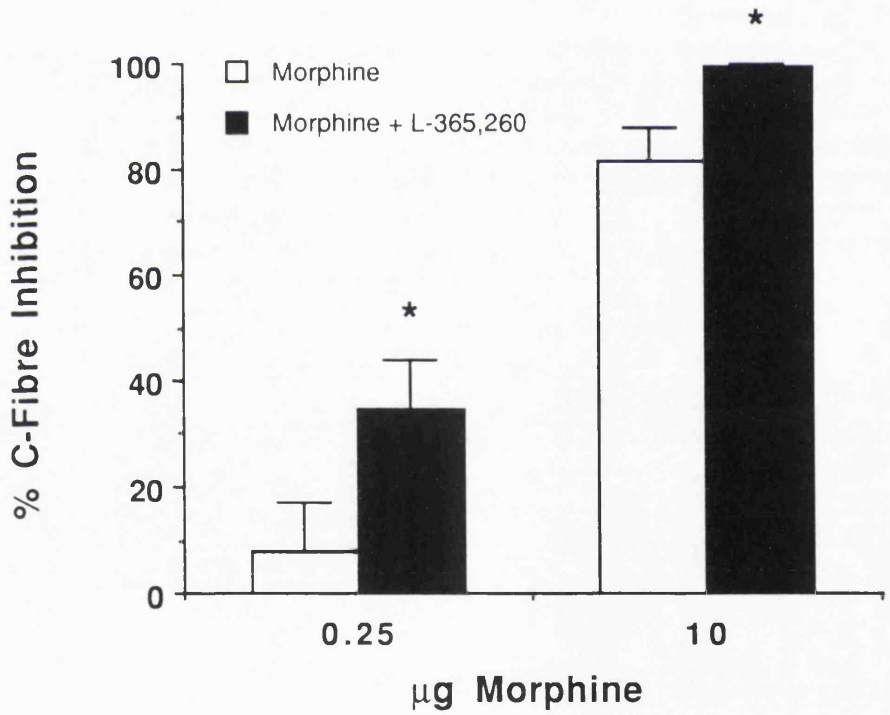


*Figure 7.2. The effect of 0.2mg/kg L-365,260 on the morphine-induced inhibitions of the C-fibre evoked response of dorsal horn neurones in normal animals. In these animals, L-365,260 produces a significant enhancement in the potency of intrathecal morphine. n=5-7 neurones per group. * p<0.05.*

In contrast to the enhancement in the potency of morphine against the C-fibre evoked response of the neurones in the presence of L-365,260, the inhibition of the Aβ-fibre evoked response by intrathecal morphine was unaffected by the CCK_B receptor antagonist (inhibition of the Aβ-fibre evoked response produced by 10μg of morphine alone 11.6 ± 15.0% versus 10.3 ± 8.8% in the presence of L-365,260).

These results indicate that in normal (control) animals the levels of endogenous CCK released physiologically in the dorsal horn are sufficient to significantly attenuate the antinociceptive effects of intrathecal morphine.

7.2.3. Effect of the CCK_B antagonist L-365,260 on the antinociceptive potency of intrathecal morphine in animals with carrageenan inflammation

In order to investigate whether endogenous CCK also attenuates the antinociceptive effects of intrathecal morphine in animals with carrageenan inflammation, two doses of morphine were chosen which produced a similar degree of inhibition of the C-fibre evoked response to those used in the normal animals. The dose response curve for intrathecal morphine is shifted to the left in animals with carrageenan-induced inflammation (chapter 5) hence these doses of morphine are lower than those used in the study undertaken in normal animals (section **7.2.2**).

Intrathecal administration of 0.01 and 2.5μg of morphine 3 hours after the injection of carrageenan into the paw produced inhibitions of the C-fibre evoked response of 11.5 ± 9.5% (n=5) and 75.4 ± 8.2% (n=9) respectively (chapter 5 and figure **7.3**). When these doses of morphine were applied cumulatively to the spinal cord 10 minutes after the subcutaneous administration of 0.2mg/kg of L-365,260, no significant enhancement was found in the antinociceptive potency of morphine (inhibitions of 12.0 ± 3.7 (n=6) and 85.2 ± 11.0 (n=5) respectively, figure **7.3**) which is in contrast to the situation in normal animals (figure **7.2**). The inhibition of the Aβ-fibre evoked response by morphine was similarly unaffected by the presence of L-365,260 in the carrageenan animals. The intrathecal administration of 1μg of naloxone reversed the inhibitions produced by 2.5μg of morphine to 78 ± 10% of control values within 20 minutes.

These findings suggest that three hours after the injection of carrageenan into the paw, a physiological change has occurred such that endogenous CCK no longer exerts an inhibitory control on the antinociceptive actions of spinal morphine in these animals.

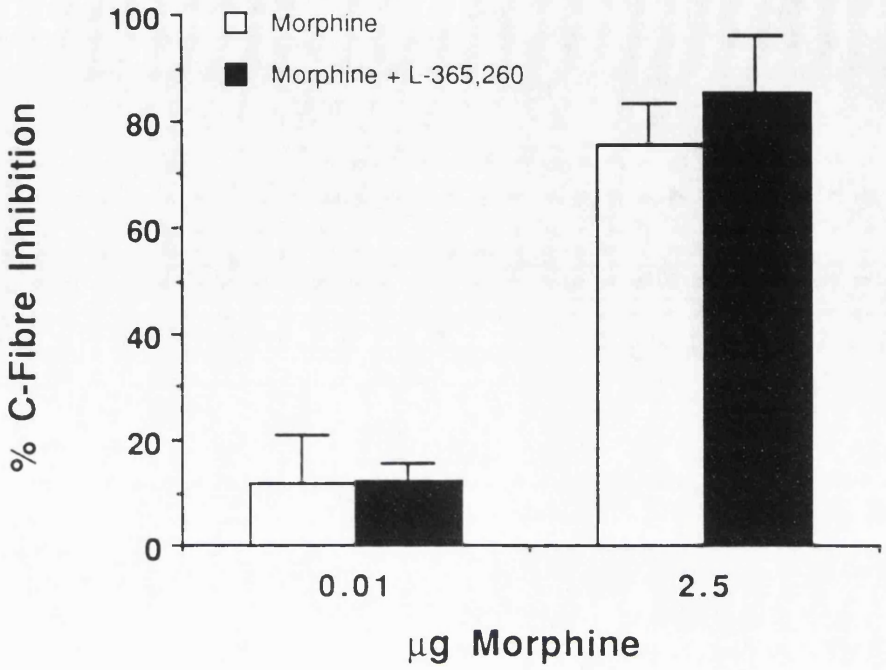


Figure 7.3. The effect of 0.2mg/kg L-365,260 on the morphine-induced inhibitions of the C-fibre evoked response of dorsal horn neurones in animals with carrageenan inflammation. In these animals with peripheral inflammation of 3 hours duration, L-365,260 no longer potentiates the antinociceptive effect of intrathecal morphine. n=5-9 neurones per group.

7.2.4. Effect of spinal CCK on morphine-induced antinociception in normal animals

1μg of CCK-8S was given intrathecally 20 minutes before the application of 10μg of morphine to the surface of the cord. There was no difference between the inhibition of the C-fibre evoked response produced by morphine alone and that produced in the presence of 1μg CCK (figure **7.4**, 2 factor ANOVA, $F_{1,52}$ = 0.004; p=0.95), with morphine alone causing an inhibition of 87.3 ± 5.4% (n=8) after 40 minutes and 86.3 ± 4.9% (n=7) when 1μg of CCK was present. These inhibitions of the C-fibre evoked response were rapidly reversed to 90±12% of control by 5μg of intrathecal naloxone.

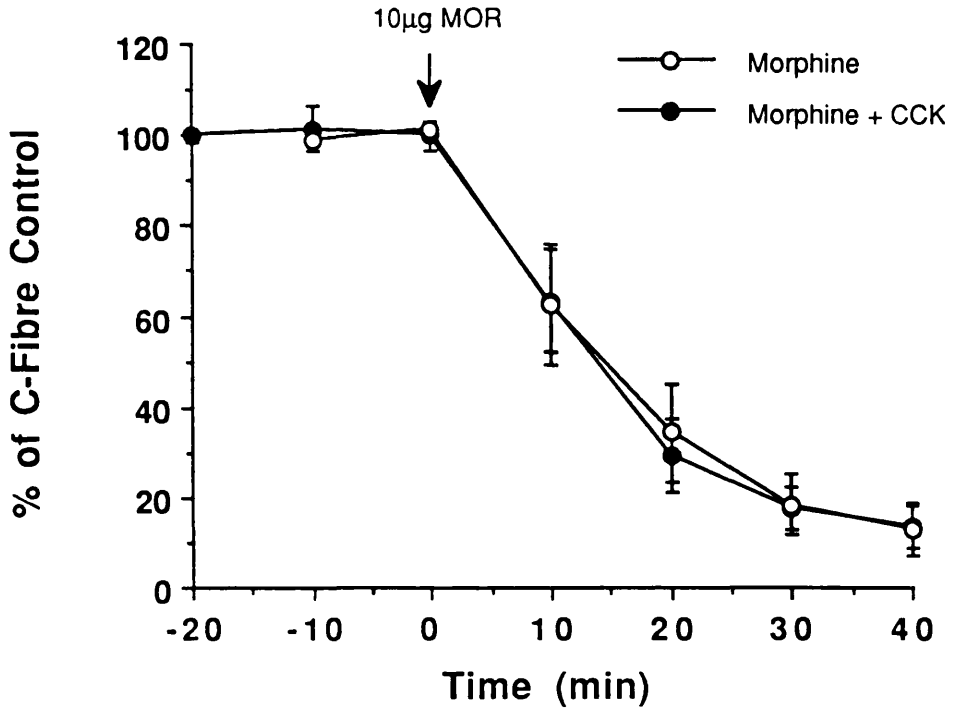


Figure 7.4. Time course for the inhibition of the C-fibre evoked response by 10μg of intrathecal morphine alone and 20 minutes after the intrathecal administration of 1μg of CCK in normal animals. In these animals, the presence of CCK had no effect on the potency of intrathecal morphine. n=7-8 neurones per group.

7.2.5. Effect of spinal CCK on morphine-induced antinociception in animals with carrageenan inflammation

The effect of 1μg of intrathecal CCK-8S (given 20 minutes before the intrathecal administration of morphine) was studied on the morphine-induced inhibition of the C-fibre evoked response of 7 dorsal horn neurones 3 hours after the injection of carrageenan into the paw. The dose of morphine used for this investigation was 2.5μg, which although smaller than that used in normal animals, produced a similar degree of inhibition of the C-fibre evoked response (chapter 5). In the presence of 1μg of CCK, 5 of these neurones showed substantially smaller inhibitions of the C-fibre evoked response with 2.5μg of morphine compared with that produced by morphine alone, whilst the morphine-induced inhibition of two neurones appeared to be unaffected by the presence of CCK. No other differences were found between the CCK sensitive and insensitive neurones in terms of their neuronal characteristics so all cells were pooled. Thus in the presence of 1μg of CCK, 2.5μg of morphine produced a significantly smaller inhibition of the C-fibre evoked response of the neurones ($F_{1,52}$ =18.5; $p<0.0001$; n=7 cells per group) (figure **7.5**).

The intrathecal administration of 1μg of naloxone reversed the morphine induced inhibition of the C-fibre evoked response to 93 ± 3% of control within 20 minutes.

Although CCK has previously been reported to excite dorsal horn neurones in this model (Magnuson et al., 1990) and in other studies (Jeftinija et al., 1981a; Willetts et al., 1985), no significant facilitation of the C-fibre evoked response was seen following the intrathecal administration of CCK in the present study. The facilitations produced by CCK in this model were previously found to be maximal 5 minutes post CCK administration (Magnuson et al., 1990). As the C-fibre evoked responses were not tested at this time point in the present study, transient excitations produced by CCK would not have been observed. Clearly, the "anti-opioid" effect of CCK is of much longer duration since it was evident throughout the 40 minute observation period.

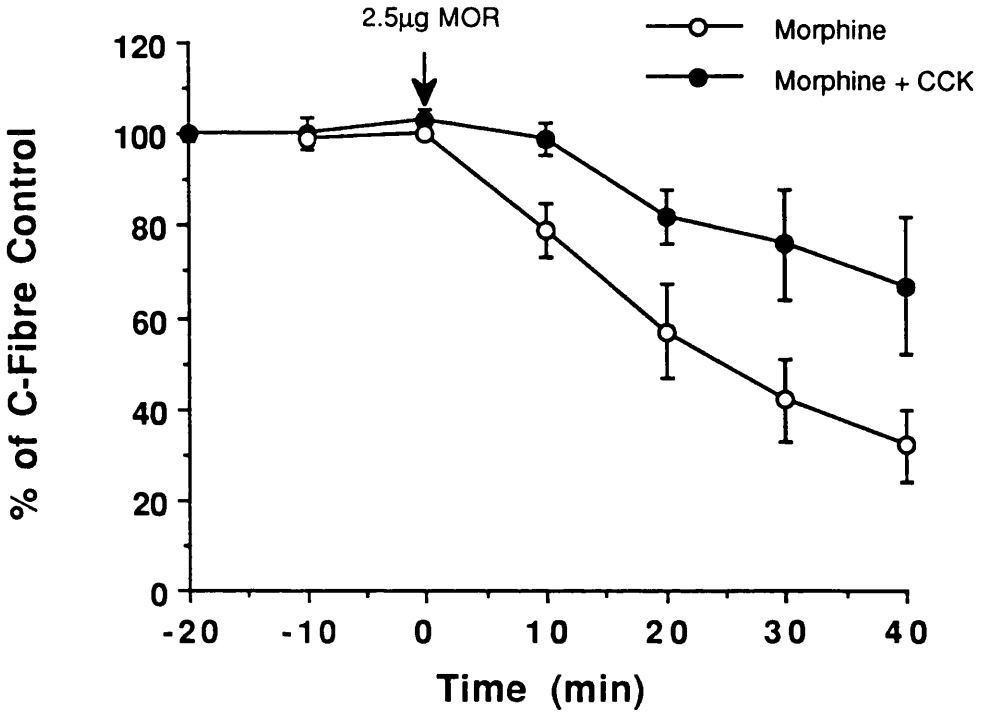


Figure 7.5. Time course for the inhibition of the C-fibre evoked response by 2.5µg of intrathecal morphine alone and 20 minutes after the intrathecal administration of 1µg of CCK in animals three hours after the injection of carrageenan into the paw. In these carrageenan animals, the presence of CCK leads to a significant reduction in the spinal potency of morphine ($p<0.0001$, ANOVA). n=7 neurones per group.

Thus exogenous CCK is still capable of reducing the antinociceptive actions of spinal morphine in animals with carrageenan inflammation.

7.2.6. Effect of L-365,260 on facilitations produced by low dose morphine in normal animals

It has been reported that i) morphine is able to cause the release of CCK in the dorsal horn of the spinal cord (Tang et al., 1984a; Benoliel et al., 1991; Zhou et al., 1993) (and see discussion), ii) CCK itself is capable of causing a facilitation of the C-fibre evoked response of dorsal horn neurones (Jeftinija et al., 1981a; Willetts et al., 1985; Magnuson et al., 1990), although this was not seen in the present study, and iii) the facilitation of the C-fibre evoked response produced by low doses of intrathecal morphine in normal animals no longer occurs in animals with carrageenan inflammation (chapter 5). The results presented in this chapter suggest that morphine may no longer be stimulating the release of CCK in animals with carrageenan induced inflammation (see discussion). These facts led me to investigate whether a morphine induced release of CCK may be responsible for the facilitations seen with the low doses of morphine in normal animals, with mu opioid receptor inhibitions then predominating as the dose is increased.

The doses of morphine chosen for this study are at the lower end of the morphine dose response curve in normal animals. The first dose (0.01μg) is a subthreshold dose and has no significant effect on the C-fibre evoked response (93.5 ± 9.6% of control, n=6) in normal animals. The second dose (0.1μg) produced a facilitation of the C-fibre evoked response to 126.2 ± 8.5% of control (n=5) when given intrathecally. As the dose of morphine was increased to 0.25μg and above, the facilitation turned to dose dependent inhibitions (see figure **7.6**). When these low doses of morphine (0.01, 0.1 and 0.25μg) were administered 10 minutes after the subcutaneous administration of 0.2mg/kg L-365,260, 0.01μg of morphine, previously a subthreshold dose, facilitated the C-fibre evoked response to 118.8 ± 7.7% of control (n=5) whilst 0.1μg of morphine failed to have a significant effect on the C-fibre evoked response (102.8 ± 8.7% of control, n=5) (see figure **7.6**). As shown previously (figure **7.2**), and included here for clarity, the inhibition produced by 0.25μg of morphine was enhanced in the presence of L-365,260 (figure **7.6**).

Thus it appears that the CCK_B receptor antagonist L-365,260 does not block the facilitations produced by low doses of morphine suggesting that this phenomenon is not due to the release of CCK. There has however been a shift in the dose response curve in the presence of the antagonist in the same way as occurs with the higher doses. As a result of this, the threshold dose of morphine required for a significant effect (with these low doses, a facilitation) on the C-fibre evoked response has been lowered.

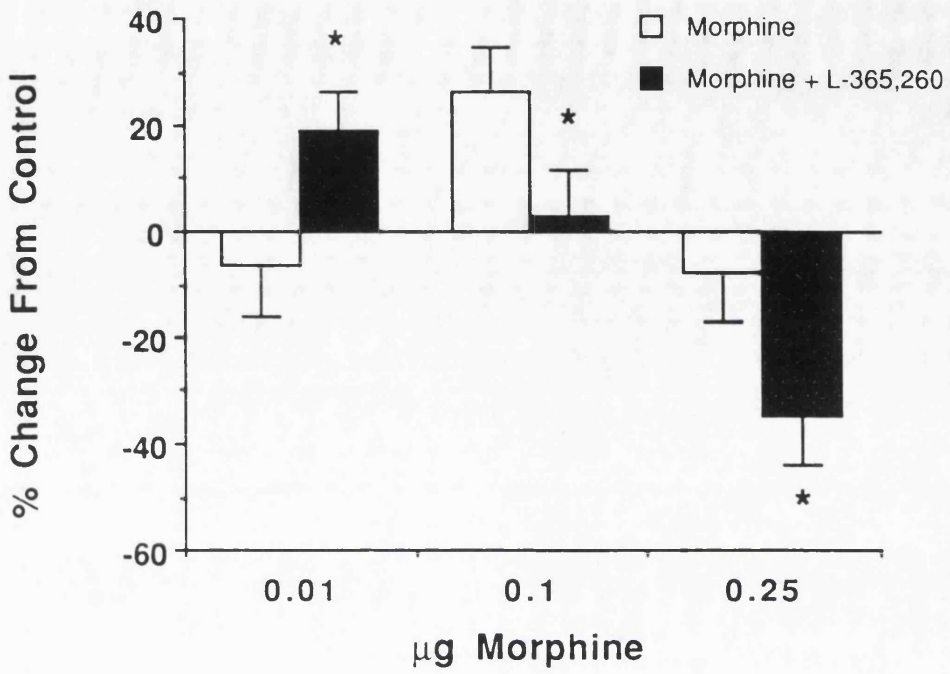


*Figure 7.6. The effects of low doses of intrathecal morphine alone and in combination with L-365,260 on the C-fibre evoked responses of dorsal horn neurones in normal animals. The vertical axis represents the % change from control so that a negative change signifies inhibitory effects and a positive change facilitation. Note that in the presence of the CCK_B receptor antagonist, the dose-response curve for morphine is shifted to the left such that the facilitation occurs with a lower dose of morphine although the magnitude of this effect remains the same. n=5-6 neurones per dose. *p<0.05 compared with morphine alone (Student's t-test).*

7.3. Discussion

The peptide CCK has been proposed to act both as a neuromodulator and neurotransmitter within the CNS. In the field of nociception, CCK has been widely heralded as an endogenous modulator of the actions of the mu opioid receptor (Itoh et al., 1982; Faris et al., 1983; Tang et al., 1984a; Watkins et al., 1984; Wiesenfeld-Hallin and Duranti, 1987; Magnuson et al., 1990; Wang et al., 1990; Kellstein et al., 1991; Zhou et al., 1993) whilst appearing to have little action on the effects mediated by delta or kappa opioid receptors (Barbaz et al., 1989; Magnuson et al., 1990; Wang et al., 1990). For this reason, it was postulated that changes resulting in a decrease in the functional activity of CCK systems within the spinal cord may lead to the greater enhancement in the potency of spinal morphine compared with the potency of the δ-opioid DSTBULET or the κ-opioid U69593 seen following carrageenan inflammation (chapter 5).

The lack of effect of the CCK_B receptor antagonist alone on the C-fibre evoked responses of dorsal horn neurones suggests that, at least in normal animals, under these experimental conditions, CCK is not playing a major role in the transmission of nociceptive information in the dorsal horn of the spinal cord. This is further supported by the fact that exogenous CCK itself produces only moderate increases in C-fibre responses, which are not seen in all cells, (Jeftinija et al., 1981a; Willetts et al., 1985; Magnuson et al., 1990) and which failed even to reach significance in the present study. Also in agreement with this conclusion are the results from behavioural studies showing that nociceptive thresholds are not altered by CCK_B receptor antagonists in all studies (Watkins et al., 1985; Dourish et al., 1988, 1990; O'Neill et al., 1989; Zhou et al., 1993) and where analgesic effects have occurred, they have been attributed to indirect opioid mechanisms (Wiesenfeld-Hallin et al., 1990). These studies were carried out in rats and employed CCK_B receptor antagonists. As the CCK receptors in the rat spinal cord are primarily of the CCK_B type (Hill et al., 1988; Hill and Woodruff, 1990; Ghilardi et al., 1992), CCK is unlikely to be playing any role in nociceptive transmission via activation of CCK_A receptors. Therefore attention was turned to the physiological role of CCK in the spinal cord being that of a (neuro)modulator.

CCK systems within the spinal cord are well placed to modulate opioid induced analgesia. Following early immunohistochemical studies, CCK was thought to be located within primary afferent C-fibres in the rat, although these findings have been challenged (Marley et al., 1982; Schultzberg et al., 1982; Ju et al., 1986; Hökfelt et al., 1988; Pohl et al., 1990; Zouaoui et al., 1990). It has been suggested that some of the early reports of the identification of CCK within rat primary afferent neurones may have in fact been due to cross-reaction of the antisera used with calcitonin gene-related peptide (CGRP) (Ju et

al., 1986; Hökfelt et al., 1988) which shares a similar C-terminal portion. In addition, oligonucleotide probes have failed to localize CCK mRNA within dorsal root ganglia in the rat (Seroogy et al., 1990; Schiffmann et al., 1991; Verge et al., 1993) suggesting that it is very unlikely that genuine CCK is found in primary afferents in normal rats. There is evidence that this is not the case after pathological damage to the afferents (Verge et al., 1993) and this is discussed later. It now seems most likely that the sources of endogenous CCK within the dorsal horn under normal conditions are the rich network of CCK-containing interneurones, found predominantly in laminae II-V and X at lumbar levels, and possibly also some of the descending fibres terminating in the dorsal horn (Fuji et al., 1985; Hökfelt et al., 1988; Verge et al., 1993). The CCK receptors activated by these fibres are mainly of the CCK_B type in the rat spinal cord (Hill et al., 1988; Hill and Woodruff, 1990; Ghilardi et al., 1992) and have recently been shown to be located primarily in laminae I and II of the spinal cord, both pre- (approximately 50-60%) and post-synaptic to the primary afferent fibres (Ghilardi et al., 1992). This distribution pattern mirrors that of the mu opioid receptor in rat spinal cord, where the highest density of binding occurs in the superficial dorsal horn, with approximately 70% of the receptors found pre-synaptically (Besse et al., 1990).

The mechanism by which CCK diminishes the antinociceptive effect of mu opioids whilst appearing to have no effect on delta or kappa opioid agonists is unknown. It has been suggested that CCK-8 suppresses the binding of selective mu and kappa, but not delta, opioid agonists to their respective receptors through an allosteric interaction between CCK receptors and opioid receptors, although the mechanism underlying this interaction is unknown (Wang and Han, 1990). However other studies have failed to find any modification of opioid binding by CCK (Slaninova et al., 1991).

Another proposal is that CCK interferes with opioid actions via post-receptor mechanisms. CCK is capable of mobilizing calcium from intracellular stores which opposes the opioid-mediated suppression of the rise in internal calcium produced by depolarisation (Wang et al., 1992). Interestingly, CCK only reversed the suppression of the K^+ induced rise in $[Ca^{2+}]i$ produced by mu and kappa, but not delta opioid agonists (Wang et al., 1992). Although this theory for the anti-opioid action of CCK seems plausible, it is hard to see how CCK is able to differentiate between the effects of mu and delta opioids by this mechanism. These opioid receptor subtypes appear to have essentially the same location within the dorsal horn (Besse et al., 1990) (which parallels that of the CCK_B receptor (Ghilardi et al., 1992)) and once the agonist has bound, are believed to activate the same G-protein link to operate the same K^+ channels (North et al., 1987). Thus at what point the interaction occurs is unclear. One speculation is that on the afferent terminals, the site of the pre-synaptic opioid control of transmitter release, there is

a close physical association of the CCK_B and mu opioid receptors, with delta opioid receptors being more remote.

The results of this study using the CCK_B receptor antagonist L-365,260 and morphine in normal animals, which was designed to probe whether spinal CCK exerts a physiological control on morphine-mediated antinociception in normal animals, demonstrates that in these animals, under these experimental conditions, the physiological activity of CCK-containing neurones within the cord is sufficient to attenuate the antinociceptive actions of intrathecal morphine. This finding is in agreement with the electrophysiological study of Kellstein et al. (1991) and the behavioural studies of Watkins et al. (1985) and Zhou et al. (1993) which also found a potentiation of morphine analgesia by CCK receptor antagonists at the level of the spinal cord in normal animals when tested against acute nociceptive stimuli. Potentiation of morphine analgesia by CCK_B receptor antagonists has also been seen following systemic administration of the drugs (Watkins et al., 1984, 1985; Dourish et al., 1988, 1990; Baber et al., 1989; O'Neill et al., 1989; Wiesenfeld-Hallin et al., 1990) although in this case, the interaction between morphine and endogenous CCK may occur at supraspinal as well as at spinal sites.

Thus in normal animals endogenous CCK does play a physiological role in negatively modulating the antinociceptive actions of exogenous mu opioid agonists applied to the spinal cord. However this interaction has never been studied in models of inflammatory pain, a state more relevant to clinical situations such as post-operative pain, where mu opioids are the major class of analgesics used. When this interaction was tested here, in animals with carrageenan induced inflammation, the CCK_B receptor antagonist produced no enhancement in the potency of spinal morphine which is in direct contrast to the situation in normal animals. This lack of effect of L-365,260 on the potency of spinal morphine in the carrageenan animals suggests that endogenous CCK is no longer producing a physiological antagonism of morphine in these animals, a change which occurs within three hours of the development of inflammation in the periphery. The nature of this change is such that it could lead to the enhanced potency of spinal morphine following carrageenan-induced inflammation (chapter 5).

What form could this change in the role of CCK in the inflammatory state take?

Exogenous CCK applied to the spinal cord of animals with carrageenan-induced inflammation was still able to attenuate the antinociceptive effects of intrathecal morphine, so the effector mechanism by which CCK is able to reduce the actions of morphine must still be intact. This implies that a decreased availability of CCK within the spinal cord following carrageenan inflammation, either due to a decreased release of CCK or reduced

/ depleted levels of the peptide within the dorsal horn, is most likely to explain the lack of effect of the CCK antagonist in these animals.

The exact sequence of events which triggers the release of CCK is not known, although there is evidence that morphine itself stimulates the release of CCK within the dorsal horn (Tang et al., 1984a; Benoliel et al., 1991; Zhou et al., 1993). It may at first appear paradoxical that morphine, which acts at an inhibitory receptor, can stimulate as well as inhibit the release of substances within the dorsal horn. However this stimulatory action may be explained through a simple disinhibition mechanism whereby a tonic inhibition of a CCK-containing neurone is blocked by morphine. A potential physiological role of this morphine-induced release of CCK is a mechanism whereby an endogenous 'brake' is applied to the antinociceptive actions of morphine or the endogenous ligand of the mu opioid receptor in times of acute pain. However in animals with carrageenan-induced inflammation the CCK_B receptor antagonist no longer produced an enhancement in the antinociceptive effect of morphine suggesting that this CCK 'brake' has been removed. It is possible that under these conditions of subchronic inflammation morphine no longer evokes the release of CCK in the spinal cord allowing more effective analgesia to occur against this more protracted pain. The absence of this autolimiting control could lead to the leftward shift in the dose-response curve for the inhibitory effects of morphine seen in the carrageenan animals (chapter 5).

Another explanation which must be considered for the lack of effect of the CCK_B receptor antagonist in the carrageenan animals is that a reduced opioid-induced release of CCK occurs as a result of the use of lower doses of morphine (0.25 and 2.5μg) in these animals, with some other factor being entirely responsible for the enhancement in morphine potency. This is unlikely since the CCK_B receptor antagonist still produced an enhancement in the responses to the even lower doses (0.01 and 0.1μg) of morphine used in normal animals (section **7.2.6.**). The use of different doses of morphine cannot therefore explain the lack of effect of L-365,260 in the carrageenan animals, leaving the presence of inflammation as the causal factor. How the development of inflammation in the periphery triggers this putative reduction in the morphine-stimulated release of CCK in the spinal cord is unknown at the present time.

A recent study by Xu et al. (1993) also suggests that changes in CCK systems may underlie changes in spinal opioid sensitivity. In this case, it was shown that an *increase* in spinal CCK (due to novel synthesis of the peptide in primary afferent fibres, Verge et al., 1993) leads to a *reduction* in the potency of spinal morphine in a rat model of neuropathic pain (Xu et al., 1993). In addition, it was suggested that an up-regulation of CCK may underlie the relative opioid insensitivity of neuropathic pain in man (Xu et al., 1993). This study in neuropathic animals is an example of changes in spinal CCK

systems in the opposite direction to those suggested in this chapter leading to changes in opioid sensitivity opposite to those seen in the carrageenan model. Thus it appears that the availability of CCK within the spinal cord, and consequently its ability to modify opioid analgesia, is not fixed but can be modified and furthermore, that this availability appears to be altered differently in different pain states.

Behavioural studies have suggested that the release of endogenous CCK is even governed by the environment to which a normal (non-suffering) rat is exposed, although at present the reported effects of environmental stimuli on CCK release are conflicting. One group suggests that CCK is released in 'safe' situations to prevent the analgesic actions of morphine (in acute nociceptive testing) whilst this release is suppressed by stimuli which signal aversive or dangerous events (Wiertelak et al., 1992). In contrast, another study reports that CCK receptor antagonists do not enhance morphine antinociception in rats in 'familiar' situations which could be equated to 'safe' situations but does so in animals exposed to a novel environment (Lavigne et al., 1992) suggesting that CCK release is associated with 'stress'. Whatever the case, these findings serve to indicate that the release of CCK is not fixed but varies, in both directions, from its normal levels according to circumstances.

The studies described above and the antagonist studies presented in this chapter demonstrate that endogenous CCK exerts a physiological variable control on the actions of morphine determined by a number of factors which can include environment and pathology. In the present study exogenous CCK clearly attenuated the effects of morphine in the animals with carrageenan inflammation. The negative results obtained in this chapter with the combination of CCK and morphine in normal animals at first appears to conflict with reports by others showing that exogenous CCK blocks the antinociceptive actions of morphine (Faris et al., 1983; Wiesenfeld-Hallin and Duranti, 1987; Kellstein et al., 1991). However Barbaz et al. (1989) reported CCK to be effective in blocking morphine antinociception in the tail flick test but not in the hot plate or abdominal stretch test in the mouse suggesting that the interaction between morphine and CCK is not a simple one.

Magnuson et al. (1990) have previously reported that in normal animals, 1μg of exogenous CCK caused a marked inhibition of the antinociceptive effect produced by the potent mu agonist [D-Ala^2,MePhe^4,Gly-ol^5]enkephalin (DAGOL) using the same electrophysiological model as that used in the present study. The differential effects of exogenous CCK on DAGOL- and morphine-mediated antinociception in normal animals may be due to morphine stimulating the release of endogenous CCK to such an extent that maximal CCK_B receptor activation results and consequently exogenous CCK can have no additional effect. Unlike morphine, DAGOL does not stimulate the release of CCK but in

contrast has been shown to reduce the release of CCK at the level of the spinal cord (Rodriguez and Sacristan, 1989; Benoliel et al., 1991). Thus when exogenous CCK is applied with DAGOL, attenuation of the analgesic effect of this opioid can occur. It is not clear why there should be this difference between morphine and DAGOL with regard to stimulation of the release of CCK, although it has been suggested that morphine may produce this effect through an action on the delta receptor (Benoliel et al., 1991) on the basis that DTLET, a delta agonist, but not DAGOL stimulates the release of CCK. This explanation does not appear to apply in this case since cross tolerance studies in this model have shown that morphine does not have any actions at the delta receptor, at least at the doses used in the present study (Kalso et al., 1993).

Another possible explanation is that morphine and DAGOL may have different efficacies at the putative subtypes of the mu receptor such as the μ_1 and μ_2 receptors (Pasternak and Wood, 1986). Indeed CCK has been reported to block the antinociceptive action of meptazinol (a μ_1 agonist) more successfully than that of morphine which binds to both μ_1 and μ_2 receptors (Barbaz et al., 1989). However the idea of μ_1 and μ_2 receptors is a matter of controversy and there is evidence for another mu opioid receptor sub-classification involving μ-δ receptor complexes (Rothman et al., 1989). Finally, there is a suggestion that DAGOL and morphine can be distinguished on the basis that different agonist-receptor complexes may couple with different G-proteins (Sanchez-Blazquez and Garzon, 1991). These possibilities need further study before conclusions can be drawn.

Is CCK responsible for the facilitations produced by low doses of morphine in normal animals but absent in animals after carrageenan?

CCK is capable of facilitating nociceptive transmission in the spinal cord (Jeftinija et al., 1981a; Willetts et al., 1985; Magnuson et al., 1990). A feature of the morphine dose response curve in normal animals is the facilitation of the C-fibre evoked response produced by low doses of morphine (see chapter 5). It was speculated that this might be due to morphine stimulating the release of CCK which in turn produces facilitations of the neurones. These morphine-evoked facilitations of the neuronal response are absent in animals with carrageenan-induced inflammation, a situation where it appears that the availability of CCK in the cord is diminished, possibly due to morphine no longer stimulating the release of CCK (this chapter). However since these morphine-induced facilitations still occur in normal animals in the presence of L-365,260 it is unlikely that CCK is involved. This facilitation may then be due to a morphine-induced release of other excitatory neuropeptides such as substance P as has been suggested by Wiesenfeld-

Hallin et al. (1991), the release of which in inflammatory states may be altered in a similar way to that of CCK. In addition there are reports of direct opioid receptor mediated increases in the duration of action potentials in dorsal root ganglion cells (Crain & Shen, 1990). This is discussed more fully in chapter 5. Both mechanisms could be altered in inflammation.

To summarize, this chapter presents evidence to show that the availability of CCK within the spinal cord during morphine-mediated analgesia is reduced following the onset of carrageenan inflammation. One mechanism which could account for this reduction in CCK levels is the abolition of the morphine-stimulated release of CCK seen in the spinal cord of normal animals (Tang et al., 1984a; Benoliel et al., 1991; Zhou et al., 1993). Regardless of the nature of the reduction in the availability of spinal CCK following carrageenan inflammation, this finding provides a basis for the enhancement in the potency of spinal morphine seen following carrageenan inflammation (chapter 5). In support of this hypothesis, not only does CCK have no effect on delta opioid mediated antinociception (Magnuson et al., 1990; Wang et al., 1990) but the potency of delta agonists in carrageenan inflammation is only moderately enhanced (see chapter 5). In more general terms, on the basis of studies with CCK antagonists, increases in CCK have been implicated in morphine tolerance (Watkins et al., 1984; Tang et al., 1984a; Panerai et al., 1987; Dourish et al., 1988, 1990; Xu et al., 1992) and neuropathic pain states (Xu et al., 1993) where there is a reduced effectiveness of morphine. These findings, together with the results presented in this chapter indicate that the enhanced and reduced effects of mu opioids seen under different conditions may be mediated through opposite extremes of the same mechanisms, in this case involving the peptide CCK. Hence further studies on the control of CCK function, such as the driving force behind the changes in CCK systems proposed to occur following inflammation, may provide interesting insights into the role of CCK in the modulation of nociceptive processing in the dorsal horn under different conditions.

CHAPTER 8

INCREASED ALPHA$_2$-ADRENERGIC CONTROLS ARE NOT A FACTOR IN THE ENHANCED POTENCY OF MORPHINE FOLLOWING CARRAGEENAN INFLAMMATION

8.1. Introduction

There is much evidence to suggest that noradrenaline is involved in modulating nociception at sites within the central nervous system. The dorsal horn of the spinal cord is one such site, a conclusion which is supported by the following evidence.

1. *The presence of noradrenergic terminals in the dorsal horn of the spinal cord.*

A moderately dense concentration of noradrenergic axons and terminals are present in the dorsal horn of the spinal cord, reaching all laminae, although they are particularly dense in the superficial dorsal horn (Carlsson et al., 1964; Fuxe, 1965; Schrøder and Skagerberg, 1985). Although a few studies have demonstrated that a tiny proportion of dorsal root ganglion cells at the lumbar level are immunoreactive for tyrosine hydroxylase (Katz et al., 1983; Price and Mudge, 1983), the rate-limiting enzyme in the synthesis of catecholamines, the noradrenergic terminals found in the dorsal horn arise almost exclusively from brainstem loci. Early studies used retrograde tracing from the spinal cord combined with immunocytochemistry for dopamine-β-hydroxylase or tyrosine hydroxylase to establish that spinal projections from noradrenergic cell groups arise from the A5 cell group, located in the region of the superior olive, the more dorsal locus coeruleus, the subcoeruleus and the laterally located A7 cell group (Satoh et al., 1977; Westlund et al., 1981, 1982, 1983, 1984). Despite the clear evidence that the brainstem is the origin of noradrenergic terminals in the spinal cord, the exact structures responsible for innervating the various regions of the spinal cord, particularly the dorsal horn, is a matter of some debate. A complicating factor in identifying the brainstem structures supplying noradrenergic innervation to the dorsal horn of the spinal cord in rats is the finding that anatomical differences exist between substrains of Sprague Dawley rats. Thus, in Sprague Dawley-derived rats obtained from Sasco Inc., noradrenergic neurones in the locus coeruleus and A7 cell groups project unilaterally to innervate the ventral and dorsal horns of the spinal cord respectively (Clark and Proudfit, 1991; Clark et al., 1991). In contrast, in Sprague Dawley-derived rats obtained from Harlan Sprague Dawley Inc., the locus coeruleus bilaterally innervates the dorsal horn of the spinal cord (Fritschy and Grzanna, 1990; Clark et al., 1991), whilst the A7 cell group projects to the ventral horn (Lyons et al., 1989; Clark et al., 1991).

2. *Alpha$_2$-adrenoceptors are located on neurones involved in the spinal transmission of nociceptive information.*

The location of α_2-adrenoceptors (the adrenergic receptor subtype involved in nociceptive processing - see below) within the dorsal horn of the spinal cord has not been

fully resolved. Autoradiographic techniques have demonstrated that α_2-adrenoceptor binding sites, although found throughout the grey matter, are concentrated in the superficial dorsal horn (Young and Kuhar, 1980; Unnerstall et al., 1984; Sullivan et al., 1987).

However, whether or not these receptors are found on primary afferent terminals is subject to controversy, with evidence both for and against pre-synaptic α_2-adrenoceptors. Unilateral rhizotomy has been reported to reduce spinal α_2-adrenoceptor binding by 20% (Howe et al., 1987a). Additionally, mRNA coding for the α_{2C}-adrenoceptor (one of the subtypes of the α_2-adrenoceptor - see discussion) is found in dorsal root ganglia in the rat suggesting some α_2-adrenoceptors in the rat spinal cord may be located on primary afferent terminals (Nicholas et al., 1993). Electrophysiological studies have shown that noradrenaline is able to increase the threshold for antidromic activation of primary afferent fibres which again suggests that α_2-adrenoceptors are located presynaptically (Jeftinija et al., 1981b; Calvillo and Ghignone, 1986). Finally, α_2-adrenoceptor agonists can inhibit the release of primary afferent transmitters (Takano et al., 1993) which again is likely to be mediated through α_2-adrenoceptors located on primary afferent terminals.

By contrast, the binding of [^{3}H]clonidine has been shown to be unaffected following the destruction of small diameter primary afferents by neonatal capsaicin treatment (Wikberg and Hajós, 1987). In support of the hypothesis that α_2-adrenoceptors are not located on the primary afferent terminals themselves is the apparent lack of synaptic contacts between noradrenergic nerve terminals and primary afferent terminals (Satoh et al., 1982; Hagihira et al., 1990). Having said this, there are suggestions (Ridet et al., 1993) that noradrenaline in the spinal cord may act via the mechanism of "volume transmission" (Fuxe and Agnati, 1991), involving a diffuse release of noradrenaline by non-junctional varicosities which could then diffuse through the extracellular fluid to reach α_2-adrenoceptors located on primary afferent terminals.

The destruction of the descending noradrenergic fibres failed to reduce α_2-adrenoceptor binding in the spinal cord (Howe et al., 1987b), implying that spinal α_2-adrenoceptors are not autoreceptors. Thus it appears that α_2-adrenoceptors in the dorsal horn of the spinal cord are located on intrinsic dorsal horn neurones and primary afferent terminals.

3. *Electrical stimulation of the brainstem region from which noradrenergic fibres project to the dorsal horn of the spinal cord produces analgesia.*

The spinal antinociception produced as a consequence of activation of descending noradrenergic pathways has been shown in many species (see refs. in Proudfit, 1992), and can be blocked by spinally administered α_2- (Jones and Gebhart, 1986; Miller and Proudfit, 1990; Yeomans et al., 1992), but not α_1- (Jones and Gebhart, 1986; Miller and Proudfit, 1990; Burnett and Gebhart, 1991), adrenoceptor antagonists.

4. *The direct spinal application of α_2-adrenergic agonists produces antinociception.*

It has been shown in a number of species, including man, that activation of spinal α_2-adrenoceptors by noradrenaline or synthetic α_2-adrenoceptor agonists leads to analgesia. In rats, intrathecal administration of α_2-adrenoceptor agonists leads to behavioural antinociception in acute nociceptive tests such as the tail flick and vocalization test (Yaksh, 1985). In electrophysiological studies, topical spinal (Sullivan et al., 1987; Sullivan et al., 1992b) and iontophoretic (Headley et al., 1978; Fleetwood-Walker et al., 1985) administration of α_2-adrenoceptor agonists, but not selective α_1-adrenoceptor agonists (Fleetwood-Walker et al., 1985) leads to the inhibition of nociceptive responses of convergent dorsal horn neurones.

The lack of effect of α_1-adrenoceptor agonists on dorsal horn neurones may at first appear surprising considering the relatively large number of α_1-adrenoceptor sites demonstrated following binding studies in spinal cord homogenates (Jones et al., 1982). However autoradiographic studies using [^{3}H]prazosin found only weak signals, with no clear laminar localization of α_1-adrenoceptor binding sites (Young and Kuhar, 1980; Dashwood et al., 1985). Whilst α_1-adrenoceptor agonists can influence motor events in the cord (Yaksh, 1985), one possible explanation for the function of α_1-adrenoceptors in the spinal cord is that rather than playing a major role in the control of nociception or motor events, the α_1-adrenoceptor may in fact be located on blood vessels within the spinal cord, thus explaining their diffuse localization.

As a result of these findings it is widely accepted that descending noradrenergic projections have the potential to modulate the spinal transmission of nociceptive information via activation of α_2-adrenoceptors, although the physiological role played by these systems has received relatively less attention.

In addition to the direct modulation of spinal nociception, α_2-adrenoceptor agonists have been shown to potentiate the antinociceptive effects of spinal opioids in

both behavioural (Yaksh and Reddy, 1981; Hylden and Wilcox, 1983; Wilcox et al., 1987; Loomis et al., 1988; Ossipov et al., 1990; Plummer et al., 1992; Roerig et al., 1992) and electrophysiological studies (Sullivan et al., 1987; Wilcox et al., 1987; Omote et al., 1991; Sullivan et al., 1992a). Thus, when a dose of an opioid agonist is combined with a dose of an α_2-adrenoceptor agonist, the resulting antinociception is greater than would be expected if the antinociceptive effects of the drugs were simply additive.

Whether the interaction between α_2-adrenoceptor agonists and opioids involves the mu or delta opioid receptor is a matter of some debate. Several studies have set out to investigate which opioid receptor subtype is involved in these interactions, with conflicting results. An electrophysiological study by Sullivan et al. (1992a) implicates the mu but not the delta opioid receptor in synergistic interactions with α_2-adrenoceptors, whilst another electrophysiological study (Omote et al., 1991) and a behavioural study employing the substance P behavioural test in mice (Roerig et al., 1992) suggest that delta opioid agonists but not mu opioids lead to synergy with α_2-adrenoceptor agonists.

However, the majority of studies showing synergistic interactions between opioids and α_2-adrenoceptor agonists have been carried out using morphine as the opioid agonist. Morphine acts preferentially on the mu opioid receptor, so there is clear evidence that mu opioid receptor agonists at least can interact synergistically with α_2-adrenoceptor agonists. The interaction between α_2-adrenoceptor agonists and kappa opioid agonists has been the subject of one study (Gordon et al., 1992); the results were inconclusive.

At the present time, the evidence strongly supports the ability of mu opioid agonists to interact synergistically with α_2-adrenoceptor agonists, whereas evidence for α_2-adrenergic interactions with delta and kappa opioids is less well established. An increased release of noradrenaline in the cord following the development of carrageenan inflammation could, by virtue of its ability to potentiate the actions of mu but not delta or kappa opioid agonists, lead to the selective enhancement in the potency of intrathecal morphine seen in the carrageenan animals (chapter 5). The enhanced potency of spinal opioids following carrageenan inflammation has been demonstrated by Hylden et al. (1991b) in a behavioural study and evidence has been presented for a possible role of noradrenaline in this phenomenon. This chapter examines whether an increase in the release of noradrenaline in the spinal cord is indeed contributing to the enhanced antinociceptive actions of spinal morphine following the development of carrageenan inflammation.

An increase in the turnover of noradrenaline in the spinal cord has been reported in arthritic rats (Weil-Fugazza et al., 1986). In addition, electrophysiological experiments performed in cats have demonstrated that the tonic descending inhibition of dorsal horn neurones is enhanced following the development of acute inflammation of the knee joint

(Cervero et al., 1991; Schaible et al., 1991b). In these experiments, the degree of tonic descending inhibition was assessed using a reversible cold block at thoracic or lumbar levels of the spinal cord, thus descending controls other than those arising from noradrenergic brainstem nuclei would also be blocked, for example those mediated by 5HT. Nevertheless, these studies suggest that enhanced spinal noradrenergic activity may occur following the development of an inflammatory state.

In order to investigate whether endogenous noradrenaline acting at spinal α_2-adrenoceptors plays any part in the enhanced antinociceptive effects of intrathecal morphine following carrageenan inflammation, α_2-adrenoceptor antagonists, atipamezole and idazoxan, were used to reveal whether changes occur in the noradrenergic control of spinal nociceptive transmission following the development of carrageenan inflammation. In addition, the spinal potency of the relatively new and highly selective α_2-adrenoceptor agonist dexmedetomidine (Virtanen et al., 1988) was examined in the carrageenan animals and compared with that seen in normal animals (Sullivan et al., 1992b) to judge whether the potency of noradrenaline (opposed to the release of noradrenaline) is altered in the carrageenan animals. Dexmedetomidine was used for these studies since this agonist has far higher selectivity for α_2- over α_1-adrenoceptors than other more traditional α_2-adrenoceptor ligands such as clonidine (Virtanen et al., 1988) which are often used in studies such as this. In addition, dexmedetomidine is more efficacious than clonidine (Takano and Yaksh, 1991), which behaves as a partial agonist in this electrophysiological model (Sullivan et al., 1987). Finally, the potency of intrathecal morphine in carrageenan animals was examined in the presence of the α_2-adrenoceptor antagonists atipamezole and idazoxan, to conclusively reveal any contribution of α_2-adrenoceptor mediated events to the antinociceptive actions of spinal morphine in this inflammatory state.

8.2. Results

The convergent neurones used in this study were located in the deeper laminae of the dorsal horn (500-1000μm, mean depth = 749 ± 37μm (n=54)), and formed a similar population to those used to investigate the potency of morphine in normal and carrageenan animals (chapter 5).

8.2.1. Effects of intrathecal dexmedetomidine in carrageenan animals

Intrathecal dexmedetomidine, in the dose range 0.1-10μg was tested on the evoked responses of 15 dorsal horn neurones 3 hours after the injection of carrageenan into the ipsilateral hind paw. Dexmedetomidine produced dose dependent inhibitions of the C-fibre evoked response, with the highest dose tested (10μg) producing a maximum inhibition of 70.0 ± 13.9% (n=5) (figure **8.1**). The Aβ-fibre evoked responses were inhibited to a lesser extent than the C-fibre responses, although the highest dose of dexmedetomidine tested (10μg) produced an inhibition of 44.5 ± 3.7%. The inhibitions of both the C- and Aβ-fibre evoked response were reversed by the intrathecal administration of 15μg of atipamezole (C-fibres reversed to 91.8 ± 5.7% of control (n=7)) or 10μg of idazoxan (C-fibres reversed to 96.4 ± 4.0% of control (n=4)).

In order to reduce the number of animals used in the study, I did not attempt to obtain a dose-response curve for the inhibition of the C-fibre evoked response by dexmedetomidine in normal animals myself, but instead choose to use the dexmedetomidine dose-response curve obtained by Sullivan et al. (1992b) in normal animals in a parallel study in the laboratory. When the dose-response curve obtained in carrageenan animals for the inhibition of the C-fibre evoked response by dexmedetomidine is compared with that obtained in normal animals (Sullivan et al., 1992b) it can be seen that there is a non-parallel leftward shift in the curve for dexmedetomidine in the carrageenan animals (figure **8.1**). In the carrageenan animals the low doses of dexmedetomidine were more potent than in the normal animals (6 fold shift at doses producing 20% inhibition of the C-fibre evoked response). However, as the dose was increased this difference disappeared, such that the highest dose of dexmedetomidine tested (10μg) was less potent in the carrageenan animals than in normals (figure **8.1**). Thus overall, the dose of dexmedetomidine required to produce a 50% inhibition of the C-fibre evoked response in both cases was very similar (estimated at 2.5μg in normal animals (Sullivan et al., 1992b) and 1μg following carrageenan inflammation), a 2.5-fold enhancement in potency.

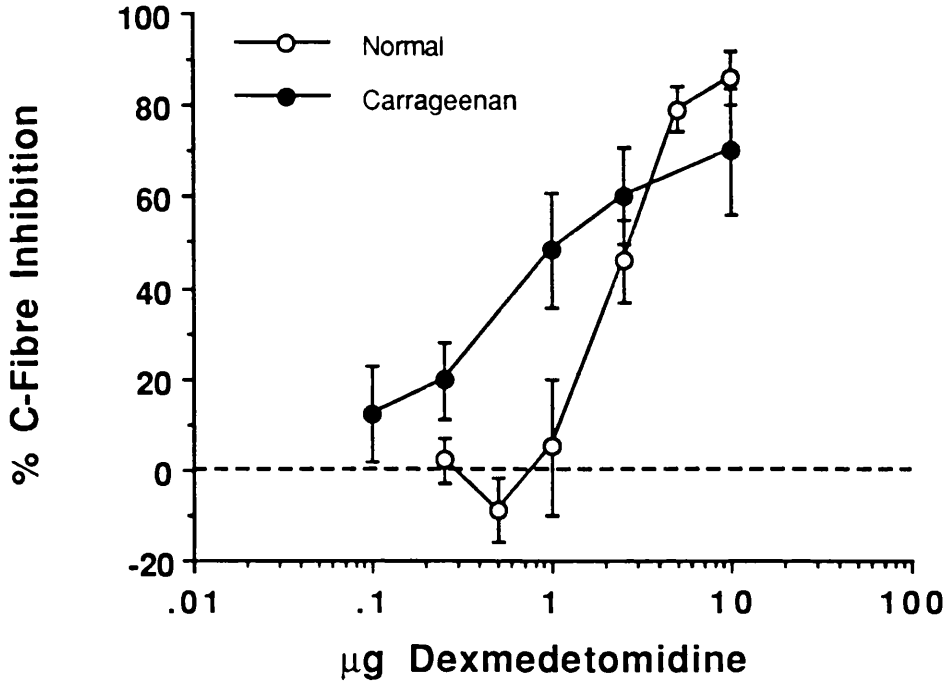


Figure 8.1. Dose-response curves for the inhibition of the C-fibre evoked response by intrathecal dexmedetomidine in normal animals (data from Sullivan et al., 1992b) and three hours after the injection of carrageenan into the paw. Note the non-parallel leftward shift in the dose-response curve for dexmedetomidine in the carrageenan animals. n=5-9 neurones per dose.

8.2.2. Anaesthetic sparing effect of intrathecal dexmedetomidine

During the intrathecal administration of high doses of dexmedetomidine evidence of the anaesthetic sparing effect of the drug was seen. This anaesthetic sparing effect has been reported previously following both systemic (Segal et al., 1988) and intrathecal (Nagasaka and Yaksh, 1990) administration of dexmedetomidine. To compensate for this anaesthetic sparing effect of spinal dexmedetomidine, the level of halothane in the inspired gases was adjusted to maintain a state in which reflexes were just abolished. Thus, following spinal application of the highest dose of dexmedetomidine (10µg), the level of halothane in the inspired air was reduced to an average of 0.5-0.7% from the control level of around 1.5%. Following the intrathecal administration of 15µg of atipamezole or 10µg of idazoxan, the anaesthetic requirements of the animals rapidly returned to control levels (~1.5%). This anaesthetic sparing effect may represent some systemic leakage of this highly lipophilic drug, although it has been shown to be partly due to an action of dexmedetomidine at α_2-adrenoceptors in the spinal cord (Nagasaka and Yaksh, 1990).

8.2.3. Effect of the α_2-adrenoceptor antagonist atipamezole on the evoked neuronal responses

The effect of the intrathecal administration of 50μg of the selective α_2-adrenoceptor antagonist atipamezole was studied on the C- and Aβ-fibre evoked responses of 6 dorsal horn neurones 3 hours after the injection of carrageenan into the ipsilateral hind paw. The evoked response of the neurones was followed for 60 minutes after the spinal application of atipamezole during which time the C-fibre evoked response showed slight facilitations, reaching 116.7 ± 10.2% of control after 20 minutes, although these failed to reach significance (Student's paired t-test, n=6) (figure **8.2**). The Aβ-fibre evoked response of the neurones was not altered by more than 5% by atipamezole.

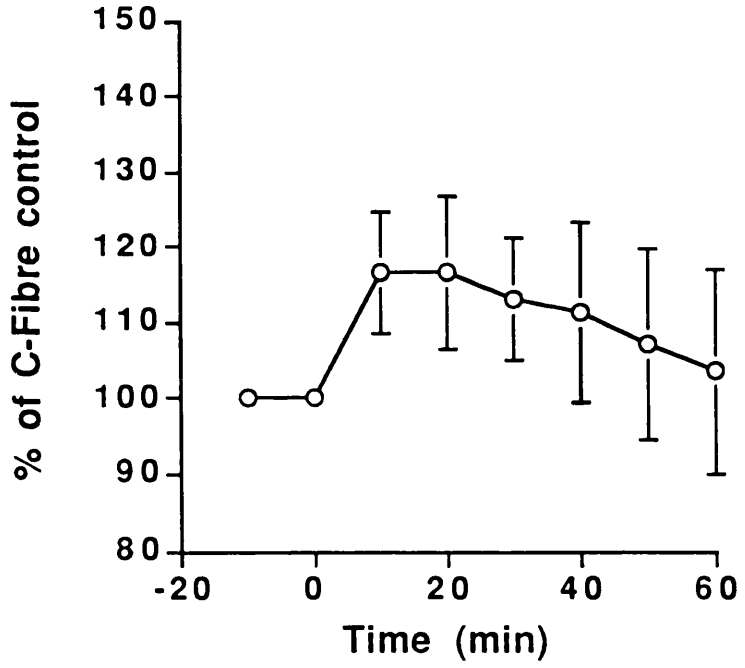


Figure 8.2. Time-course of the effects of intrathecal atipamezole (50μg) on the C-fibre evoked response of dorsal horn neurones three hours after the injection of carrageenan into the paw. Atipamezole was applied to the cord at t=0, and the effects followed for 60 minutes. Atipamezole did not produce a significant effect on the neuronal response at any of the time points tested. n=6.

The dose of atipamezole used (50μg) is approximately four times higher than that which completely reversed the effects of the highest dose of dexmedetomidine in the same

animals (see section **8.2.1.**). Therefore, this lack of a significant effect of atipamezole is unlikely to be due to the dose being insufficient for antagonism of the spinal actions of noradrenaline, the endogenous ligand for the spinal α_2-adrenoceptor.

The effects of intrathecal atipamezole alone on the evoked neuronal responses of dorsal horn neurones in normal animals were tested in a parallel study in this laboratory by Sullivan et al. (1992b). In these animals also, the presence of atipamezole failed to alter the C- or Aβ-fibre evoked responses of the neurones.

8.2.4. Effect of the α_2-adrenoceptor antagonist idazoxan on the evoked neuronal responses

In contrast to the findings with atipamezole, 100μg of idazoxan, applied intrathecally, produced a rapid and long lasting facilitation (>90 minutes) of the C-fibre evoked response in animals with carrageenan inflammation (figure **8.3**). In these animals, idazoxan produced a moderate but marked facilitation of the C-fibre evoked response which was significant at all of the time points tested ($p<0.05$) except the 30 minute point ($p=0.06$) (paired Student's t-test, n=4). This facilitation, which typically elevated the response to around 125% of the control value, did not diminish in the 90 minute period over which it was studied and by contrast, tended to increase slightly over the period.

In normal animals, idazoxan also produced a facilitation of the C-fibre evoked response to around 118% of control, however this facilitation was not significant at any of the time points tested (paired Student's t-test, n=5) (figure **8.3**). In normal animals, the facilitation of the response produced by idazoxan remained constant for approximately 50 minutes after administration of the drug after which point the evoked C-fibre response began to decline back to the control level, reaching control levels by 80 minutes. This is in contrast to the actions of idazoxan seen in the animals with carrageenan inflammation, where the effects of idazoxan did not show any signs of diminishing over the 90 minute period studied (figure **8.3**).

The idazoxan induced facilitation of the C-fibre evoked response in animals with carrageenan induced inflammation was more prolonged and significantly greater than that produced in normal animals (ANOVA, $F_{1,66}=10.39$, $p=0.002$).

The Aβ-fibre evoked response was not altered by more than 5% in either the animals with carrageenan inflammation or in normal animals.

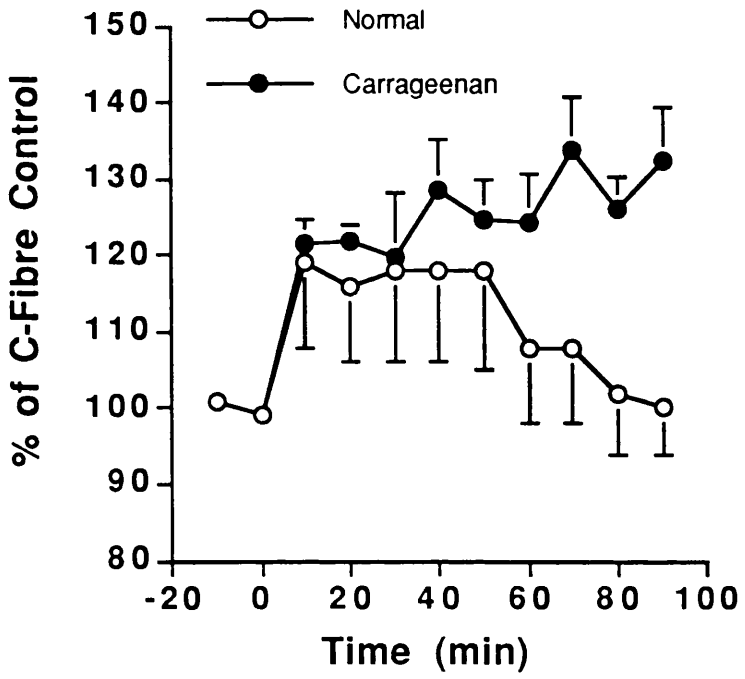


Figure 8.3. Time-course of the effects of intrathecal idazoxan (100μg) on the C-fibre evoked response of dorsal horn neurones in normal animals and three hours after the injection of carrageenan into the paw. Idazoxan was applied to the cord at t=0, and the effects followed for 90 minutes. In normal animals, the facilitation of the response by idazoxan did not reach significance at any of the time points tested. In contrast, in the carrageenan animals, the facilitations of the evoked response were significant at all time points except 30 minutes. Note the altered time course of the drug in the carrageenan animals. n=4-5.

The results obtained with intrathecal idazoxan in normal animals and animals with carrageenan inflammation suggest that the spinal α_2-adrenoceptor mediated control of nociception is enhanced in carrageenan animals compared with normals. This change in spinal α_2-adrenoceptor mediated controls could account for the enhancement in the potency of morphine following carrageenan inflammation via a synergistic interaction.

8.2.5. Effect of α_2-adrenoceptor antagonists on morphine-induced antinociception following carrageenan inflammation

The antinociceptive effect of two doses of intrathecal morphine, alone and in combination with the α_2-adrenoceptor antagonist atipamezole or idazoxan, were tested on the C-fibre evoked response of dorsal horn neurones 3 hours after the injection of carrageenan into the paw. The two doses of morphine used for this study (0.25 and 2.5μg) produced inhibitions of 16.8 ± 4.7% and 68.0 ± 8.2% when given alone (n=6).

The intrathecal administration of 50μg of atipamezole (a dose approximately 4 times greater than that required to reverse profound dexmedetomidine-mediated inhibitions (see section **8.2.1.**) 10 minutes prior to the application of 0.25 or 2.5μg of morphine had no effect on the antinociceptive potency of morphine in animals with carrageenan inflammation. Figure **8.4** shows that in the presence of 50μg of atipamezole, 0.25 and 2.5μg of morphine produced inhibitions of the C-fibre evoked response of 16.0 ± 8.4% and 71.6 ± 6.6% respectively (n=6-7) which are identical to those produced by intrathecal morphine alone in carrageenan animals.

The intrathecal administration of 100μg of idazoxan (a dose 10 times greater than that required to reverse profound dexmedetomidine-mediated inhibitions in the normal animals (**8.2.1.**)) 10 minutes prior to the application of 0.25 and 2.5μg of morphine in animals with carrageenan induced inflammation was also without effect on the morphine induced inhibitions of the C-fibre evoked response (inhibitions of 10.0 ± 15.2% and 68.4 ± 12.8% respectively, n=5) (figure **8.4**). These percentage inhibitions of the C-fibre evoked response produced by morphine in the presence of idazoxan were calculated by using the number of C-fibre evoked responses obtained before the administration of idazoxan as controls. When the control response was taken as the number of evoked neuronal responses obtained post-idazoxan, to take into account the significant facilitation of the response produced by idazoxan alone, the inhibitions produced by morphine were 14.0 ± 12.4% and 73.2 ± 10.6%. Thus there is no reduction in the spinal inhibitory effects of morphine in the presence of idazoxan.

The inhibitory effects of morphine in both the atipamezole and idazoxan experiments were completely reversed by intrathecal naloxone (1μg).

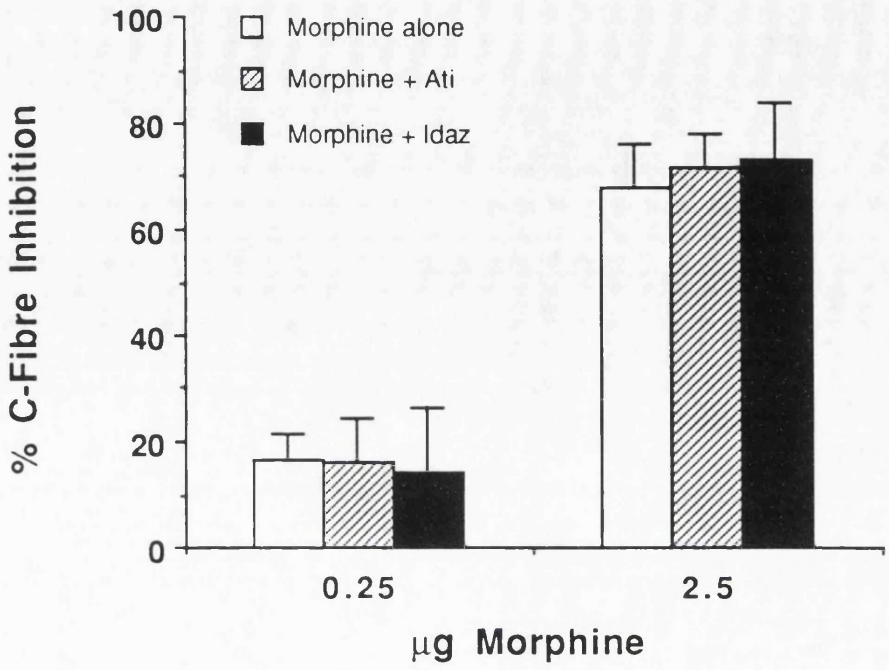


Figure 8.4. Mean maximal inhibition of the C-fibre evoked responses of dorsal horn neurones in carrageenan animals produced by two doses (0.25 & 2.5μg) of intrathecal morphine alone, and in the presence of 50μg of atipamezole (Morphine + Ati) or 100μg of idazoxan (Morphine + Idaz), also applied intrathecally. Neither α_2-adrenoceptor antagonist reduced the ability of morphine to inhibit the nociceptive responses in these animals. n=5-7 neurones per combination.

These results demonstrate that the enhanced potency of morphine following carrageenan inflammation is independent of any changes that may occur in spinal noradrenergic systems.

8.3. Discussion

In the introduction it was proposed that the greater enhancement in the spinal antinociceptive potency of morphine (a mu opioid) compared with that seen with selective delta and kappa opioid agonists following carrageenan inflammation (chapter 5) may be explained by increased activity of noradrenergic descending controls in the inflammatory state. Many studies have reported that synergism occurs between the spinal antinociceptive actions of morphine and those of selective α_2-adrenoceptor agonists such as clonidine and dexmedetomidine (Yaksh and Reddy, 1981; Hylden and Wilcox, 1983; Sullivan et al., 1987; Wilcox et al., 1987; Loomis et al., 1988; Ossipov et al., 1990; Plummer et al., 1992; Sullivan et al., 1992a), whilst the combination of dexmedetomidine with the delta opioid agonist used in the present study, DSTBULET, produces a purely additive antinociceptive effect (Sullivan et al., 1992a). In addition, there is no convincing evidence that anything other than an additive antinociceptive effect occurs between the antinociceptive actions of kappa opioid agonists and α_2-adrenoceptor agonists (Gordon et al., 1992). This, together with the findings that both the tonic descending inhibition of dorsal horn neurones (Cervero et al., 1991; Schaible et al., 1991b) and the turnover of noradrenaline in the spinal cord (Weil-Fugazza et al., 1986) are enhanced in inflammatory states, forms the basis for the theory that noradrenaline may be involved in the enhanced antinociceptive potency of spinal morphine following carrageenan-induced inflammation.

In the electrophysiological model used in the present study, the spinal antinociceptive potency of the selective α_2-adrenoceptor agonist dexmedetomidine is only modestly enhanced in animals with carrageenan inflammation when compared with normal animals (Sullivan et al., 1992b). This suggests that marked changes in the number or affinity of α_2-adrenoceptors in the spinal cord are unlikely to have occurred within the first three hours following the injection of carrageenan. There is evidence from an autoradiographic study that an increase in the number of α_2-adrenoceptor binding sites in the sheep spinal cord occurs following 3 weeks of chronic inflammatory nociception (Brandt and Livingstone, 1990). Thus although changes in spinal α_2-adrenoceptor numbers may occur at a later stage after the injection of carrageenan, the results with dexmedetomidine suggest that this does not seem to be involved in the enhanced potency of spinal morphine seen in this study,which occurs within hours of the injection of carrageenan (chapter 5).

Other authors have demonstrated in behavioural studies that the antinociceptive potency of both systemic (Hylden et al., 1991b; Kayser et al., 1992) and spinal (Hylden et al., 1991b) clonidine is enhanced in inflammatory conditions. One difference between

these behavioural studies and the electrophysiological one presented here may lie with the fact that the behavioural studies look primarily at changes in nociceptive thresholds, whereas the electrophysiological study presented in this chapter examines changes in nociceptive responses produced by suprathreshold noxious stimulation. This difference in intensity may have some bearing on the range of doses used. It could be envisaged that in a behavioural study examining nociceptive thresholds with a cut-off point imposed the range of doses used may be at the lower end of the range used in this electrophysiological study where doses sufficient to abolish a suprathreshold stimulus were used. This is likely to be an important factor since in the present study the antinociceptive effects of low doses of dexmedetomidine show some enhancement whereas those produced by high doses are unaltered (see figure **8.1**). Consequently, it could be predicted that in a behavioural study looking at nociceptive thresholds a mild increase in the effectiveness of an α_2-adrenoceptor agonist would be seen over the whole of the dose range studied. However with this electrophysiological study using suprathreshold stimuli, and with no restrictions imposed, although a shift was seen at the lower end of the dose response curve, this difference disappeared as the dose was increased resulting in only a small reduction in the ED_{50} dose (2.5 fold) for intrathecal dexmedetomidine in the carrageenan animals.

In support of the concept that differences in testing methods may lead to a different range of doses being used is the finding that in a behavioural study by Takano and Yaksh (1992) using paw withdrawal to noxious heat, intrathecal clonidine was able to increase the paw withdrawal latency to the same degree (i.e. up to the time point at which testing was terminated to avoid tissue damage) as dexmedetomidine. In contrast, in electrophysiological studies employing suprathreshold noxious electrical stimulation, clonidine was clearly a partial agonist, only being able to inhibit the evoked nociceptive response by 50% (Sullivan et al., 1987), whereas dexmedetomidine was able to completely inhibit the evoked response (Sullivan et al., 1992b).

Another possible factor in the difference in results between the behavioural and electrophysiological studies may be that different α_2-adrenoceptor agonists were used, with clonidine being used for both behavioural studies (Hylden et al., 1991b; Kayser et al., 1992), whereas dexmedetomidine was used in this electrophysiological study. Although there are now known to be subtypes of α_2-adrenoceptor receptors in the rat (see Bylund, 1988), with both α_{2A}- and α_{2C}-adrenoceptors found in the rat spinal cord (Nicholas et al., 1993), the evidence to date suggests that in the spinal cord clonidine and dexmedetomidine act on predominantly the same α_2-adrenoceptor subtype, the α_{2A}-adrenoceptor (Takano and Yaksh, 1992). However, clonidine may additionally act on an α_2 "non-A" subclass of receptors (Takano and Yaksh, 1993), which could potentially

show differential alterations in the inflammatory state, leading to the observed differences between the results of the behavioural studies employing clonidine (Hylden et al., 1991b; Kayser et al., 1992) and the present electrophysiological study using dexmedetomidine.

Thus studies with α_2-adrenoceptor agonists are as yet unable to provide firm evidence either for or against a role for the noradrenergic system, at least via an action on α_2-adrenoceptors, in the enhanced potency of spinal morphine following inflammation.

The results presented in this chapter indicate that despite some small changes in the actions of dexmedetomidine following carrageenan inflammation, changes in spinal α_2-adrenoceptor number or function on the scale needed to account for the 18-fold shift in the antinociceptive potency of morphine seen in this model following inflammation (chapter 5) are unlikely. Thus if α_2-adrenoceptors do play a role in the enhanced potency of morphine following the development of inflammation it must be due to increased spinal levels of noradrenaline rather than receptor changes, a theory that was tested here by the use of α_2-adrenoceptor antagonists.

The level of tonic α_2-adrenoceptor mediated control of spinal nociceptive transmission was gauged in both normal animals and 3 hours after the onset of carrageenan inflammation using the spinal application of α_2-adrenoceptor antagonists to ascertain whether an increase occurs in the functional activity of the descending noradrenergic controls after inflammation. The lack of significant effect of both atipamezole and idazoxan on nociceptive responses in normal animals in this electrophysiological model suggests that in the anaesthetized rat at least, descending noradrenergic controls are not playing a major role in the tonic control of spinal nociceptive processing. This conclusion is supported by the lack of effect of intrathecally administered idazoxan and atipamezole on nociceptive thresholds in behavioural studies in normal animals (Nagasaka and Yaksh, 1990; Hylden et al., 1991b; Takano and Yaksh, 1992). Another behavioural study, using a different α_2-adrenoceptor antagonist, yohimbine, did find hyperalgesia following intrathecal administration of the antagonist (Sagen and Proudfit, 1984); other studies using this antagonist have not found any change in nociceptive thresholds following intrathecal administration (Howe et al., 1983; Takano and Yaksh, 1992).

In the carrageenan animals however, intrathecal administration of idazoxan, but not atipamezole, produced a significant facilitation of the C- but not Aβ-fibre evoked response suggesting that nociceptive but not innocuous responses may be subject to enhanced α_2-adrenergic controls in inflammatory states. In contrast, a behavioural study using intrathecal idazoxan in animals with carrageenan inflammation has failed to demonstrate any effect of idazoxan on nociceptive thresholds (Hylden et al., 1991b), whilst a similar study in arthritic animals has shown paradoxical analgesia after spinal

idazoxan (Kayser et al., 1992). Thus from the results of these studies, whether or not an increased noradrenergic control of spinal nociceptive processing occurs following the development of inflammation in the periphery is a matter of some confusion. Alternative approaches to this question have shown that an increase in the turnover of spinal noradrenaline occurs in arthritic animals (Weil-Fugazza et al., 1986) and a reversible cold block of descending controls in cats with inflamed knee joints has been used to demonstrate that dorsal horn neurones receiving input from the inflamed joint are subject to greater descending influence than those receiving input from normal joints (Cervero et al., 1991; Schaible et al., 1991b), suggesting an increased activity of descending controls. However, in the latter experiments it must be remembered that a cold block will produce a non-specific block of all descending controls, not just those arising from noradrenaline containing nuclei.

The reason for the differential effects of atipamezole and idazoxan on the evoked neuronal responses in the carrageenan animals seen in the present electrophysiological study is not clear. The doses of antagonist used were greater than those required to completely reverse the maximal effects of the α_2-adrenoceptor agonist dexmedetomidine on the neuronal responses so the doses used are not likely to be responsible for the differing effects of the two antagonists. It may be that idazoxan and atipamezole act on different receptor subtypes. As well as acting on α_2-adrenoceptors, idazoxan also has high affinity for non-adrenergic or imidazoline binding sites (Michel et al., 1989). Atipamezole is also an imidazole-type compound and so also has the potential to bind to this site, although to my knowledge no study to date has specifically investigated this. These imidazoline sites have no known function in the spinal cord at present but it is conceivable that they may have some enhanced function in inflammation. Another factor may be the existence of subtypes of the α_2-adrenoceptor (Bylund, 1988). Although both atipamezole and idazoxan have been speculatively identified as α_{2A}-adrenoceptor antagonists (Takano and Yaksh, 1992), idazoxan was found to have significantly different ID_{50}'s for the spinal antagonism of dexmedetomidine and clonidine, whereas atipamezole was not able to discriminate between these two drugs (same ID_{50} value for each) (Takano and Yaksh, 1992). This slight difference in profile between atipamezole and idazoxan may be a factor in the difference found between the two antagonists following carrageenan inflammation.

The difference in the time course of the actions of idazoxan in normal and carrageenan animals is difficult to explain but may result from altered metabolism or reuptake of noradrenaline in inflammatory states.

The results presented here in this electrophysiological study and those obtained in behavioural studies (Nagasaka and Yaksh, 1990; Hylden et al., 1991b; Takano and

Yaksh, 1992) showing the lack of effect of α_2-adrenoceptor antagonists alone in normal animals suggest that noradrenaline in the spinal cord plays very little role in the modulation of spinal nociceptive transmission in normal animals. In view of this, the findings from studies in normal animals showing that the antinociceptive effects of morphine are unaltered by the systemic or spinal administration of α_2-adrenoceptor antagonists (Solomon and Gebhart, 1988; Sullivan et al., 1992b) and thus that synergy with noradrenaline does not contribute to the antinociceptive actions of morphine in the normal animal is not surprising.

Although the results of the present study with α_2-adrenoceptor antagonists alone in animals with carrageenan inflammation show that only a modest increase occurs in the level of noradrenaline in the spinal cord following inflammation, this does not preclude the occurrence of synergy between spinal morphine and noradrenaline under these conditions since is has been shown that synergy between α_2-adrenoceptor agonists and mu opioid agonists can occur with subthreshold doses of the respective agonists (Sullivan et al., 1992a). Thus, the only way to demonstrate whether a synergistic interaction between the increased levels of noradrenaline in the cord and exogenous morphine is responsible for the enhanced potency of spinal morphine seen inflammation (chapter 5) is to examine the potency of spinal morphine in these animals in the presence of an α_2-adrenoceptor antagonist.

In the present study in animals with carrageenan inflammation I used high doses of two α_2-adrenoceptor antagonists in conjunction with morphine and found that the potency of intrathecal morphine was unaltered. This is in contrast to the findings of Hylden et al. (1991b) in a behavioural study who found that the intrathecal coadministration of 10μg of idazoxan with 5μg of morphine in animals with unilateral carrageenan inflammation blocked the antinociceptive actions of morphine on the inflamed paw. However in this study idazoxan also blocked the actions of morphine on the non-inflamed paw. Since the effects of idazoxan were bilateral yet the increased potency of morphine was unilateral in this study, α_2-adrenoceptors cannot be responsible for the enhancement in opioid effects on the inflamed side. However, Hylden et al. (1991b) did find a unilateral effect of idazoxan on morphine analgesia after systemic administration of the drugs.

This latter study indicates a role for α_2-adrenoceptors in the enhancement of morphine antinociception following carrageenan-induced inflammation, but at peripheral and / or supraspinal sites of action. Thus whereas there is no evidence for an enhanced action of morphine due to interactions with endogenous noradrenaline at the spinal level in animals with carrageenan inflammation, alterations in α_2-adrenergic systems may occur at non-spinal sites in inflammation.

To summarize the results with idazoxan alone indicate that α_2-adrenergic systems are activated by inflammation and play a role in the control of nociceptive neuronal responses. There is good evidence that mu opioids can synergize with α_2-adrenoceptor agonists in a number of models of nociception (Yaksh and Reddy, 1981; Hylden and Wilcox, 1983; Sullivan et al., 1987; Wilcox et al., 1987; Loomis et al., 1988; Ossipov et al., 1990; Plummer et al., 1992; Sullivan et al., 1992a). However, despite the enhanced role played by α_2-adrenoceptors in the control of nociceptive responses in inflammation, the results of the present study do not support the theory that alterations in the α_2-adrenergic system are responsible for the enhanced potency of spinal morphine following inflammation. Relevant to this conclusion are the results of a recent behavioural study, in which the spinal administration of the selective α_2-adrenoceptor agonist clonidine, but not noradrenaline, was able to potentiate the actions of spinal morphine (Plummer et al., 1992). Thus it may be the case that at the spinal level endogenous noradrenaline, unlike synthetic agonists, is not sufficiently potent to potentiate the actions of morphine, suggesting that α_2-adrenergic systems play at most a minor role in the enhanced spinal potency of morphine following inflammation.

These results, together with those from the previous chapter (chapter 7), suggest that the enhanced potency of intrathecal morphine following the peripheral injection of carrageenan results, at least in part, from changes in CCK systems within the spinal cord, whilst changes in spinal α_2-adrenergic systems do not contribute to this phenomenon.

CHAPTER 9

STUDIES ON THE SPINAL ROLES OF THE ENDOGENOUS OPIOIDS IN CARRAGEENAN INFLAMMATION

9.1. Introduction

The endogenous opioids consist of three families of peptides, the endorphins, the enkephalins and the dynorphins which act primarily on the μ-, δ- and κ-opioid receptors respectively, although none of the opioid peptides are entirely selective for a particular receptor type (Kosterlitz, 1985). The peptides are synthesized as pro-peptides in the cell body and then transported to the nerve terminals where they are cleaved to their active fragments. Although pro-opiocortin produces only one active opioid peptide, β-endorphin, preproenkephalin and preprodynorphin are capable of producing a number of active fragments, each with differing affinity at the opioid receptors (Kosterlitz, 1985). Once the peptides have been released from the neurones, they are degraded into inactive fragments by peptidases.

Within the spinal cord there is an apparent mismatch in the distribution of the opioid peptides and their respective receptors. In the rat spinal cord, mu opioid receptors comprise the greatest proportion of opioid receptors, making up 69% of the opioid receptors in the superficial layers of the cervical dorsal horn (Besse et al., 1990). Despite the predominance of the mu opioid receptor in the spinal cord, immunofluorescent staining for β-endorphin, the endogenous ligand for the mu receptor, is absent within the spinal cord of adult rats (Bloom et al., 1978; Haynes et al., 1982). Although the enkephalins are not totally selective for the delta receptor, their physiological actions in the spinal cord can all be explained by actions at this receptor (Dickenson et al., 1986, 1988), leaving us with an apparent lack of an endogenous ligand for the mu opioid receptor in the spinal cord. It is possible that endorphins released from sites in the brain may reach mu opioid receptors in the spinal cord via the cerebrospinal fluid and there may be as yet undiscovered mu opioid ligands. However the physiological function played by the large population of spinal mu opioid receptors in the apparent absence of an endogenous ligand remains unresolved.

The enkephalin family of peptides comprise the largest population of opioid peptides within the spinal cord. Enkephalin-immunoreactivity is found throughout the dorsal horn of the spinal cord, although primarily in the superficial laminae, and originates from both intrinsic neurones (see refs. in Todd and Spike, 1993) and also from some of the descending fibres terminating in the dorsal horn (Hökfelt et al., 1979). These descending enkephalin systems have been implicated in the descending controls involved in phenomena such as stimulation produced analgesia (Besson and Chaouch, 1987) and diffuse noxious inhibitory controls (DNIC) (Le Bars et al., 1981). Delta opioid receptors, which are the target for the enkephalins in the spinal cord (Dickenson et al., 1986, 1988), comprise 24% of spinal opioid receptors (Besse et al., 1990). Although

over half (61%) of the δ-opioid receptors are located presynaptically (Besse et al., 1990), and evidence has been presented for a presynaptic action of enkephalin (Jessell and Iversen, 1977; Sastry, 1979; Hori et al., 1992), until recently axoaxonic synapses between enkephalin-immunoreactive fibres and primary afferent terminals in the rat spinal cord had been reported to be rare (Bresnahan et al., 1984; Ribeiro-da-Silva et al., 1991), or absent (Hunt et al., 1980; Sumal et al., 1982). Enkephalinergic contacts directly onto delta opioid receptors on small-diameter primary afferent terminals have recently been demonstrated in the rat spinal cord using immunostaining directed against the recently cloned delta opioid receptor DOR-1 together with antisera directed against leu-enkephalin (Dado et al., 1993), providing further evidence for the suggestion that the endogenous enkephalins may exert a δ-receptor mediated pre-synaptic control of the release of primary afferent transmitters. As well as this pre-synaptic action, the enkephalins also act postsynaptically to produce inhibitions of dorsal horn neurones (Zieglgänsberger and Tulloch, 1979; Yoshimura and North, 1983; Jeftinija et al., 1987).

The remaining family of opioid peptides, the dynorphins, are found in relatively small amounts in the spinal cord of normal rats, with the pool of pro-dynorphin-derived peptides being 10-20 times lower than that of pro-enkephalin-derived peptides in these animals (Iadarola et al., 1988b; Pohl et al., 1990). However, this situation changes dramatically after the onset of inflammation (see discussion). Dynorphin containing neurones in normal animals are located throughout the spinal cord, with the highest concentrations being found in laminae I, II and V (Khachaturian et al., 1982; Vincent et al., 1982; Cruz and Basbaum, 1985; Miller and Seybold, 1987; Ruda et al., 1988). Most of the staining for dynorphin in the spinal cord of the rat is thought to originate from intrinsic dorsal horn neurones, although some dynorphin containing neurones project to the diencephalon and brain stem (Nahin, 1987; Leah et al., 1988). The presence of dynorphin in primary afferent fibres in the rat is controversial. Although low levels of staining for dynorphin have been found in dorsal root ganglia in the rat (Botticelli et al., 1981) and dorsal rhizotomy has been reported to reduce the levels of dynorphin staining in the spinal cord (Tuchscherer and Seybold, 1989), other studies have failed to find a reduction in the levels of dynorphin in the dorsal horn the following dorsal rhizotomy (Botticelli et al., 1981; Pohl et al., 1990). The target opioid receptor for dynorphin is the kappa receptor which comprises only 7% of the opioid receptors in the rat spinal cord and is located both pre- and post-synaptic to the C-fibre primary afferent terminals (Besse et al., 1990).

The proportions and locations of the opioid receptors given above are a result of studies on rat spinal cord, however a limited number of studies of human spinal cord have also been carried out. With regard to the opioid peptides, studies on human spinal cord

have revealed that like the rat, the enkephalins and dynorphin, but not β-endorphin are found within the spinal cord (Przewocki et al., 1983a). The majority of opioid receptors in the human spinal cord are concentrated in the superficial layers of the dorsal horn, predominantly in lamina II (Faull and Villiger, 1987) which corresponds with the opioid receptor distribution found in rat spinal cord. The proportions of the various opioid receptors in the human spinal cord have also been examined using the sequential displacement of [^{3}H]diprenorphine by β-casomorphin-4-amide and (D-Ala^2-D-Leu^5)-enkephalin (Czlonkowski et al., 1983). At first glance it appears that kappa rather than mu opioid receptors predominate in the human spinal cord. However in this study, the authors also used the same ligands to examine the proportions of the opioid receptors in the rat spinal cord where they found the proportion of kappa receptors to be 35%, 5x higher than that obtained by Besse et al. (1990) using [^{3}H]ethylketocyclazocine in the presence of DAGO and DTLET to label kappa opioid sites. It is possible that the less selective ligands used by Faull and Villiger (1987) produce an overestimation in the number of kappa opioid sites at the expense of mu and delta opioid sites. When the proportions of the different opioid receptors in the rat and human are compared in the study by Faull and Villiger (1987), they appear similar. Thus if an overestimation of the number of kappa sites in the rat spinal cord is occurring, then the proportion of kappa sites in humans may be less than first thought.

Since the discovery of the endogenous opioid peptides in the mid-seventies (Hughes et al., 1975; Lord et al., 1977; Goldstein et al., 1979), attempts have been made to determine the role played by these endogenous opioid peptides in the control of pain mechanisms, particularly in protracted pain states. These studies have still left many questions unanswered, particularly with regard to the role played by dynorphin.

Many studies have been devoted to investigating the changes that occur in the levels of the endogenous opioid peptides in the spinal cord following the development of inflammation in the periphery (Cesselin et al., 1980; Millan et al., 1985, 1986, 1987, 1988; Iadarola et al., 1988a,b; Ruda et al., 1988; Nahin et al., 1989; Weihe et al., 1989; Noguchi et al., 1991; Przewlocka et al., 1992) and in the levels of mRNA coding for these peptides (Höllt et al., 1987; Iadarola et al., 1988a,b; Ruda et al., 1988; Draisci and Iadarola, 1989; Weihe et al., 1989; Draisci et al., 1991; Noguchi et al., 1991, 1992; Przewlocka et al., 1992). However, the functional consequences of these alterations are still relatively unclear.

Behavioural studies have attempted to gauge the consequences of the inflammation-induced alterations in opioid levels on nociceptive processing by looking at the effects of opioid antagonists on the hyperalgesia that is associated with the development of peripheral inflammation (Kayser and Guilbaud 1981, 1990, 1991; Millan

et al. 1985, 1987, 1988; Kayser et al., 1988; Millan and Colpaert 1990, 1991). In addition, a limited number of electrophysiological studies have examined the effects of opioid antagonists on neuronal activity in animals with inflammation (Kayser et al., 1988; Lombard and Besson, 1989; Stiller et al., 1993). However, with the exception of Stiller et al. (1993) these studies have relied on systemic administration of the drugs and hence their site of action is not always clear, possibly involving actions at peripheral and supraspinal as well as spinal sites. In addition, the majority of the studies have used the non-selective opioid antagonist naloxone making it difficult to determine the opioid receptor and therefore the putative endogenous opioid peptide involved.

This chapter aims to further elucidate the question of whether changes in endogenous opioid systems, which are functionally relevant to nociceptive processing, occur as a result of the development of inflammation in the periphery. By concentrating on one specific site of opioid modulation, the spinal cord, and by using, in addition to the non-specific antagonist naloxone, tools better able to differentiate between the different endogenous opioids / opioid receptors, I have attempted to determine whether there is increased modulation of spinal nociceptive transmission by endogenous opioids following three hours of carrageenan-induced inflammation. Hence I have addressed whether endogenous opioid peptides play a role, and whether this is an anti- or even pro-nociceptive role, in the early central response to the development of inflammation in the periphery.

In the first instance increasing spinal doses of the non-selective opioid antagonist naloxone, intended to cumulatively antagonize μ-, δ- and finally κ-opioid receptors, were used to determine whether the controls on spinal nociceptive processing exerted by endogenous opioids via actions at any of the opioid receptors were altered following the development of carrageenan inflammation.

Alterations in spinal enkephalinergic activity alone were then gauged by using the mixed peptidase inhibitor kelatorphan (Fournié-Zaluski et al. 1984) applied directly to the spinal cord in animals with carrageenan inflammation and comparing these actions to those seen in normal animals. The activity of dynorphin containing neurones in the spinal cord was examined using the selective kappa opioid antagonist nor-binaltorphimine (nor-BNI) (Portoghese et al. 1987), again applied directly to the spinal cord.

9.2. Results

9.2.1. Effect of intrathecal naloxone in normal animals

Four doses of naloxone (0.01, 1, 20 and 250μg), given intrathecally, were tested on the C- and Aβ-fibre evoked responses of 11 dorsal horn neurones in normal animals. Three of the doses used (1, 20 and 250μg) were shown in chapter 5 to reverse the effects of selective μ-, δ- and κ-agonists respectively and hence should be sufficient to reverse the effects of any endogenous opioids acting at these receptors. None of the doses of naloxone had a significant effect on the C- or Aβ-fibre evoked response of the neurones when followed over a 40 minute time course (the maximum effect of doses on the C-fibre evoked responses of individual neurones is shown in figure **9.1**). The greatest effect of naloxone in these animals was seen with the intermediate dose of 1μg which reduced the C-fibre evoked response to 90.5 ± 8.3% (n=6) (see figure **9.2**). The Aβ-fibre evoked response was not altered by more than 5% by any of the doses of naloxone tested.

9.2.2. Effect of intrathecal naloxone in carrageenan animals

Intrathecal naloxone was tested on the C- and Aβ-fibre evoked responses of 16 dorsal horn neurones three hours after the injection of carrageenan into the ipsilateral hind paw. In contrast to the lack of significant effects produced by any of the doses of naloxone (0.01, 1, 20 and 250μg) when applied to normal animals, two of these doses produced significant alterations in the C-fibre evoked neuronal responses in the carrageenan animals. The lowest dose of naloxone tested in the carrageenan animals, 0.01μg, paradoxically produced a moderate, but significant inhibition of the C-fibre evoked response, reducing it to 79.8 ± 3.1% of control (n=8) (p=0.0004, 1-tailed paired t-test) (figures **9.1** and **9.2**). As the dose of naloxone was increased to 1 and then 20μg, this inhibition was lost, turning to a significant facilitation of the C-fibre evoked response (115 ± 6.8% of control) as the dose was increased to 250μg, (p=0.03, one-tailed paired t-test, n=6) (figures **9.1** and **9.2**). None of the doses tested had a significant effect on the Aβ-fibre evoked response: for example, the maximum change produced in the Aβ-fibre evoked response by 0.01μg of naloxone was an inhibition of 4.2 ± 9.7% (n=5).

Both 0.01 and 250μg of naloxone produced significantly greater changes in the C-fibre evoked response of the neurones in the carrageenan animals than in the normal animals (p=0.009 and 0.046 respectively, Mann-Whitney test, 1-tailed) (figure **9.2**).

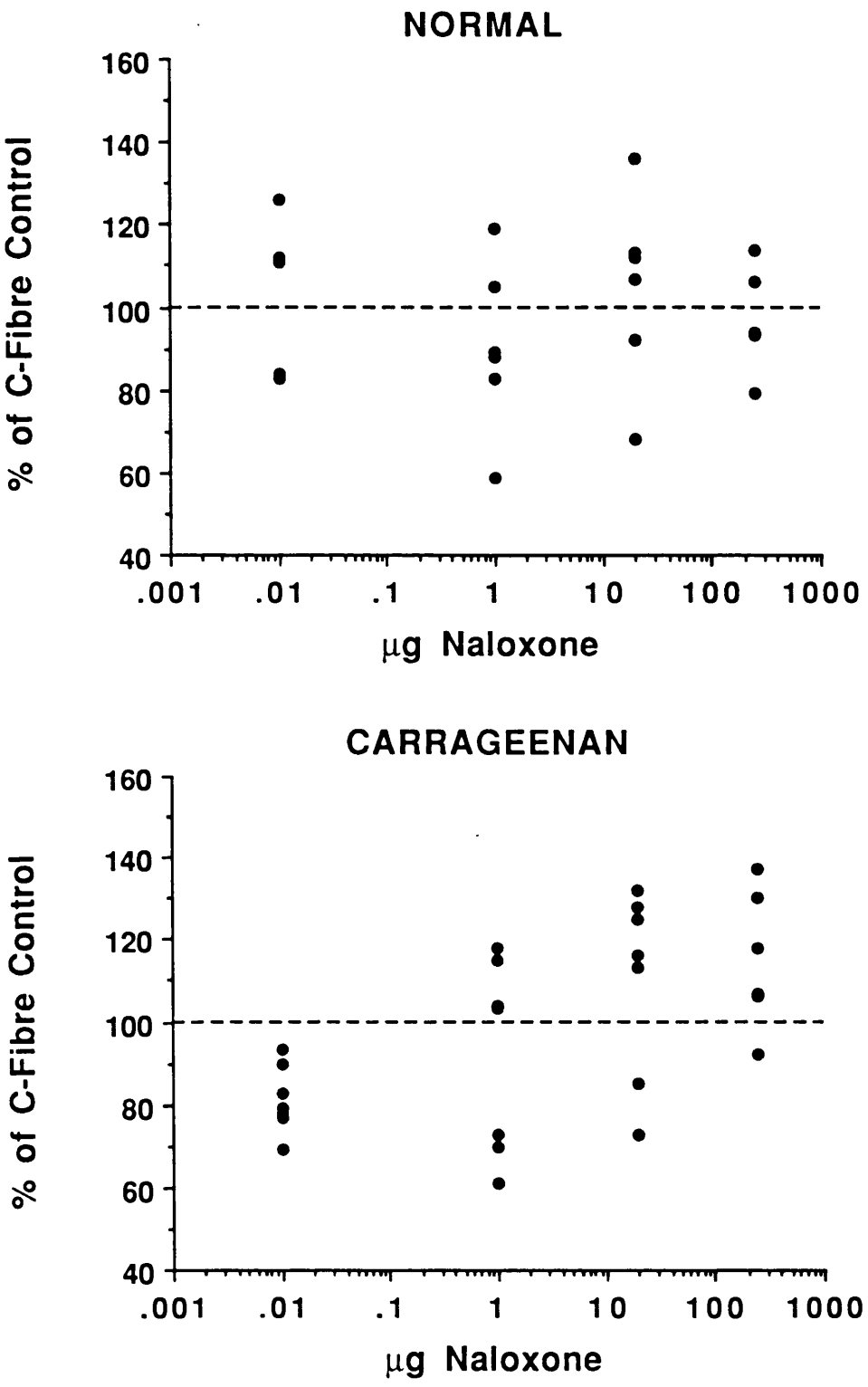


Figure 9.1. Effects of intrathecal naloxone on individual dorsal horn neurones in normal (upper panel) and carrageenan (lower panel) animals. Note that whilst the effects of naloxone on individual neurones in normal animals are unpredictable, clear dose related effects are produced in the carrageenan animals.

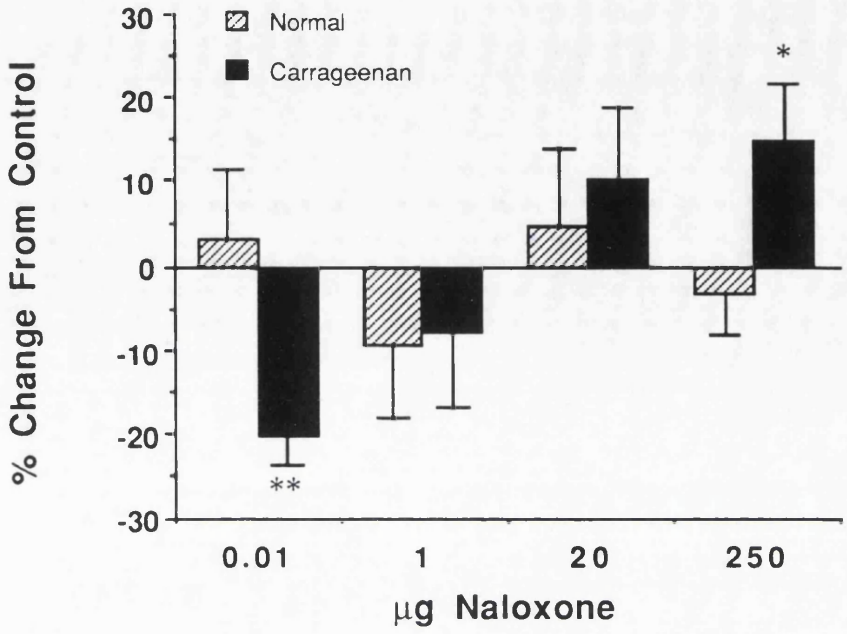


*Figure 9.2. Mean change in the C-fibre evoked neuronal response produced by intrathecal naloxone in normal and carrageenan animals. n=5-8 neurones per group. * $p<0.05$, ** $p<0.01$ compared with normal animals.*

9.2.3. Effect of intrathecal kelatorphan in normal animals

Three doses of kelatorphan (5, 50 and 250μg applied to the spinal cord) were tested on the C- and Aβ-fibre evoked responses of 12 dorsal horn neurones in normal animals. The lowest dose tested produced a significant inhibition of the C-fibre evoked response of 22.2 ± 5.5% (p=0.01, n=5, paired t-test), which increased to 42.4 ± 10.1% (n=5) with the next dose of 50μg. However when the dose of kelatorphan was increased to 250μg, no further increase in the inhibitory effect of kelatorphan was seen, with the inhibition of the C-fibre evoked response plateauing at 42.6 ± 9.2% (n=5) (figure **9.3**). The Aβ-fibre evoked responses were almost insensitive to the effects of kelatorphan, with 250μg of kelatorphan producing a maximum inhibition of only 7.0 ± 5.6%.

9.2.4. Effect of intrathecal kelatorphan in carrageenan animals

The same doses of kelatorphan (5, 50 and 250μg), given intrathecally, were tested on the C- and Aβ-fibre evoked responses of 10 dorsal horn neurones 3 hours after the injection of carrageenan. The lowest dose tested (5μg) produced a significant inhibition of the C-fibre evoked response of 15.1 ± 4.5% (p=0.02, n=7) which is similar to that seen in normal animals (figure **9.3**). As the dose of kelatorphan was increased to 50 and then 250μg the inhibitions of the C-fibre evoked response increased to 32.1 ± 5.4% (n=8) and 39 ± 12.5% (n=4) respectively (figure **9.3**). The inhibitions produced by kelatorphan in the carrageenan animals were not significantly different to those seen in normal animals although they tended to be slightly smaller. As in the normal animals, the inhibitory effects of kelatorphan appeared to reach a plateau, with 250μg of kelatorphan failing to produce a significantly greater inhibition than that seen with 50μg. These results demonstrate that 3 hours after the onset of carrageenan inflammation, kelatorphan is neither more potent (as judged by the effect of individual doses) or capable of producing a greater response (as judged by the maximum effect) than in normal animals.

The Aβ-fibre responses were not affected by kelatorphan, with a maximum inhibition of 4.2 ± 6.2% produced by 50μg.

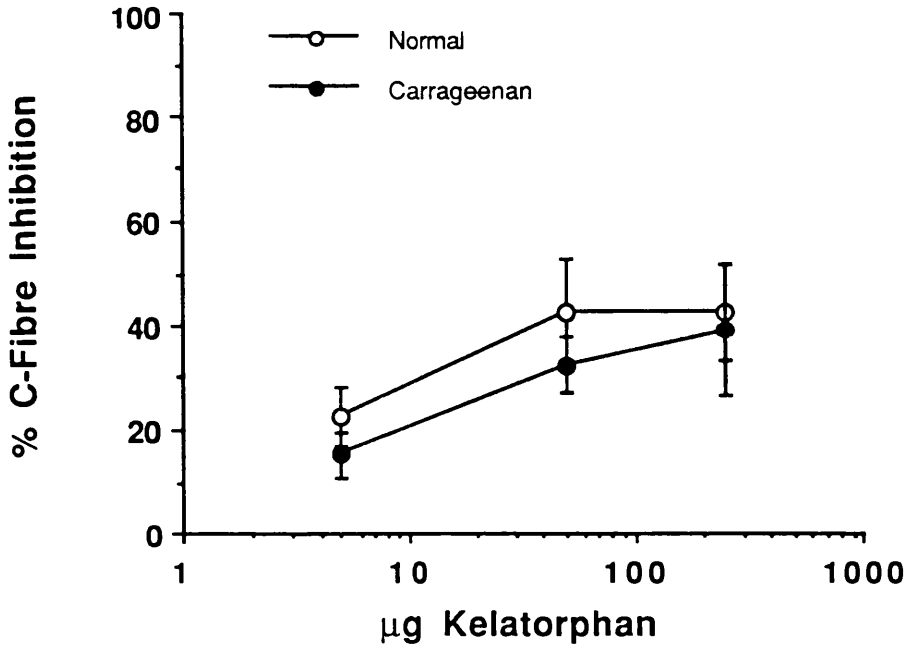


Figure 9.3. Dose-response curves for the inhibition of the C-fibre evoked response of dorsal horn neurones by intrathecal kelatorphan in normal and carrageenan animals. Note that the inhibitory effect of kelatorphan reaches a plateau in both groups of animals, with the inhibitions not exceeding 50%. n=4-8 neurones for each point.

9.2.5. Effect of intrathecal nor-binaltorphimine in normal animals

The effects of two doses of the kappa antagonist nor-binaltorphimine (nor-BNI) (10 and 100μg), given intrathecally, were tested on the C- and Aβ-fibre evoked responses of 11 dorsal horn neurones in normal animals. The response of the neurones to nor-BNI was not uniform although the properties and locations of recording sites of the neurones were indistinguishable. The C-fibre evoked responses of 4/11 (36%) of the neurones were facilitated by both doses of nor-BNI, whilst 3/11 (27%) neurones showed purely inhibitory responses following both doses of nor-BNI. The remaining four neurones (36%) showed a mixed response to nor-BNI, being inhibited by the low dose of 10μg and then facilitated by 100μg of nor-BNI (figure **9.4**, upper panel). When the responses of the neurones following the administration of nor-BNI were expressed as a mean percentage of control, it appears that nor-BNI (at either dose) had no net effect on the C-fibre evoked response, with the mean response after administration of 10 and 100μg of nor-BNI remaining at 96.1 ± 5.5% and 108.5 ± 5.3% of control respectively. However, this is clearly not a full representation of the actions of nor-BNI since it can be seen from figure **9.4** (upper panel) that nor-BNI is having a marked effect on the neuronal responses, albeit in a bidirectional fashion. Therefore it seems more appropriate to look at the responses of the neurones in terms of the magnitude of the effect produced by nor-BNI, regardless of the direction of effect, in much the same way as for the altered neuronal responses produced by carrageenan (see chapter 3). 10μg of nor-BNI produced a mean change (in either direction) from control of 15.7 ± 2.7% (n=11) with 100μg of nor-BNI producing no further increase in effect (mean change from control = 15.6 ± 3.3%, n=11). The magnitude of the change in the C-fibre evoked response produced by 10μg, but not 100μg of nor-BNI was significantly greater than that produced by the intrathecal administration of saline alone (mean change from control produced by saline, in either direction = 7.0 ± 1.5%, n=6) (Mann-Whitney test, one-tailed p-value = 0.032 for 10μg) (figure **9.5**).

The changes produced in the Aβ-fibre response by nor-BNI were also bidirectional but of a smaller magnitude than those produced in the C-fibre response with 10μg producing a change from control of 6.2 ± 2.6% (n=9) which increased to 10.7 ± 2.3% with 100μg (n=9).

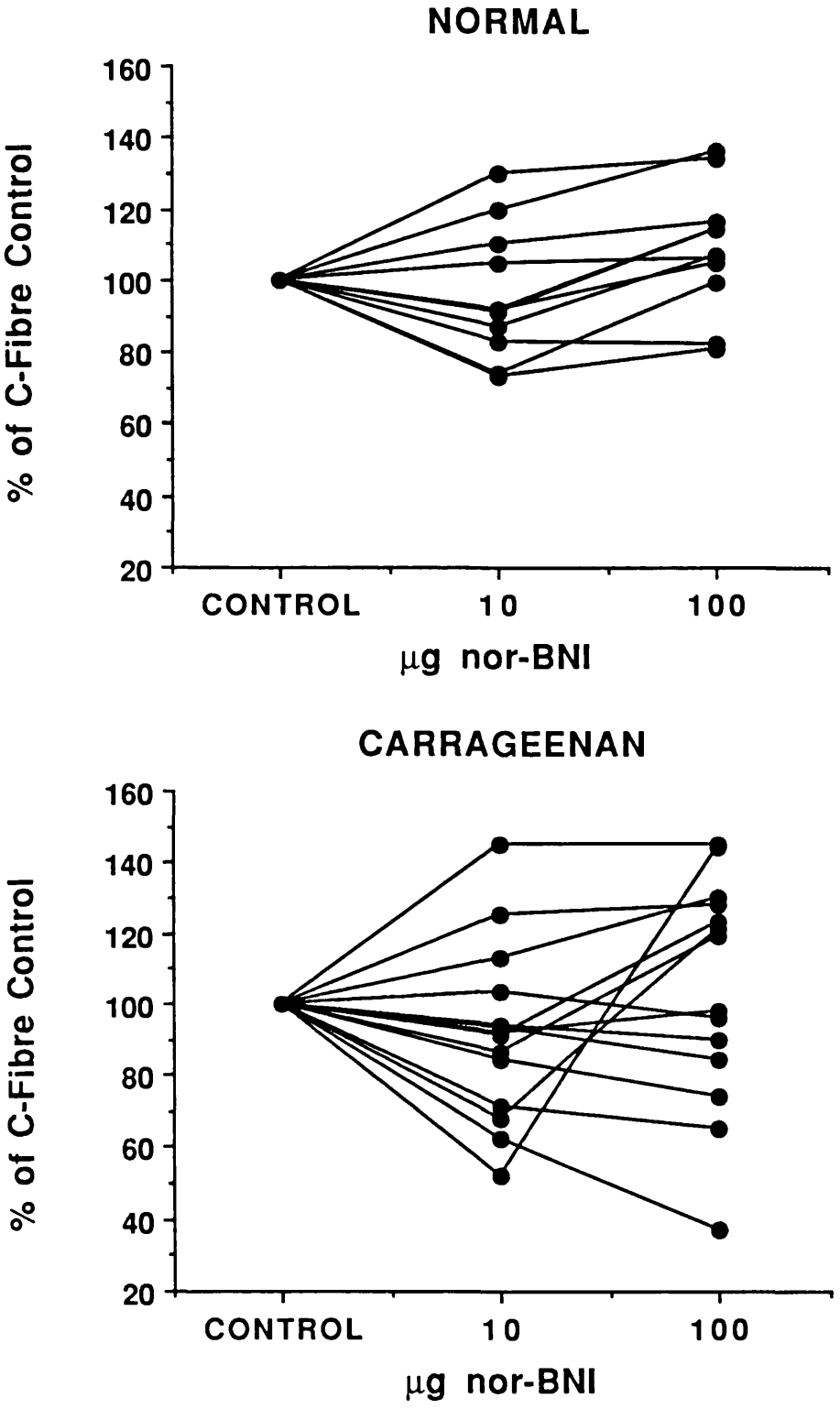


Figure 9.4. Effects of 10 and 100μg of nor-BNI, given intrathecally, on the C-fibre evoked responses of individual dorsal horn neurones in normal (top, n=11) and carrageenan (bottom, n=14) animals.

9.2.6. Effect of intrathecal nor-binaltorphimine in carrageenan animals

The effects of the same two doses of nor-binaltorphimine (10 and 100μg) were tested on the C- and Aβ-fibre evoked responses of 14 neurones 3 hours after the injection of carrageenan into the paw. As in normal animals, the response of the neurones to nor-binaltorphimine was not uniform (figure **9.4**). The C-fibre evoked responses of 3/14 (21%) of the neurones showed an enhanced response following both doses of nor-BNI. However, 6/14 (43%) of the neurones had a decreased response following both doses of nor-BNI, whilst the remaining 5 (36%) neurones had a decreased response following 10μg of nor-BNI, which was then facilitated above the control levels when the dose was increased to 100μg (figure **9.4**, lower panel).

As in normal animals, when the neuronal responses after nor-BNI were expressed as a mean percentage of control, nor-BNI produces no net change in the C-fibre evoked response, with the responses after 10 and 100μg of nor-BNI being 91.4 ± 6.7% and 103.9 ± 8.5% of control respectively (n=14). However, when the effect of nor-BNI was expressed as the degree of change in the neuronal response from the control level, regardless of direction, significant changes were seen, with 10μg and 100μg of nor-BNI producing a mean change in the C-fibre evoked response of 20.9 ± 4.1% (n=14) (Mann-Whitney test, one-tailed p-value = 0.03 compared with saline) and 26.1 ± 4.5% (n=14) (p=0.009) respectively (figure **9.5**). Furthermore, the magnitude of the change in the C-fibre evoked response produced by 100μg of nor-BNI in the carrageenan animals was significantly greater than that seen in normal animals (Mann-Whitney test, one-tailed p-value = 0.047) (see figure **9.5**).

The change in the Aβ-fibre evoked response produced in these animals by nor-BNI was again bidirectional. Unlike the C-fibre evoked response, the magnitude of the change in the Aβ-fibre evoked response produced by nor-BNI was not enhanced in the inflammatory state (mean change from control 7.8 ± 2.2% and 7.9 ± 1.8% with 10 and 100μg of nor-BNI respectively (n=11)).

The enhanced effects of nor-BNI on C-fibre evoked neuronal activity in the carrageenan animals compared with that seen in normal animals suggests that there is a greater release of the endogenous kappa ligand, presumably dynorphin, in the inflamed state. This is in contrast to the enkephalins, which are the endogenous ligands for the delta opioid receptor, which do not appear to be altered by the development of carrageenan inflammation, at least not at this point in the inflammatory process (section **9.2.4**).

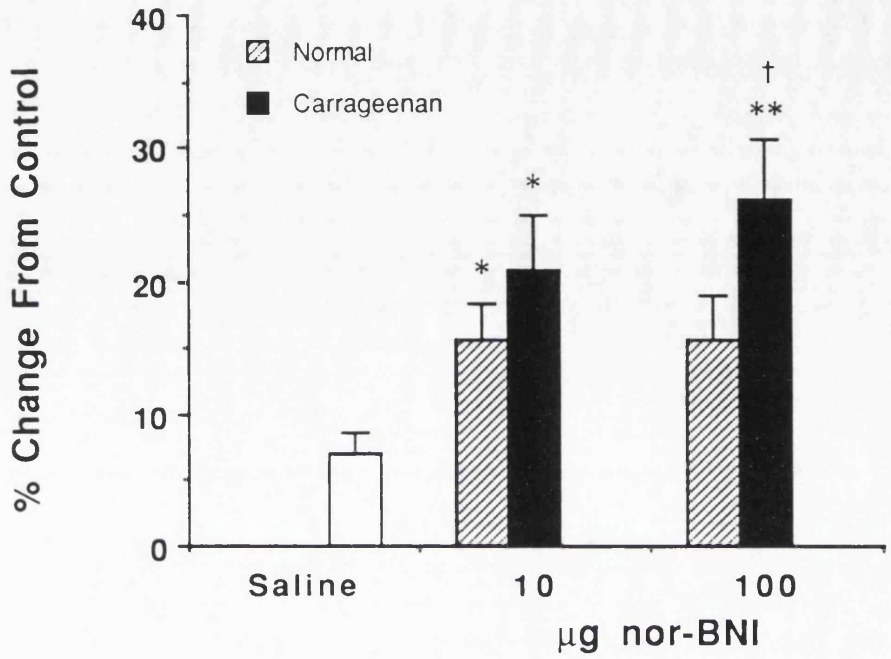


*Figure 9.5. Magnitude of change in the C-fibre evoked response of the dorsal horn neurones (regardless of direction of change) produced by 10 and 100µg of nor-BNI in normal and carrageenan animals and by intrathecal saline alone. * $p<0.05$, ** $p<0.01$ compared with saline alone. † $p<0.05$ compared with normal animals. n=6-14 neurones per group.*

9.2.7. Correlation between neuronal responses post-carrageenan and the effects of nor-BNI

The application of exogenous dynorphin to the spinal cord produces both facilitations and inhibitions of the responses of dorsal horn neurones (Knox and Dickenson, 1987; Hylden et al., 1991a), and the bi-directional effects of nor-BNI in this study suggest that endogenous dynorphin is also having this dual effect. In view of the enhanced actions of nor-BNI in the carrageenan animals and the bi-directional changes in the evoked neuronal response following the injection of carrageenan into the paw (chapter 3), it was proposed that an increased release of dynorphin in the spinal cord following the injection of carrageenan into the paw might be driving the changes in the evoked neuronal responses. However there was no correlation between the direction of change in the response of the cell in the 3 hours following the injection of carrageenan, i.e. whether the neurone showed a facilitated or inhibited response, and the direction of the subsequent

response to nor-BNI. Thus nor-BNI failed to return the altered neuronal responses induced by carrageenan to control levels, effectively ruling out dynorphin, at least via actions at kappa opioid receptors, as the cause of the altered neuronal responses post carrageenan.

However there was a significant correlation between the magnitude of the change in the neuronal response of a cell following the injection of carrageenan into the paw and the magnitude of the change in the neuronal response, regardless of direction, subsequently produced by nor-BNI (Pearson correlation, r = 0.722, p=0.018 with 100μg of nor-BNI). Figure **9.6** shows that neurones exhibiting the largest change in response post carrageenan also showed the largest change in response after 100μg of nor-BNI.

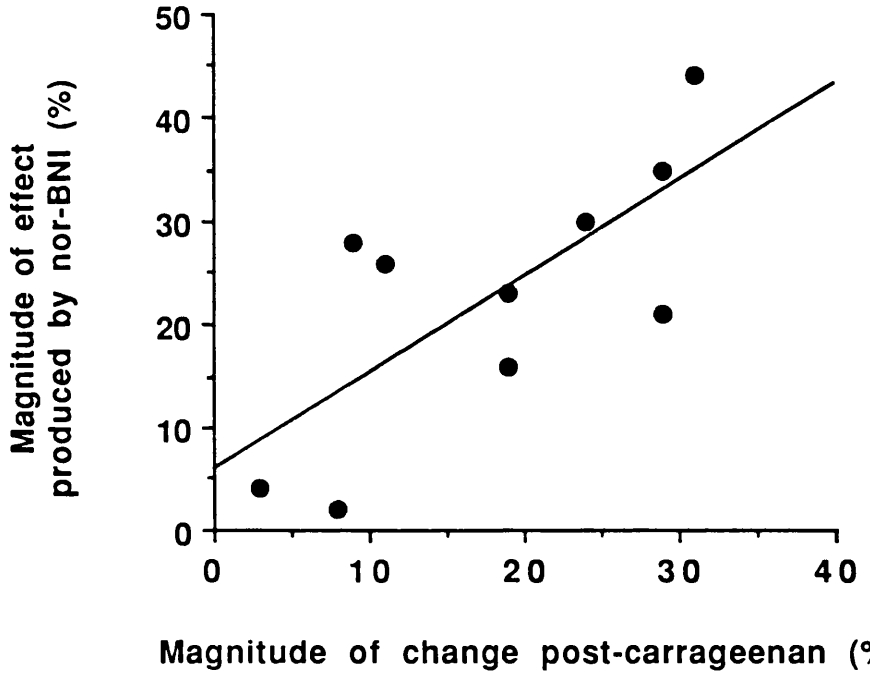


Figure 9.6. Correlation between the magnitude of the change in the neuronal response produced by carrageenan inflammation and the magnitude of the change subsequently produced in the same neurones by 100μg of nor-BNI. A significant correlation exists between these two factors (r = 0.722, p=0.018) suggesting a link exists between the causal mechanisms (see discussion for details).

9.3. Discussion

Despite numerous studies investigating the alterations in the levels of mRNA for the endogenous opioid peptides and the levels of the peptides themselves in the spinal cord following the development of inflammation in the periphery (see refs. in **9.1**), the functional consequences of these changes have received relatively less attention. This chapter has attempted to use pharmacological means to determine firstly whether changes in the physiological release of the opioid peptides occurs at the time when the hyperalgesia associated with the development of carrageenan inflammation in the periphery is at its peak, and secondly to unravel the consequences of any alterations in the spinal levels of the opioid peptides by using a number of tools to attempt to differentiate between the particular opioid peptides involved.

Many of the studies attempting to determine whether alterations in the levels of opioid peptides have any effect on the hyperalgesia associated with peripheral inflammation have used the mixed opioid antagonist naloxone. Hence naloxone was used in the present study as a starting point to investigate changes in the endogenous opioid peptides by cumulatively antagonizing first μ, then including δ and finally κ-opioid receptors with increasing doses of naloxone.

The intrathecal administration of increasing doses of naloxone in normal animals had no significant effect on the C-fibre evoked response. Although naloxone appeared to produce changes in the C-fibre evoked response that were greater than would be expected from saline alone, these changes were variable and not dose related in any way. The lack of a significant overall effect of intrathecal naloxone in normal animals seen in the present study is in contrast to the findings of Woolf (1980) and Dickenson et al. (1981) who found dose related effects of naloxone following intrathecal administration in behavioural studies. This difference may be related to the use of threshold noxious stimuli in the behavioural studies of Woolf (1980) and Dickenson et al. (1981), compared with the suprathreshold noxious stimuli used in the present study, against which the endogenous opioids may show lower efficacy. However, other behavioural studies have not seen effects on nociceptive thresholds following systemic administration of naloxone in normal animals (Kayser and Guilbaud, 1981, 1991).

By contrast, in animals with carrageenan inflammation of only three hours duration, the effects of the same doses of naloxone are markedly altered with complex dose related effects being produced. Whilst the highest doses of naloxone tested produced an enhancement in the C-fibre evoked response, consistent with a hyperalgesic effect resulting from a block of a tonic opioid mediated inhibition in these animals, the most obvious and surprising effect on the C-fibre evoked response is the inhibition of this

response produced by extremely low doses of intrathecal naloxone. This antinociceptive effect has been reported in behavioural studies following intrathecal administration of low doses of naloxone in normal animals (Woolf, 1980; Dickenson et al., 1981) and following low systemic doses of naloxone in animals with arthritis of three weeks duration (Kayser and Guilbaud, 1981, 1990) and animals with carrageenan inflammation of between 4 and 24 hours duration (Kayser et al., 1988). Studies on post-operative pain in humans have also demonstrated analgesia following the systemic administration of low doses of naloxone (Levine et al., 1979). My results and those of Woolf (1980) and Dickenson et al. (1981) show that this action can occur at the spinal level.

The mechanism underlying this paradoxical antinociceptive effect of low doses of naloxone is unclear. It has been suggested that the analgesic effects of very low doses of naloxone in inflammatory states are due to the interaction of naloxone with putative pre-synaptic opioid receptors located on enkephalinergic terminals, which are particularly sensitive to the drug following the development of inflammation (Kayser and Guilbaud, 1987; Kayser et al., 1988). At present there is no evidence to suggest that such an autoreceptor exists on spinal enkephalinergic, or indeed on any other peptide neurones, although the existence of a presynaptic kappa opioid receptor on enkephalin neurones in the rat brain stem has been proposed following release studies (Ueda et al., 1987). Even if this was the case in the spinal cord, this presynaptic kappa receptor (which is less sensitive to naloxone than mu and delta receptors) is unlikely to be sensitive to such low doses of naloxone, leaving the mechanism of the low dose naloxone analgesia in animals with peripheral inflammation unclear. Interestingly, the facilitations of the C-fibre evoked response by low doses of morphine in normal animals, which may be the counterpart of the C-fibre inhibitions produced by low doses of naloxone, were absent in the carrageenan animals (chapter 5) which suggests that different mechanisms may underlie these two effects.

Intermediate doses of naloxone, which were sufficient to block μ- and δ-opioid receptors, produced no clear effect on the C-fibre evoked response suggesting that no increase in the spinal actions of the enkephalins occurs at the time of peak hyperalgesia following carrageenan inflammation. This hypothesis was subject to further investigation using a more selective tool to examine the spinal actions of the enkephalins following the development of inflammation. Enkephalins released in the spinal cord are rapidly degraded, primarily by neutral endopeptidase-24.11 (NEP) (Malfroy et al., 1978) and aminopeptidase-N (APN) (Hambrook et al., 1976; Waksman et al., 1985). Inhibition of these enzymes by drugs such as kelatorphan (Fournié-Zaluski et al., 1984) allows the physiological roles of the enkephalins (which act spinally at the delta receptor under these conditions (Dickenson et al 1986, 1988)) to be gauged. However, it must be said that

kelatorphan can also prevent the degradation of other peptides such as substance P (Roques et al., 1993) and thus the results obtained using this drug may reflect the protection of a number of non-opioid peptides in addition to the enkephalins.

In both normal and carrageenan animals, intrathecal kelatorphan produced a dose dependent inhibition of the C-fibre evoked neuronal response suggesting that whatever the range of peptides protected by kelatorphan, inhibitory effects predominate implicating the enkephalins. The lack of effect of kelatorphan on the Aβ-fibre evoked response of the convergent neurones is consistent with the finding that over 50% of opioid receptors are located on C-fibre terminals (Besse et al., 1990). There was no increase in either the maximum effect of kelatorphan, or the ED_{50} value for kelatorphan in the carrageenan animals compared to normals. Thus three hours into the development of carrageenan inflammation, there is no alteration in the enkephalinergic control of nociceptive transmission in the spinal cord. The lack of change in the potency of kelatorphan, which implies no change in the potency of the enkephalins themselves, agrees with the results presented in chapter 5 where the potency of the delta opioid agonist DSTBULET showed only a small enhancement in potency in carrageenan animals compared with the 18 fold enhancement in spinal potency seen with the mu opioid morphine (chapter 5).

Kelatorphan has been examined in a behavioural study in arthritic animals and in this study an enhanced antinociceptive action was found (Kayser et al., 1989). However this study used systemic administration of kelatorphan, which penetrates the blood brain barrier only poorly (Roques et al., 1993). Thus the results of Kayser et al. (1989) cannot be compared to the present study, which used the spinal route of administration, since they are likely to result primarily from enhanced actions of the drug at peripheral sites in the inflammatory state. More recently, a compound able to prevent the degradation of the enkephalins and to penetrate the blood brain barrier has been synthesized (RB 101, Fournié-Zaluski et al., 1992). This compound, together with another enzyme inhibitor (RB 38 A), which is unable to penetrate the blood brain barrier, has been used in conjunction with the peripherally acting opioid antagonist methylnaloxonium to investigate the actions of the endogenous opioids at peripheral and spinal sites in animals with inflammation produced by injection of Freund's complete adjuvant into the paw (Maldonado et al., 1994). In this study, both enzyme inhibitors produced a selective antinociceptive effect on the inflamed but not non-inflamed paw following systemic administration which was antagonized in both cases by methylnaloxonium, which acts primarily outside the brain. This indicates that the enhanced antinociceptive actions of the enkephalins in inflammation result from enhanced actions in the inflamed peripheral tissue rather than at central sites (Maldonado et al., 1994).

The lack of change seen in the actions of spinal kelatorphan or intermediate doses

of intrathecal naloxone in the present study three hours after the injection of carrageenan into the paw is not surprising since immunohistochemical studies find only a small increase in the staining for the mRNA for enkephalin even after several days of inflammation induced by the intraplantar injection of carrageenan (Draisci and Iadarola, 1989; Draisci et al., 1991, but see Noguchi et al., 1992) or complete Freund's adjuvant (Iadarola et al., 1988b; Przewlocka et al., 1992). Even when increases in the mRNA for enkephalin were seen, this increase was too small to yield a measurable increase in the levels of met-enkephalin in the spinal cord (Iadarola et al., 1988b). Indeed it has been suggested that the high level of background staining for enkephalin may indicate that the pool of enkephalin is sufficient to cope with the extra demands of inflammation (Draisci and Iadarola, 1989), although the results of the present study suggest that a greater spinal release of enkephalins in response to inflammation does not occur.

Potentially of more importance are the physiological consequences of the increase in spinal dynorphin following the development of inflammation in the periphery. The levels of mRNA for dynorphin and the levels of dynorphin itself, although relatively low in the spinal cord of normal animals, increase dramatically following the onset of peripheral inflammation (Millan et al., 1985, 1986, 1987, 1988; Höllt et al., 1987; Iadarola et al., 1988a,b; Ruda et al., 1988; Draisci and Iadarola, 1989; Nahin et al., 1989; Weihe et al., 1989; Draisci et al., 1991; Noguchi et al., 1991; Hylden et al., 1992). Immunocytochemical studies have demonstrated that an increase in the levels of preprodynorphin mRNA in the cord can be detected as early as 4 hours after the onset of carrageenan inflammation (Draisci and Iadarola, 1989), with a maximum elevation in mRNA levels of 200-400% occurring 24 hours post-injection (Iadarola et al., 1988a; Draisci and Iadarola, 1989; Draisci et al., 1991). This rise in preprodynorphin mRNA in the cord is secondary to the rise in mRNA for the proto-oncogene *c-fos*, which occurs within 30 minutes of the injection of carrageenan into the paw (Draisci and Iadarola, 1989). Indeed, the finding that preprodynorphin mRNA and Fos-like immunoreactivity co-localize after inflammation (Noguchi et al., 1991), and that both are attenuated by neonatal capsaicin treatment (Hylden et al., 1992), strongly implicates Fos in the control of dynorphin gene expression. The levels of dynorphin itself do not start to rise until later, with no detectable increase seen until 24 hours after the injection of carrageenan into the paw (Iadarola et al., 1988a).

In this chapter I have used antagonists to look at the physiological role played by spinal dynorphin released during the development of carrageenan-induced hyperalgesia. This method has the advantage of revealing changes in the levels of dynorphin released from existing stores in response to the development of hyperalgesia without having to wait for novel synthesis of the peptide. The upregulation in dynorphin synthesis lags behind the time course of the development of hyperalgesia following the injection of

carrageenan and this may represent either a late involvement of dynorphin in the inflammatory process or be a response to the enhanced release of the peptide earlier in the inflammatory process. The antagonist studies presented in this chapter reveal that the functional activity of dynorphin in the cord is enhanced at the time of peak carrageenan-induced hyperalgesia.

Although this increase in spinal dynorphin as a result of inflammation has been well documented, attempts to assign a physiological role to this upregulation during inflammation have led to conflicting ideas. Unlike enkephalin, which has never been suggested to play anything other than an analgesic role in the spinal cord, both pro- and antinociceptive roles have been proposed for dynorphin. It has been suggested by some groups following antagonist studies that dynorphin, via an action at kappa receptors, plays an analgesic role in the inflammatory state by modulating the behavioural hyperalgesia associated with inflammation (Millan et al. 1985, 1987, 1988; Kayser and Guilbaud, 1991; Millan and Colpaert, 1990, 1991). However, in addition to this analgesic role proposed for dynorphin, there is evidence to suggest that dynorphin is implicated in pro-nociceptive events, namely the expansion of the receptive fields of lamina I projection neurones that has been reported to occur following inflammation (Hylden et al. 1989, 1991a) and in the spread of pathology to the contralateral side of the cord following unilateral inflammation (Millan and Colpaert, 1990, 1991).

The results of behavioural studies where dynorphin itself has been applied spinally in normal animals do not help to clarify the possible role of dynorphin in inflammation. Although spinal dynorphin has been reported to produce analgesia in these studies (Han and Xie, 1982; Piercey et al., 1982; Przewlocki et al., 1983b; Hermann and Goldstein, 1985; Spampinato and Candelletti, 1985), profound motor effects and neurotoxicity are also produced (Przewlocki et al., 1983b; Faden and Jacobs, 1984; Hermann and Goldstein, 1985; Caudle and Isaac, 1987) throwing doubt on the analgesic results obtained using behavioural measures. When the effects of dynorphin on dorsal horn neurones have been examined, (i.e. no motor response required) dynorphin produces bi-directional changes in the neuronal responses (Knox and Dickenson, 1987; Hylden et al., 1991a); in other words, the results favour neither an analgesic or hyperalgesic role for dynorphin.

In this study, high doses of intrathecal naloxone were used in the first instance to gauge the role of dynorphin in the inflammatory state. In contrast to the effects of very low doses of naloxone in these animals, the high doses of naloxone facilitated the C-fibre evoked response, consistent with a hyperalgesic action. This is in agreement with behavioural studies in animals with peripheral inflammation where the administration of high doses of naloxone, although this time by systemic routes so allowing actions at

opioid receptors at supraspinal and peripheral sites and as well as spinal sites, potentiated the hyperalgesia (Kayser and Guilbaud, 1981, 1990, 1991; Millan et al., 1988; Millan and Colpaert, 1990, 1991). However, naloxone is a non-specific opioid antagonist and would therefore also be simultaneously blocking mu and delta receptors at the doses used. What this tells us about the actions of dynorphin is debatable, particularly since the some of the effects of exogenous dynorphin applied to the spinal cord have been shown to be naloxone insensitive (Herman and Goldstein, 1985; Knox and Dickenson, 1987).

In order to obtain a more accurate picture of the spinal actions of endogenous dynorphin following the development of inflammation, via actions at kappa opioid receptors, the selective kappa opioid receptor antagonist nor-BNI was used. Of particular interest in this study with regard to the action of dynorphin in the inflammatory state were the changes in the evoked neuronal responses which developed as a result of the inflammation in the periphery (chapter 3). The electrically evoked responses of convergent dorsal horn neurones were altered during the development of inflammation, with approximately half the cells showing enhanced responses as the inflammation developed and the other half showing decreased responses (chapter 3). This bidirectional change in the response of the neurones to the developing inflammation is similar to that seen when dynorphin is applied to the spinal cord in normal animals when both facilitations and inhibitions of the neuronal response are seen (Knox and Dickenson 1987). Hylden et al. (1991a) have also reported dual effects of dynorphin and other kappa agonists on both the responses and peripheral receptive field sizes of dorsal horn neurones. Thus in view of the reported increase in the levels of spinal dynorphin following inflammation, albeit belatedly, and the similarity between the neuronal response to the spinal application of dynorphin (Knox and Dickenson, 1987) and that seen post-carrageenan (chapter 3), it was hypothesized that an increase in the levels of spinal dynorphin in response to the inflammation might be driving the observed neuronal changes.

The results with nor-BNI do not appear to support this theory, at least in terms of an action of dynorphin at kappa opioid receptors. Nor-binaltorphimine produced a bidirectional change in the evoked response of the neurones, presumably by antagonizing the actions of endogenous dynorphin at kappa receptors, the magnitude of which was significantly greater in the carrageenan animals than in normal animals. However, there was no correlation between the direction of change in the neuronal response post-carrageenan and the direction of the subsequent change in neuronal response produced by nor-BNI. Despite the similarities between the response of spinal neurones to exogenous dynorphin (Knox and Dickenson 1987; Hylden et al. 1991a) and the change in response arising from the development of inflammation, the absence of this critical correlation argues against an increased activation of kappa opioid receptors by dynorphin being

responsible for the alterations in neuronal response seen post-carrageenan.

There was however a significant correlation between the magnitude of the change in the C-fibre evoked neuronal response induced by carrageenan (but not the direction) and the magnitude of the change in response of the same cells produced by nor-BNI. Thus the neurones that showed the largest inhibition and those with the largest facilitation of their response with the development of carrageenan-induced inflammation then showed the largest change in their response after nor-BNI, whether or not this was an enhanced or decreased response. This correlation suggests that although dynorphin acting at kappa receptors is unlikely to be driving the neuronal changes induced by carrageenan, there is likely to be a strong link between the spinal system responsible for driving both these changes and those in the spinal dynorphin system. The *N*-methyl-D-aspartate (NMDA) receptor system within the spinal cord is a likely candidate.

In chapter 3, the role of the NMDA receptor in the neuronal changes post carrageenan was proposed based on evidence that the degree of wind-up of the neurones pre-carrageenan (an NMDA mediated event) determines the direction and magnitude of the subsequent neuronal change. It is therefore of interest that some of the actions of dynorphin have been linked to non-opioid events involving excitatory amino acids and their receptors, particularly NMDA receptors. In the hippocampus, exogenous dynorphin has been shown to increase the extracellular levels of the excitatory amino acids through a non-opioid mechanism (Faden, 1992). It has also been proposed that the block of the tail-flick reflex by dynorphin applied to the spinal cord involves non-opioid, NMDA receptor mediated excitotoxic transmission in the cord (Caudle and Isaac 1988), a finding extended by Bakshi and Faden (1990) who demonstrated that both competitive and non-competitive NMDA receptor antagonists reduced dynorphin-induced hindlimb paralysis.

It could be envisaged that the increased release of dynorphin in the cord post-carrageenan (judged by the enhanced actions of nor-BNI in these animals) may lead to an increase the extracellular levels of excitatory amino acids which would in turn lead to enhanced activation of NMDA receptors. Thus if dynorphin is driving the release of excitatory amino acids within the cord which is then in turn, through activation of NMDA receptors, driving the changes in evoked neuronal response, then this could explain the observed link between the magnitude of the effect of nor-BNI (which is a measure of the kappa receptor mediated dynorphin influence on a particular neurone) and the magnitude of the change in the evoked response of that neurone. If this were the case, then blocking kappa opioid receptors with nor-BNI would not reverse the altered neuronal responses since the actions of dynorphin on excitatory amino acids are mediated through a non-opioid mechanism (Faden, 1992). In view of these non-opioid actions of dynorphin, it must be stated that the present study on the role of dynorphin in inflammation is restricted

to actions at kappa receptors as it relies on opioid receptor antagonists as tools. However these may not reveal all the possible actions of dynorphin and this must be borne in mind when interpreting these results.

Thus it appears that dynorphin plays an increased although complex role in the control of spinal nociceptive transmission following inflammation. Interestingly, the results obtained with the selective kappa opioid antagonist nor-BNI in the carrageenan animals (both facilitations and inhibitions of the evoked response) are different to those produced by high intrathecal doses of the non-selective opioid antagonist naloxone in this study (consistent facilitations of the evoked response). Whether the cumulative antagonism of μ, δ and κ-opioid receptors by naloxone as opposed to just κ-opioid receptors by nor-BNI or differential antagonism of κ-opioid receptor subtypes underlies this difference is not known. Either way, results obtained using high doses of naloxone cannot be presumed to give a true picture of the actions of dynorphin in the inflamed state.

Interestingly, the dual effects on the evoked neuronal responses seen with nor-BNI suggests that, as in normal animals (Knox and Dickenson, 1987), endogenous dynorphin is having both facilitatory and inhibitory effects on the evoked neuronal responses in the carrageenan animals. Although the kappa agonist U69593 produced both facilitations and inhibitions of the evoked neuronal responses in normal animals (chapter 5), the effects of this compound were purely inhibitory in the carrageenan animals (chapter 5). Likewise, the facilitations produced by low doses of intrathecal morphine in normal animals were absent in the carrageenan animals (chapter 5). Dynorphin has been shown to produce depolarization of dorsal root ganglion neurones by decreasing a K^+ conductance whereas the kappa ligand U-50,488H does so by increasing a calcium conductance (Shen and Crain, 1990). Thus although different modes of action, possibly reflecting actions at different kappa opioid receptor subtypes, could be put forward for the ability of dynorphin, but not U69593, to produce facilitations of the neuronal response in the carrageenan animals, it seems strange that such effects should be produced by endogenous opioid ligands (revealed by nor-BNI and low doses of naloxone) in these animals and yet not by exogenous ligands (U69593 and morphine, chapter 5), regardless of dose. The reasons for this apparent discrepancy remain elusive.

The enhanced, but bidirectional effects of dynorphin on spinal neuronal responses revealed by nor-BNI following the development of carrageenan inflammation have also been reported in a model of joint inflammation (Stiller et al., 1993). Thus the role played by dynorphin (analgesic or hyperalgesic) in the inflammatory state is unclear, and it may not simply be an endogenous analgesic response to the enhanced nociceptive state as proposed on the basis of naloxone studies (Millan et al., 1988; Millan and Colpaert, 1990, 1991).

The finding that neonatal capsaicin treatment reduces the expression of Fos-like immunoreactivity and preprodynorphin mRNA following the injection of complete Freund's adjuvant into the paw, yet has little effect on the magnitude of the thermal hyperalgesia produced (Hylden et al., 1992) does not shed any light on the possible role, whether pro- or anti-nociceptive, of dynorphin in inflammation. Increases in dynorphin have been suggested to be responsible for the transfer of the symptoms of inflammation to the contralateral side following intraplantar injection of Freund's adjuvant (Millan and Colpaert, 1990, 1991), a role which could be regarded as pro-nociceptive. However, this conclusion was reached following studies employing high doses of naloxone which, as demonstrated in this chapter, can produce different results to the selective blockade of kappa opioid receptors alone. Thus although there clearly is an upregulation in the levels of dynorphin in the spinal cord following the development of peripheral inflammation (shown by immunocytochemical studies) and presumably in the release of this peptide (judged by the enhanced actions of nor-BNI) the consequences of this still remain relatively obscure, and may involve actions at non-opioid receptors.

CHAPTER 10

CONCLUSIONS

It has been recognized for some time now that the central nervous system is capable of a great deal of plasticity in the processing of nociceptive information. Even a brief (seconds) high intensity noxious insult can trigger changes in the spinal processing of nociceptive messages which persists for some time (minutes to hours) after cessation of the stimulus, so called central hypersensitivity (Woolf, 1983). Nociception arising from pathological damage to the body, such as inflammation, is of longer duration, and is able to evoke a far greater repertoire of changes, including gene induction, which may have long term implications for the transmission and modulation of nociceptive messages.

With this in mind, the purpose of the experiments presented in this thesis was to examine the central consequences of the development of acute peripheral inflammation, a model relevant to clinical conditions such as post-operative pain, on spinal systems involved in the transmission and modulation of nociception, and how this might relate to clinical therapy. In pursuit of this, spinal neurotransmitter systems classically regarded to play excitatory, inhibitory or modulatory roles in nociceptive processing were examined using specific receptor agonists and antagonists to reveal if these systems were subjected to plastic changes as a result of the changing situation in the periphery. The experiments described in this thesis show that within just three hours of the injection of carrageenan into the paw, the time corresponding to the peak hyperalgesia seen in behavioural studies using this inflammatory agent (Kayser and Guilbaud, 1987; Hargreaves et al., 1988a; Hylden et al., 1991b), a number of spinal transmitter systems, inhibitory and modulatory, as well as excitatory, have undergone changes.

As might be predicted from the development of behavioural hyperalgesia following the peripheral injection of carrageenan, this thesis provided evidence of the development of central hypersensitivity of dorsal horn neurones following the development of carrageenan inflammation (chapter 3). Many dorsal horn neurones showed enhanced responses to electrical stimulation of their peripheral receptive field during the development of inflammation in the surrounding tissue. The use of electrical stimulation, which by-passes the sensitized peripheral nociceptors, allows the deduction that this sensitization is a result of central mechanisms. The strong correlation between the magnitude of the enhanced neuronal response post-carrageenan and the degree of NMDA receptor-mediated wind-up of the neurones (chapter 3), together with the ability of NMDA receptor antagonists to reverse the facilitated input onto the neurones (chapter 4), strongly implicates activation of the NMDA receptor in the hypersensitivity. This is consistent with the findings of Woolf and Thompson (1991) who demonstrated that the induction and maintenance of central sensitization is dependent on NMDA receptor activation, as are the events underlying the second phase of the formalin response (Haley et al., 1990; Coderre and Melzack, 1992).

However, in addition to enhanced responses of dorsal horn neurones following the development of carrageenan-induced inflammation, many dorsal horn neurones, almost 50%, displayed decreased responses to electrical stimulation as the inflammation developed. Furthermore these were the neurones exhibiting most wind-up during the control period and therefore potentially the most excitable neurones (chapter 3). The link between the decreased neuronal responses and wind-up (chapter 3) together with subsequent studies using the NMDA receptor antagonist MK-801 (chapter 4) also implicated NMDA receptor-mediated events in these inhibitions. The conclusion drawn from these results was that whilst NMDA receptor-mediated central hypersensitivity was occurring as a result of the development of inflammation in the periphery, when this led to an excessively large degree of neuronal activation, as with neurones already exhibiting a high degree of wind-up under these stimulating conditions, inhibitions were triggered to counter and damp down this activity. Activation of NMDA receptors appears to be central to the triggering of these subsequent inhibitions.

Although this may initially seem surprising, there is evidence in the literature that mechanisms exist whereby NMDA receptor activation can trigger inhibitions. Given that excessive NMDA receptor activation can lead to neuronal damage through excitotoxic mechanisms (Olney, 1990), this is perhaps not surprising. Activation of spinal NMDA receptors has been shown to inhibit spinal nociceptive reflexes via an enhancement of descending controls (Kolhekar et al., 1993). In chapter 8 of this thesis it was shown that descending noradrenergic controls are enhanced 3 hours after the injection of carrageenan into the paw (chapter 8). In addition, studies in cats using a reversible cold block have shown that the tonic descending control of dorsal horn neurones is enhanced following the induction of acute arthritis of the knee joint (Cervero et al., 1991; Schaible et al., 1991b). This inflammation-induced upregulation in the activity of descending controls may be triggered by spinal NMDA receptor activity. The spinal co-administration of SP with NMDA has been shown to produce a reduction in the response to NMDA in one-third of dorsal horn neurones tested (Dougherty and Willis, 1991a). This again suggests there is a ceiling on the activation of the NMDA receptor, after which point inhibitory circuits are triggered. Whilst this inhibitory circuit may involve activation of supraspinal mechanisms (see above), activation of inhibitory circuitry in the spinal cord itself is also likely to be involved.

Following the induction of peripheral inflammation, both the synthesis (Nahin and Hylden, 1991) and levels (Castro-Lopes et al., 1992, 1994) of GABA in the dorsal horn of the spinal cord are enhanced; this is coupled with a 3-fold increase in the potency of the exogenously administered $GABA_B$ agonist baclofen (chapter 6). It is note-worthy that much of the nitric oxide synthase (NOS) in dorsal horn neurones appears to be co-

localized with GABA (Valtschanoff et al., 1992; Spike et al., 1993). NMDA receptor activation appears to be necessary for the activation of NOS and subsequent generation of NO (see Meller and Gebhart, 1993), implying that these GABAergic interneurones are likely to have NMDA receptors located on them. It can be envisaged that under conditions where there is a very high degree of neuronal activity, NMDA receptors on GABAergic interneurones (which may need a higher level of afferent drive to produce the depolarization necessary to overcome the Mg^{2+} block of the channel than those in excitatory circuits) become activated, increasing the drive of GABA-mediated inhibitions. Activation of NMDA receptors on these neurones may then also lead to the generation of NO which could diffuse to presynaptic terminals to increase GABA release, further increasing inhibitions. Alternatively, the NO released from GABAergic neurones may act elsewhere to increase excitations within the cord.

Another potential inhibitory system which undergoes a dramatic upregulation in the spinal cord following the onset of inflammation involves the opioid peptide dynorphin (see chapter 9). In this thesis I showed, using the kappa opioid antagonist nor-BNI, that the physiological actions of dynorphin in the spinal cord are enhanced following the injection of carrageenan into the paw. Whilst the upregulation in dynorphin has been suggested, mainly on the basis of behavioural studies using the non-selective opioid antagonist naloxone, to be an endogenous analgesic response to the development of inflammation in the periphery (Millan et al. 1985, 1987, 1988; Kayser and Guilbaud 1991; Millan and Colpaert 1990, 1991), electrophysiological studies, including that presented in chapter 9, using either nor-BNI or dynorphin itself are unable to support either an anti- or pro-nociceptive role for dynorphin (Knox and Dickenson, 1987; Hylden et al., 1991a; Stiller et al., 1993). Thus the role played by the dramatic upregulation in spinal dynorphin as a consequence of the development of peripheral inflammation is unclear. The correlation between the magnitude of effect of nor-BNI on a neurone, which presumably reflects the level of dynorphin control of a given neurone, and the degree of change in the evoked response of that neurone post-carrageenan (chapter 9), which depends on the degree of NMDA receptor activation, is interesting given the possible non-opioid actions of dynorphin. Some of the actions of dynorphin are reported to be mediated through activation of NMDA receptors (Caudle and Isaac, 1988; Bakshi and Faden, 1990). Thus the enhanced actions of dynorphin in the inflamed state may involve actions through NMDA receptors as well as κ-opioid receptors. The other family of opioid peptides found in the cord, the enkephalins, do not undergo a dramatic upregulation following the development of inflammation, and the results with kelatorphan (chapter 9) suggest that their release is not altered in the inflammatory state. This suggests that the dynorphins are likely to be playing a specific function post-inflammation. However, the bidirectional nature of the changes produced by dynorphin (see chapter 9) mean that it is

unclear whether the inflammation-induced upregulation in dynorphin contributes to the development of central hypersensitivity, the inhibitions induced as a result of this, or possibly both effects.

In addition to the upregulation in the dynorphin component of the endogenous opioid system, inflammation-induced changes occur which lead to an enhancement in the spinal potency of exogenously administered opioids. This enhancement in potency was far greater for the μ-opioid morphine, than for the δ- or κ-opioids tested (chapter 5), which implicates changes in specific systems within the spinal cord. This enhancement in μ-opioid potency following the development of inflammation was linked to a down-regulation in the spinal CCK system, which can interfere with the inhibitory actions of μ- but not δ- or κ-opioids in normal animals (chapter 7).

From the evidence presented above it appears that the upregulation in inhibitory systems, either direct inhibitory systems such as GABA and the descending controls, or indirect via the downregulation in CCK, form an important part of the central response to the development of inflammation in the periphery, and do much to counter the negative aspects to this phenomenon such as central hypersensitivity. There is evidence that the induction of inhibitions may play an important role in another model of inflammatory nociception, the formalin model. This model differs from the carrageenan model used in the present study in that the injection of formalin into the paw leads to a biphasic response in both behavioural studies, where this is manifest as spontaneous flinching and paw licking by the animal, and in electrophysiological studies where it presents as ongoing firing of the dorsal horn neurones (see Tjølsen et al., 1992). The second phase of this response is NMDA receptor-mediated (Haley et al., 1990; Coderre and Melzack, 1992) and known to have an inflammatory basis, yet despite the continuing development of inflammation, this response stops within 1 hour of the injection of formalin into the paw (see Tjølsen et al., 1992). The reason for this abrupt end to the response is unknown, but it is possible that the high level of activation of the NMDA receptor during the second phase of the response rapidly leads to the induction of inhibitions which damp down the excessive neuronal activity.

Given the importance of the inhibitory response to counter the excitations produced as a result of the development of inflammation in the periphery, which leads to a manageable, physiologically appropriate pain state, is it then a failure of inhibitions, rather than an excess of excitations that leads to inappropriate, difficult to treat pain states arising from nerve damage and related events? There is evidence for the former.

Allodynia, which is a feature of conditions involving nerve damage, is difficult to treat using conventional analgesics and seems to result from a failure of GABA and glycine mediated inhibitions in the spinal cord (Yaksh, 1989). It has been proposed that

the injury discharge generated at the time of nerve injury and / or tonic afferent input may be sufficient to cause an NMDA receptor-mediated excitotoxic death of inhibitory interneurones in the dorsal horn (see Woolf and Doubell, 1994). A mechanism such as this may underlie the loss of Aβ-fibre evoked inhibitions seen following nerve section (Woolf and Wall, 1982). In contrast, during the development of carrageenan inflammation, excessive NMDA receptor activation appears to trigger inhibitions (chapter 3), which presumably protect against the possibility of excitotoxic damage. Furthermore, in inflammatory conditions, the levels of GABA in the spinal cord are elevated (Castro-Lopes et al., 1992, 1994).

The insensitivity of neuropathic pains to opioid analgesics has been attributed to an upregulation in the levels of the 'anti-opioid' peptide CCK in the spinal cord (Xu et al., 1993); the mirror image of the situation following inflammatory nociception (chapter 7). Upregulations in endogenous CCK in the spinal cord have also been implicated in the manifestation of allodynia in spinally injured rats. In this model it is suggested that the abnormal sensory processing in the spinal cord is under tonic opioidergic control, and it is the blockade of this by upregulated CCK which leads to the occurrence of painful sensations in these rats (Xu et al., 1994).

Why should different causes of pain evoke such different responses in the spinal cord? One possibility is that inflammation of peripheral tissue can be considered as a natural process, which forms part of the body's response to injury and is involved in the healing process, and therefore the central changes are part of the 'pre-programmed' inflammation response. Even when a failure of the immune system leads to an inappropriate inflammatory response, as in the case of rheumatoid arthritis, the messages reaching the central nervous system still signal inflammation, and so lead it to respond with both an excitatory component to signal damage and thereby protect the injured zone, and an inhibitory component to keep activity in check. In the case of nerve damage, which often arises through trauma, and so cannot be considered a natural process, the response of the nervous system is one-sided, with excitations being produced as a result of damage in the periphery, which can lead to central sensitization, but without the countering increase in inhibitions, which may even be lost as a result of pathological change. This situation may arise from events such as normal central connections being lost following nerve degeneration or central sprouting, or the generation of ectopic discharges from a neuroma which may trigger an aberrant central response.

The commonly held view is that plasticity in spinal sensory processing is detrimental, leading to central hypersensitivity and related phenomenon which leads to difficult to treat pain states. Although the evidence to date suggests this would appear to be true following nerve damage (see above), the evidence presented in this thesis shows

that this is not always the case with nociception arising from inflammation. The demonstration that some plasticity in the nervous system is beneficial to the body has implications for clinical therapy, namely pre-emptive analgesia.

There is little doubt that a barrage of nociceptive impulses is capable of triggering a number of changes in spinal systems. This has led to the popularity of the concept of pre-emptive analgesia - blocking the pain pathways with local anaesthetics or analgesics before they are bombarded with impulses from tissue damaged during surgery on the assumption that these will lead to central hypersensitivity and therefore increased pain which requires more analgesic management. Pre-emptive trials testing the benefits of administering opioids before surgery compared with afterwards have failed to find the expected clinical benefit (McQuay, 1994). Given the results presented in this thesis, this is perhaps not surprising. Post-operative pain has a strong inflammatory component which can trigger the generation of functional opioid receptors in the periphery (Joris et al., 1987; Hargreaves et al., 1988b; Stein et al., 1988a,b) and, as shown in chapter 5, an enhancement in opioid potency at spinal sites. The development of both these controls, and maybe also the non-opioid controls such as the enhancement in the descending noradrenergic system and the upregulation in GABA, may be blocked by pre-emptive analgesia. Hence in cases of post-operative pain the benefits of blocking the development of central hypersensitivity may be outweighed by the disadvantages of potentially blocking an enhancement in opioid potency in situations where the opioids are the major class of analgesic drugs used. This may not be the case for surgery where there is intentional nerve section, for example amputations, where preventing central changes with pre-emptive analgesia is likely, on the basis of the evidence available to date, to be beneficial.

Some of the animal studies investigating the benefits of pre-emptive analgesia using models of inflammatory nociception may be potentially misleading to clinical practice due to the short duration of the animal model used. The inflammatory model used in these studies is often the formalin test where, for example, opioids administered after the first phase are clearly less effective at inhibiting the inflammatory second phase of the response than those administered before the first phase (Dickenson and Sullivan, 1987b). This would appear to be clear evidence for the benefit of pre-emptive analgesia against pains of inflammatory origin. However, this is not likely to relate well to clinical situations. In the formalin test, pre- vs post-administered opioid analgesia was only being examined over the period 30-60 minutes post-insult. At this early stage, the mechanisms leading to the enhanced opioid potency post-inflammation are only just developing, so there is no benefit to post-administered analgesia at this early time point. In clinical situations however, nociception is of much longer duration, and the patient is likely to

require analgesics for many days. Over this longer time period, the benefits of inflammation-enhanced opioid potency are likely to be large. Indeed, one clinical trial investigating pre- vs. post-surgery administration of opioids for surgical pain reported that although patients given morphine pre-operatively needed less morphine in the first 24hrs post surgery than those given morphine only after the surgery, in the subsequent 24 hours this was reversed (Richmond et al., 1993). This may be a reflection of a greater opioid potency in those patients in whom central plasticity was not blocked by the prior administration of opioids.

Given the pros and cons of pre-emptive analgesia under different nociceptive situations, perhaps the future of this strategy lies in trying to predict which conditions are most likely to benefit from it. As has been shown in this thesis, plastic changes promoting nociception, which have attracted much attention, are more than matched by increases in inhibitory controls in this inflammatory model. Another direction may come from trying to unravel further the mechanisms behind the changes in spinal nociceptive processing to see if it is possible to selectively block only those changes which are likely to be considered detrimental to the patient. It remains to be seen if it is possible to separate the underlying causes in this way.

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