AN ELECTROPHYSIOLOGICAL STUDY IN THE RAT ON THE ROLE OF CENTRAL AND PERIPHERAL MEDIATORS IN PROLONGED NOCICEPTION

by

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This thesis is dedicated to the memories of

my late father, Miles Haley and my late fiancé, Michael Barrett.

The love and support they gave me during their lives will remain with me forever.

ABSTRACT

This *in vivo* pharmacological study has used electrophysiological techniques to investigate the mechanisms and control of prolonged nociception. Formalin injection into the receptive field was used as a prolonged stimulus and the activity of nociceptive neurones located within the spinal cord was recorded in the halothane anaesthetized intact rat. Acute electrically evoked activity of these neurones resulting from both A and C fibre inputs was used as a comparison. The response of dorsal horn neurones to formalin was biphasic and lasted about 60 minutes. The first peak firing began immediately following injection of the formalin and lasted for about 5 minutes. Following a period of inactivity the neurone once again started firing, usually about 25 minutes following the formalin administration and this second peak persisted for approximately 35-40 minutes. This profile of activity is very similar to the pain related behaviour observed in conscious animals following formalin administration. Correlations were sought between the electrically evoked properties of the neurones and their responses to formalin. Intravenous and intrathecal administration of mu opioids inhibited both peaks of the formalin response.

Using peripheral and intrathecal administration of bradykinin and selective B1 and B2 receptor antagonists, evidence was provided for a role of the bradykinin B2 receptor in the peripheral generation of the second peak of the response.

The role of excitatory amino acids within the spinal cord was investigated. The non-selective antagonist γ-D-glutamyl glycine (DGG) reduced both the electrically evoked A and C fibre responses and both peaks of the formalin response. Selective antagonists at the N-methyl-D-aspartate (NMDA) receptor, 5-DL-amino phosphonovaleric acid (AP5), ketamine and MK801 were administered intrathecally and intravenously. Ketamine reduced the 'wind up' of the neurones (the frequency dependent potentiation of dorsal horn neurone responses to repetitive C fibre stimulation) and all the compounds selectively reduced the second peak of the formalin response with little effect on the first peak.

Nitric oxide (NO) has been implicated in both bradykinin and NMDA mediated

events. An inhibitor of nitric oxide synthesis administered into the receptive field and intrathecally reduced both peaks of the formalin response whilst systemic administration resulted in a selective inhibition of the second peak. However, intrathecal administration of the NO precursor L-arginine unexpectedly reduced both the electrically evoked A and C fibre responses and both peaks of the formalin response. Although these results are suggestive of a role for NO in both peripheral and spinal mediation of nociceptive events, the high doses required, route dependent effects and contradictory result with L-arginine suggest the involvement of this molecule is complex.

Whilst administration of selective mu and delta opioid agonists into the receptive field had no effect on either peak of the formalin response, a selective kappa opioid agonist inhibited both peaks of the formalin response whilst having no effect on the acute electrically evoked A or C fibre responses.

Thus the peripheral generation of the formalin response involves the actions bradykinin, via the B2 receptor, during the second phase and this may also involve the generation of nitric oxide. The afferent input is mediated within the spinal cord by excitatory amino acids acting at non-NMDA receptors during the first peak of the response, but during the prolonged activity of the second peak NMDA receptor involvement becomes important as well. It is possible that the NMDA receptor may be responsible for amplifying the response. This formalin evoked activity within the spinal cord may also involve nitric oxide. The formalin response can be modulated by opioids acting at the spinal level and at the peripheral level where only the kappa receptor appears to be effective. These results indicate some of the mechanisms involved in the generation and transmission of prolonged nociception and may provide future directions for the development of novel analgesics.

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CONTENTS

CHAPTER 1:

INTRODUCTION

1.1 Do we need new analgesics?

The management of pain, ranging from 'mild' to 'severe', still relies on the use of a small range of drug types. Mild to moderate pain is generally controlled using non-steroidal anti-inflammatory drugs (NSAID's; e.g. aspirin and paracetamol) and 'weak' opioids such as codeine and these are often used in combination. Moderate to severe pain relies on 'strong' opioids, especially morphine, and local anaesthetics for relief. The treatment of short duration mild to moderate pain is reasonably successful and even if relief is not provided, the condition can often be tolerated by the patient until it subsides. However the treatment of severe or chronic pain states is more critical since failure to relieve the condition would result in tremendous suffering by the patient.

The use of opioids in chronic pain management is often inadequate and this stems from fears and misconceptions regarding the actions of these drugs. A major factor influencing the use of opioids is the fear of addiction. Whilst non-medical use of opioids can indeed lead to addition there is no evidence to suggest that even prolonged treatment with opioids leads to addiction. However this fear still leads to strict government controls on the use of these drugs and may subsequently deny patients the relief they require (Schuster, 1989; Twycross and McQuay, 1989; Melzack, 1990). Another restraint on the use of opioids as analgesics is the misconception that prolonged use leads to tolerance. Whilst tolerance to opioids does occur in the absence of a pain state the requirement for higher doses in chronic pain is usually the result of an advancing disease state. A similar situation exists with the respiratory depression caused by opioids; whilst this can occur, the presence of an ongoing pain state appears to prevent this problem. A major drawback (from the patient's viewpoint) in the use of opioids is the occurrence of unpleasant side effects such as constipation and nausea and this could result in difficulties with patient compliance. In addition, opioids are not effective in alleviating every pain state and some conditions are considered to be 'opioid-insensitive' (McQuay, 1988).

The problems associated with the use of opioids, be they political, resulting from

unpleasant side effects or due to ineffectiveness limits their therapeutic potential. If people are to receive the pain relief they require, the search for better analgesics must continue. Elucidating the mechanisms underlying the sensation of pain is necessary if we are to avoid 'fishing in the dark' when developing novel analgesics. By determining the chemicals involved at the various stages of pain transmission it may then be possible to develop selective antagonists which could be therapeutically useful.

1.2 The morphology of nociceptive pathways

1.2.1 Primary afferent neurones

Primary afferent neurones are essentially divided into three main categories: Aß, Aδ and C fibres on the basis of size, myelination and conduction velocity (Gasser and Erlanger, 1927; Burgess and Perl, 1973). The Aß fibres are fast conducting, low threshold myelinated neurones and are believed to respond to mainly innocuous stimuli. By contrast the thin myelinated Aδ fibres and the slowly conducting, fine unmyelinated high threshold C fibres are thought to transmit nociceptive information. The primary afferent neurones are unipolar and whilst the cell soma is located in a dorsal root ganglion (DRG) they have axons that divide outside the DRG and project both to the peripheral tissue and to the spinal cord. Upon entry into the spinal cord afferent fibres form synapses with spinal neurones. However in peripheral tissue unmyelinated C fibres branch extensively and terminate in 'free' nerve endings (see Yaksh and Hammond, 1982 and Besson and Chaouch, 1987). Slowly conducting unmyelinated C fibres are classed according to the profile of response elicited by various peripheral stimuli. The majority of nociceptors (around 73% in the rat; Lynn and Carpenter, 1982) appear to be polymodal, responding to pressure, heat and chemical stimuli (Bessou and Perl, 1969). Other unmyelinated cutaneous C fibres present in the rat respond to either non-noxious stimuli (sensitive mechanoreceptors), cold stimuli (cold thermoreceptors) or appear to be unresponsive.

1.2.2 The dorsal horn of the spinal cord

The dorsal horn has been divided into distinct laminae based on the cytoarchitectonic organization of the grey matter. The original laminae were proposed by Rexed (1952) for the cat dorsal horn but a similar organization has also been proposed for the rat (Molander et al., 1984) and monkey (Price and Mayer, 1974). Lamina I (marginal zone) consists mainly of small neurones with dendritic trees that spread out horizontally, parallel to the surface of the dorsal horn, although in the rat spread of the dendrites into the substantia gelatinosa (SG; Lamina II) has been observed in a proportion of neurones (Ralston, 1979; Brown, 1982; Lima and Coimbra, 1986).

Lamina II (SG) of the cat and monkey contains two main types of cell, both of which have small cell bodies. Stalked cells have dendrites that project down from their soma located in the dorsal (outer) part of the SG (II_o) and occasionally project into the inner, more ventral, region of the SG (II_i). Islet cells have dendrites that extend in a rostralcaudal plane and are generally confined to the II_i region of the SG (Brown, 1982). The rat differs from the cat in that it appears to have, in addition to the stalked and islet cells, either atypical cells or additional cell types in this region some of which have dendrites that extend into Laminae I and III (Beal, 1983; Woolf and Fitzgerald, 1983; Bicknell and Beal, 1984; Todd and Lewis, 1986; Todd, 1988; Todd and McKenzie, 1989).

The cells in Laminae III-VI are generally larger than those found in the more superficial laminae in both the rat and the cat. In addition there appears to be extensive overlap between neurones located in these laminae (Brown, 1982; Woolf and King, 1987). Horseradish peroxidase staining of Lamina V neurones in the rat reveals extensive dendritic branching which occasionally extends as far as Lamina II (Woolf and King, 1987). Thus many neurones appear to have dendrites that are confined either to the laminae in which the cell somas reside or extend only to adjacent laminae. However a proportion of neurones do appear to have dendrites that extend beyond these regions with a few Lamina I and Lamina V cells projecting dendrites in to the Lamina II region and a small number of Lamina II cells that extend into Laminae I and III.

1.2.3 The termination of primary afferent neurones in the dorsal horn

Prior to entry into the dorsal horn, afferent fibres bifurcate and send collaterals in both rostral and caudal directions. The unmyelinated fibres join the tract of Lissauer and project for only a couple of segments from their original dorsal root entry zone before terminating in the dorsal horn (Réthelyi, 1977; Yaksh and Hammond, 1982). Studies using histochemical and degeneration techniques have demonstrated in cat, rat and monkey the termination of unmyelinated (C fibre) afferents in Laminae I and II. The thin myelinated (Aδ fibre) afferents terminate both in the superficial laminae (I and II_o) of the dorsal horn and also within the deeper laminae (V and VI). By contrast the thick myelinated (Aß) fibres only appear to terminate in the deeper regions of the dorsal horn (III-VI) (Réthelyi, 1977; Light and Perl, 1979 a, b; Jancsó and Király, 1980; Ralston and Ralston, 1982; Swett and Woolf, 1985; Maxwell and Réthelyi, 1987; Light and Kavookjian, 1988)

Neurones responding to both Aß and C fibre inputs are located in all regions of the dorsal horn and it seems unlikely that the responses all arise from monosynaptic connections with the afferent fibres since these terminate in different regions of the dorsal horn. A few neurones located within the deeper laminae in the rat have dendrites that can extend as far as the SG region where the C fibre afferents terminate but the majority do not appear to extend that far (Hunt et al., 1982; Woolf and King, 1987). It therefore seems likely that many deep neurones receive polysynaptic C fibre inputs via interneurones located within the SG region (Fitzgerald and Wall, 1980; Ralston and Ralston, 1982). Similarly superficial neurones probably receive Aß inputs via polysynaptic connections from deeper neurones.

1.2.4 Ascending nociceptive information

In addition to any local circuitry, dorsal horn neurones also project axons to higher centres of the central nervous system via ascending columns of fibres which occupy the anterolateral quadrant of the spinal cord. Three main tracts have so far been

identified; the spinothalamic, spinoreticular and spinocervicothalamic. The presence of dorsal horn neurones with ascending projections has been demonstrated in many studies. Horseradish peroxidase and fluoro gold have been used as retrograde tracers where injection into the thalamus resulted in staining of neurones in the superficial and deep laminae of the dorsal horn (Giesler et al., 1979; Kevetter and Willis, 1983; Burstein et al., 1990). In addition, studies have used antidromic stimulation techniques to characterize the trajectories and response properties of ascending tract neurones (see Besson and Chaouch, 1987). The general electrophysiological properties of projection neurones and convergent interneurones within the dorsal horn appear to be similar (Dickenson and Le Bars, 1983) as are their responses to iontophoretically applied excitatory amino acid receptor agonists (Aanonsen et al., 1990). In addition morphine inhibition of projection and other convergent neurones in the dorsal horn appears similar (Hylden and Wilcox, 1986), suggesting that the pharmacology of nociceptive neurones is similar for both output and interneurones. Since there is claimed to be no correlation between morphology, histochemistry nor physiology of most dorsal horn cells (Wall, 1988) the most useful criteria to classify these cells will then be functional responses to peripheral stimuli. This has been used in this thesis.

1.3 The responses of dorsal horn neurones

1.3.1 The responses of dorsal horn neurones to peripheral stimulation

Dorsal horn neurones respond in different manners to innocuous and noxious stimulation of their peripheral receptive fields. However, based on these responses, they can be broadly divided into three groups. Neurones responding to only innocuous stimulation have been termed class 1 cells whereas neurones that respond to noxious stimulation only are called nociceptive specific, noxious-only or class 3 neurones (see Le Bars et al., 1986 and Besson and Chaouch, 1987). Neurones that respond to both innocuous and noxious stimuli have been termed Class 2 neurones. These 'convergent'

neurones have also been called wide dynamic range, Lamina V cells, polymodal and multireceptive cells and are found in both superficial and deeper laminae of the rat dorsal horn (Menétrey et al., 1977; Fitzgerald and Wall, 1980; see Besson and Chaouch, 1987). The neurones recorded in this study were all convergent neurones.

1.3.2 Evidence supporting a role for convergent neurones in nociceptive processing

Electrophysiological recordings of dorsal horn convergent neurones have been utilized as a means of investigating the mechanisms underlying nociceptive transmission. However this demands the assumption that convergent neurones are involved in nociceptive transmission and reflect the 'pain' perceived by a conscious animal. Convergent neurones clearly respond to a variety of peripheral noxious stimuli as well as to innocuous stimulation (see Le Bars et al., 1986). Attempts have been made to correlate the activity of these neurones with the behavioural responses of awake monkeys and were found to be effective at coding the intensity of thermal stimuli (Dubner et al., 1981). In addition, many convergent neurones show an increasing response to repetitive stimulation of the receptive field with a constant intensity stimulus. This phenomena has been termed 'wind up' and has also been described by human subjects following repetitive heat stimulation (Mendell, 1966; Price et al., 1977).

So, convergent neurones respond to noxious stimulation and their activity may reflect the sensation described as 'pain' in animals and humans. In addition these neurones have ascending projections and this implies a role in the transmission of nociceptive information to higher centres. Finally, the activity of these neurones can be reduced by procedures that result in analgesia in animals and humans (Le Bars et al., 1986). Thus convergent dorsal horn neurones appear to be involved in the transmission of nociceptive information in the spinal cord and appear suitable candidates for the investigation of the mechanisms underlying nociception.

1.3.3 Modulation of dorsal horn neurones

The activity of dorsal horn neurones can be modulated by both segmental and supraspinal control mechanisms. Local segmental inhibition is believed to result from Aß fibre activation of inhibitory interneurones probably located in the SG region. These interneurones project onto convergent neurones and exert an inhibitory influence in addition to the excitatory effect of the Aß fibres. A counterbalance to this indirect inhibition is the indirect increase in cell excitability elicited by the C fibre inputs. The activation of C fibres results in the stimulation of convergent neurones. However, in addition to this action, C fibre inputs may also either 1) stimulate an excitatory interneurone or 2) inhibit the activity of inhibitory interneurones (probably via the stimulation of another inhibitory interneurone). Whilst it is not known which mechanism predominates, both could result in an increase in the excitability of the convergent cell. These ideas and observations arise from the 'gate theory' for the segmental control of nociceptive transmission within the spinal cord (Wall, 1978; see Besson and Chaouch, 1987). In addition to these segmental controls dorsal horn convergent neurones are sensitive to supraspinal modulation. These descending inhibitions take two forms 1) tonic inhibitory controls, 2) inhibitions evoked by the noxious stimulation of distant body regions (diffuse noxious inhibitory controls; DNIC; Le Bars and Villanueva, 1988). These descending inhibitions are interesting as they are believed to be involved in the analgesia elicited by supraspinal morphine as well as in acupuncture, stress analgesia and may provide a link between activity in higher centres and sensory processing in the spinal cord (Le Bars and Villanueva, 1988).

1.4 Pharmacology of nociceptive pathways

1.4.1 Peripheral tissue

The activation of peripheral afferent fibres can result from mechanical, heat or chemical stimulation. The responses to mechanical stimuli such as pinch result from

direct physical distortion of the free nerve endings generating a local potential and eliciting ion flow in the nerve ending. The mechanism by which heat stimulates the free nerve endings is unknown; it still remains to be determined whether this is a direct or indirect effect (see Yaksh and Hammond, 1982 and Besson and Chaouch, 1987). Within the peripheral tissue there are many candidates as chemical stimulators of primary afferent fibres. Bradykinin and 5HT both stimulate C fibres and produce pain in man when applied onto a blister base (Horton, 1963; Beck and Handwerker, 1974; Richardson et al., 1985; Mense, 1986; Whalley et al., 1987a). Interestingly 5HT appears to potentiate the effects of bradykinin both on nociceptor activity and in man. The role of histamine in peripheral events appears uncertain with claims that it elicits pain in man (see Magerl and Handwerker, 1988). However very little activation of afferent fibres occurs following peripheral administration and some human studies find it only elicits itch (Fjällbrant and Iggo, 1961; Kumazawa and Mizumura, 1977; LaMotte et al., 1988; Magerl and Handwerker, 1988).

Rather than stimulating the afferent nerves some chemicals whilst having no effect alone, potentiate the effects of bradykinin. Prostaglandins potentiate the bradykinin induced activation of C fibres and inhibitors of their synthesis (e.g. aspirin) are analgesic in behavioural studies (Juan and Lembeck, 1974; Hunskaar and Hole, 1987). Interestingly bradykinin itself can release prostaglandins (Lembeck et al., 1976). More recently leukotrienes have been implicated as mediators of hyperalgesia although their actions may be dependent on the presence of polymorphonuclear leukocytes (Levine et al., 1985). Another category of inflammatory mediators are those that act on the vasculature to cause vasodilatation and thereby increase the infiltration of the area with mediators such as bradykinin. Substance P (SP) is believed to be involved in this manner as it has no effect on the afferent fibres and is not algogenic (Kumazawa and Mizumura, 1977; Lembeck and Gamse, 1977). However SP is present in peripheral terminals and released following antidromic stimulation of the nerves. In addition it induces local plasma extravasation when injected into the skin (see Yaksh and Hammond, 1982 and Besson and Chaouch, 1987).

Bradykinin is a potent activator of peripheral nociceptors, elicits pain in man and

its actions are potentiated by other inflammatory mediators. It is generated from a large precursor peptide present in circulating plasma, lymph and interstitial fluid and is known to bind to two different receptor subtypes, B1 and B2 (Regoli and Barabé, 1980). Cleavage of the precursor to release the peptide can occur following contact with either plasma kallikrein (produced via the Hageman factor pathway), tissue kallikrein or cellular proteases. The continual presence of the bradykinin precursor and the cleavage enzymes means the system is permanently available for activation following a tissue damaging insult (Proud and Kaplan, 1988). Thus bradykinin may be a critical peptide in the peripheral generation of nociception especially when associated with inflammation. Indeed noxious peripheral stimulation results in the generation of bradykinin (Rocha e Silva and Antonio, 1960; Ohara et al., 1988). With the recent development of specific antagonists at bradykinin receptors (Schachter et al., 1987) it is now possible to investigate the role of bradykinin in peripheral nociceptive events. Recently it has been suggested that bradykinin may mediate some of its actions via the generation of nitric oxide, and this molecule has also been implicated in central events following the activation of excitatory amino acid receptors (Palmer et al., 1987; Garthwaite et al., 1988). The availability of inhibitors of nitric oxide synthesis now allows investigation into the role of this molecule in both peripheral and central nociceptive events (see 1.4.2).

1.4.2 Primary afferent fibres

Primary afferent nociceptive neurones contain many peptides and amino acids, all of which could be putative neurotransmitters at the peripheral or central terminals of the afferent neurones. The list includes Substance P (SP), excitatory amino acids (glutamate and aspartate), cholecystokinin (CCK), vasoactive intestinal peptide (VIP), somatostatin, calcitonin gene-related peptide (CGRP), bombesin and adenosine 5'-trisphosphate (ATP) (see Yaksh and Hammond, 1982 and Salt and Hill, 1983). Investigation has focussed on the role of substance P and glutamate in nociceptive transmission within the spinal cord dorsal horn since antagonists are available at these receptors.

Immunoreactive staining for SP has demonstrated the presence of this peptide in dorsal root ganglia and in primary afferent terminals located within the dorsal horn of the rat (Battaglia and Rustioni, 1988; DeBiasi and Rustioni, 1988). However whilst dorsal rhizotomy depletes SP levels in the dorsal horn, the peptide has also been shown to be present within intrinsic spinal neurones (Hökfelt et al., 1980; Jessell, 1983). The release of SP has been demonstrated *in vitro* following stimulation of the dorsal roots at C and Aδ intensities and *in vivo* following noxious stimulation of the peripheral tissue (Yaksh et al., 1980; Kantner, et al., 1986). This release of SP may occur from both primary afferent neurones and intrinsic neurones since release has also been observed in dissociated dorsal root ganglion cells (Mudge et al., 1979). In electrophysiological experiments SP has been shown to produce mainly excitatory effects on dorsal horn nociceptive neurones although it has also been shown to excite non-nociceptive neurones (see Salt and Hill, 1983). Historically SP has been considered to be the primary afferent neurotransmitter however the role of this peptide in nociception is now being reconsidered. Iontophoretic administration of SP onto dorsal horn neurones results in a delayed but sustained excitation, a time course that differs from the excitation observed with nociceptive stimulation. In addition nociceptive stimuli can still elicit excitations in dorsal horn neurones following depletion of spinal SP levels (see Besson and Chaouch, 1987). However whilst SP may not be the neurotransmitter in nociception a 'neuromodulatory' role has been proposed. Thus SP may be released alongside an alternative neurotransmitter, perhaps an excitatory amino acid, thereby modifying the neural activity elicited by nociceptive events (see Yaksh and Hammond, 1982; Urbán and Randic, 1984; Randic et al., 1990).

Immunoreactive experiments have demonstrated the presence of the excitatory amino acids glutamate and aspartate in the dorsal root ganglia and primary afferent terminals of the rat and monkey. Interestingly glutamate and SP were often seen to coexist in these afferent fibres (Battaglia and Rustioni, 1988; DeBaisi and Rustioni, 1988; Westlund et al., 1989). In addition both glutamate and aspartate are present within dorsal horn interneurones since the loss of these cells coincides with a reduced

level of both these amino acids in the dorsal horn (Davidoff et al., 1967) and iontophoretically applied excitatory amino acid receptor agonists stimulate nociceptive neurones in both deep and superficial laminae (Headley et al., 1987; Aanonsen et al., 1990). The receptors to which these excitatory amino acids bind as agonists, have been divided into three main types: The AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; previously termed the quisqualate receptor) and kainate receptors are also both referred to as non-NMDA receptors to distinguish them from the N-methyl-D-aspartate (NMDA) receptor. These receptors are defined by their selectivity for the agonists AMPA (and quisqualate), kainate and NMDA respectively. Binding sites for all three receptor subtypes have been demonstrated in both the human and rat dorsal horn and are restricted to the SG region (Monaghan and Cotman, 1982, 1985; Jansen et al., 1990).

Excitatory amino acids have been implicated in the transmission of nociceptive information within the dorsal horn since iontophoretic administration of both glutamate and aspartate excites dorsal horn neurones *in vivo* in the cat and rat (Curtis et al., 1960; Biscoe et al., 1976). In addition glutamate release in the spinal cord has been demonstrated *in vivo* following prolonged noxious stimulation of the receptive field in the rat (Skilling et al., 1988). Of particular interest is the role of the NMDA receptor subtype in nociception since in other regions of the central nervous system this receptor is involved in the neural plasticity associated with long term potentiation (Bliss and Lynch, 1988).

1.4.3 Modulation by opioids

Opioid receptors have been divided into three subtypes based on the profile of agonist binding at each site: mu (μ), delta (∂) and kappa (κ) (see Kosterlitz, 1985). Endogenous peptides acting at these receptors are cleaved from large precursors; however these often bind to more than one receptor subtype and investigation into the actions of each subtype has utilised synthetic agonists. Opioid receptors undergo axonal transport in the afferent fibres and travel both towards the periphery and the central terminals (Young et al., 1980; Laduron, 1984). Autoradiographic studies have shown

binding of mu and kappa receptors within the SG region whilst delta binding appears to be more diffuse throughout the dorsal horn (Gouardères et al., 1985; Morris and Herz, 1987). Rhizotomy results in an approximately 50% loss in the numbers of opioid receptors within the dorsal horn suggesting that a large proportion are located on the presynaptic afferent terminals (LaMotte et al., 1976). However the remaining receptors are likely to be located within the dorsal horn and may be located postsynaptically (see Yaksh, 1987). Autoradiographic studies have also located mu, delta and kappa receptors in discrete regions of the brain (Mansour et al., 1986, 1987).

Behavioural studies have demonstrated antinociceptive actions for mu, delta and kappa agonists acting in the periphery during models of inflammatory nociception (Stein et al., 1989). In addition agonists at all three receptor subtypes appear to be antinociceptive at the level of the spinal cord in most models of nociception (see Yaksh, 1987) with mu and delta agonists also being effective in supraspinal regions (see Yaksh et al., 1988). *In vivo* electrophysiological studies have demonstrated the spinal antinociceptive effects of selective mu and delta and kappa (higher doses are required) agonists (see Duggan and North, 1984; Dickenson et al., 1987; Sullivan et al., 1989; Sullivan and Dickenson, 1991). Thus the modulation of nociceptive information by opioids may occur at the peripheral, spinal or supraspinal levels.

1.5 Plasticity within the spinal cord

Prolonged hypersensitivity arising from peripheral tissue injury has been attributed to changes in the threshold of peripheral nociceptors and also alterations in nociceptive transmission within the spinal cord. Both the increase in flexor reflex response and expansion of the receptive field observed following peripheral thermal injury are attributed to central events: administration of local anaesthetic into the site of injury whilst blocking all sensory input from the ipsilateral paw, has no effect on the expanded receptive field or enhanced responses to nerve stimulation from the contralateral paw (Woolf, 1983). Some dorsal horn neurones increase the size of their response to successive peripheral stimuli and this has been termed 'wind up' (Mendell, 1966). This

'wind up' is C fibre and frequency dependent since stimulation at Aß fibre thresholds or at frequencies lower then 0.5 Hz does not elicit this phenomenon. The NMDA receptor has been implicated in this amplification of dorsal horn neuronal activity since antagonists at this receptor block the 'wind up' of the neurones without altering the initial responses to the stimulus (Davies and Lodge, 1987; Dickenson and Sullivan, 1987b). It is therefore interesting to determine if spinal NMDA receptors are also involved in the responses of dorsal horn neurones to prolonged nociceptive inputs from the peripheral receptive field since this may be expected to elicit 'wind up'.

1.6 Do we need new animal models of nociception?

There are a large number of animal models for investigating both the mechanisms of nociception and the effectiveness of analgesics. However the majority of these models, be they behavioural or electrophysiological, utilize short duration stimuli and this may not reflect the clinical situation. This is highlighted by the development of tolerance; a loss of effectiveness of a drug following repetitive administration. This phenomena is often observed with opioids when short duration stimuli such as the hotplate test are used but is not observed in the clinic when opioids are administered in chronic pain states (Stevens and Yaksh, 1989; Twycross and McQuay, 1989). Thus fundamental differences may exist between the mechanisms involved in short and long term pain states and better animal models of nociception are required if we are to investigate these.

The search for behavioural models of prolonged nociception has lead to the development of models using polyarthritis and unilateral arthritis (Dubner, 1989). These models have several problems associated with them, including ethical considerations. The measurement of nociception is based on the development of hyperalgesia and is therefore quantified by examining alterations in the nociceptive thresholds to additional noxious stimuli (e.g. noxious pressure on the paw). The development of these

hyperalgesic states often takes days or even weeks and this makes them unsuitable for electrophysiological studies.

The formalin test has been developed as a behavioural model and is rather different from the hyperalgesic models already described. The administration of a dilute formalin solution into the paw of a rodent, cat or monkey results in spontaneous biphasic biting and licking of the injection site which lasts about 60 minutes (Dubuisson and Dennis, 1977; Alreja et al., 1984). The biphasic response is thought to represent an initial short duration direct effect of the chemical on the afferent endings followed by a distinct prolonged period of behaviour arising from the development of inflammation in the periphery. This spontaneous biphasic behaviour is mirrored in the firing pattern of dorsal horn convergent neurones *in vivo* which also undergo two quite distinct periods of firing over about 60 minutes (Dickenson and Sullivan 1987a).

Interestingly opioid inhibition of the formalin response in behavioural studies shows little development of tolerance. It therefore differs from more acute tests and may be a better model of the prolonged pain observed in the clinic (Melzack, 1990). Thus the formalin response is potentially a useful model for examining the differences between acute and prolonged responses since both are elicited by the same injection. In addition the reasonably short time course of the response allows the whole response to be studied using *in vivo* electrophysiological techniques.

1.7 The general aims of this project

The basic aim of this project was to determine whether there were any fundamental differences in the peripheral generation and spinal transmission of nociceptive inputs of short duration 'acute' stimuli and longer duration stimulation. The generation of bradykinin during peripheral inflammatory events and its ability to stimulate afferent fibres makes it an attractive candidate for investigation in the periphery. Interestingly bradykinin has also been implicated in central nociceptive events and this aspect has also been investigated. The recent development of selective antagonists at the bradykinin receptors has provided a tool for the investigation of the role of this peptide in both

peripheral and spinal events during the acute and prolonged phases of the formalin response.

Excitatory amino acids excite dorsal horn neurones and appear to be involved in the spinal transmission of nociceptive information. The possible involvement of the NMDA receptor in central plasticity events makes it an exceptionally good candidate for any changes in nociceptive processing that may occur in the spinal cord during prolonged nociception. Interestingly, excitatory amino acid receptors have been observed undergoing axonal transport in the peripheral afferent fibres. Thus the role of non-NMDA and NMDA receptors in both the peripheral and central events that occur following a prolonged stimulus can be investigated using selective antagonists for these receptors.

Recently nitric oxide has been implicated in the vasodilatory effects of bradykinin and the actions of excitatory amino acid receptors in the cerebellum. The possible role of both these chemicals in nociception may therefore also involve the generation of nitric oxide in both the periphery and spinal cord during nociception. Inhibitors of nitric oxide synthesis are now available and these have been used to investigate the role of nitric oxide in the peripheral generation and central processing of nociceptive information during the formalin response.

Finally, opioid modulation of prolonged nociceptive responses may differ from the acute situation. Opioid receptors undergo axonal transport in the afferent nerve and may therefore have peripheral antinociceptive effects as well as spinal. Using agonists at the mu, delta and kappa subtypes of opioid receptor the ability of these agonists to inhibit the prolonged formalin response was investigated following both peripheral and spinal administration.

CHAPTER 2:

MATERIALS AND METHODS

The main technique employed in these studies was the measurement of the single unit activity of convergent dorsal horn neurones in the intact, anaesthetized rat using extracellular recording.

2.1 Preparation of the animals

2.1.1 Animals

Male Sprague-Dawley rats (200-250g) were used and all rats were obtained from the University College London animal house.

2.1.2 The use of low dose atropine to prevent excessive tracheal secretions

Low doses of atropine administered intraperitoneally prior to or during surgery have been used to prevent excessive tracheal secretions which can be life threatening in an animal maintained for several hours in a static and anaesthetized state. Due to some degree of variability in the general health of the animals available for experimentation atropine was used routinely prior to surgery in an effort to prevent deterioration due to airway obstruction. Atropine has been shown to be antinociceptive in the rat tail flick test when administered intraperitoneally at similar doses to those used in these studies. However this antinociceptive effect has a short duration, being over within 45 minutes (Ghelardini et al., 1990). It would therefore be unlikely to alter the responses of the dorsal horn neurones to noxious stimulation. Indeed the administration of atropine (100μg in 100μl) intraperitoneally prior to surgery had no effect on the subsequent response of dorsal horn neurones to formalin injection into the receptive field (Figure 2.1). The latency to arrival of the second peak of the formalin response was also unaltered: control: 32.3 ± 2.1 min (n=61); atropine: 33.4 ± 2.4 min (n=44).

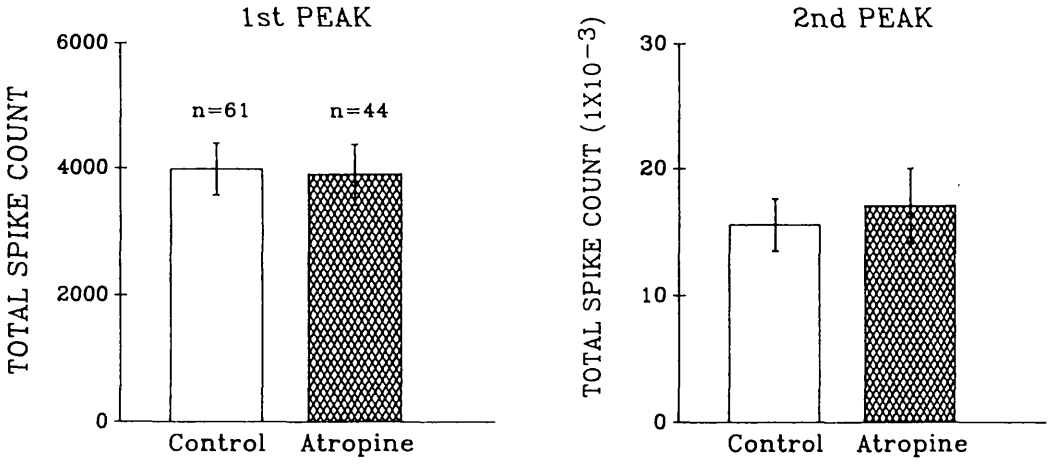

Figure 2.1 Intraperitoneal administration of atropine (100μg in 100μl) prior to surgery had no effect on the size of either the first or second peaks of the formalin response.

2.1.3 Anaesthetic

The initial induction of anaesthesia was obtained by placing the conscious animal in a sealed perspex box and delivering 3% halothane (Fluotec,mark 3 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 300 cm^3min^{-1} respectively. Once the animals had lost their righting reflex they were removed and placed ventral side up on an animal heating blanket (Animal Blanket Control Unit, Harvard) where a nose cone was fitted and anaesthetic delivery continued. When the animals had lost all reflex withdrawal activity the surgical procedures were begun. A tracheal cannula was inserted and the animal was then placed in the headholder of a stereotaxic frame using the earbars and a nosebar to keep the head steady. Anaesthetic delivery was maintained via a closed delivery system consisting of a small polythene Y-piece: one link was connected to the halothane dispenser, the other to rubber tubing that was vented to the outside. The surgical procedures were performed with a halothane level of 2-2.5% and the animal was then maintained at 0.8-1.2% for the remainder of the experiment. This produced a state of complete areflexia in the animal for the duration of the experiment.

2.1.4 The choice of anaesthetic and the effects on the responses of dorsal horn neurones

The requirements for any anaesthetic employed in studies on intact animals are that it provides an adequate depth of anaesthesia whilst not suppressing the activity of the neurones under study or interfering with the actions of exogenously administered pharmacological agents. It is essential that the animal is maintained in a state of complete areflexia, thus permitting stable recordings of neurones in addition to ensuring the animal under study does not feel discomfort or pain.

The dissociative anaesthetic ketamine was considered to be unsuitable in these studies following evidence clearly indicating that it interacts in a non-competitive manner with the N-methyl-D-aspartate (NMDA) receptor at subanaesthetic doses (Lodge and

Johnson, 1990). NMDA receptors have been implicated in nociceptive transmission and ketamine does inhibit the responses of dorsal horn neurones to noxious stimulation (Davies and Lodge, 1987). Barbiturate anaesthetics were also considered unsuitable since they have been shown to depress the activity of dorsal horn neurones in the cat (Wall, 1967; Kitahata et al., 1975) and also cause increases in their activity in the cat and monkey (Hori et al., 1984; Collins and Ren, 1987). The systemically active anaesthetic urethane has been used in electrophysiological experiments although it too has been shown to depress activity in the spinal cord (Shapovalov, 1965). α-Chloralose is also a systemically administered anaesthetic however in rats it results in inconsistent inhibitions of dorsal horn neurones by morphine compared to halothane anaesthetized animals (Le Bars et al., 1980a). In addition none of these anaesthetics are volatile and it is therefore likely that the level of anaesthesia may vary throughout the experiment with top up injections being required. Many studies utilize spinalization in combination with decerebration in order to avoid the problems associated with anaesthetics. However the loss of descending influences on the neurones as a result of this surgery will inevitably alter the responses of dorsal horn neurones.

Halothane is a volatile anaesthetic that is usually delivered in a gaseous mixture of nitrous oxide and oxygen, therefore allowing continuous delivery of a constant level of anaesthetic. It has been shown to inhibit the activity of dorsal neurones in the cat at doses ranging from 0.5-3% (de Jong et al., 1970; Kitahata et al., 1975; Namiki et al., 1980). However the activity of rat dorsal horn neurones appears very similar in halothane anaesthetized (0.5%) and non-anaesthetized animals (Le Bars and Chitour, 1983). In addition there is no change in the brain levels of glycine, GABA or glutamate in the rat at concentrations up to 3% although a small increase in aspartate levels occurs (Arai et al., 1990). Thus the levels of brain amino acids are unlikely to alter during these experiments since the level of halothane required to produce complete areflexia in these present studies in the rat was within the 0.8-1.2% range.

The carrier gas nitrous oxide also produces analgesia and an interaction with endogenous opioid systems has been suggested as a mechanism of action for this compound (Gillman, 1986). Behavioural studies in the rat have demonstrated nitrous

oxide (67% in oxygen) induced analgesia in the tail-flick test can be blocked by systemic naloxone (Berkowitz et al., 1977; Lawrence and Livingston, 1981). However it is unlikely that nitrous oxide interferes with exogenously applied opioids since the inhibition of dorsal horn neurones by morphine is very similar in anaesthetized (with nitrous oxide in the gas mixture) and unanaesthetized rats (Le Bars et al., 1983). In addition whilst nitrous oxide has been shown to reduce the activity of lamina V dorsal horn neurones in the cat the doses required for this inhibition are greater than those used in the present studies. The spontaneous activity of neurones located within other laminae are unaffected by 75-80% nitrous oxide in oxygen (de Jong et al., 1970; Kitahata et al., 1971).

Previous studies have demonstrated that anaesthesia via inhalation of halothane in a nitrous oxide/oxygen mixture is able to maintain the rat in a state of complete areflexia whilst both the arterial blood pressure and end expiratory carbon dioxide level remain within normal limits, showing no spontaneous fluctuations. Furthermore EEG recordings show the animals have regular slow waves which are unaltered by noxious stimuli (Dickenson and Le Bars, 1987). The responses of dorsal horn neurones to noxious stimulation of the receptive field are very similar to those seen in the unanaesthetized rat and morphine produces consistent inhibitions of the dorsal horn neurones in rats anaesthetized in this manner (Le Bars et al., 1980b; Le Bars and Chitour, 1983). Thus halothane inhalation in a mixture of nitrous oxide and oxygen appears to provide adequate anaesthesia for the animal whilst allowing stable recordings of the activity of dorsal horn neurones to noxious stimulation.

2.1.5 Maintenance of core temperature

Throughout the duration of the experiment the body temperature of the animal was monitored and maintained. A rectal thermal probe was inserted and the rectal temperature maintained within normal limits (36.5-37°C) by automatic feedback control to the heating blanket on which the animal was placed (Animal Blanket Control Unit, Harvard).

2.2 Surgery

2.2.1 Lumbar laminectomy

A mid-line skin incision was made over the dorsal surface of the rat and any underlying connective tissue was removed to expose the dorsal musculature surrounding the vertebral canal. Incisions were made on both sides of the vertebral canal running from lower thoracic to lower lumbar level and the muscle was deflected to either side. Care was taken to avoid any damage to either dorsal or ventral roots at their points of entry into the spinal cord. The rostral end of the isolated region of vertebral canal and associated musculature was raised and clamped. The superficial muscle covering the dorsal surface of the vertebral canal was scraped off to expose individual vertebrae. Surgical rongeurs were used to perform the laminectomy which normally extended from L1-L3. If the underlying dura mater remained intact this was very carefully removed from the surface of the cord; the pia mater was left intact. Removal of the dura mater was necessary in order to allow insertion of the microelectrode and to facilitate drug access to the target neurones following administration directly onto the surface of the spinal cord. Care was taken to avoid damage to the central or superficial blood vessels located on the dorsal surface of the spinal cord. The caudal end of the exposed vertebral column was clamped, with a small degree of tension applied in order to increase the stability of the laminectomy. Additional support was provided by metal rods placed laterally on either side of the vertebral canal and musculature in the region of the laminectomy. The completed laminectomy formed a small "trough" which allowed the intrathecal application of drug solutions.

2.2.2 Venous cannulation

For experiments requiring systemic administration of a drug the animals were implanted with a jugular cannula prior to being mounted in the stereotaxic frame. The volume of injection for drugs administered via the cannula was 0.2-0.25 ml.

2.3 Electrophysiological recording of dorsal horn neurones

2.3.1 Finding the neurones

Glass-coated tungsten microelectrodes were used to record action potentials extracellularly from the dorsal horn. Electrodes were inserted into a hypodermic needle fitted with a gold pin connector and directly coupled to the A terminal of the recording headstage (Neurolog NL 100). This was already mounted on a stereotaxic frame micromanipulator and connected to a SCAT microdrive (Digitimer) system which was used to lower the electrode into the dorsal horn in 10μm steps and measure the depth of the neurones being recorded. The electrode tip was brought into contact with the surface of the spinal cord and very carefully lowered into the dorsal horn. The surface of the dorsal horn was determined by tapping the receptive field until noise was observed in response to the stimulus but no spikes could be detected. If the electrode 'burst' through it was carefully brought back to the surface so that the cell depth could be estimated. The neurones were located by lightly tapping the receptive field and occasionally applying pinch stimulation whilst simultaneously lowering the electrode into the dorsal horn. Non-noxious stimulation was used to initially locate neurones in order to avoid excessive tissue damage in the receptive field.

2.3.2 Extracellular recording of single dorsal horn neurones

Following insertion of the electrode into the dorsal horn, differential recording between the electrode and an indifferent electrode attached to the skin of the animal, was performed using a Neurolog headstage (Neurolog NL 100, A-B configuration). The voltage signal was amplified (Neurolog NL 104 x5K, Neurolog NL 106 x80) and filtered (Neurolog NL 125). The filtered signal was fed into a storage oscilloscope (Tektronix, 5013N), allowing visual display (Figure 2.2), and into the window discriminator of a spike trigger (Neurolog NL 200). The potentiometers of the NL 200 were set to gate only those spikes whose amplitude crossed a pre-set threshold. Each gated spike triggered a brightening (BRIT) pulse which, when fed into the second

channel of the storage oscilloscope, appeared as an intense 'dot' above each of the gated action potentials. This allowed the single unit activity of the neurone being studied to be visually discriminated if there was background multiple unit recording and the signal was monitored throughout the experiment to ensure accurate recording of the single neurone. In addition the filtered signal was fed via an audio-amp module (NL120) allowing audio discrimination of single neurones. A box diagram of the recording system in shown in Figure 2.3.

When the electrode tip was in close proximity to a cell the signal from that particular neurone was of larger amplitude than the background activity allowing its response to be gated as previously described. The cell was characterised by noting the responses to light brush, prod, radiant heat and sustained pinch of the receptive field. The responses to electrical stimulation of the receptive field were also tested as described below.

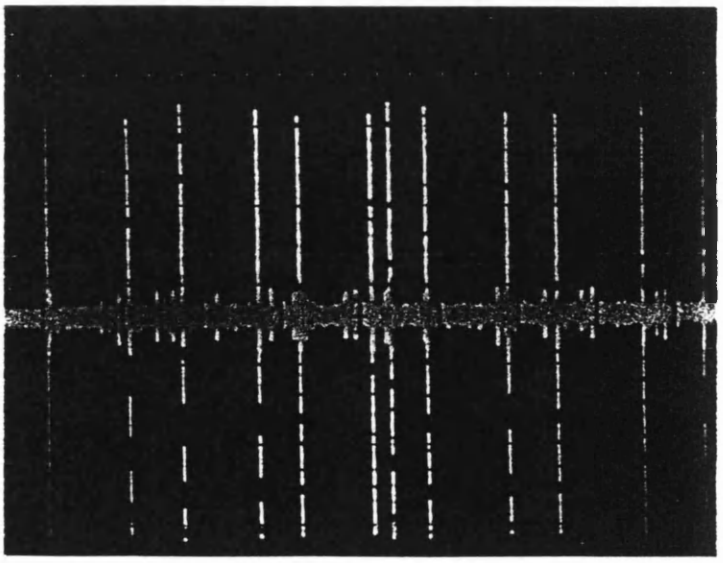

Figure 2.2 Photographic example of an extracellular recording of a dorsal horn neurone as displayed by a storage oscilloscope. Note the high signal to noise ratio.

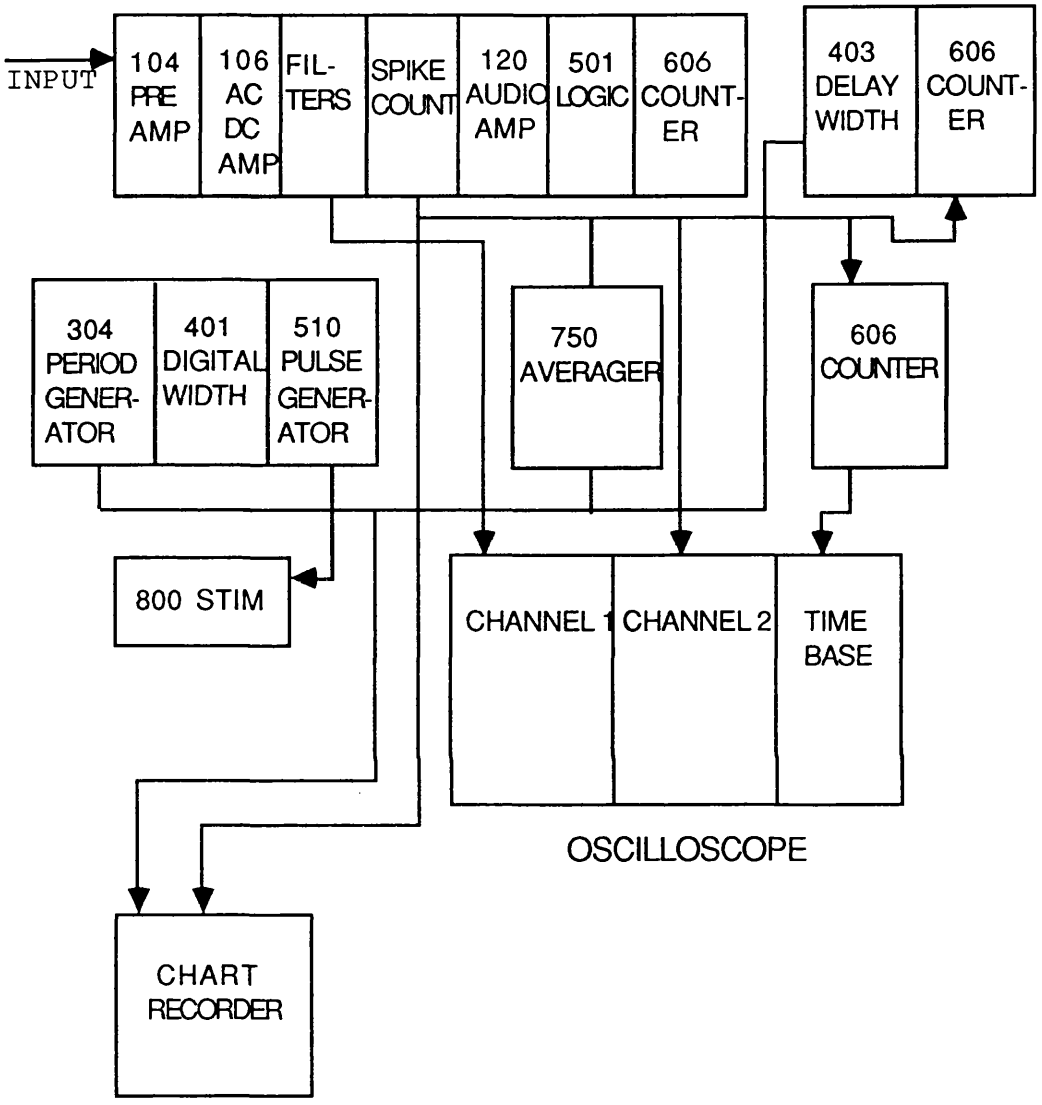

Figure 2.3 A simplified schematic representation of the equipment used to record, analyse and display the single unit activity from convergent dorsal horn neurones. The INPUT arises from the output of the recording electrode and headstage. For further details see text (2.3.2 and 2.4.1).

2.4 Stimulation of the receptive field

2.4.1 Transcutaneous electrical stimulation of the receptive field

A pair of small stimulating needles were inserted subdermally in the centre of the receptive field and single pulses of electricity (2ms wide square wave pulse) were delivered at a frequency of 0.5 Hz. This frequency of stimulation was chosen as the conduction of impulses along C fibres is known to fail at high frequency stimulation. The stimulation threshold for the A fibre evoked responses (0.01-0.1 mA) is lower than for C fibre evoked responses (0.1 -1.5mA) and the latencies are also different. The A fibre evoked responses are apparent within 90ms of the stimulus whilst the C fibre evoked response occurs about 100ms after the stimulus. Thus the A and C fibre evoked responses can be clearly separated on the basis of both stimulation threshold and latency. Stimulation of the receptive field at three times the threshold for either A fibre or C fibre activation elicited reproducible A or C fibre evoked responses in the convergent neurones and were therefore adopted for all the experiments involving transcutaneous electrical stimulation. The A and C fibre evoked responses were quantified according to the following procedure:

A period generator (Neurolog NL 304) and digital width module (Neurolog NL 401) controlled the frequency and duration of the electrical stimulus. These fed into a pulse buffer module (Neurolog NL 510) which controlled the amplitude of the stimulus delivered by a stimulus isolator (Neurolog NL 800) to the stimulating needles. In addition the period generator triggered the sweep of the storage oscilloscope. The averager (Neurolog NL 750) was set to 16 sweeps in order to construct a post stimulus histogram (PSTH), receiving the output of the NL 200 (BRIT pulses), and was triggered by the period generator. The output from the averager was fed into Neurolog logic gates (NL 501) which have two output channels. One output was fed directly into a latch counter (Neurolog NL 606) whilst the other output was fed via a delay module (Neurolog NL 403) into another latch counter. The delay was set at 90ms. Counter 1

therefore registered the total number of counts in a 1s sweep whilst counter 2 only captured spikes occurring after a delay of 90ms. The latency of A fibre evoked spikes falls within 0-90ms and a simple subtraction therefore yields the A and C fibre evoked counts. The logic gates were controlled by the averager so counting and stimulating only proceeded for the 16 stimuli making up the train. The output from the averager could also be fed into a chart recorder (JJ Instruments CR 650S) in order to produce hard copies of the PSTH's.

2.4.2 Formalin administration into the receptive field

Formalin (50µl of a 5% solution in 0.9% saline; BDH) was administered directly into the centre of the receptive field as a subcutaneous injection using a Hamilton 50µl syringe. Neuronal activity was measured continuously for 60 minutes: the pulse integrator was set to external timer and the stimulating system switched off. The output from the averager was continuous, fed into the logic gates and then, via output 1, directly into the latch counter. Time was measured with an external clock. A ratemeter recording was also produced by feeding the spike trigger output via a pulse integrator (Neurolog NL 601) into the chart recorder. On average 2-3 injections of formalin were administered per animal, separated by intervals of about 2 hours.

2.4.3 Bradykinin administration into the receptive field

Bradykinin (0.1µg-10µg in 0.9% saline; Sigma) was administered in a volume of 10µl via a 50µl Hamilton syringe. The syringe and needle were placed subcutaneously in the receptive field and remained there for the duration of the experiment. This was to reduce the activity evoked in the neurone by injection of the needle rather than the bradykinin itself. This was not a problem with formalin as a longer duration response was produced (about 5 min) compared to the short duration response seen with bradykinin (about 2 min). In addition some of the experiments with bradykinin required repetitive injections of the peptide into the same area of the receptive field. It was therefore desirable to keep the position of the injection as constant as possible.

2.5 Plasma extravasation induced by formalin

Halothane (0.8-1.5%) anaesthetized rats fitted with tracheal and jugular cannulae were mounted in stereotaxic frames. Evans blue (50mg/kg in 0.9% saline; Gurr) was administered intravenously in a volume of 0.2-0.25 ml. Formalin was administered into the hindpaw toe 10 minutes following the administration of Evans blue in order to investigate the distribution of plasma extravasation evoked. Saline was administered into the same digit on the opposite paw and used for comparison. The inflammation evoked by formalin was allowed to develop for 2 hours and the distribution of dye leakage assessed visually and noted.

In the experiments investigating the effects of the kappa agonist U50488H on the formalin induced plasma extravasation the following protocol was used. Ten minutes following the administration of Evans blue, U50488H (100µg in 50µl in H_20) was injected into digit V on one hindpaw whilst distilled H_20 (50µl) was injected into the same digit on the opposite hindpaw. A further ten minutes later formalin was administered into the same toes and the subsequent inflammation was allowed to develop over 2 hours. The level of anaesthesia was increased until a heartbeat was no longer detected in the rat. The glabrous and hairy skin was removed from the whole paw and digits and incubated as described below. The plasma extravasation elicited by U50488H and H_2O alone was measured by allowing the extravasation to develop for 120 minutes and then the skin was removed as previously described.

2.5.1 Measurement of Evans blue content in the hindpaw skin

The hairy and glabrous skin of the paws was removed and weighed. They were incubated in 4mls of formamide (100%; Sigma,) at 60°C overnight (Saria and Lundberg, 1983). The absorbence of the resulting solution was measured in a spectrophotometer at a wavelength of 620nm. A standard curve of absorbence was constructed with increasing concentrations of Evans blue in formamide (Figure 2.4). The Evans blue content of the tissue was measured by reading the concentration of the solution off the standard curve. This was then expressed as mg dye per mg tissue.

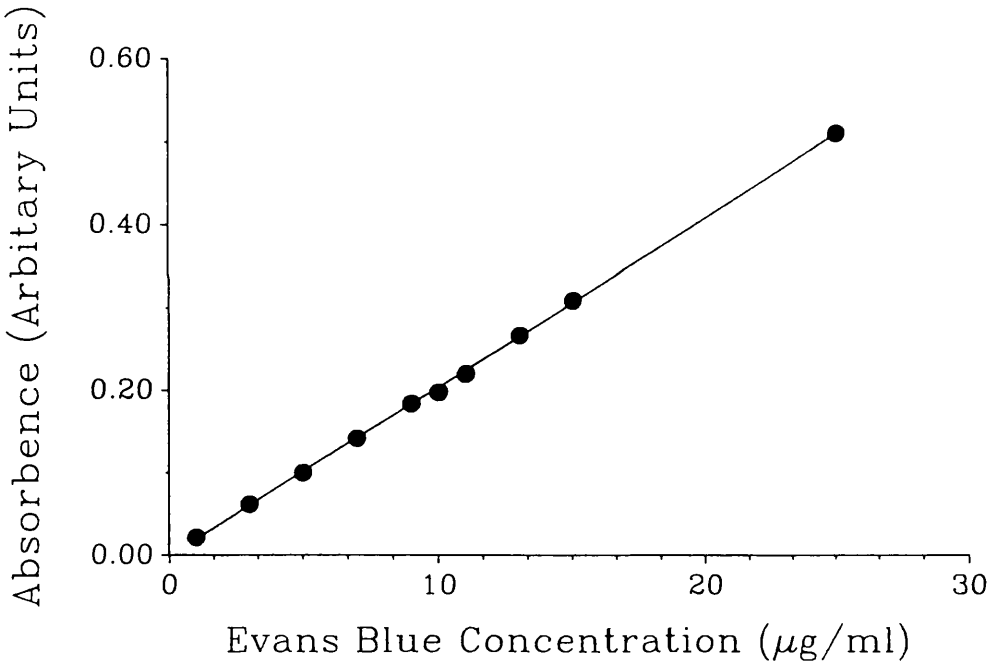

Figure 2.4 Standard curve showing the absorbence (as read on a spectrophotometer) of increasing concentrations of Evans blue dye in formamide measured at a wavelength of 620nm. n=3 for each point, S.E.M. lies within the symbol.

2.6 Drugs

The drugs used (and their sources) were as follows:

5-DL-amino phosphonovaleric acid (AP5; Sigma); L-arginine hydrochloride (Sigma); Atropine sulphate (Sigma); Bradykinin (Sigma); $DArg^0[Hyp^3Thi^{5,8}DePhe^7]$bradykinin (B4162; kindly donated by Prof. M. Schachter, Kings College London, UK); $DesArg^9[Leu^8]$bradykinin (Sigma); trans ±3,4-dichloro-N-methyl-N-[2-(1-pyrolidinyl)-cyclohexyl] benzeneacetamide (U50488H; Upjohn); Evans blue (Gurr); Formalin (BDH); Formamide (Sigma); γ-D-glutamyl glycine (DGG; Sigma); Ketamine hydrochloride (Ketalar®; Parke-Davis); Lignocaine hydrochloride (Phoenix Pharmaceuticals); (+)-5-methyl-10,11-dihydro-5H- dibenzo[a.d]cyclo-hepten-5,10-imine maleate (MK801; kindly donated by Dr. L. Iversen, Merck Sharp and Dohme Neuroscience Research Centre, Harlow UK.); Morphine hydrochloride (Sigma); Naloxone hydrochloride (Sigma); Nitro-L-arginine methyl ester (L-NAME; Sigma); Tyr-D-Ala-Gly-MePhe-Gly-ol (DAGOL; kindly donated by Prof. B. P. Roques, Université René Descartes, Paris); Tyr-D-Ser(Otbu)-Gly-Phe-Leu-Thr (DSTBULET; kindly donated by Prof. B. P. Roques).

2.7 Analysis of results

The electrically evoked A and C fibre responses of individual cells were calculated as the mean number of spikes elicited by pre-drug tests. Responses elicited following the administration of a drug were calculated by reference to the pre-drug values and expressed as a percentage of these control values. Statistical significance of the effect of a drug was tested using Student's paired t test.

Formalin evoked activity was expressed as the mean counts ± standard error of the mean (S.E.M.) for the first and second peak of the response over a population of neurones. The effect of a drug on the formalin response elicited in a group of cells was

compared to a separate group receiving saline or H_2O in place of the drug. The means of these two groups of cells were compared and statistical significance was tested using the Mann-Whitney U-test. This non-parametric test compares the medians of each group rather than the mean, but in all the groups tested the median value lay within the standard error of the mean.

Where a drug (or saline) was added during the ongoing activity of the formalin response second peak, the change in the rate of firing of the neurone was measured. This was expressed as the mean maximal inhibition, compared to the mean predrug rate of firing. The statistical significance between the change in the rate of firing produced by saline compared to the drug over a population of neurones was tested using the Mann-Whitney U-test.

The plasma extravasation results were expressed as the mean Evans blue content per mg of tissue ± S.E.M.. The data were analysed using Student's t-test where paired or unpaired tests were used as appropriate.

CHAPTER 3:

THE PROPERTIES OF CONVERGENT NEURONES AND THEIR RESPONSES TO FORMALIN

3.1 Introduction

The responses of dorsal horn neurones to both innocuous and noxious peripheral stimuli has been studied for many years and the majority of studies have focussed on the cat spinal cord. The first recordings of rat dorsal horn neurones were made by Wall and colleagues in 1967, but it was not until a decade later that the rat once more became the subject of investigation (Menétrey et al.; 1977). Rodents are commonly used in behavioural studies examining the actions of analgesics and many nociceptive tests are applied to both mice and rats (Wood, 1984). These animal models utilize:

a) Heat stimulation (Hot-plate test, tail flick test and tail withdrawal test)

b) Electrical stimulation (Tail stimulation, Flinch-Jump assay)

c) Noxious pressure or pinch

d) Chemical stimulation (abdominal constriction test, formalin test)

e) Inflammation induced hyperalgesia (yeast, carrageenan or adjuvant-induced).

The effects of application of some of these noxious stimuli have been investigated in electrophysiological recordings in the rat but these concentrate mainly on acute stimuli such as heat, pressure or electrical stimulation (Menétrey et al., 1977; Schouenborg and Sjölund, 1983; Dickenson and Sullivan, 1986). In these short duration stimuli the activity of the convergent neurones recorded compares well with the activity displayed by the rats in behavioural studies. In addition, these responses can be modulated by analgesics which are known to be active in behavioural studies (see Le Bars and Chitour, 1983). However very little is known about the role of these dorsal horn neurones during more prolonged stimuli such as inflammatory conditions.

The formalin response is a well established behavioural test for assessing the effectiveness of analgesics. It differs from other inflammatory conditions being of reasonably short duration and evoking spontaneous nociceptive behaviour in the animals. Unlike the arthritic models available it is therefore possible to assess the effect

of analgesics in the absence of additional nociceptive stimuli such as noxious pressure. This chapter describes some of the properties of the convergent neurones and compares the responses elicited by formalin with the behaviour evoked in conscious animals. In addition mu opioid agonists have been administered both systemically and intrathecally in order to determine whether the dorsal horn neuronal activity elicited by formalin could be modulated by known analgesics.

3.2 Results

3.2.1 The responses of convergent neurones to natural stimuli

Light brushing, prod, pinch and radiant heat were used to stimulate the hindpaw receptive fields of the dorsal horn neurones. Whilst the majority of these neurones responded well to light prod, noxious pinch and heat the responses to brush were variable (Figure 3.1A, B). Out of a total of 650 neurones, 90 neurones were located within the superficial laminae (0 - 250μm) and 54% of these responded to brush, 52 were within the intermediate laminae (250 - 500μm, presumed to include substantia gelatinosa neurones) and 65% responded to brush whilst out of 508 deeper neurones (500 -1000μm) only 44% responded to brush of the receptive field.

All neurones responded to pinch with a sustained response lasting the duration of the stimulation although some adaptation from an initial peak of firing to a lower rate of discharge was observed. Prolonged firing that outlasted the stimulus for tens of seconds was also seen (Figure 3.1C, D). Occasionally neurones were encountered that responded only to noxious stimuli although these were rare. An example of the responses of this type of cell is given in Figure 3.1D.

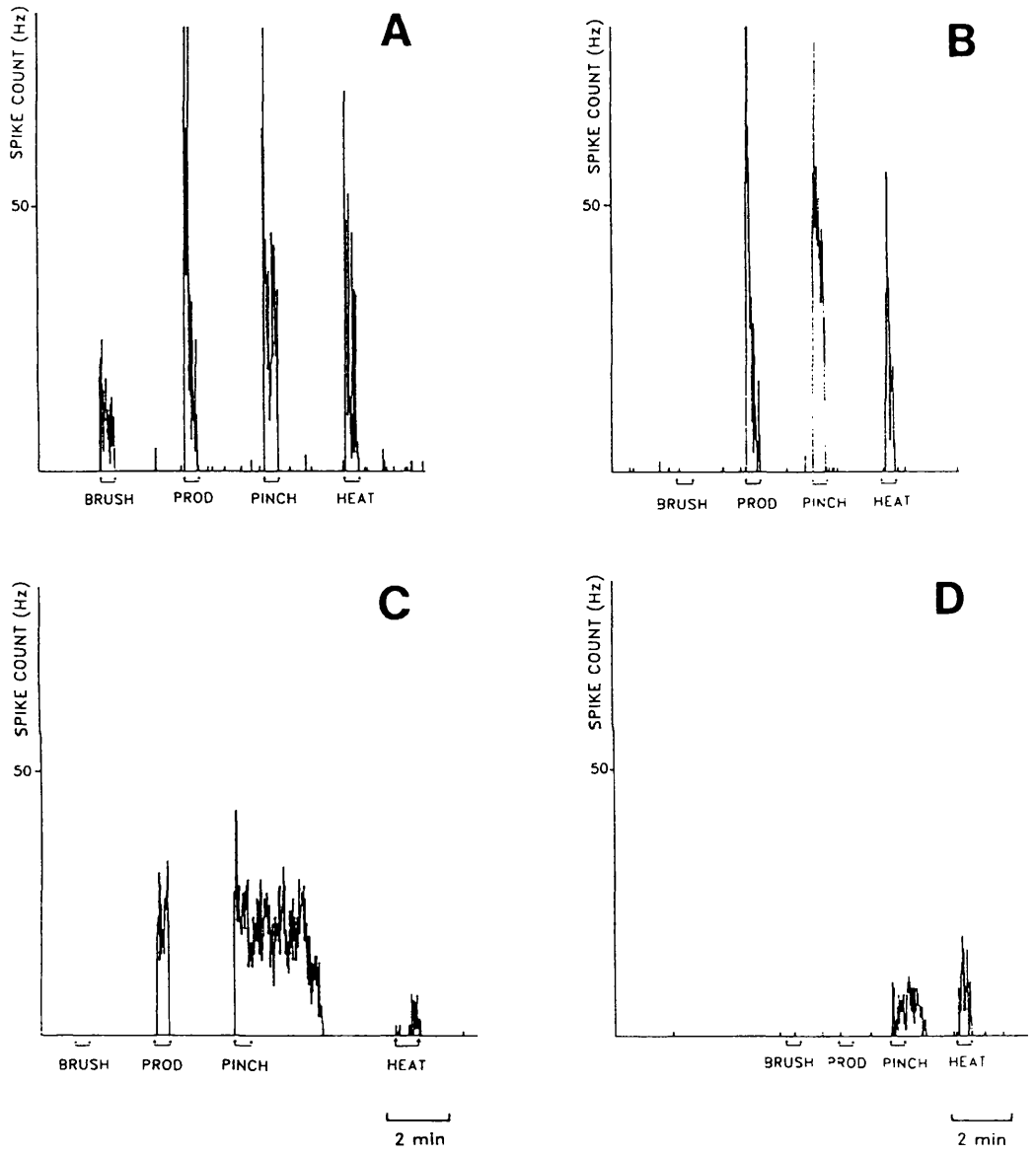

Figure 3.1 Ratemeter recording of the responses of single dorsal horn neurones to brush, prod, pinch and noxious radiant heat. A, B, C) convergent neurones responding to innocuous stimuli (brush and/or prod) and noxious stimuli (heat and pinch). D) Noxious only cell responding to only heat and pinch. Note that C) and D) respond to pinch with a prolonged firing that persists even after the stimulus has ended.

3.2.2 Responses of convergent neurones to electrical stimulation of the receptive field

Transcutaneous administration of a short electrical pulse (. 2 ms wide) across the receptive field via small intradermal stimulating electrodes resulted in firing of the dorsal horn neurones. A low amplitude stimulus (0.01 - 0.1 mA) produced only short latency (0 - 90 ms) responses resulting from fast conducting Aß fibre inputs. Increasing the stimulus intensity (0.1 - 1.5 mA) produces an additional longer latency (90 - 1000 ms) response in the neurones resulting from slower conducting C fibre inputs. Following a train of 16 stimuli (0.5 Hz) a post stimulus histogram was constructed (see 2.4.1) (Figure 3.2).

Many neurones responded to electrical stimulation with A fibre activity only, often firing in response to joint movement and innocuous events but not to noxious stimuli. These neurones were not studied any further. The majority of the neurones recorded were convergent, as defined by their responses to natural stimuli. These convergent neurones responded with both A and C fibre evoked activity. Occasionally, but rarely, a cell was found that responded with C fibre activity but without an A fibre evoked response and these cells were unresponsive to innocuous stimuli such as brush or prod.

The convergent neurones responded to electrically evoked C fibre inputs in two broad manners. Some of the neurones produced a discreet band of activity with a narrow range of latencies and very little activity beyond this range (Figure 3.2A). Other neurones exhibited a more diffuse C fibre response with a wider range of latencies and activity occurring beyond the main range; this prolonged response has been termed post-discharge (Figure 3.2B). This post-discharge is likely to result from activity within the dorsal horn rather than arising from the inputs of very slowly conducting afferent fibres. This post discharge could not be quantified separately and was counted with the C fibre activity.

Repetitive stimulation of the receptive field at 0.5 Hz with a constant stimulus elicited two profiles of response in the dorsal horn neurones. Some cells responded

with a constant size response whilst others increased their response with each successive stimulus usually plateauing after about 10 - 12 stimuli (Figure 3.3). This phenomena of increasing response to a constant peripheral stimulus is known as 'wind up'. The proportions of cells displaying 'wind up' varied with depth in the dorsal horn. Sixty-three percent of superficial neurones displayed 'wind up' although it was less pronounced than that seen in deeper neurones (Figure 3.3). Seventy-four percent of intermediate cells and 68% of deep neurones displayed 'wind up'.

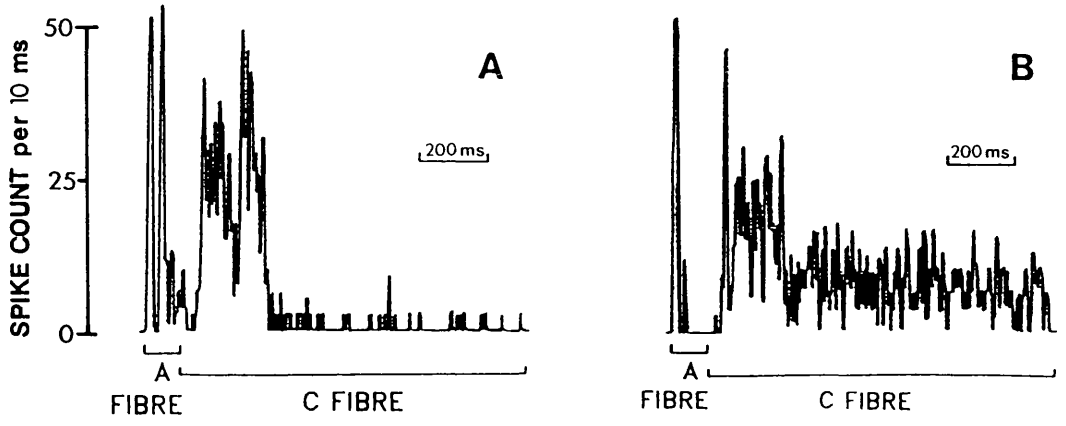

Figure 3.2 Post stimulus histograms (PSTH) of the responses of two dorsal horn neurones following peripheral electrical stimulation. Stimulation was at three times the C fibre thresholds at 0.5 Hz and the PSTH's are for responses over 16 stimuli. A) A neurone displaying both A and C fibre responses but no post discharge. B) A neurone with post discharge in addition to the A and C fibre responses.

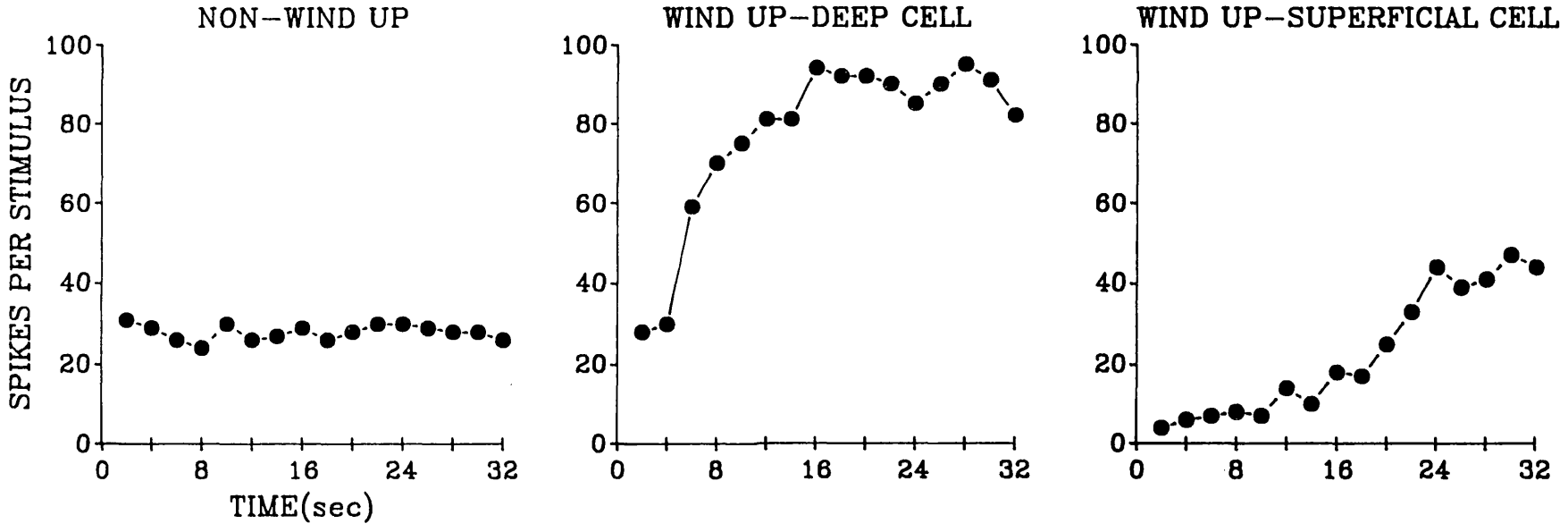

Figure 3.3 Examples of the responses of single dorsal horn neurones to repetitive electrical stimulation of the receptive field. Stimulation was at three times the C fibre threshold for 16 stimuli at 0.5 Hz. Neurones that do not display 'wind up' (non-'wind up') respond to each stimulation with a constant number of action potentials. 'Wind up' neurones display increased firing in response to the constant peripheral stimulation which tends to be more dramatic in deep neurones compared to superficial cells.

3.2.3 The relationship between the depth of a neurone and its responses to electrical stimulation of the receptive field

The relationship between the various properties of neurones resulting from electrical stimulation of the peripheral receptive fields was examined. Correlation coefficients were calculated using information gathered from 635 neurones. There was no correlation between the A fibre threshold of the neurones and the A fibre counts elicited by electrical stimulation (correlation coefficient r= 0.002). Similarly, the C fibre threshold of the cells and the C fibre count evoked by electrical stimulation were independent of one another (r= -0.168). Additionally, no correlation existed between the A fibre threshold of the cell and its C fibre threshold (r= 0.192).

As calculated by the correlation coefficient there was no relationship between the depth of a neurone within the dorsal horn and its A fibre threshold (r= 0.01), C fibre threshold (r= 0.026) or in the A fibre evoked counts (r=0.077) or C fibre evoked counts (r= 0.197). However rather than looking at a linear change in depth, if the neurones were grouped into three regions (superficial (0 - 250μm), intermediate, presumed to include substantia gelatinosa (250 - 500 μm) or deep (>500μm)) some differences were apparent.

The mean A fibre threshold was unchanged in all three regions: superficial: 0.11 ± 0.006 mA (n=79); substantia gelatinosa: 0.12 ± 0.02 mA (n=49); deep: 0.12 ± 0.005 mA (n=460). However a small reduction in the C fibre threshold of substantia gelatinosa neurones relative to the other regions was apparent: superficial: 1.14 ± 0.05 mA; substantia gelatinosa: 1.00 ± 0.06 mA; deep: 1.12 ± 0.02 mA. Over the whole population of neurones this reduction was not statistically significant but if 'wind up' and non-'wind up' neurones were separated a clear difference was observed (Figure 3.4). Neurones that did not 'wind up' in response to C fibre strength electrical stimulation did not display any variation in their C fibre thresholds. However, substantia gelatinosa neurones that displayed 'wind up' had significantly lower

thresholds than either superficial (p=0.008) or deep (p=0.013) 'wind up' neurones (Figure 3.4).

Over the whole population of neurones, there was no significant difference in the A fibre evoked response between the substantia gelatinosa and deep neurones, however both these regions produced responses that were greater than those in the superficial region: superficial: 110 ± 12 counts (n=79); substantia gelatinosa: 184 ± 34 counts (n=49, p=0.016 compared to superficial); deep: 157 ± 6 counts (n=460, p=0.00006 compared to superficial). This small increase in response compared to superficial cells was observed in both the deep 'wind up' (p=0.002) and deep non-'wind up' neurones (p=0.001). However the substantia gelatinosa neurones were no longer significantly different from the superficial neurones in either the 'wind up' or the non-'wind up' groups, probably reflecting the smaller sizes of the groups (Figure 3.5).

The C fibre evoked responses of the substantia gelatinosa and deep neurones were also increased relative to the superficial neurones but were not significantly different from each other over the whole population of neurones: Superficial: 360 ± 24 counts (n=79); substantia gelatinosa: 566 ± 51 counts (n=49, p=0.0001 compared to superficial); deep: 536 ± 16 counts (n=460, p=0.0006 compared to superficial). However separation of the 'wind up' and non-'wind up' neurones revealed a striking difference between the two groups. There was a significant difference in the size of the C fibre evoked response between 'wind up' and non-'wind up' neurones in superficial ('wind up': 417 ± 32 counts (n=51); non-'wind up': 248 ± 23 counts (n=25, p=0.0009)), substantia gelatinosa ('wind up': 653 ± 65 counts(n=34); non-'wind up': 310 ± 35 counts (n=12, p=0.004)) and deep regions ('wind up': 655 ± 21 counts (n=273); non-'wind up': 363 ± 16 counts (n=158, p=0.0000)). 'Wind up' neurones in both the substantia gelatinosa and deep regions produced significantly larger responses than the superficial 'wind up' cells (Figure 3.5). However there was a much smaller difference between the responses of non-'wind up' cells in these these regions with only the deep neurones producing significantly greater responses.

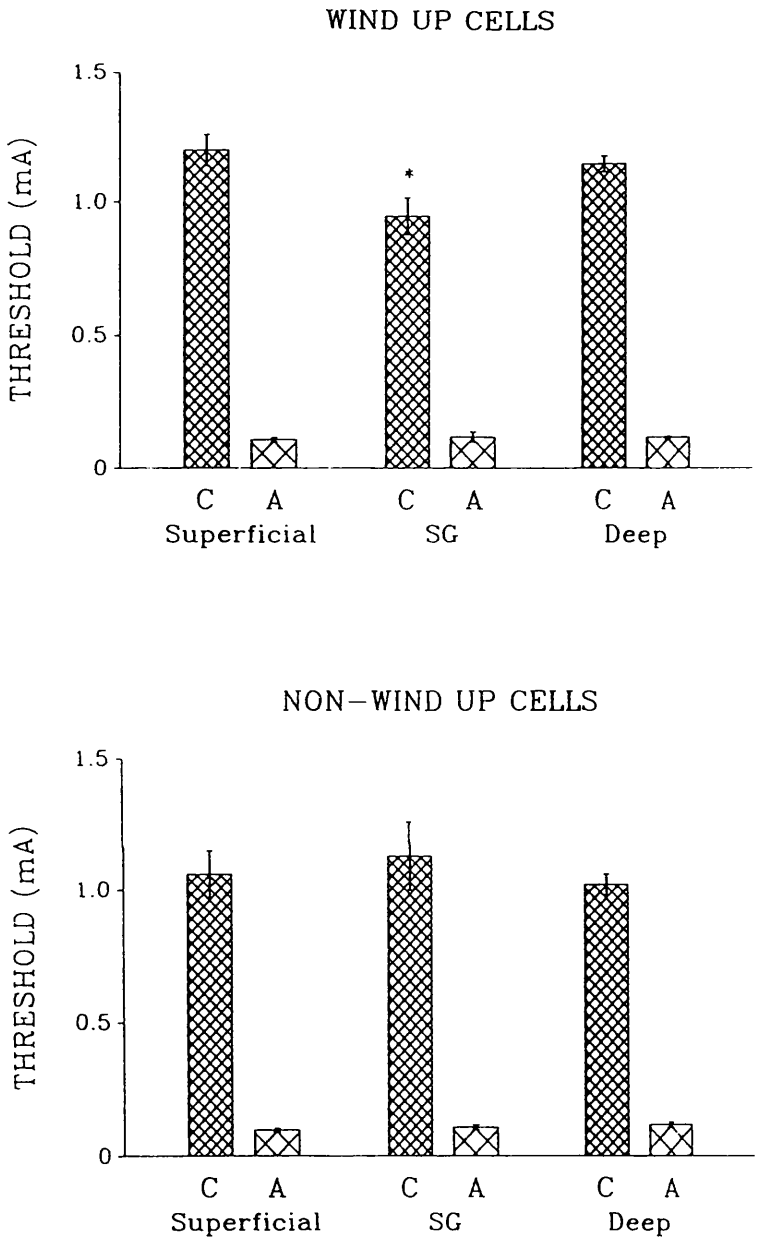

Figure 3.4 Comparison of the A and C fibre thresholds for the electrically evoked activity of neurones located in the superficial, intermediate (substantia gelatinosa, SG) and deep regions of the dorsal horn. These neurones have been divided into those that display 'wind up' (upper graph) and those that do not (lower graph). 'Wind up' neurones located in the SG region had lower thresholds than other neurones. n ranges between 34 and 273 for 'wind up' cells and between 12 and 158 for non-'wind up' cells. *p=0.008 compared to superficial neurones and 0.013 compared to deep neurones, Mann-Whitney U-test.

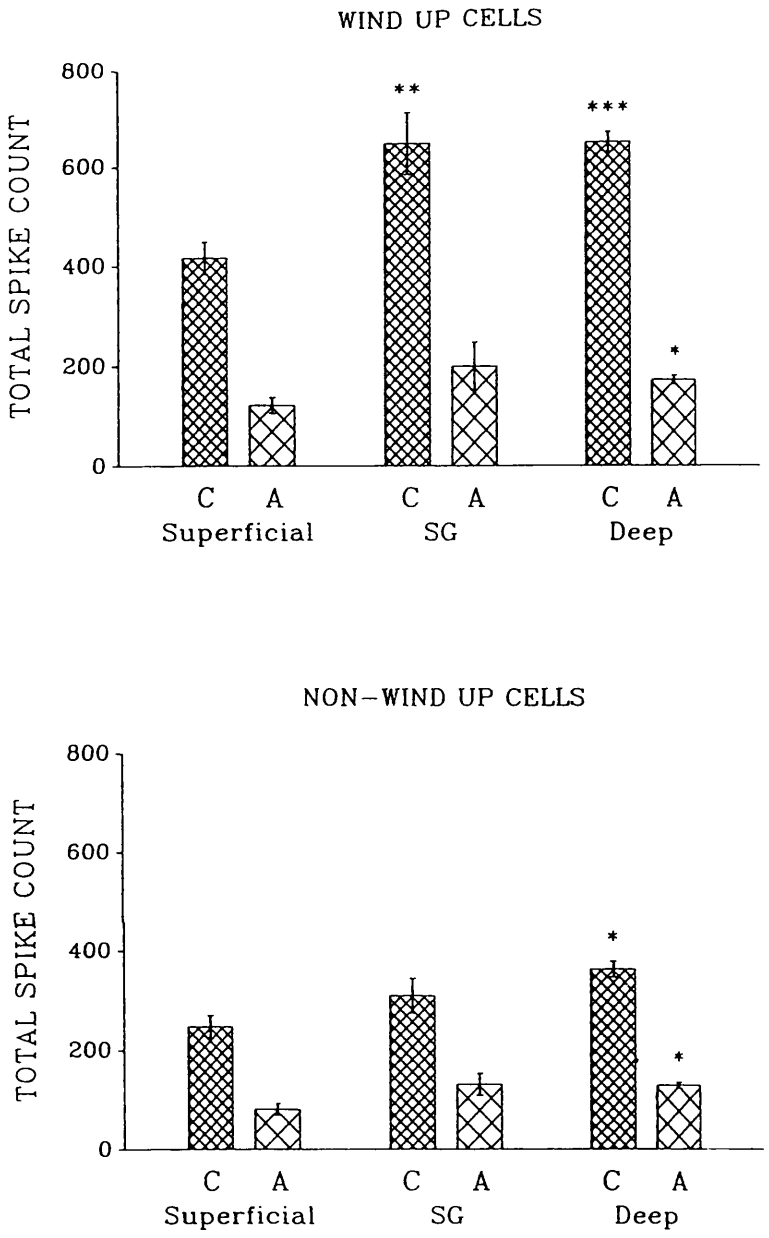

Figure 3.5 Comparison of the A and C fibre counts for the electrically evoked activity of neurones located in the superficial, intermediate (substantia gelatinosa, SG) and deep regions of the dorsal horn. These neurones have been divided into those that display 'wind up' (upper graph) and those that do not (lower graph). 'Wind up' neurones located in the SG and deep regions had larger C fibre evoked responses than superficial cells or non-'wind up' cells in the same regions. The changes in A fibre evoked responses were small and the same for both 'wind up' and non-'wind up' cells. n ranges between 34 and 273 for 'wind up' cells and between 12 and 158 for non-'wind up' cells. *$p<0.004$ **$p=0.001$ ***$p<0.00006$ compared to superficial neurones, Mann-Whitney U-test.

3.2.4 Responses of dorsal horn neurones to peripheral (s.c.) administration of formalin

Formalin (50µl of a 5% solution in saline) was injected subcutaneously into the centre of the receptive field and this elicited a biphasic firing in the dorsal horn neurones over a period of about 60 minutes. Firing began immediately following the injection of formalin, this activity lasted for about 5 minutes and was over within 10 minutes of the injection. The rate of firing of the neurones during this phase was very variable but only occasionally exceeded 50 Hz. Following a period of inactivity lasting about 15 - 20 minutes the neurones began to fire again. Over a period of about 40 minutes the firing rate would initially increase until it reached a plateau (rarely greater than 50 Hz) and then gradually decline until the neurone was once again inactive. Administration of 50µl of saline had only a very transient excitatory effect on the neurones (Figure 3.6.). This biphasic profile is also observed in the behaviour of rodents and primates injected with formalin into the paw (Figure 3.7).

Generally the activity finished within about 60 minutes and in order to quantify the response this was taken as a cut off time. All action potentials counted during the first 10 minutes following the formalin injection were termed the first peak whilst the activity occurring in the remaining 50 minutes of the response was assigned to the second peak.

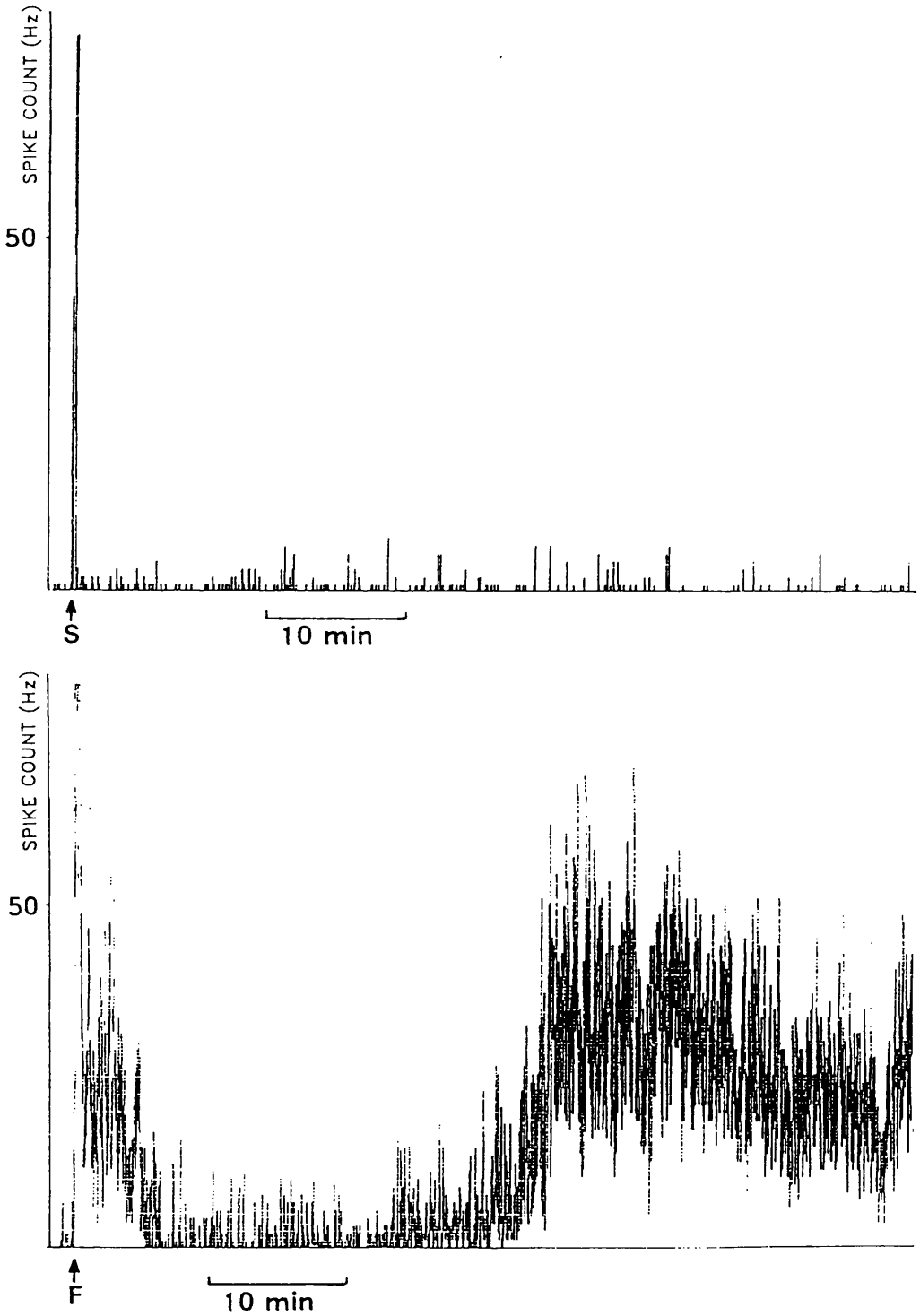

Figure 3.6 Examples of the ratemeter recordings of single dorsal horn neurones firing in response to injections of saline (S) and formalin (F) into the receptive field as indicated by the arrows. The duration of the recordings is 60 minutes; activity in the first ten minutes is assigned to the first peak whilst the second peak consists of all firing during the remaining 50 minutes.

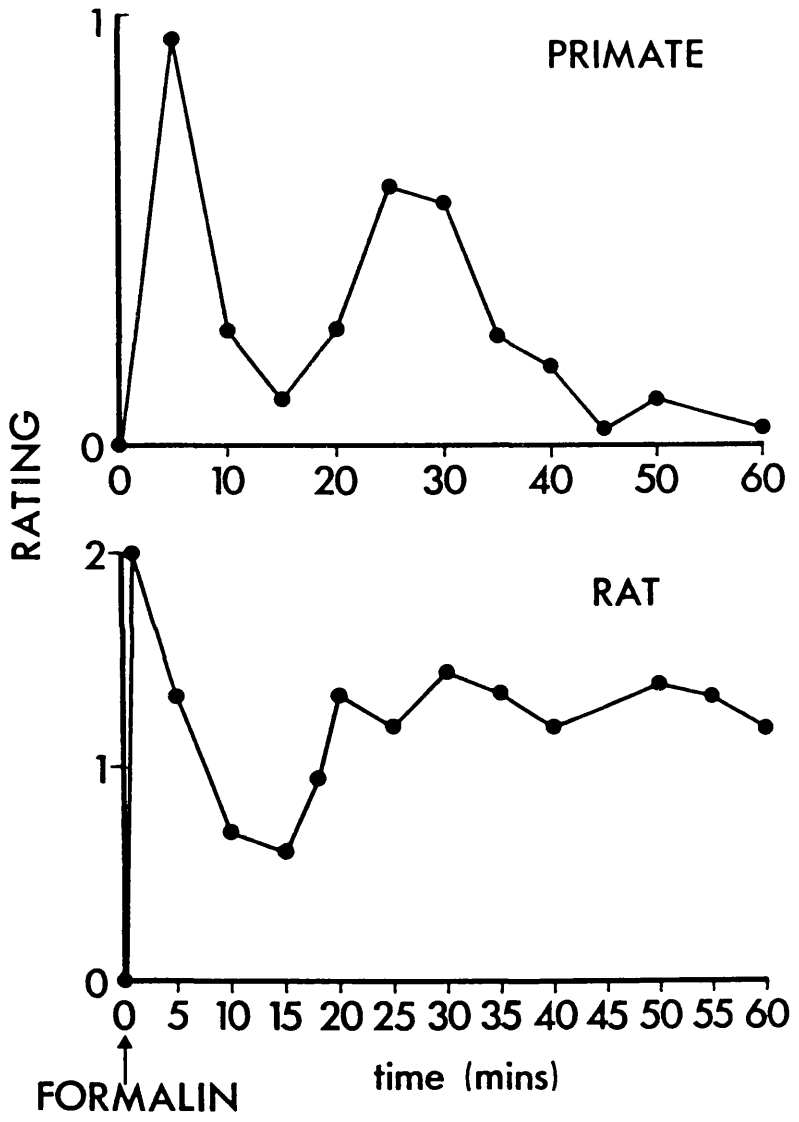

Figure 3.7 Examples of the behavioural responses of primates (upper graph) and rats (lower graph) to an injection of formalin (arrow) into the hand or paw. The ordinate represents a rating scale reflecting behaviour indicative of pain in these animals. Taken from Alreja et al., 1984 (primate) and Dubuisson and Dennis, 1977 (rat).

3.2.5 Relationship between the depth, electrically evoked activity of the cells and their responses to formalin

Responses to formalin were recorded in 159 dorsal horn neurones. No correlation was found between the A fibre threshold of the cells and the size of either the first (r=0.002) or second peaks (r=0.042) of the formalin response. Similarly there was no correlation between the C fibre threshold and the first (r=-0.126) or second peak (r=-0.219), the A fibre evoked count and the first (r=0.176) or second peak (r=0.107) or the C fibre evoked count and the first (0.516) or second peaks (r=0.378) of the formalin response.

There was no significant difference in the size of the formalin responses elicited in either superficial (1st peak: 3686 ± 692 counts; 2nd peak: 15,159 ± 3460 counts, n=19), substantia gelatinosa (1st peak: 6160 ± 1649 counts; 2nd peak: 26,989 ± 8100 counts, n=13) or deep neurones (1st peak: 4467 ± 298 counts; 2nd peak: 19,090 ± 1842 counts, n=127). Separating the neurones into those that display 'wind up' and those that do not revealed consistently larger responses in the 'wind up' neurones (Figure 3.8). However this difference was only statistically significant in the deep neurones where the first but not the second peak of the response in 'wind up' cells was larger than in non-'wind up' neurones (p=0.001).

It is interesting to determine if the size of the second peak of the formalin response is in any way related to the size of the first peak elicited in a given neurone. Examination of the size of the first and second peak responses of 194 neurones to formalin revealed a correlation coefficient of 0.731 which was not sufficiently close to one to allow a direct linear relationship to be concluded. However regression analysis yields a slope of 4.125 ± 0.278 which is significantly different from zero (p=0.0000), allowing us to conclude that some kind of relationship between the two peaks is likely but more detailed analysis would be required to determine its exact nature.

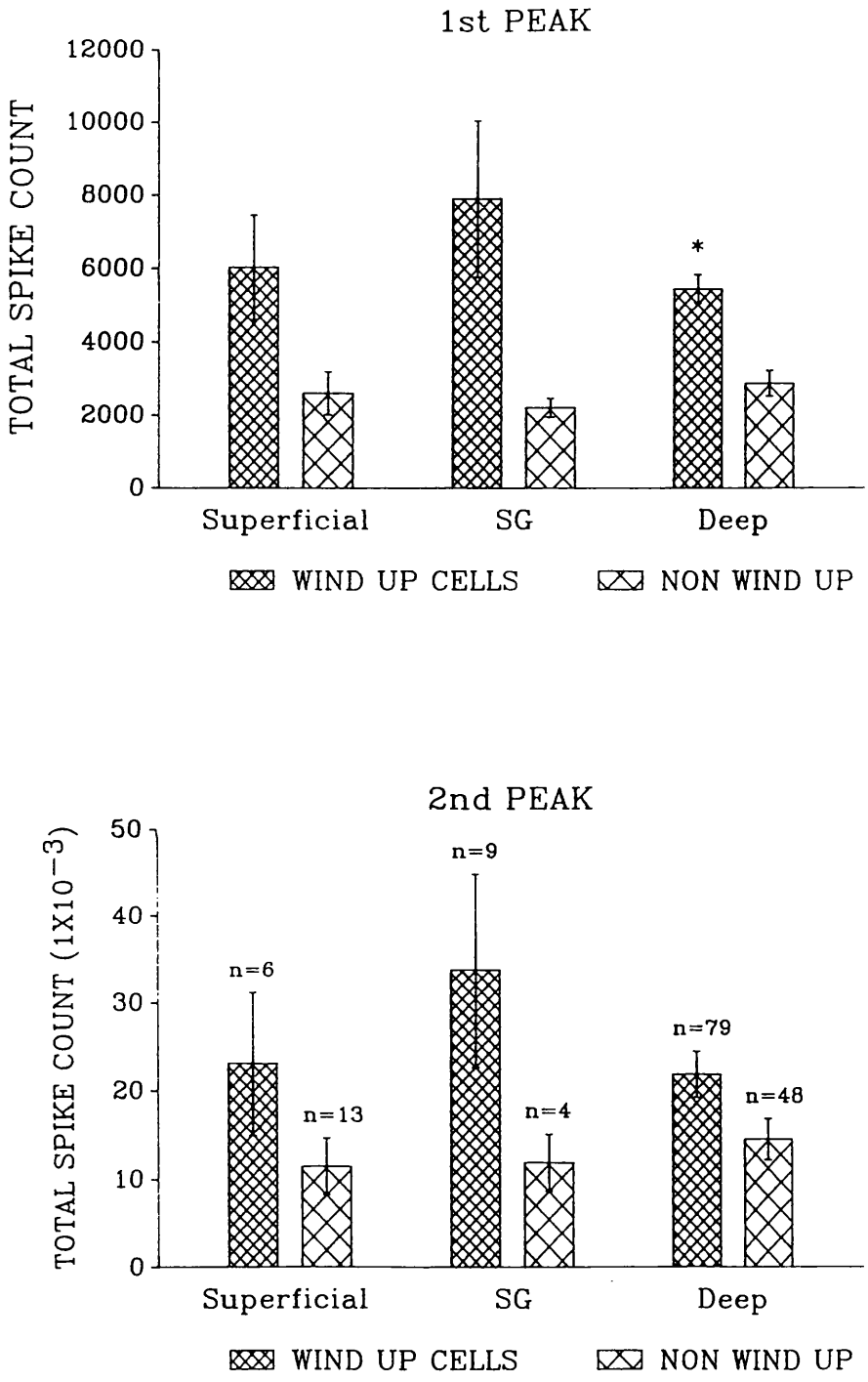

Figure 3.8 The size of the formalin response first and second peaks was generally greater with neurones that display 'wind up' compared to those that do not in superficial, substantial gelatinosa (SG) and deep regions of the dorsal horn. n values are the same for the first and second peaks. *p=0.001 compared to the deep non-'wind up' neurones, Mann-Whitney U-test.

3.2.6 Administration of formalin into two separate areas of the receptive field has no effect on the response of the neurones to formalin

Following the injection of formalin into a region of the paw (usually an individual toe) and monitoring the subsequent neuronal response for 60 minutes it was possible to elicit another formalin response from the same paw. Recording from a different cell with a receptive field remote from the site of the original administration resulted in a response no different in either size (Figure 3.9) nor latency to the second peak (1st cell: 31.3 ± 1.3 minutes; 2nd cell: 28 ± 2.2 minutes) from that produced by the first formalin injection . The time that elapsed between the first and second injections of formalin was just enough to monitor the response of the cell to the initial injection and then find a different cell. This time lapse never exceeded 2 hours.

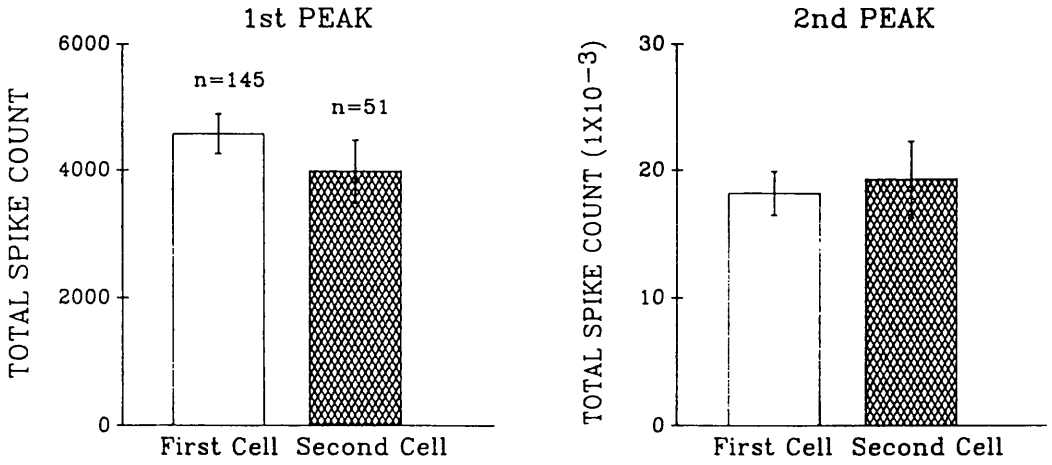

Figure 3.9 Injection of formalin into the receptive areas of two different neurones one after the other had no effect on the subsequent size or profile of the responses recorded. The receptive fields were located on the same paw but remote from one another and the time between the two injections was never greater than two hours.

3.2.7 Local plasma extravasation elicited by formalin injection into the receptive field

Halothane anaesthetized rats were injected with an Evans blue solution (50mg/kg) via an intravenous jugular cannula 10 minutes prior to formalin administration into a toe on the hind paw. Plasma extravasation was visualized as an increase in the dye content of the tissue surrounding the injection site. Saline injection was unable to elicit any visible plasma extravasation (Figure 3.10). The distribution of the plasma extravasation elicited by formalin varied depending on the toe into which the formalin had been administered but it tended to follow the path of nerve innervation for that region (Figures 3.10 & 3.11). The time course of the plasma leakage was slower than the response observed in the dorsal horn neurones becoming visible at about 40 minutes and plateauing around 2 hours after formalin injection.

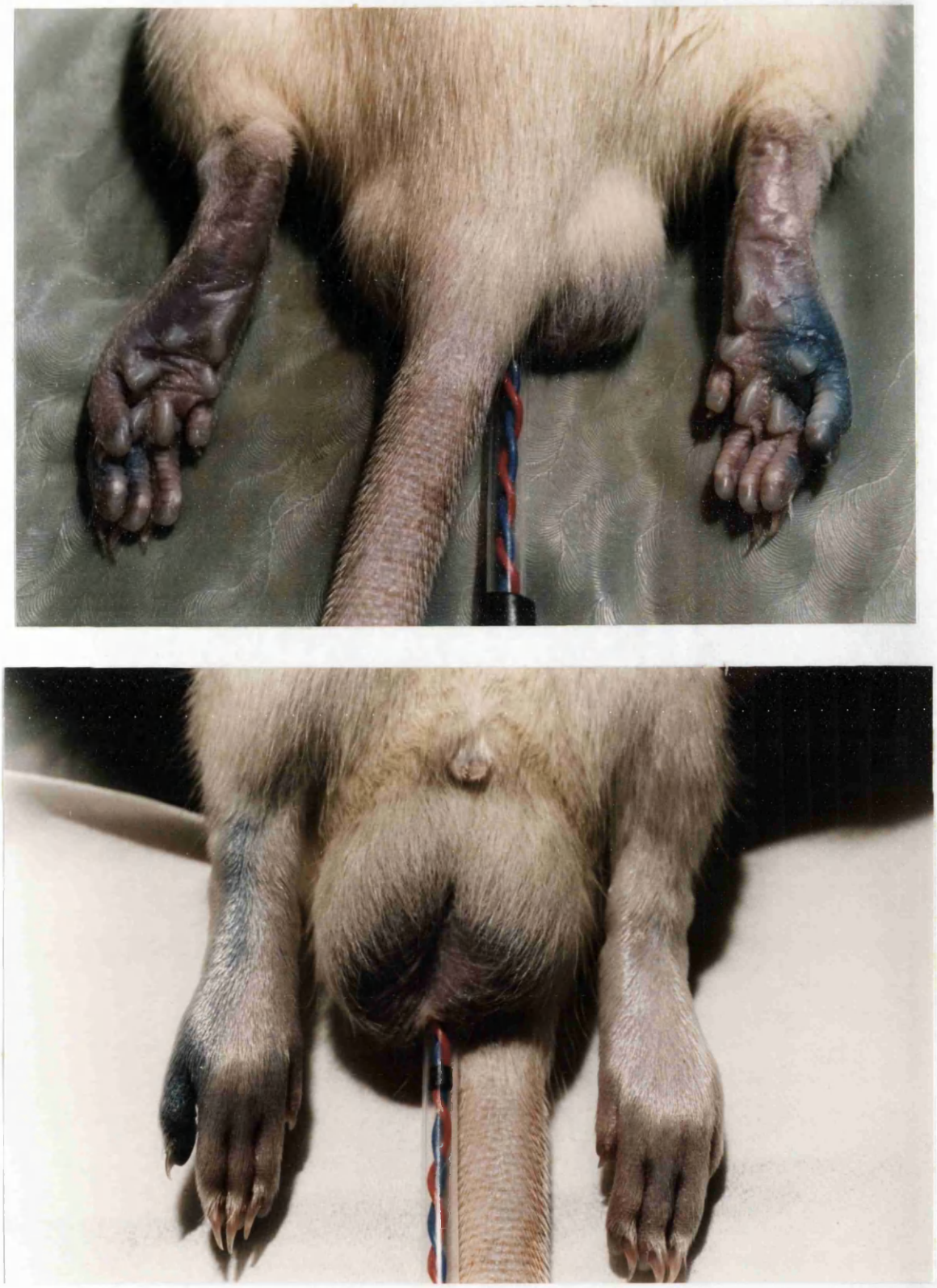

Figure 3.10 Photographic example of plasma extravasation elicited 2 hours after injection of formalin into digit V on the right paw; saline was administered into the left paw for comparison. Note that whilst extravasation remained local to the site of injection in the glabrous skin (upper photograph), the hairy skin (lower photograph) displayed distinct tracts of dye extravasation.

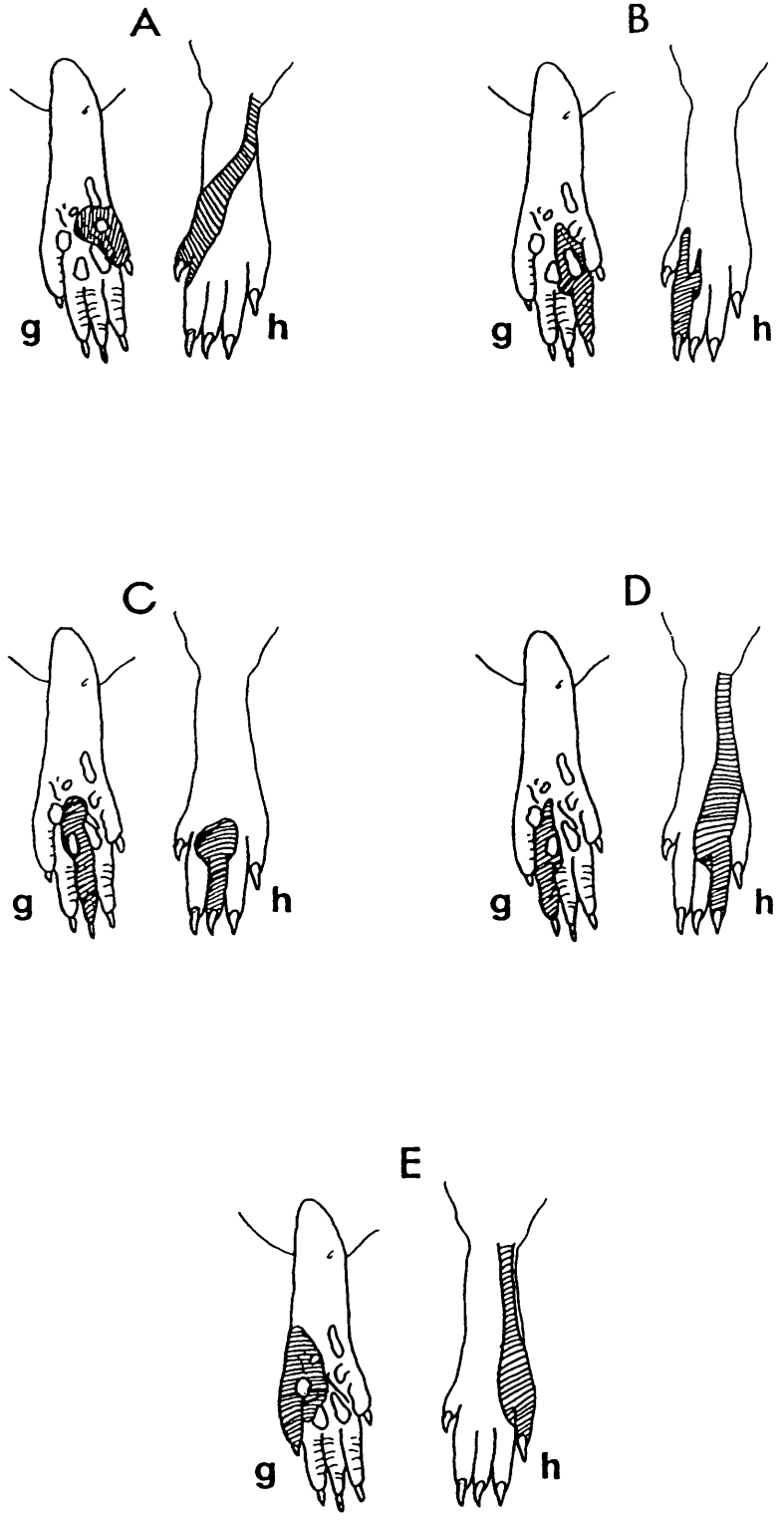

Figure 3.11 Distribution of plasma extravasation, as visualized using Evans blue, following formalin administration into individual toes. A,B,C,D,E represent the areas of Evans blue accumulation 120 minutes following formalin injection into individual toes (digits I to V) in both the glabrous (g) and hairy skin (h). Note the lack of extravasation in the foot pads.

3.2.8 The response of dorsal horn neurones to bradykinin administration into the hindpaw

Bradykinin (10μl) injected subcutaneously directly into the receptive field on the hindpaw, via a fixed needle, resulted in a short duration firing of the dorsal horn neurones. Both the size and duration of the response increased with increasing dose: 0.1μg bradykinin (n=6) produced a mean response size of 995 ± 435 counts and a duration of 1.52 ± 0.43 minutes whilst the mean response to 10 μg bradykinin was 3647 ± 1055 counts and the duration was 3.92 ± 0.72 minutes (Figure 3.12).

Repetitive administration of bradykinin resulted in a rapid tachyphylaxis in the responses of the dorsal horn neurones. This desensitization to bradykinin occurred at even the lowest doses of the peptide and with saline (Figure 3.13). The third injection resulted in reductions, compared to the first response, of 96%, 99% and 62% in the responses to saline, 0.1μg and 10μg bradykinin respectively.

3.2.9 The relationship between the bradykinin and electrically evoked responses of dorsal horn neurones

The responses of 47 neurones to bradykinin administration into the receptive field were recorded. No correlation was found between the size of the response to bradykinin and the A (r=0.009) or C fibre (r=-0.270) thresholds or the size of the A (r=0.592) or C fibre (0.487) counts of the cells. The responses of the neurones to bradykinin could not be examined with relation to their depth within the dorsal horn due to the small number of cells recorded.

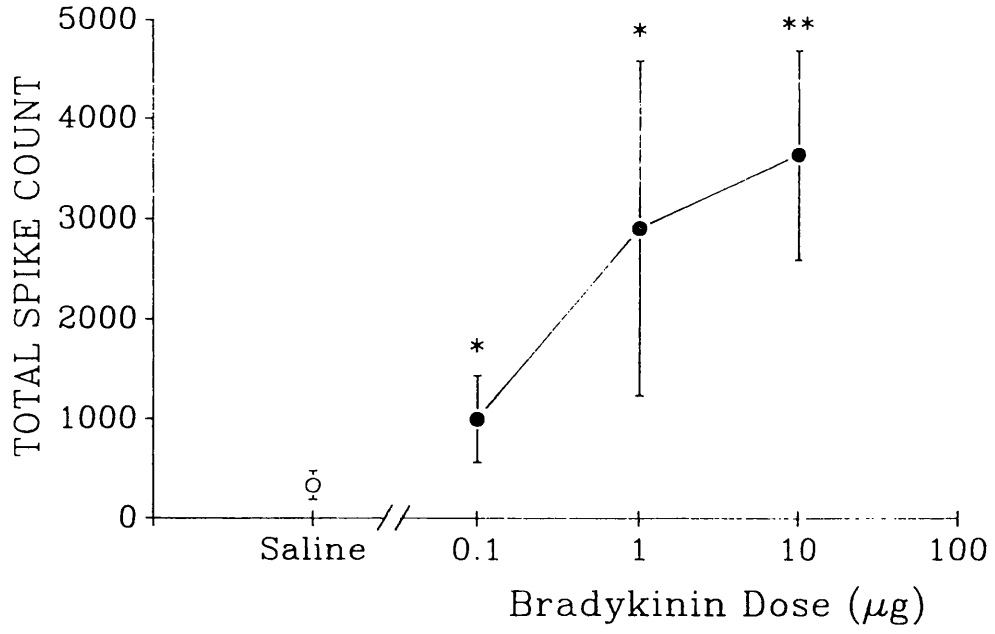

Figure 3.12 Bradykinin injected in a fixed volume of 10μl directly into the receptive field resulted in responses greater than those produced by the same volume of saline. Increasing the dose of bradykinin resulted in greater responses of the neurone. n ranges from 6 to 11. *p<0.05 **p=0.001 compared to the saline group, Mann-Whitney U test.

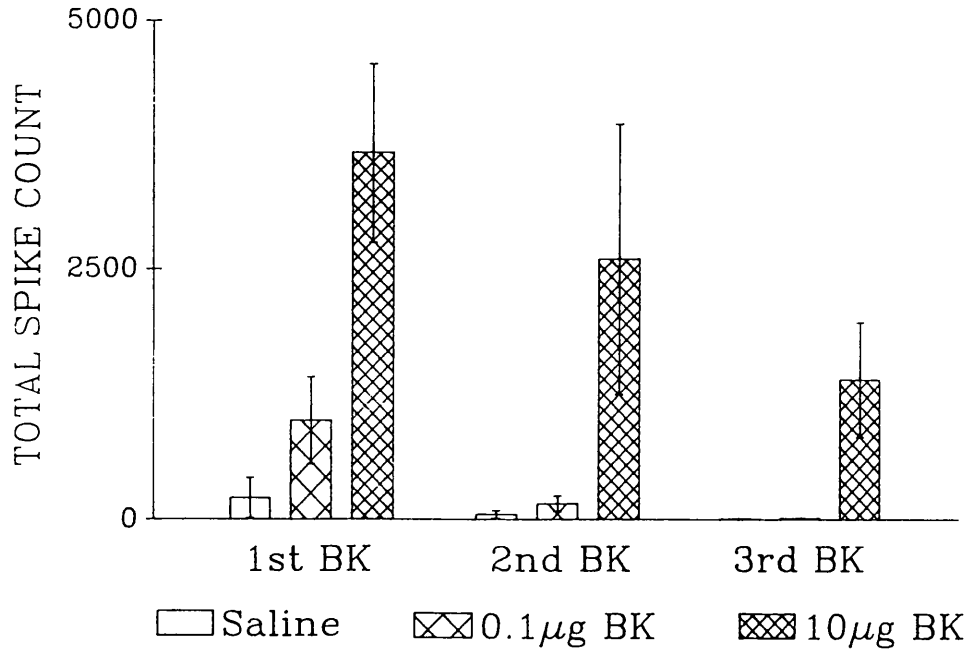

Figure 3.13 Repetitive injections of saline or bradykinin (BK) into the receptive field produces a rapid loss in the size of the response, irrespective of the dose of bradykinin administered. n ranges from 4 to 14.

3.2.10 Modulation of the formalin response by intrathecal and systemic mu opioid agonists

Intrathecal administration of the mu agonist Tyr-D-AlaGlyMePheGly-ol (DAGOL) as a 20 minute pretreatment resulted a dose-related inhibition of both the first and second peaks of the formalin response (Figure 3.14). The top dose of 5μg DAGOL produced inhibitions of the first and second peak of 85% (Control: 3401 ± 564 counts, n=10; DAGOL: 504 ± 363 counts, n=3; $p<0.05$) and 98% (Control: 9770 ± 3051 counts; DAGOL: 206 ± 251 counts; $p<0.02$) respectively.

DAGOL administered during the second peak was less effective at inhibiting the activity although the top dose of 5μg was still able to produce an inhibition in the firing rate of 90% ± 8 (n=6; p=0.0005). However the lower dose of 0.25 μg resulted in only 20% ± 15 (n=6; $p>0.1$) inhibition in the firing rate during the second peak compared with 90% inhibition when administered as a pretreatment (Figure 3.14).

Intravenous morphine administered during the second peak activity resulted in a reduction in the rate of firing of the neurone. The highest dose administered (0.2 mg/kg) produced an inhibition of 59% (Figure 3.15).

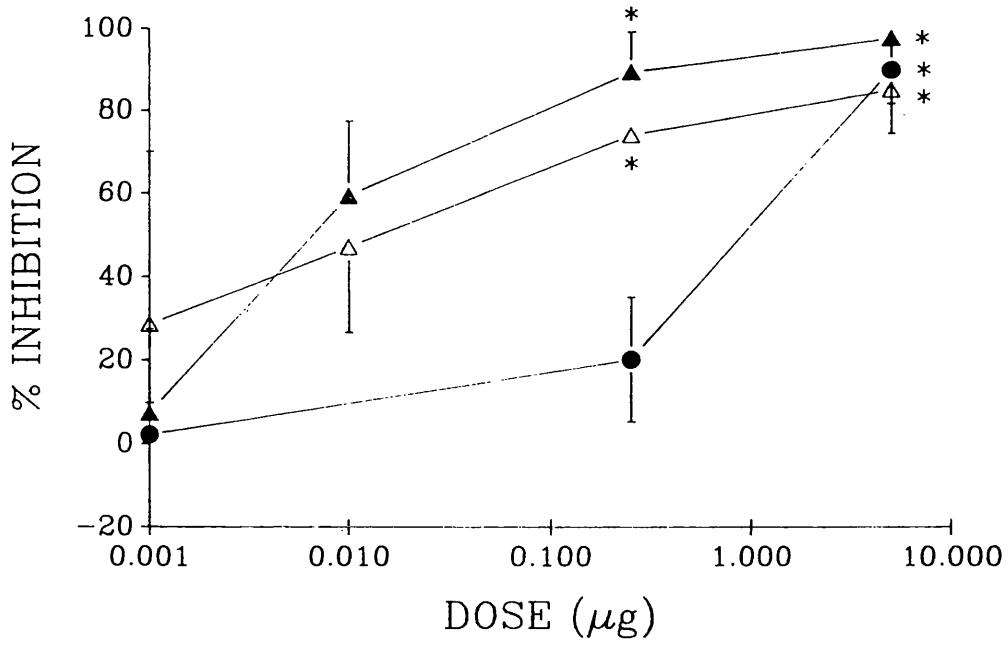

Figure 3.14 Inhibition of the formalin response by intrathecal DAGOL administered 20 minutes prior to formalin (1st peak: Δ-Δ; 2nd peak: ▲-▲) and during the second peak (●-●). n ranges from 3 to 7. *p< 0.05, Mann-Whitney U-test.

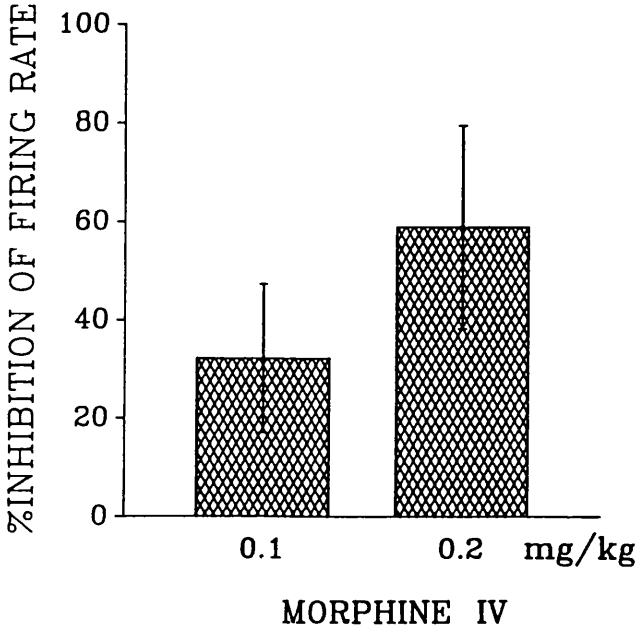

Figure 3.15 Inhibition of the rate of firing of the neurones by intravenous morphine administered during the second peak of the formalin response. n ranges from 3 to 6.

3.3 Discussion

3.3.1 The responses of dorsal horn neurones to natural stimuli

The majority of neurones recorded within the dorsal horn were convergent neurones responding to innocuous stimuli such as prod or brush as well as to noxious heat or pinch. Rarely were noxious only neurones encountered although this may result from the location procedure used (see 2.3.1); the receptive field was prodded and occasionally pinched as the electrode was lowered into the dorsal horn. Noxious pinch was not used continuously as a search stimulus in order to keep local tissue damage to a minimum.

The neurones recorded always responded to innocuous prod, noxious pinch and heat but the response to brush was varied. This is in agreement with previous studies which found subclasses of convergent (class 2) neurones; those that responded to brush and those that did not (Menétrey et al., 1977; Woolf and King, 1987). Neurones located in the intermediate, substantia gelatinosa, region of the dorsal horn were most likely to be activated by brushing of the receptive field with 65% responding. Those least likely to respond were located deep within the dorsal horn with only 44% of neurones in this region being activated by brush. Previous studies agree well with the proportion of superficial neurones responding to brush being around 50% but disagree regarding the deeper neurones with values of 33% and 68% (Menétrey et al., 1977; Woolf and King, 1987). These studies did not distinguish between substantia gelatinosa neurones and deep neurones and varying proportions of each may explain the different percentages seen responding to brush.

Some of the neurones responded to noxious pinch with sustained firing that outlasted the 30 second stimulus by tens of seconds. This after discharge has been observed in recordings of rat spinal neurones following pressure or pinch stimuli in the rat (Woolf and King, 1987; Schouenborg and Dickenson, 1988). Noxious pressure or

pinch produces firing in afferent neurones that generally does not exceed the stimulus (see Yaksh and Hammond, 1982 and Besson and Chaouch, 1987). It therefore seems likely that the prolonged after discharge results from mechanisms within the spinal cord rather that from excessive activity in the afferent fibres (Schouenborg and Sjölund, 1983).

3.3.2 The responses of dorsal horn neurones to electrical stimulation

Electrical stimulation of the receptive field resulted in a short latency (0-90ms) response and a longer latency (>90ms) response arising from the inputs of fast conducting myelinated fibres and slowly conducting unmyelinated fibres respectively. The A fibre response may represent inputs from both Aß and A∂ fibres since these could not be separately measured although A∂ fibre inputs generally have a higher threshold than the Aß fibre inputs. The average latency to arrival of the A fibre response is around 4 to 20ms, representing conduction velocities of between $6ms^{-1}$ to $30ms^{-1}$ (assuming the distance from the receptive field to the spinal cord is around 12cm), confirming that this response is likely to represent inputs from A fibres. The average latency of the delayed response is around 100 to 200ms, corresponding to conduction velocities of about $0.6ms^{-1}$ to $1.2ms^{-1}$, confirming that the responses result from C fibre inputs (Gasser and Erlanger, 1927; Burgess and Perl, 1973).

'Wind up' has been observed in dorsal horn recordings in both the cat and rat (Mendell, 1966; Schouenborg and Sjölund, 1983) and increasing pain sensation to a constant stimulus has been demonstrated in man (Price et al., 1977). The proportion of neurones within the dorsal horn that display 'wind up' has been estimated at 52% and 42% (Cervero et al., 1984; Woolf and King, 1987). This is lower than the proportion found to display 'wind up' in this study. The greater amount of 'wind up' displayed by deep cells compared to superficial neurones has also been observed previously in the rat (Schouenborg and Sjölund, 1983).

Interestingly there is no relationship between the A and C fibre thresholds of the neurones and the size of the responses elicited by these inputs. However there are some differences between neurones located at different depths within the dorsal horn. Whilst the average A fibre threshold of the neurones does not vary there is a small drop in the C fibre threshold of substantia gelatinosa neurones compared to superficial or deep. This appears to result from a lower threshold of 'wind up' neurones since there is no difference between the non-'wind up' cells in these regions.

The average size of the A fibre evoked responses observed in substantia gelatinosa and deep neurones are slightly larger compared to superficial cells and this does not appear to be related to the ability of a cell to 'wind up' since both 'wind up' and non-'wind up' neurones show a similar increase. The average size of the C fibre evoked responses is larger in both the substantia gelatinosa and deep compared to the superficial neurones. However this is due almost entirely to the larger responses of 'wind up' cells in these regions since the non-'wind up' neurones show only small regional changes.

3.3.3 The responses of dorsal horn neurones to peripheral formalin

Injection of formalin into the receptive field produces a biphasic response in the dorsal horn neurones whilst saline only elicited a transient firing in the neurone lasting about 1 -2 minutes. This response to formalin is remarkably similar to the profile of response observed in behavioural studies in mice, rats and monkeys following formalin injection (Dubuisson and Dennis, 1977; Alreja et al., 1984; Hunskaar et al., 1985). All these species exhibit a biphasic pattern of response behaviour although the time courses differ slightly. In these dorsal horn recordings the first peak began immediately, was over within 10 minutes and this was then followed by a silent period lasting about 15 minutes. At about 20 - 25 minutes following the injection of formalin the neurones began firing again and this lasted between 30 - 60 minutes. This time course mirrors the pain related behaviour observed in rats following formalin administration (Dubuisson

and Dennis, 1977) but is slightly more prolonged than the 30-40 minute biphasic response observed in mice (Hunskaar et al., 1985). A previous study recording dorsal horn neurones in the cat did not observe a biphasic response to peripheral formalin but instead saw continuous firing of the neurones for between 10 and 55 minutes (Banna et al., 1986). This mirrors the behaviour observed in cats following formalin injection as this too consists of continuous nociceptive activity for about 50 minutes (Dubuisson and Dennis, 1977).

The dose of formalin administered in behavioural studies seems to vary with species. However in studies using Sprague-Dawley rats 50µl of a 5% formalin solution has been used as the noxious stimulus (Dubuisson and Dennis, 1977; North, 1977; Sugimoto et al., 1986; Wheeler-Aceto et al., 1990). A recent study in mice has indicated that the maximum size and duration of the formalin response is elicited with 5% formalin although a smaller volume of injection was used in this species (Rosland et al., 1990).

Interestingly, the size of the neuronal response to formalin does not depend on the A or C fibre threshold of the neurones nor on the size of the acute A or C fibre evoked response. In addition there is no significant difference between the size of the formalin response between superficial, substantia gelatinosa and deep neurones. Whilst 'wind up' neurones generally produced larger first and second peaks to formalin compared to non-'wind up' neurones, this difference was only significant within the deep region. The relationship between the first and second peaks of the formalin response appears to be complicated. Whilst there is no direct correlation between the size of the first peak and the size of the second peak, a correlation coefficient of 0.73 suggests that they may be linked in some way. Regression analysis produces a slope of 4.13 ± 0.28, significantly greater than zero, again indicating that some relationship exists between the two peaks but that it is not simply linear.

Recording consecutive formalin responses from remote regions of the same paw had no effect on the size or profile of the response of the second neurone compared to the first neurone. This tends to imply that any changes occurring centrally are either short lasting or are not influencing cells with inputs from alternative regions in the paw.

The consistent size and profile of formalin responses following repetitive administration into the same paw has also been observed in behavioural studies in the mouse and monkey although the interval between injections ranged from days to weeks rather than hours (Alreja et al., 1984; Rosland et al., 1990).

The time course for emergence of formalin evoked plasma extravasation, as visualized using Evans blue, is similar to the development of oedema following formalin administration. Initially a rapid rate of oedema formation results in an increase in paw volume of about 50% within 2 hours followed by a slower rate leading to a plateau by about 9 hours (Brown et al., 1968); Evans blue accumulation was visible at about 40 minutes, developed rapidly and appeared to plateau around 2 hours after formalin injection. The distribution of plasma leakage induced by formalin was confined to an area local to the injection site in the glabrous skin but often formed a narrow tract running the length of the paw in the hairy skin. These tracts appear to follow the path of nerve innervation to the toes (Brenan et al., 1988; Wiesenfeld-Hallin, 1988) and are therefore likely to represent formalin stimulated axon reflex mediated extravasation.

3.3.4 The responses of dorsal horn neurones to peripheral bradykinin

Most neurones recorded responded to bradykinin administration into the receptive field. The dose response curve for bradykinin activation of dorsal horn neurones compares well with the bolus intra-arterial doses shown to be effective at exciting nociceptors *in vivo* in the rat, cat and dog (0.1 - 30 μg) (Beck and Handwerker, 1974; Franz and Mense, 1975; Kumazawa and Mizumura, 1977; Kumazawa and Mizumura, 1980; Haupt et al., 1983; Kanaka et al., 1985; Martin et al., 1987). *In vivo* activation of dorsal horn neurones by peripheral intra-arterial bradykinin has been demonstrated in the cat and monkey with similar doses of bradykinin (2.5 - 30 μg) (Besson et al., 1972; Belcher, 1979; Foreman et al., 1979; Hong et al., 1979).

Repetitive injections of bradykinin into the receptive field resulted in a rapid

tachyphylaxis in the response of the dorsal horn neurones. This loss of response is likely to result from a peripheral mechanism since neurones no longer responding to the peptide respond normally to bradykinin injected into an adjacent, naive, part of the receptive field. Tachyphylaxis to bradykinin has also been demonstrated in peripheral afferent fibre recordings in several species (Beck and Handwerker, 1974; Franz and Mense, 1975; Kumazawa and Mizumara, 1977; Baker et al., 1980; Kanaka et al., 1985; Kirchoff et al., 1990). This desensitization to bradykinin appears to result from a very local action at the free nerve ending: application of bradykinin to a naive ending restores activity to an axon no longer responding to bradykinin application at a separate ending on the same fibre (Baker et al., 1980). Tachyphylaxis to bradykinin has also been observed in dorsal horn neuronal recordings in the cat (Guilbaud et al., 1977; Belcher, 1979) although this was not seen in the monkey (Foreman et al., 1979). Tachyphylaxis appears to occur even at the lowest dose of bradykinin administered as has been observed in peripheral fibre recordings (Kanaka et al., 1985). The highest dose of bradykinin administered produced the most sustained response over a series of injections as well as producing responses that were consistently higher than those produced by saline. For these reasons the 10μg dose of bradykinin was used in later experiments. Interestingly there appeared to be no relationship between the response of the neurones to bradykinin and the threshold or size of the neuronal response to electrically evoked A or C fibre stimulation.

3.3.5 Modulation of the formalin response by opioids

Both peaks of formalin evoked neuronal firing were inhibited by intrathecal or intravenous administration of mu opioid agonists. The dose of intravenous morphine required to inhibit the second peak of the formalin response (59% inhibition with 0.2mg/kg) is approximately ten fold lower than that required to produce a similar degree of inhibition in the acute electrically evoked C fibre response (63% inhibition with 3 mg/kg i.v.; Le Bars et al., 1980b). Behavioural studies in mice, rats, cats and monkeys

have all demonstrated the ability of systemically administered morphine to reduce the pain related behaviour elicited by formalin (Dubuisson and Dennis, 1977; Alreja et al., 1984; Hunskaar et al., 1985). However the doses of subcutaneous morphine found to be effective in rats against formalin evoked behaviour were equieffective in the acute tail flick and hotplate tests (Dennis et al., 1980; Abbott et al., 1986). Interestingly the inhibition, by intravenous morphine, of responses to nociceptive stimuli in arthritic rats occurs in the dose range 0.1-1 mg/kg (see Kayser et al., 1987) which is comparable to the doses effective in this study against the formalin response second peak.

Intrathecal administration of DAGOL produced a dose related inhibition of both peaks of the formalin response when administered as a pretreatment. The ED50 for this inhibition was about 0.01 μg, approximately ten fold lower than the dose required to produce 50% inhibition of electrically evoked C fibre activity (Dickenson et al., 1987). However if the DAGOL is administered during the second peak of the formalin response the dose response curve is almost identical to that seen against electrically evoked C fibre activity. This loss in sensitivity of the formalin response to inhibition by DAGOL is unlikely to result from the time of onset of DAGOL since this is reasonably rapid and the maximum inhibition should have been observed prior to the end of the formalin response. In addition the reduction in the efficacy of DAGOL has also been observed if the opioid is administered only 3 minutes following the formalin injection (Dickenson and Sullivan, 1987).

In conclusion, these convergent neurones respond to acute stimulation of the receptive field such as transcutaneous electrical pulses or exogenous administration of bradykinin and more prolonged stimuli such as formalin. In addition the formalin injection elicits a profile of activity in these neurones that mirrors the response behaviour observed in conscious animals. This neuronal activation can also be modulated by both systemic and intrathecal administration of opioids which have been shown to reduce formalin evoked behaviour in rodents and primates. Thus the responses of dorsal horn neurones to peripheral administration of formalin are sensitive to inhibition. However,

the ability of intrathecal opioids to inhibit the second peak of the formalin response appears to be reduced if they are administered after the injection of formalin. It is therefore possible that alterations may be occurring within the dorsal horn during the prolonged response that result in a reduction in the ability of opioids to inhibit the response. Thus, the prolonged neuronal activity of dorsal horn neurones evoked by formalin may prove to be a useful model in determining the peripheral and central mechanisms underlying prolonged nociception.

CHAPTER 4:

THE ROLE OF BRADYKININ IN THE FORMALIN RESPONSE: STUDIES AT BOTH PERIPHERAL AND CENTRAL LEVELS

4.1 Introduction

4.1.1 Bradykinin in the periphery

The peptide bradykinin has long been postulated as a peripheral mediator in both inflammatory and nociceptive events. Kinins are present in inflammatory exudates elicited by carrageenan (Ferreira et al., 1974; Capasso et al., 1975; Uchida et al., 1983; Barlas et al., 1985; Damas et al., 1990), noxious heat (Rocha e Silva and Antonio, 1960) and pinching the skin (Ohara et al., 1988). Bradykinin injected into the paw produces nociceptive behaviour in the mouse (Shibata et al., 1986) and intra-arterial injection elicits vocalization in both the dog and cat (Guzman et al., 1962). In addition, application of bradykinin onto a blister base in the human elicits a pain response which can be blocked by selective antagonists (Horton, 1963; Whalley et al., 1987a).

Direct application of exogenous bradykinin stimulates polymodal nociceptors in muscle (Franz and Mense, 1975; Kumazawa and Mizumura, 1977; Foreman et al., 1979; Baker et al., 1980), viscera (Juan and Lembeck, 1974; Kumazawa and Mizumura, 1980; Haupt et al., 1983) and skin (Beck and Handwerker, 1974; Kanaka et al., 1985; Martin et al., 1987; Reeh, 1986) in several species. Neurones located within the spinal cord of the cat and monkey have also been shown to respond following close-arterial administration of bradykinin (Besson et al., 1972; Randic and Yu, 1976; Belcher, 1979; Foreman et al., 1979; Hong et al., 1979; Soja and Sinclair, 1980). In addition ventral root depolarizations have been elicited by peripheral administration of bradykinin onto the tail in an *in vitro* preparation of rat spinal cord with attached tail (Dray et al., 1988). The involvement of peripheral inflammatory mechanisms in the formalin response has been postulated following behavioural experiments demonstrating the inhibitory effect of non-steriodal anti-inflammatory compounds on the second phase of this response (Hunskaar and Hole, 1987; Shibata et al., 1989).

4.1.2 Bradykinin in the spinal cord

The presence of both bradykinin and binding sites for this peptide has been demonstrated in various regions of the brain (Corrêa et al., 1979; Perry and Snyder, 1984; Kariya et al., 1985). Although the overall level of bradykinin binding within the rat spinal cord is low it appears to be localized within the substantia gelatinosa region (Perry and Snyder, 1984; Steranka et al., 1988). This has lead to speculation regarding a possible role for this peptide within the spinal cord during nociceptive events.

Bradykinin appears to be excitatory within the spinal cord producing activation of dorsal horn neurones *in vivo* in the cat following microelectrophoretic application (Henry, 1976; Randic and Yu, 1976). Similarly spinal administration of bradykinin results in ventral root depolarizations *in vitro* in the neonatal rat (Dray et al., 1988). However behavioural studies in the rat have implicated a spinal *antinociceptive* role for bradykinin (Laneuville et al., 1989).

This study aimed to investigate the role of endogenous bradykinin during the inflammatory events occurring in the periphery during the formalin response. In addition selective antagonists were used to determine whether bradykinin was involved within the spinal cord during the formalin response.

4.2 Results

4.2.1 Desensitization of the receptive field to bradykinin inhibits the formalin response

Injection of the peptide bradykinin (10μg in 10μl) into the peripheral receptive field via a fixed needle under the skin resulted in an initial response of the dorsal horn neurones which was short lasting, being finished within 5 minutes. Repetitive administration of the same dose of bradykinin at 10 minute intervals resulted in a loss of response of the central neurones until there was little or no response at all by the 4th and 5th injections (Figure 4.1). Desensitization was a result of peripheral mechanisms since responses could always be elicited in the neurones by administration of the peptide into other areas of the receptive field.

This selective peripheral desensitization to bradykinin was utilized in order to investigate the role of the peptide in the periphery during the formalin response. Bradykinin (10μg in 10μl) was administered into the receptive field at 10 minute intervals over 50 minutes. The 5th administration of bradykinin resulted in a mean response of 7 % ± 3 (n=6) compared to the first injection response. Subsequent formalin administration into this area resulted in a response with a reduced first peak and a markedly reduced second peak (Figures 4.1 & 4.2). The second peak reduction (to 31% of control) did not quite reach statistical significance compared to controls (p=0.07, Mann-Whitney U-test) although the response was clearly reduced over the population of neurones. Interestingly the latency to arrival of the second peak was not altered despite the reduced size of the response. Control: 23.6 ± 5.6 minutes, n=8; Desensitized: 22.2 ± 2.6 minutes, n=6.

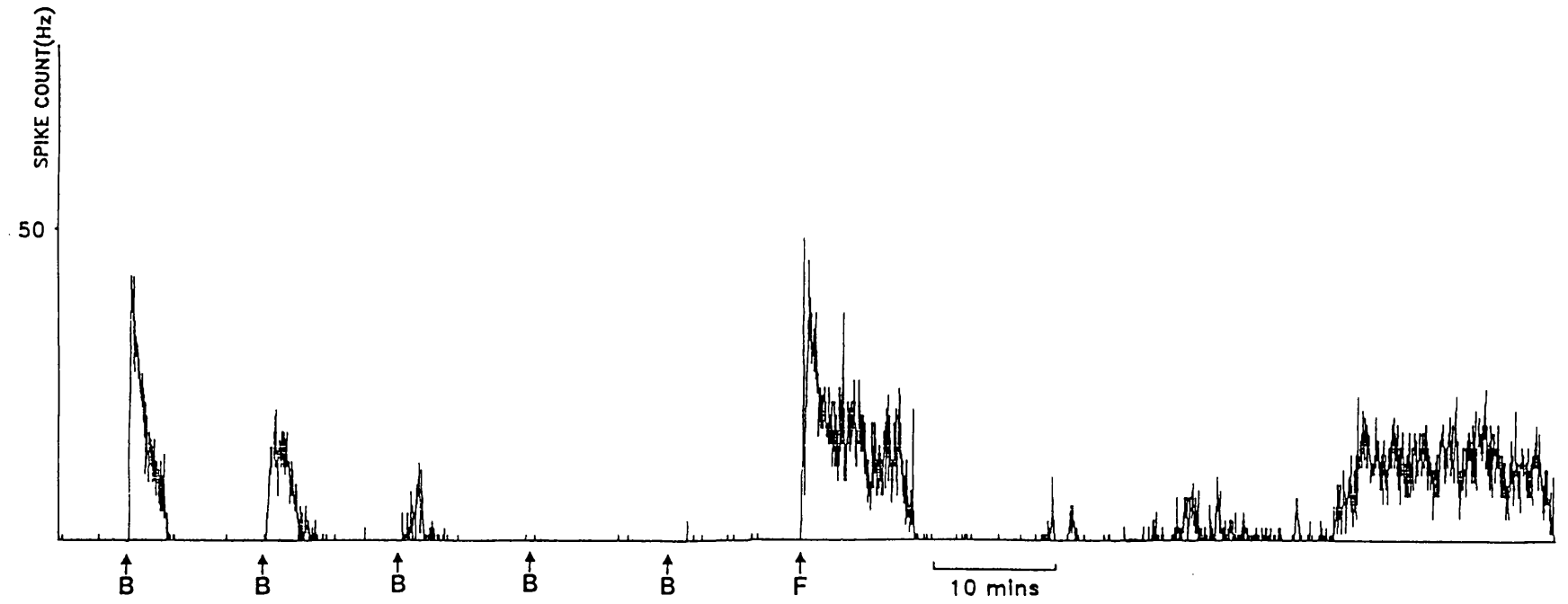

Figure 4.1 A ratemeter recording of the firing rate (Hz) of a single dorsal horn neurone. Repetitive injections of bradykinin (B) into the receptive field resulted in a rapid loss of the response to this constant peripheral stimulus. The subsequent administration of formalin (F) resulted in a response with a reduced second peak but an unchanged first peak.

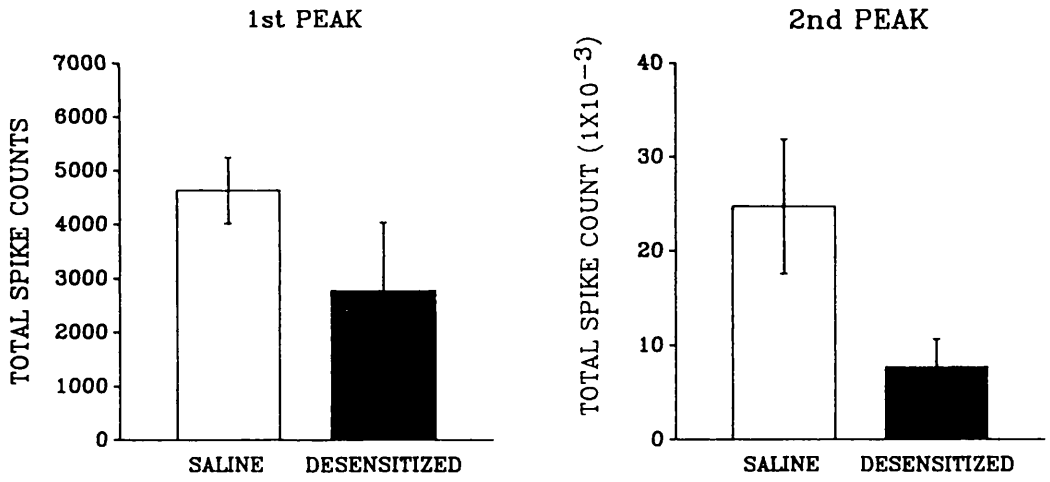

Figure 4.2 Prior desensitization of the peripheral receptive field to bradykinin reduced the second peak of the formalin response with a smaller reduction in the first phase of the response. n ranges from 6 to 8.

4.2.2 Peripheral (s.c.) administration of a bradykinin B1 receptor antagonist fails to inhibit the formalin response

The B1 receptor antagonist DesArg^9[Leu^8]bradykinin upon injection into the receptive area resulted in spontaneous firing of the dorsal horn neurones but this was not statistically different from that produced by saline alone. Injection of 50µl of saline produced a mean count of 1843 ± 502 (n=11) and mean duration of 2.9 ± 0.6 min whilst the top dose (200µg) of the B1 antagonist resulted in a mean count of 3329 ± 1014 (n=6) and mean duration of 4.2 ± 0.6 minutes.

The B1 receptor antagonist DesArg^9[Leu^8]bradykinin administered directly into the receptive field (in a volume of 50µl) 10 minutes prior to formalin did not inhibit either peak of the subsequent response. However, a dose related increase in both peaks of the response was observed. The top dose of 200µg caused a 72% increase in the size of the first peak and a significant increase of 136% (n=6; p=0.03) in the second peak (Figure 4.3).

The latency to the start of the second peak was unaltered by the B1 antagonist. The control group mean latency was 25.3 ± 4.1 minutes whilst for the 200µg DesArg^9[Leu^8]bradykinin group it was 23.2 ± 3.7 minutes.

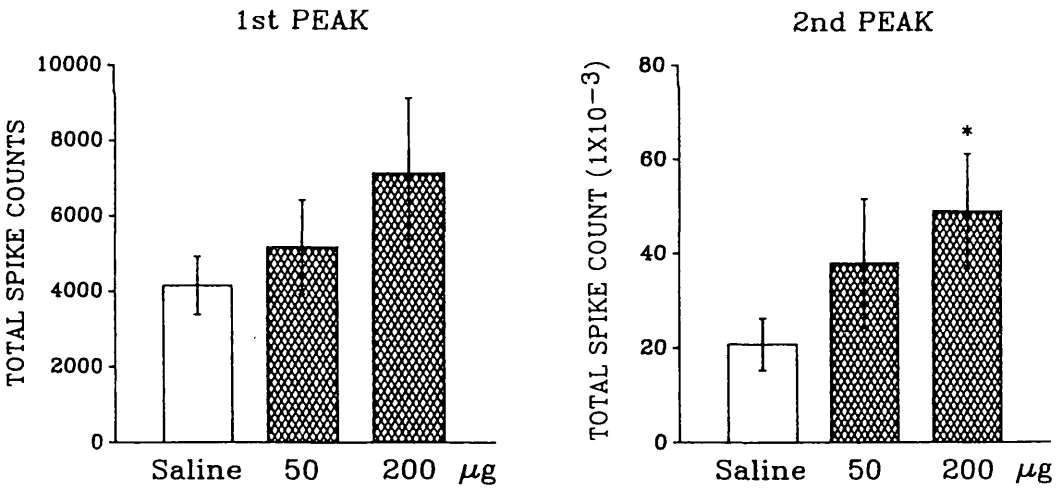

Figure 4.3 Peripheral administration of the B1 receptor antagonist DesArg^{9}[Leu^{8}]bradykinin directly into the receptive field 10 minutes prior to formalin increases both the first and second peaks of the response. n ranges from 6 to 12. *p=0.03, Mann-Whitney U-test.

4.2.3 Peripheral (s.c.) administration of a bradykinin B2 receptor antagonist inhibits the second peak of the formalin response

Injection of the bradykinin B2 receptor antagonist [Hyp^{3}Thi^{5,8}DePhe^7]bradykinin (B4162) directly into the peripheral receptive field in a volume of 50µl resulted in some spontaneous firing of the neurones. However, both the size and duration of the response were similar to the responses produced by saline alone (Saline: 1971 ± 414 counts, 3.1 ± 0.6 minutes, n=14; B4162: 1815 ± 383 counts, 3.6 ± 0.7, n=13).

Administration of B4162 directly into the peripheral receptive field (in a volume of 50µl) 10 minutes prior to formalin resulted inaninhibition of the second but not the first peak of the response. The highest dose administered (50µg in 50µl) inhibited the second peak by 61% (n=13; P=0.02) whilst producing only a 4% alteration in the first peak of the formalin response (Figure 4.4) The mean latency to arrival of the second peak was not significantly altered even at the highest dose of B4162: Control: 25.4 ± 3.2 minutes (n=16); 50µg B4162: 30.3 ± 4.2 minutes (n=13).

. B4162 is a peptide analogue of bradykinin and may therefore be rapidly degraded within the tissue following injection into the skin. Administration of the antagonist directly into the receptive field 10 minutes after the injection of formalin resulted in a small increase in the effectiveness of the compound (Figure 4.5). Whilst 25µg of B4162 had no effect on the second peak of the response when administered 10 minutes prior to formalin, as a posttreatment it produced an inhibition of 55% (n=5). The top dose of 50µg produced a similar inhibition as both a posttreatment and pretreatment with inhibitions of 51% and 61% respectively (n=5). The reductions in the second peak do not reach statistical significance, probably due to the large error limits present on the control group (Figure 4.5). The latency to arrival of the second peak was not significantly altered even with the top dose of B4162: Control: 27.6 ± 3.3 minutes (n=6); 50µg B4162: 22.6 ± 2.8 minutes (n=5).

It therefore appears that whilst some degradation of the antagonist may occur, as indicated by the posttreatment studies, this has only a small effect on the potency of the compound, only halving the dose required to maximally inhibit the response. The ability of B4162 to inhibit the second peak of the response appears to reach a maximum at around 50-60% inhibition. Thus the posttreatment study shows no increased inhibition with the top dose of 50μg, compared to the pretreatment experiments. In addition both the 25μg and 50μg doses produced a similar level of inhibition (55% and 51% respectively) in the posttreatment experiments.

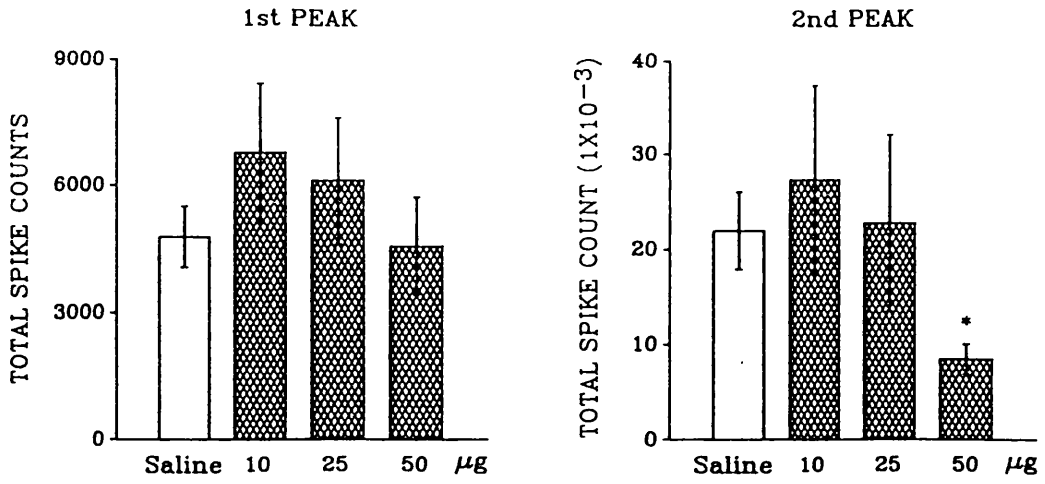

Figure 4.4 Peripheral administration of B4162 directly into the receptive field 10 minutes prior to formalin injection had no effect on the first peak of the response but significantly inhibited the second peak with the highest dose tried. n ranges from 7 to 16. *p=0.02, Mann-Whitney U-test.

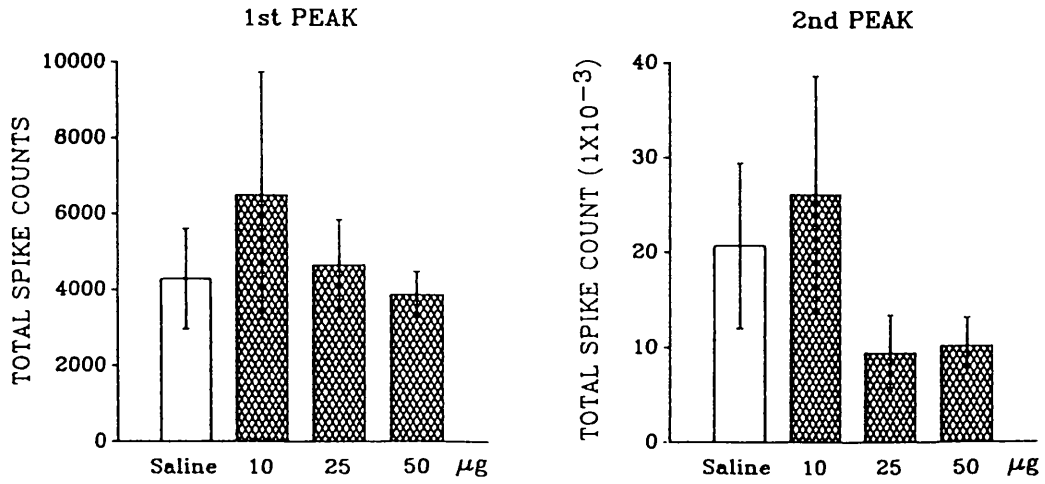

Figure 4.5 Peripheral administration of B4162 directly into the receptive field 10 minutes after formalin injection inhibited the second peak of the response with maximal inhibition being achieved with the 25μg dose. B4162 is not present during the first peak of the response. n ranges from 5 to 6.

4.2.4 Inability of a B2 receptor antagonist to inhibit electrically evoked A and C fibre responses

Administration of the top dose (50μg) of the B2 receptor antagonist B4162 directly into the receptive field (and site of electrical stimulation) did not inhibit the electrically evoked A and C fibre responses of the dorsal horn neurones. A small increase in the C fibre evoked response occurred, however this reached statistical significance at only one point, 30 minutes following the injection of B4162 where an increase of 24 ± 7% (n=5, $p < 0.05$) was observed

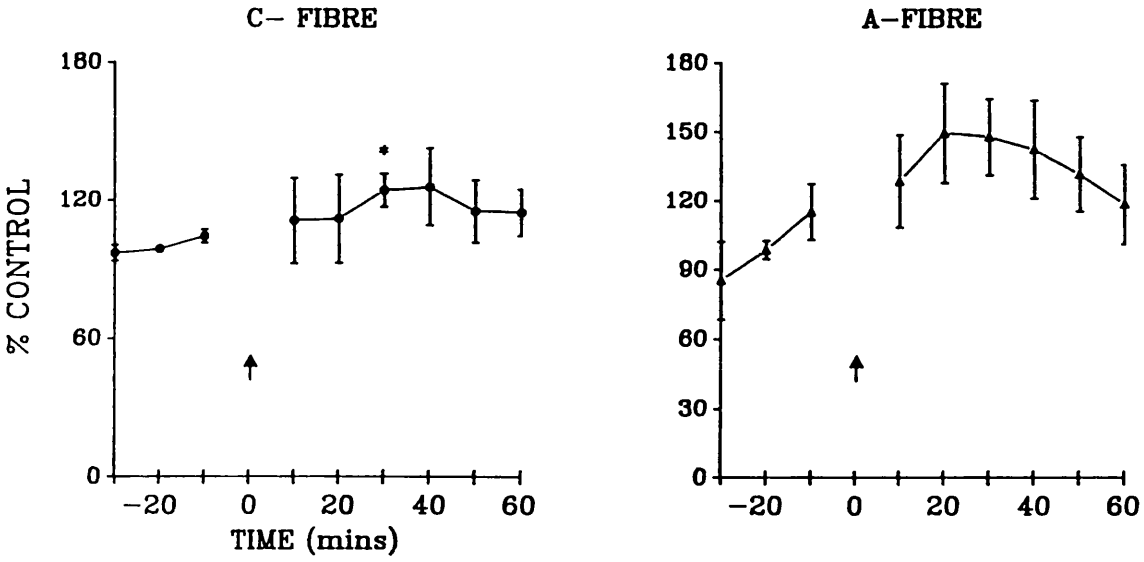

Figure 4.6 The effect of 50 μg B4162 on the electrically evoked A and C fibre responses when injected directly into the site of stimulation. n=5 for all points. *p<0.05, Student's t-test.

4.2.5 A B2 receptor antagonist inhibits responses evoked by exogenous bradykinin

The effects of the B2 receptor antagonist B4162 on the formalin response were presumed to result from antagonism at the bradykinin receptor. The ability of B4162 to inhibit the responses of the neurones to exogenously applied bradykinin was therefore determined.

Repetitive subcutaneous injection of 10μg bradykinin (in 10 μl) via a fixed needle, into the centre of the receptive field resulted in a rapid loss in the neuronal response to this stimulus (see 4.2.1). Subcutaneous administration of 50μl saline into the receptive field 10 minutes following the first administration of bradykinin and 10 minutes prior to the second administration (20 minutes have therefore elapsed between the first and second bradykinin responses) resulted in an increase in the size of the second and third bradykinin responses. This apparant sensitization was only transient with the fourth and fifth injections of bradykinin showing profound desensitization. If 50μg B4162 (in 50μl) was administered in place of saline the sensitization was blocked and all the subsequent responses to bradykinin were small (Figure 4.7).

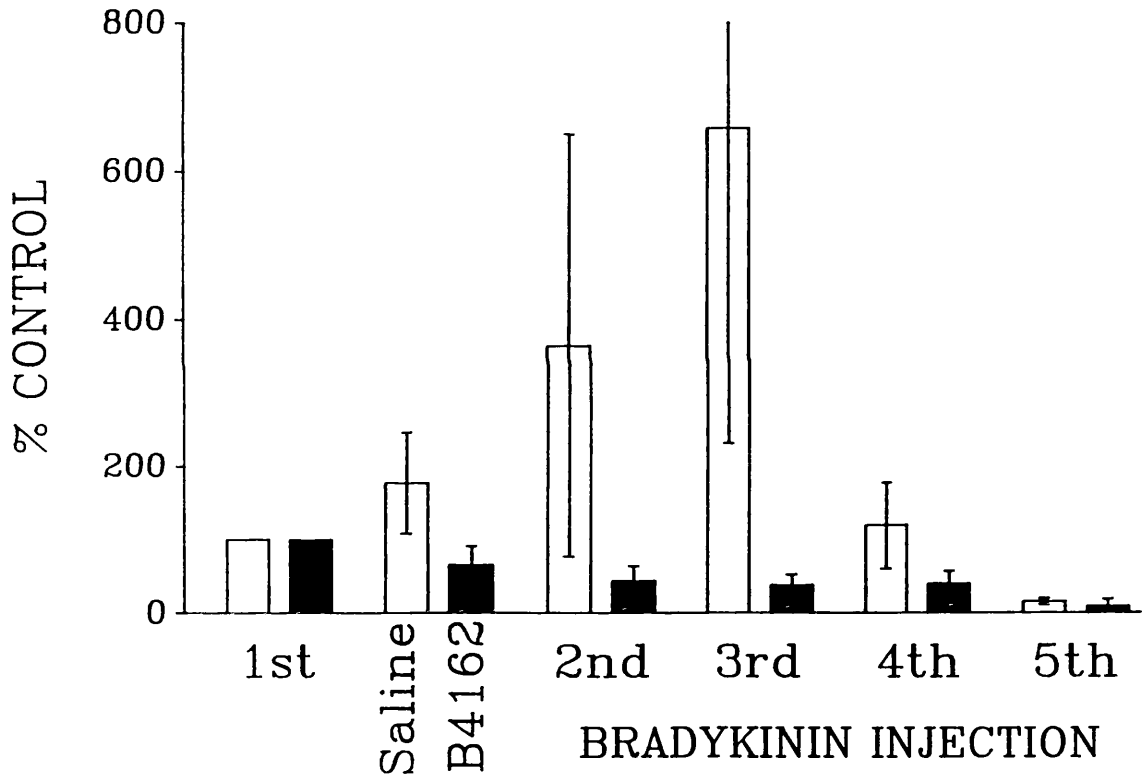

Figure 4.7 Inhibition of the responses of dorsal horn neurones to bradykinin by the B2 antagonist B4162. All results are expressed as a percentage of the first response to bradykinin (taken as 100%). Fifty µl of either saline or 1mg/ml B4162 were injected into the receptive field ten minutes after the first injection of bradykinin and ten minutes prior to the second injection. n ranges from 5 to 7.

4.2.6 The B2 receptor antagonist is unlikely to be an agonist

The effects of B4162 on formalin-induced and bradykinin-induced responses were presumed to result from antagonism at the bradykinin receptor. However it is possible that these effects may result from agonist induced desensitization of the bradykinin receptor. B4162 was examined for agonist properties in this system.

The administration of increasing doses of the B2 antagonist B4162 into the receptive field resulted in an increase in the size of the neuronal responses. However due to the small quantities of B4162 available it was not possible to increase the dose and retain the injection volume of 10μl. The increase in B4162 dose was therefore obtained by increasing the volume of injection of the 1mg/ml solution. Equivalent volumes of saline and B4162 elicited equivalent responses at each volume injected (Figure 4.8).

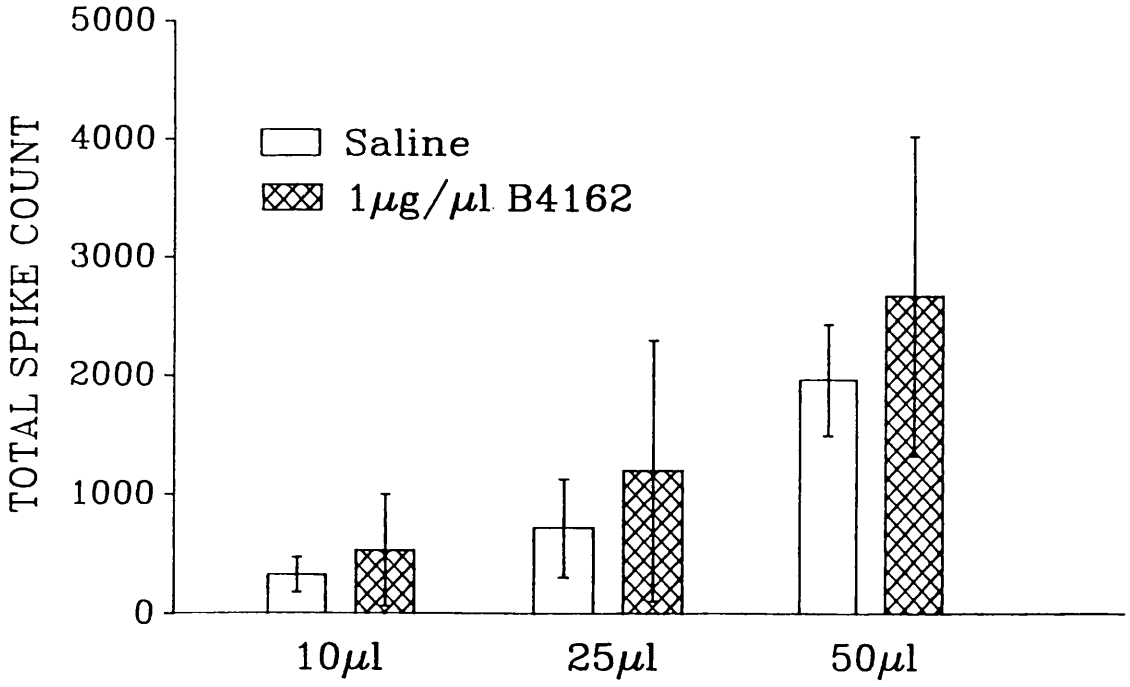

Figure 4.8 The dose-response curve for B4162 injected into the receptive field. The response for 1mg/ml B4162 was no greater than that observed with the same injection volume of saline. n ranges from 5 to 10.

4.2.7 Low dose 'desensitization' with the B2 receptor antagonist has no effect on the formalin response

Repetitive administration of either B4162 (5μg in 10μl) or saline (10μl) into the receptive field resulted in a loss of response to subsequent injections (Figure 4.9). The fifth injection of B4162 and saline produced responses only 43% and 14% the size of the first injection, respectively. The responses elicited by 5μg B4162 were significantly greater than those evoked by the same volume of saline at every administration (Figure 4.9).

Subsequent injection of formalin resulted in responses that were the same for both the saline and B4162 groups. Neither the first $\overset{nor}{\lambda}$ second peaks were changed (Figure 4.10) and the latency to arrival of the second peak was unaltered: Control: 27.5 ± 2.8 minutes (n=5); B4162: 19.5 ± 3.0 minutes (n=5). Thus 'desensitization' of the receptive field with the B2 receptor antagonist had no effect on the formalin response.

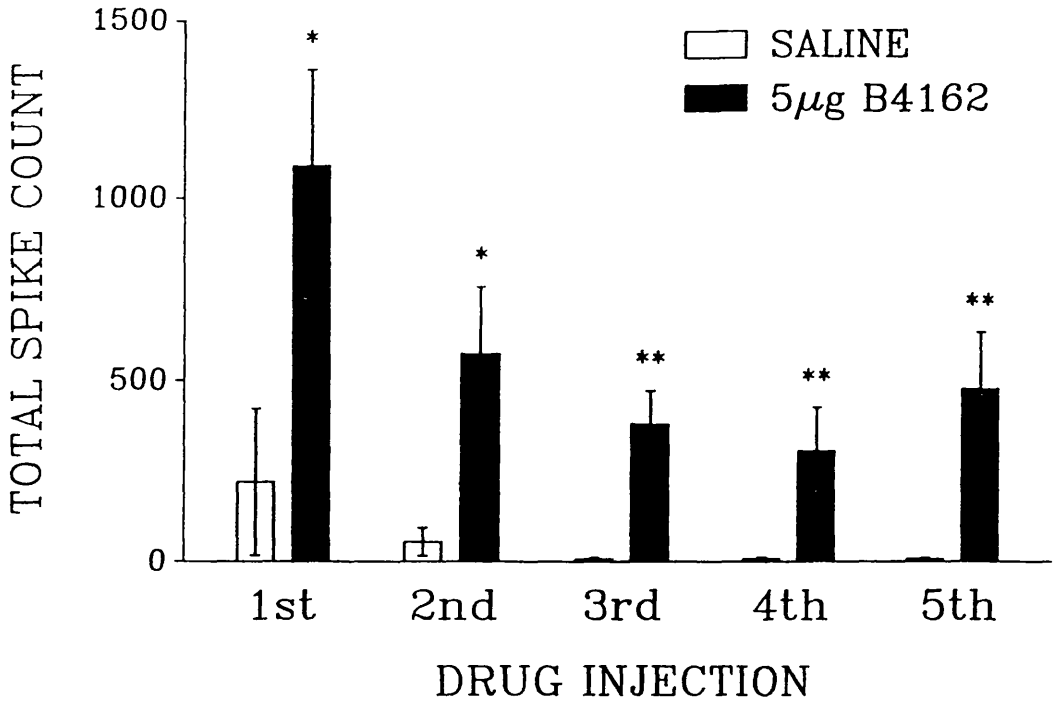

Figure 4.9 Repetitive administration of either B4162 (5μg in 10 μl) or saline (10μl) into the receptive field resulted in a loss in the size of the response of the dorsal horn neurones with successive injections. B4162 evoked greater responses than saline at all the injections. n=5 for all points. *p< 0.05 **p< 0.01; Mann-Whitney U-test.

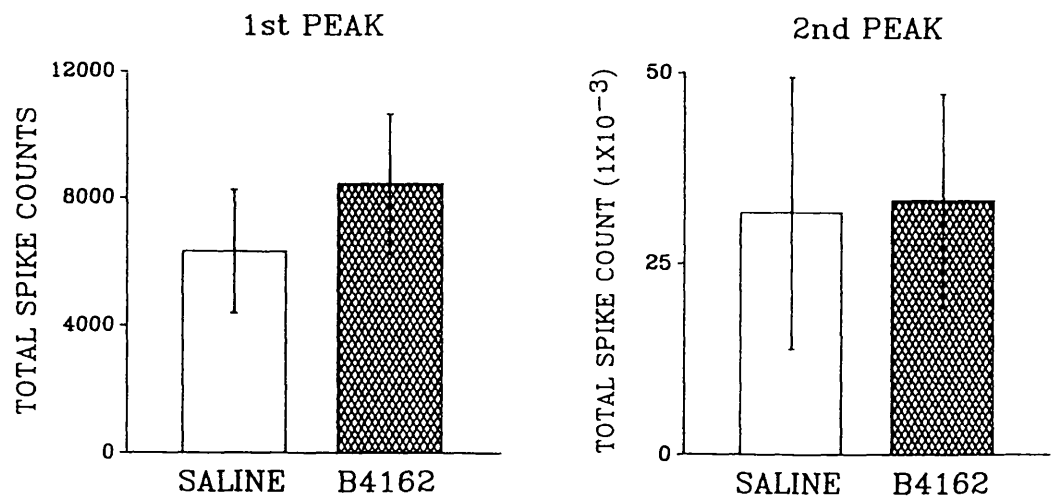

Figure 4.10 Following 'desensitization' of the receptive field with the B2 antagonist B4162 the subsequent injection of formalin resulted in a response that was no different from the control where saline was administered in place of B4162. n=5 at all points.

4.2.8 Intrathecal administration of a B1 antagonist inhibits the second peak of the formalin response

Intrathecal administration of the B1 receptor antagonist DesArg^9[Leu^8]Bradykinin as a 10 minute pretreatment had no effect on the first peak of the formalin response. However the top dose administered (200μg) produced a significant 63% (n=7; $p<0.05$) inhibition of the second peak (Figure 4.11). The latency to arrival of the second peak was not significantly altered by this treatment: Control: 25.3 ± 3.9 minutes (n=13); 200μg DesArg^9[Leu^8]Bradykinin: 30.3 ± 2.8 minutes (n=7).

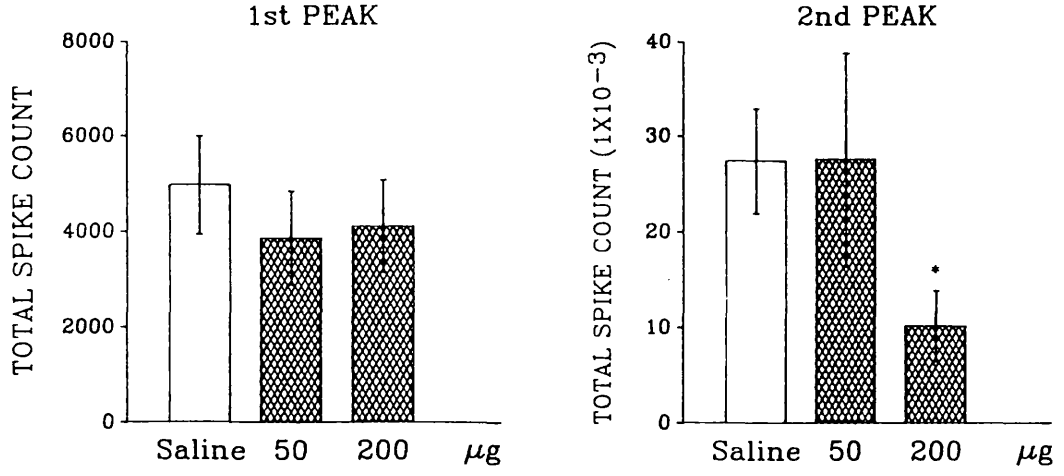

Figure 4.11 Intrathecal administration of the B1 receptor antagonist DesArg^9[Leu^8]Bradykinin 10 minutes prior to formalin had no effect on the first peak of the response but significantly inhibited the second peak at the highest dose applied. n ranges from 6 to 13. *$p< 0.05$, Mann-Whitney U-test.

4.2.9 Intrathecal administration of a B2 receptor antagonist has no effect on the formalin response

The B2 receptor antagonist B4162 administered intrathecally as a 10 minute pretreatment had no significant effect on either peak of the subsequent formalin response (Figure 4.12). The latency to arrival of the second peak was not significantly altered by the highest dose applied: control: 25.3 ± 3.9 minutes (n=13); 200μg B4162: 38.2 ± 7.8 minute (n=6).

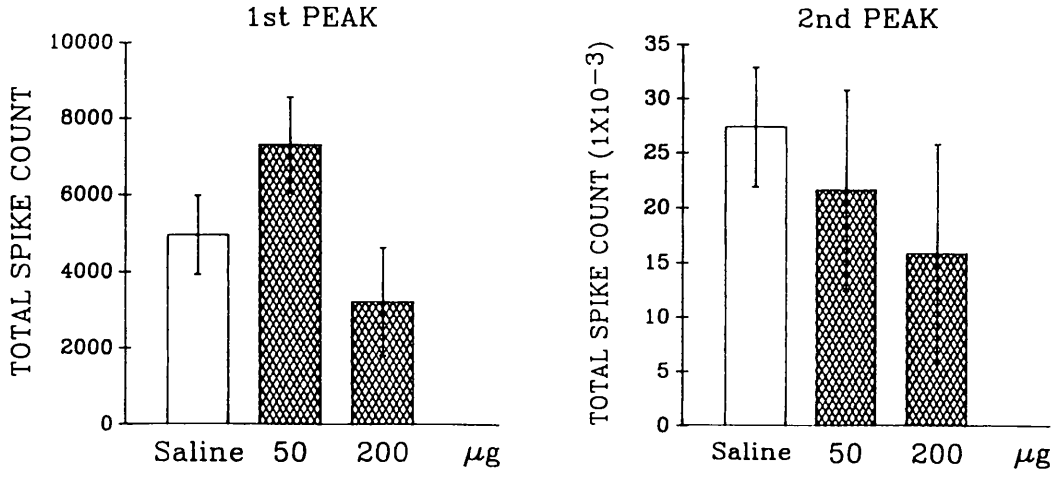

Figure 4.12 Intrathecal administration of the B2 antagonist B4162 as a 10 minute pretreatment had no significant effect on either phase of the formalin response at the doses tried. n ranges from 6 to 13.

4.3 Discussion

4.3.1 The role of bradykinin in the periphery during the formalin response

Repetitive injections of bradykinin into the receptive field resulted in a rapid tachyphylaxis in the response of the dorsal horn neurones. This loss of response is likely to result from peripheral mechanisms since neurones no longer responding to the peptide in one area of the receptive field responded normally to bradykinin injected into an adjacent, naive, area. This desensitization to bradykinin has been discussed in more detail in a previous chapter (see 3.3.4). Injection of formalin into the receptive field following desensitization with bradykinin resulted in a 69% reduction in the size of the second peak of the neuronal response to formalin and a 40% reduction in the first peak of the response. Thus the ability of the receptive field to respond to bradykinin appears to be important in the peripheral nociceptive events occurring during the formalin response, especially during the second peak.

The use of bradykinin analogues as selective antagonists at the bradykinin B1 and B2 receptors allowed the receptors involved in the formalin response to be determined. The selective B1 receptor antagonist DesArg^9[Leu^8]bradykinin (Regoli and Barabé, 1980) was unable to inhibit either phase of the formalin response and resulted instead in a significant increase in the size of the second peak. Enhanced responses to bradykinin have been observed with this B1 antagonist in visceral afferent fibre recordings in the dog and may result from agonist actions of the compound (Mizumura et al., 1990).

The failure of DesArg^9[Leu^8]bradykinin to inhibit the formalin response is not in agreement with a recent behavioural study in mice (Shibata et al., 1989) in which both the first and second peaks of the formalin response were inhibited by a low dose (150pmol, equivalent to 0.2μg) of the B1 antagonist injected into the plantar region prior to formalin. The failure of the study by Shibata and colleagues to investigate the effects of a B2 antagonist makes conclusions regarding the receptor subtype involved in the

inhibition tentative. The dose of B1 antagonist used in their behavioural model is 250 times lower than the smallest dose administered in this electrophysiological study. As some bradykinin antagonists possess agonist activity at high doses (Regoli et al., 1990; Griesbacher and Lembeck, 1987; Schachter et al., 1987; Brass et al., 1988), it is possible that lower doses of the B1 antagonist may have been inhibitory in these formalin experiments. However in peripheral recordings of dog visceral afferent fibres lower doses of the B1 antagonist were still unable to inhibit the activity induced by bradykinin (Mizumura et al., 1990). In addition the ventral root depolarizations induced by peripheral administration of bradykinin in an *in vitro* rat tail-cord preparation were unaltered by either the B1 agonist or antagonist (Dray et al., 1988).

By contrast peripheral injection of the putative B2 receptor antagonist D-Arg-[Hyp^{3}Thi^{5,8}DePhe^{7}]bradykinin (B4162) (Schachter et al., 1987; Braas et al., 1988; Regoli et al., 1990) prior to formalin inhibited the second peak of the response without altering the first peak. The potency of this compound was slightly increased when it was administered after formalin suggesting that this peptide analogue may undergo some degradation following injection into the skin. The maximum inhibition of the second peak observed with either desensitization or B4162 was always about 50-60% and complete inhibition was never observed. This residual activity may represent the actions of peripheral mediators other than bradykinin. Interestingly Shibata et al. (1989) were also unable to completely inhibit either the first or second peak of the formalin response with increasing doses of the B1 antagonist.

The selective inhibition of the formalin response second peak by B4162 indicates its effects are not the result of a local anaesthetic action since it leaves the first peak of the response unaltered. This is confirmed by the inability of the antagonist to inhibit the electrically evoked A and C fibre responses. B2 receptor antagonists have been shown to selectively inhibit bradykinin induced contraction of the ileum *in vitro* (Vavrek and Stewart, 1985; Griesbacher and Lembeck, 1987) and vascular permeability *in vivo* (Whalley et al., 1987b) whilst having no influence on the responses produced by angiotensin II, substance P, histamine and acetylcholine. B4162 inhibition of the bradykinin induced activity indicated that in this system the antagonist is capable of

inhibiting responses to the peptide. However, the possibility that B4162 may also inhibit responses elicited by other mediators was not investigated in this study.

In addition to its antagonist activity, high doses of B4162 have been shown to exert agonist actions *in vivo* causing increased vascular permeability in rabbit and rat skin (Griesbacher and Lembeck, 1987; Schachter et al., 1987; Whalley et al., 1987b) and hyperalgesia when injected into the rat paw (Steranka et al., 1988). These agonist actions in the rat were observed with doses of B4162 that ranged from 10 to 20 nmol injected into the skin. This is comparable to the doses of B4162 administered in this study (10 - 50 μg which is equivalent to 5 - 26 nmol). Agonist activity has also been demonstrated in various *in vitro* systems (Steranka et al., 1988; Regoli et al., 1990) although other *in vitro* studies have failed to demonstrate any agonist actions for this compound (Braas et al., 1988). However 'desensitization' by B4162 does not appear to be the basis of the inhibition of the formalin response observed.

It has been assumed that the actions of these antagonists arise from their ability to block the bradykinin B2 receptor. However it is possible, especially at the doses used in this study, that the inhibitions produced result from desensitization of the receptive field by the antagonist acting as an agonist. However this explanation appears unlikely since direct administration of the antagonist into the receptive field was unable to produce firing that was in excess of that produced by saline. In addition, tachyphylaxis induced by repetitive injections of the B2 antagonist was unable to influence the subsequent formalin response. The cumulative dose of B4162 administered into the paw would have been 25μg, a dose that was ineffective when administered as a single pretreatment injection. Since tachyphylaxis induced by bradykinin inhibits the second phase of the response it appears the inhibitory actions of B4162 are probably not the result of agonist actions. In addition it is unlikely that the B2 antagonist is inhibiting the formation of bradykinin from its plasma precursor (Costello and Hargreaves, 1989) confirming that its actions probably result from receptor antagonism.

Behavioural studies in rats have demonstrated the effectiveness of B2 receptor antagonists at inhibiting the hindpaw hyperalgesia elicited by bradykinin (Steranka et al., 1988), urate crystals (Steranka et al., 1988) and carrageenan (Costello and Hargreaves,

1989). In addition acetic acid induced writhing in mice is also inhibited by B2 antagonists (Steranka et al., 1987). The effective doses of B4162 administered in the hyperalgesia studies (2 - 20 nmol) are very similar to those inhibiting the second peak of the formalin response (26 nmol) Unfortunately none of these behavioural studies have investigated the effect of a B1 antagonist on hyperalgesia thus making classification of the receptor subtype involved tentative. A human study has confirmed the antinociceptive activity of bradykinin antagonists; B2 antagonists blocked the pain response elicited by bradykinin administered onto a blister base whilst the B1 antagonist was ineffective. Additionally a B1 agonist was unable to elicit pain when applied directly onto the blister base (Whalley et al., 1987a).

The mechanism by which the bradykinin antagonists inhibit the formalin response is as yet undetermined. They may be blocking a direct action of bradykinin on the afferent fibres. Alternatively they may be blocking the local vascular actions of the peptide since the same dose range of B4162 blocks bradykinin induced plasma extravasation in the rabbit (Griesbacher and Lembeck, 1987; Schachter et al., 1987; Whalley et al., 1987b) and reduces hyperalgesia in the rat (Steranka et al., 1988). However bradykinin induced plasma extravasation in the rat was not blocked by B2 antagonists (Whalley et al., 1987b) although the oedema produced by carrageenan was reduced by a B2 antagonist in the dose range that inhibited the hyperalgesia produced by the same stimulus (Costello and Hargreaves, 1989). It is therefore possible that the antagonists may have a duel mode of action: blocking direct afferent stimulation by bradykinin and reducing the plasma extravasation induced by the peptide during inflammatory events. This could additionally prevent inflammatory and nociceptive mediators from arriving at the site of insult via the plasma.

The inability of the B2 antagonist to completely inhibit the second peak of the formalin response implicates the involvement of mediators in addition to bradykinin. The presence of prostaglandins in the exudate elicited by carrageenan (Willis, 1969; Ohuchi et al., 1976; Barbieri et al., 1977; Higgs et al., 1983) indicates a potential role for these cyclo-oxygenase products (Samuelsson et al., 1978) in peripheral

inflammatory events. Prostaglandins have been shown to potentiate bradykinin induced activity in afferent fibres whilst having no effect by themselves (Juan and Lembeck, 1974; Chahl and Iggo, 1977; Mense, 1981; Mizumura et al., 1987; Kirchoff et al., 1990). In addition bradykinin itself can release prostaglandins (Lembeck et al., 1976; Juan, 1977) and this can be blocked by B2 antagonists (Griesbacher and Lembeck, 1987). Thus a loop may exist by which the receptor that mediates the activation of afferent fibres could also result in the release of sensitizers that potentiate the responses of the nerves to bradykinin.

Behavioural studies in mice have implicated a role for prostaglandins in the formalin response; inhibitors of the prostaglandin forming enzyme cyclo-oxygenase reduced nociceptive behaviour during the second peak with mixed effects on the first peak (Hunskaar and Hole, 1987; Shibata et al., 1989). It is likely therefore that bradykinin and prostaglandins act together during the second peak of the formalin response producing sensitization and activation of the afferent endings. Substance P also potentiates the nociceptive activity of bradykinin following co-administration into the paws of mice (Shibata et al., 1986) and this peptide has also been proposed as a peripheral mediator during the formalin response, along with histamine and 5HT (Shibata et al., 1989). Whilst bradykinin appears to be a peripheral mediator during the formalin response it is likely that it is only one among many interacting mediators that are generated during inflammatory states. Despite this, bradykinin could be of considerable importance since blocking its actions could also prevent the release of other sensitizing molecules in addition to preventing the stimulation of afferent neurones.

4.3.2 The role of bradykinin in the spinal cord during the formalin response

Previous studies investigating the role of bradykinin within the spinal cord have yielded conflicting results. *In vivo* and *in vitro* studies have indicated that the peptide is excitatory, activating dorsal horn neurones in the cat (Henry, 1976; Randic and Yu,

1976) and causing ventral root depolarizations in the neonatal rat (Dray et al., 1988). However behavioural studies have demonstrated antinociceptive activity with intrathecal bradykinin in the tail flick test (Laneuville and Couture, 1987; Laneuville et al., 1989). Despite this apparent conflict in results both the *in vitro* neonatal study and the behavioural study agree the effects of bradykinin are mediated via the B2 receptor since both a B1 agonist and a B1 antagonist are ineffective.

By contrast, in this present study intrathecal administration of a B2 receptor antagonist had no effect on the formalin response even at very high doses. However the highest dose of the B1 antagonist selectively inhibited the second peak of the response. The inhibition produced by the B1 antagonist may result from non-specific actions since the doses used (200μg = 154 nmol) are about 15 times higher than those shown to be ineffective against intrathecal bradykinin in a behavioural study (10.3 nmol) (Laneuville and Couture, 1987). It is possible that the prolonged nature of the formalin stimulus may result in the emergence of B1 receptors within the spinal cord since the appearance of B1 receptors at the site of stimulation following noxious events has been observed *in vivo* (see Regoli and Barabé, 1980).

The synthesis of new B1 receptors has been postulated to explain increases, with time, in the sensitivity of peripheral tissues *in vitro* to B1 agonists and antagonists (Regoli and Barabé, 1980). Bradykinin binding within the rat spinal cord appears localized to the substantia gelatinosa region and to be of the B2 receptor type (Steranka et al., 1988). However changes in the levels of bradykinin receptors within the spinal cord have not been investigated following prolonged noxious stimulation and it is possible that synthesis of B1 receptors may occur during prolonged noxious events. The time course for the emergence of peripheral B1 receptors *in vivo* appears to be in the order of hours (see Regoli and Barabé, 1980); which is probably too long to explain the selective inhibition of the second peak of the formalin response by the B1 antagonist.

Intrathecal administration of a B2 receptor antagonist was unable to inhibit the formalin response confirming previous observations. Ventral root depolarizations elicited by intrathecally administered bradykinin *in vitro* could be blocked by a B2 receptor antagonist (Dunn and Rang, 1990). However neither B1 nor B2 antagonists

administered intrathecally could reduce the ventral root depolarizations elicited by peripheral noxious heat applied to an attached tail *in vitro* (Dray et al., 1988). Similarly whilst the antinociceptive effects of bradykinin in the tail flick test could be blocked by a B2 antagonist, neither a B1 nor a B2 antagonist was able to influence the latency to tail flick by themselves (Laneuville and Couture, 1987). So whilst the antagonists appear able to reduce the effects of exogenous bradykinin the role of endogenous receptors during 'natural' stimuli may be questionable.

Whilst the effects of intrathecal bradykinin appear to be contradictory, exciting dorsal horn neurones and yet producing antinociception in behavioural tests, they can be rationalized. Prior to any antinociceptive effects in the tail flick study, nociceptive behaviour such as squeaking, urinating and efforts to escape was initially observed following intrathecal administration of bradykinin (Laneuville and Couture, 1987). Bradykinin has been shown to elicit depolarizations in dorsal roots in the spinal cord slice (Dunn and Rang, 1990) and intrathecal bradykinin produces peripheral vasodilatation which is thought to occur via the stimulation of C fibre terminals and therefore ascending pathways (Jacques and Couture, 1990). It is possible that the antinociceptive effects observed *in vivo* with intrathecal bradykinin result from the stimulation of ascending pathways, leading to the activation of descending noxious inhibitory controls (DNIC). DNIC can be activated following the stimulation of ascending nociceptive pathways and they act to inhibit convergent dorsal horn neurones in various segments of the spinal cord. In behavioural studies noxious stimulation of one body region results in reduced response (i.e. antinociception) to noxious stimulation of another body region and this is thought to occur via the stimulation of DNIC (Le Bars and Villanueva, 1988). These descending inhibitory influences on the spinal cord are believed to be mediated via the release of noradrenaline and 5HT within the spinal cord (Evans, 1989). Interestingly intrathecal administration of an alpha 2 adrenoceptor antagonist inhibits the bradykinin elicited antinociception in the tail flick test, implying that activation of these descending inhibitions may occur (Laneuville et al., 1989). Thus the antinociceptive effects of bradykinin could simply result from the activation of

nociceptive pathways within the spinal cord which in turn produce descending inhibitions.

Thus whilst bradykinin has been implicated in mediating nociceptive events both within the periphery and within the spinal cord, evidence only exists to support a physiological role during peripheral events. Bradykinin may be involved in spinal mechanisms but at present no physiological role for this peptide has been demonstrated in nociception.

CHAPTER 5:

THE ROLE OF EXCITATORY AMINO ACIDS WITHIN THE SPINAL CORD DURING THE FORMALIN RESPONSE

5.1 Introduction

The excitatory amino acids glutamate and aspartate, and the peptide substance P have all been proposed as neurotransmitters within the spinal cord since they are present both in afferent fibres and within the dorsal horn (see 1.4.2). That dorsal horn neurones are excited by iontophoretic application of glutamate and aspartate *in vivo* is well established in the cat and rat (Curtis et al., 1959, 1960; Biscoe et al., 1976) and has been demonstrated in the mouse also (Biscoe et al., 1977). However research into the transmission of nociceptive information within the spinal cord has, until recently, focused on the role of substance P in these events. The development of selective antagonists at excitatory amino acid receptors during the 1980's has recently stimulated interest in the role of glutamate (and aspartate) in nociceptive events within the spinal cord. It was originally concluded by Curtis and colleagues in 1959 that "glutamic and aspartic acids have a non-specific excitatory action upon certain spinal neurones". However, the evidence in favour of a transmitter role for glutamate within the spinal cord has continued to accumulate.

Glutamate levels in the dorsal root, dorsal root ganglion and dorsal grey matter are higher than in the ventral regions in both the cat and rat (Graham et al., 1967; Duggan and Johnston, 1970; Roberts et al., 1973). In addition an uptake system for a glutamate precursor (glutamine), and a glutamate synthesizing enzyme (glutaminase) are both present in rat dorsal root ganglion (Duce and Keen, 1983; Cangro et al., 1985; Battaglia and Rustioni, 1988). The presence of glutamate, and aspartate, in afferent fibres and terminals has now been demonstrated in rats, cats and monkeys (DeBiasi and Rustioni, 1988; Miller et al., 1988; Westlund et al., 1989; Maxwell et al., 1990) and these amino acids may also be present within interneurones in the dorsal horn (Davidoff et al., 1967).

The excitatory amino acid receptors to which glutamate and aspartate bind have been divided into three main types: the N-methyl-D-aspartate (NMDA) receptor and two non-NMDA receptors; the kainate and AMPA receptors (formerly termed the

quisqualate, Quis, receptor) (see Watkins et al., 1990). Antagonists have been developed that act non-selectively at these receptor subtypes (e.g. γ-D-glutamyl glycine, DGG; Davies and Watkins, 1981) and selectively at the NMDA receptor (e.g. 5-DL-amino phosphonovaleric acid, AP5; Davies and Watkins, 1982) and this allows investigation into the involvement of these receptors within the spinal cord. A summary of the antagonists used in this study and the receptors at which they act is presented in Table 5.1. Studies in the rat have demonstrated glutamate binding localized to the substantia gelatinosa region of the dorsal horn (Greenamyre et al., 1984). Further work with selective agonists in both the rat and human has shown the presence of NMDA, Kainate and AMPA receptors within this same region (Monaghan and Cotman, 1982, 1985; Jansen et al., 1990).

NON-NMDA	NMDA
DGG	DGG
	AP5
	KETAMINE
	MK801

TABLE 5.1 The excitatory amino acid antagonists used in this study and the receptors they act at. Abbreviations used: NMDA (N-methyl-D-aspartate), DGG (γ-D-glutamyl glycine), AP5 (5-DL-amino phosphonovaleric acid), MK801((+)-5-methyl-10,11-dihydro-5H-dibenzo[a.d]cyclo-hepten-5,10-imine maleate)(See Collingridge and Lester, 1989).

For many years a neurotransmitter role for glutamate within the mammalian spinal cord has been proposed based on the excitatory actions of this amino acid on spinal cord neurones and the selective blockade of these actions with receptor antagonists (see Watkins and Evans, 1981). Unfortunately the early *in vivo* studies did not determine

the responses of these neurones to peripheral stimulation of the receptive field. Thus whilst it became clear that dorsal horn neurones responded to glutamate and aspartate and this could be blocked with antagonists, the role of these amino acids in nociceptive events within the dorsal horn was unknown. However with the development of selective antagonists, the role of excitatory amino acids in the spinal mediation of nociception has more recently become a focus of investigation (see Dickenson, 1990 and Headley and Grillner, 1990).

Particular interest has focused on a possible neurotransmitter role for glutamate in central hypersensitivity events within the spinal cord; following a peripheral thermal injury enhanced flexor reflex responses and expanded receptive fields are observed which appear to result from central mechanisms (Woolf, 1983). Whilst the sensitization of peripheral nociceptors has been clearly demonstrated (see Besson and Chaouch, 1987) there is accumulating evidence indicating that hypersensitivity events can also be rapidly elicited within the spinal cord (Woolf, 1983; Wall, 1984; Cook et al., 1986; Wall et al., 1988; Hylden et al., 1989). Glutamate has been implicated in central plasticity events in other regions of the central nervous system such as the development of long term potentiation (LTP) within the hippocampus. In addition the role of the NMDA subtype of receptor appears to be pivotal in the development of the enhanced synaptic transmission observed in LTP since it can be blocked by selective NMDA antagonists (see Bliss and Lynch, 1988). Dorsal horn neurones often respond to repetitive electrical stimulation of their receptive fields with a sequential increase in firing and this augmented response following constant peripheral stimulation has been termed 'wind up' (Mendell, 1966). This phenomenonis dependent on the frequency of the peripheral stimulation, being observed at 0.5 Hz or greater. In addition the 'wind up' of dorsal horn neurones is probably mediated by C fibres input since Aß fibre stimulation does not induce 'wind up' even at stimulation intensities and frequencies that elicit 'wind up' after C fibre stimulation (Dickenson and Sullivan, 1987b). Whilst opioids are less effective at inhibiting 'wind up' compared to constant responses, the NMDA antagonists AP5 and ketamine reduce this enhanced response of the neurones (Davies and Lodge,

1987; Dickenson and Sullivan, 1986,1987b). Thus excitatory amino acid receptors and in particular the NMDA receptor, appear to be involved in the amplification of nociceptive events in the spinal cord.

This study aimed to determine whether the excitatory amino acid system within the spinal cord was involved in the prolonged activity elicited in the dorsal horn neurones by peripheral administration of formalin. Using the selective NMDA antagonists AP5, ketamine (Anis et al., 1983) and MK801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a.d]cyclo-hepten-5,10-imine maleate; Wong et al., 1986) in addition to the non-selective NMDA and non-NMDA antagonist DGG, the role of the NMDA receptor within the spinal cord during the prolonged firing produced by formalin was investigated.

In addition to the excitatory amino acid receptors present within the dorsal horn, axonally transported kainate, NMDA and AMPA receptors have been demonstrated within the afferent nerve itself (Lewis et al., 1987). Kainate depolarized dorsal roots and dorsal root ganglion cells from immature rats *in vitro* (Agrawal and Evans, 1986; Evans et al., 1987; Huettner, 1990) whilst dorsal root ganglion cells from adult rats appear to be more selective for NMDA with kainate being relatively ineffective (Lovinger and Weight, 1988). The axonal transport of glutamate receptors towards the peripheral, as well as central, terminals of the afferent nerve raised the possible involvement of these receptors in peripheral nociceptive events. The non-selective excitatory amino acid receptor antagonist DGG was used to investigate the role of these receptors during the formalin response.

5.2 Results

5.2.1 Inhibition of the electrically evoked A and C fibre response by intrathecal DGG

The non-selective excitatory amino acid receptor antagonist DGG rapidly reduced the electrically evoked C and A fibre responses of the neurones (Figure 5.1). The A fibre response appeared more sensitive to inhibition by DGG with the lowest dose of 100μg reducing the A fibre evoked response by 24% ($p \leq 0.025$) within 20 minutes whilst having no effect on the C fibre evoked response (100% of control). 250 μg DGG produced an inhibition of the A fibre evoked count of 34% although this did not reach statistical significance, but no change in the the C fibre evoked response (110% of control). The top dose of 1000μg reduced both the A and C fibre evoked responses to 43% ± 8 ($p \leq 0.005$) and 32% ± 22 ($p \leq 0.05$) of control values respectively (Figure 5.1).

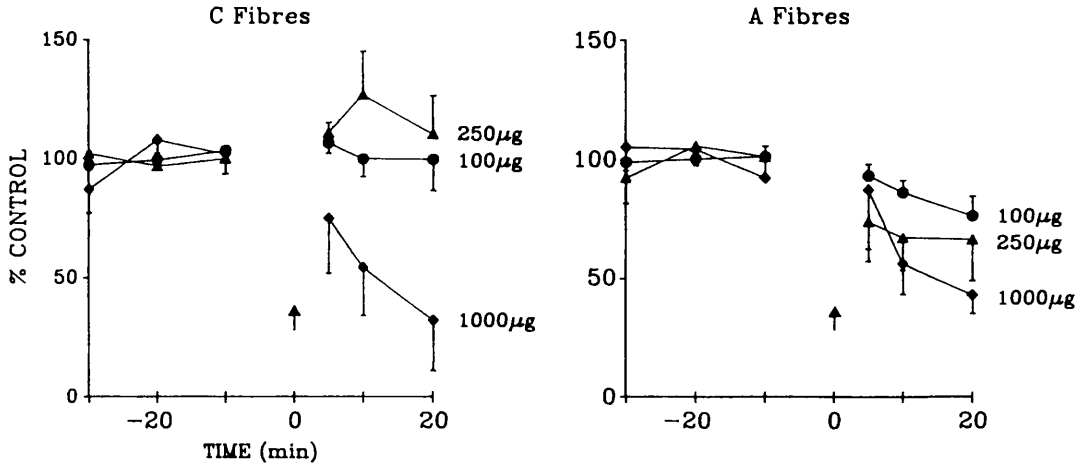

Figure 5.1 Intrathecal administration of DGG (as indicated by the arrow) inhibits the electrically evoked A and C fibre responses of the neurones. n ranges from 4 to 6.

5.2.2 Inhibition of the formalin response by intrathecal DGG

Intrathecal administration of DGG 20 minutes prior to the injection of formalin resulted in a dose-related reduction in both the first and second peaks of the subsequent neuronal response (Figure 5.2). Both peaks were inhibited to a similar extent with the top dose of 1000μg producing inhibitions, compared to controls, of 99.8% (p=0.004) and 98.4% (p=0.004) for the first and second peaks respectively.

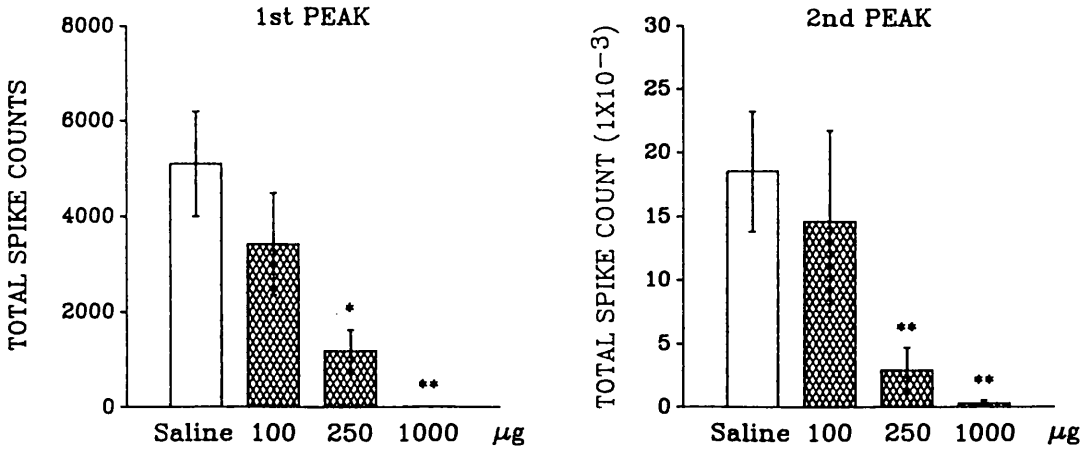

Figure 5.2 Intrathecal administration of DGG (in 50μl) reduces the size of both the first and second peaks of the formalin response compared to controls. n ranges from 4 to 8. * p=0.008 ** p=0.004, Mann-Whitney U-test.

5.2.3 Loss of effectiveness of intrathecal DGG when administered after the injection of formalin

DGG (1000μg) administered intrathecally 3 minutes after the injection of formalin resulted in a small 32% reduction in the second peak compared to controls but this was not statistically significant (Figure 5.3). However, the first peak of the response, which is ongoing at the time of administration, was significantly increased: Control counts: 2557 ± 780, n=4; DGG: 5321 ± 549, n=4; p=0.02.

DGG (1000μg) administered intrathecally during the neuronal activity of the second peak resulted in a 78% reduction in the spike count over the following 30 minutes compared to saline administration (Figure 5.3). This reduction is significant when compared to controls (p=0.03) but is less than the 98% inhibition observed with the same dose as a pretreatment (Figure 5.3).

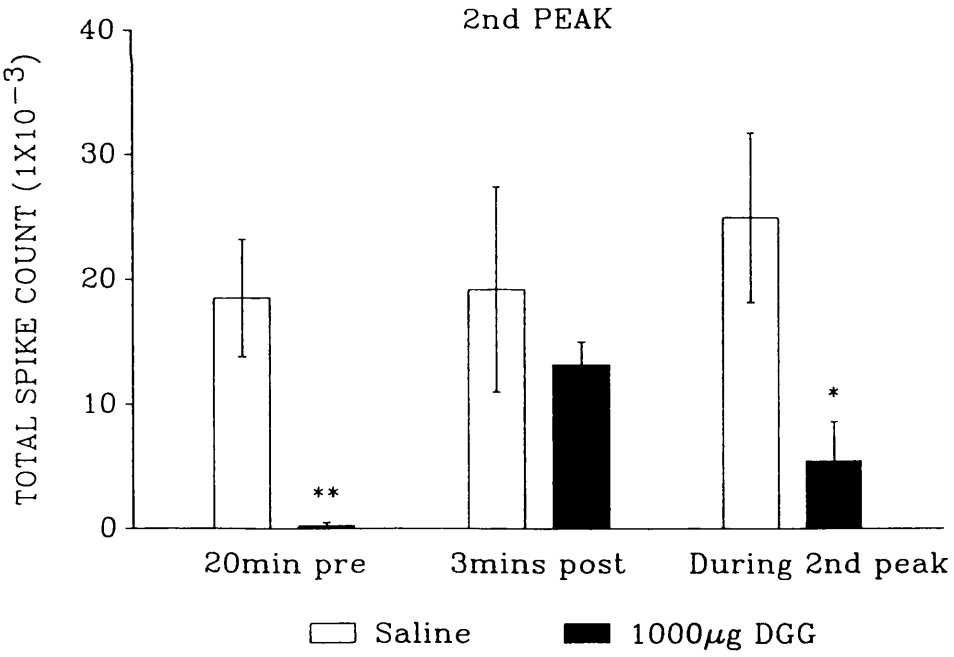

Figure 5.3 Intrathecal DGG (1000μg) administered at varying times relative to the administration of formalin. DGG was given as a 20 minute pretreatment (see previous figure), 3 minutes after formalin injection or during the second peak firing. n ranges from 4 to 6. * p=0.03 ** p=0.004 compared to the control for each group, Mann-Whitney U-test.

5.2.4 Effect of intrathecal AP5 on the electrically evoked A and C fibre responses

The selective NMDA receptor antagonist AP5 was applied intrathecally 40 minutes prior to formalin. There was no reduction in the electrically evoked C fibre response with either the 250μg or 500μg dose. There was a significant reduction in the A fibre response with the top dose of AP5 and a maximum inhibition of 26% ($p<0.03$) was obtained (Figure 5.4).

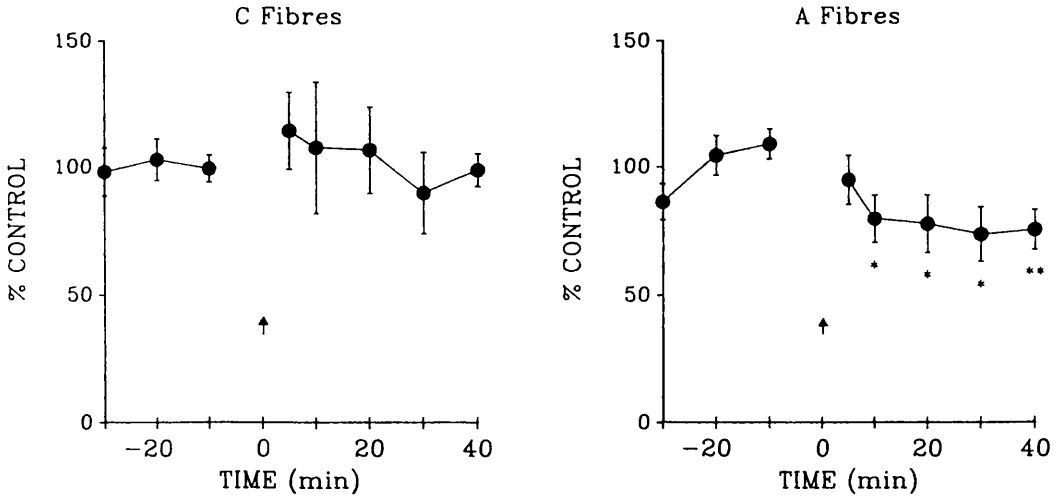

Figure 5.4 Intrathecal administration of 500μg AP5 (as shown by arrow; n=6) has no effect on the electrically evoked C fibre responses of the cells but has a small effect on the evoked A fibre responses. *$p<0.03$ **$p<0.01$, Student's t-test.

5.2.5 Inhibition of the formalin response by intrathecal AP5

The NMDA receptor antagonist AP5 administered intrathecally as a 40 minute pretreatment reduced the size of the first peak with both the 250μg and 500μg dose, but these changes were not statistically significant, probably due to the large standard errors present on both the control and treatment groups, reflecting the inherent variability in the response (Figure 5.5).

The second peak of the formalin response was inhibited in a dose-related manner by pretreatment with intrathecal AP5 and this reached statistical significance with the top dose of 500μg (Figure 5.5). The second peak of the response appeared more sensitive to inhibition by AP5 since 500μg resulted in a 51% reduction of the first peak compared to a second peak reduction of 83% ($p=0.006$) with the same dose. It should be noted that some neurones appeared insensitive to AP5 with 3/8 neurones unaltered after 250μg whilst at 500μg AP5 2/9 neurones were unaffected. These cells have been omitted from the above results.

Interestingly, no changes in the latency to arrival of the second peak were observed (control 33 ± 4 mins; AP5(250μg) 35 ± 9 mins; AP5(500μg) 29 ± 7 mins) despite the significant reduction in the size of the second peak activity.

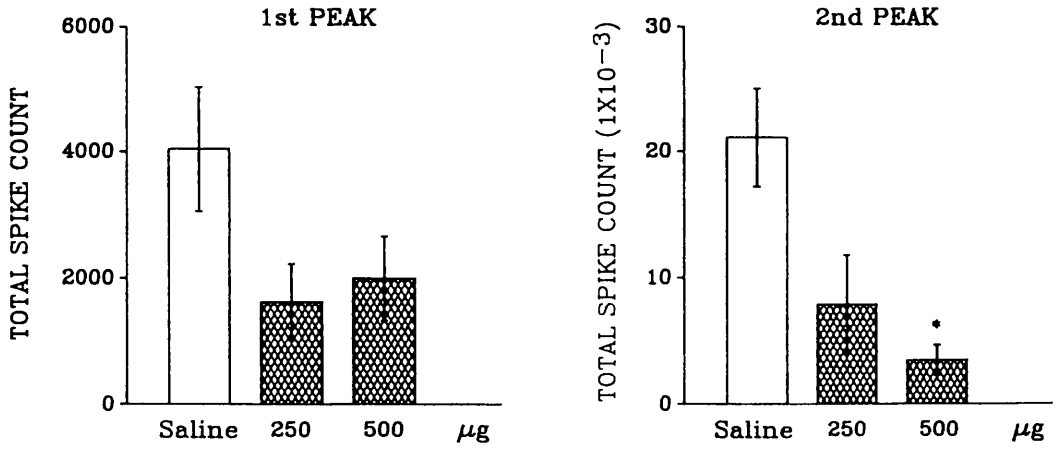

Figure 5.5 Intrathecal administration of AP5 (in 50μl) reduces the first peak of the formalin response slightly but markedly inhibits the second peak. n ranges from 5 to 14. * p=0.006, Mann-Whitney U-test.

5.2.6 Inhibition by ketamine of electrically evoked 'wind up'

Intravenous administration of ketamine (4-8 mg/kg) 4 minutes prior to electrical stimulation had no significant effect on the overall C fibre evoked activity of the neurones with a response 95% ± 15 of control with 8mg/kg. However, ketamine was able to inhibit the sequential increase in the electrically evoked C fibre response of the neurone, termed 'wind up', in 5/6 neurones located within the deeper laminae. The neurones that did show reduced'wind up' with 8mg/kg had their C fibre counts reduced to 58% ± 7 of control. Neurones located within more superficial laminae were less affected by this NMDA antagonist and the wind up of 4/6 cells was unaltered. Of the neurones located within the intermediate region and presumed to be substantia gelatinosa cells, 2/3 displayed an increase in 'wind up' (Figure 5.6).

The response of these neurones to A fibre activation was essentially unaltered by intravenous ketamine (4-8 mg/kg) administered 4 minutes prior to stimulation. The higher dose of 8mg/kg reduced the response to 82% ± 12 (n=7) of control but this small reduction was not significant.

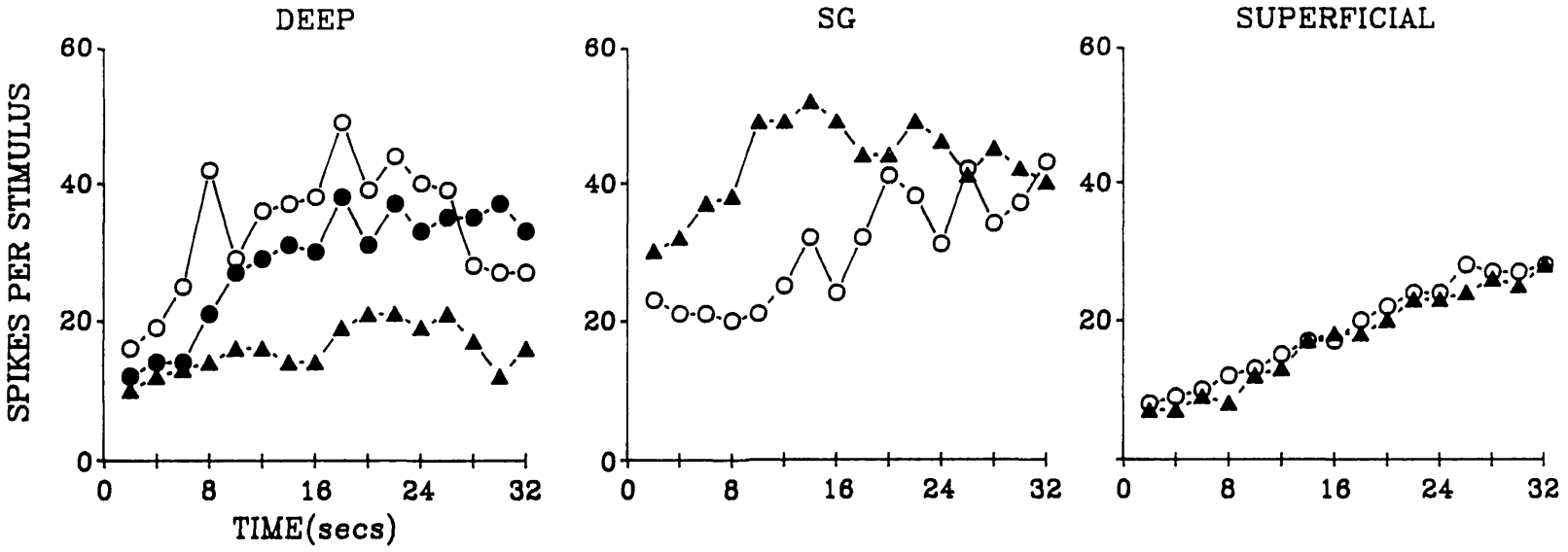

Figure 5.6 Intravenous ketamine (4-8mg/kg) administered 4 minutes prior to electrical stimulation of the receptive field has differing effects on the C fibre stimulated 'wind up' of neurones located within different regions of the dorsal horn. The examples are from single cells located within the deep (>500μm), substantia gelatinosa (SG; 250-500μm) and superficial (0-250μm) regions. The 'wind up' of deep neurones was inhibited by increasing doses of ketamine whilst substantia gelatinosa neurones were enhanced. Superficial neurones that displayed 'wind up' were generally unaffected by ketamine.

O-O Control ●-● 4 mg/kg ketamine ▲-▲ 8mg/kg ketamine.

5.2.7 The effect of intravenous ketamine on the second peak of the formalin response

When administered during the formalin response second peak, intravenous ketamine (1-8mg/kg) inhibited the rate of firing of 11/11 neurones located within both the deeper and superficial laminae in a dose-related manner (Figure 5.7 and 5.8). The top dose of 8mg/kg resulted in a 66% ± 12 (n=4, p=0.06) inhibition in the rate of firing. Saline administered intravenously had only a minor and transient effect on the population of neurones (Figure 5.8). Ketamine was administered during the second phase of the formalin response in only two intermediate, substantia gelatinosa, neurones. Of these, one displayed an increase in activity and the other was inhibited.

The inhibitions produced by ketamine were very transient and this made expression of the results during the second peak problematic. The results have therefore been expressed as the mean maximal inhibition in the rate of firing of the neurones following ketamine administration regardless of the duration of this inhibition (which ranged from 3.1 ± 0.9 mins; n=4 at 1mg/kg to 5.6 ± 1.3 mins; n=4 at 8mg/kg) as a percentage of the control firing prior to ketamine. The results for MK801 have also been expressed in this manner.

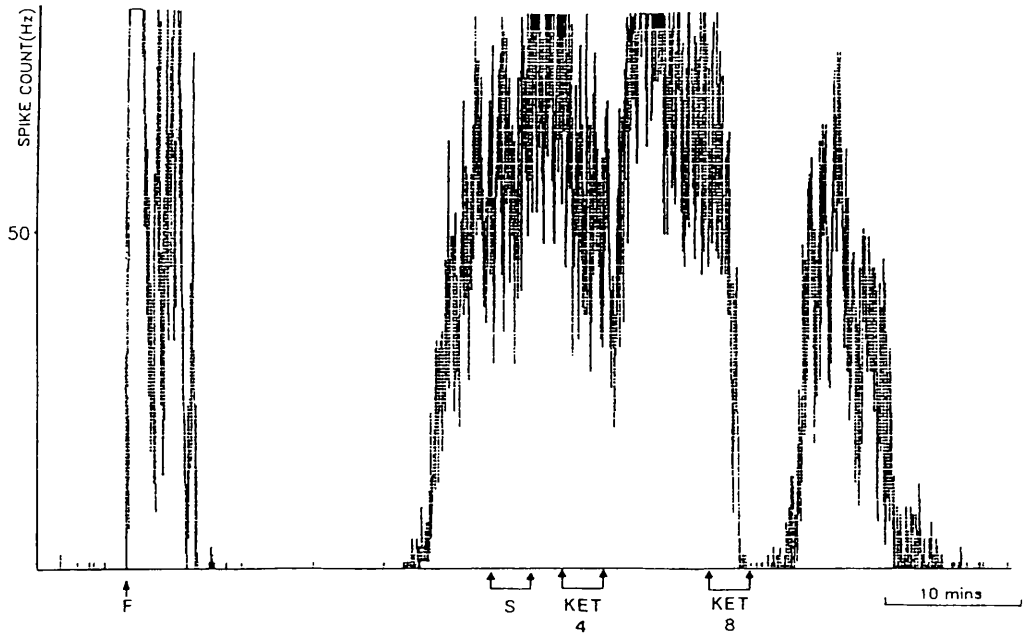

Figure 5.7 Ratemeter recording of the firing (Hz) of a single dorsal horn neurone responding to the administration of formalin into the receptive field. Intravenous ketamine (KET, 4 and 8 mg/kg) as indicated inhibited the firing of the neurone although the duration of action was short. Saline (S) had little or no effect on this neurone.

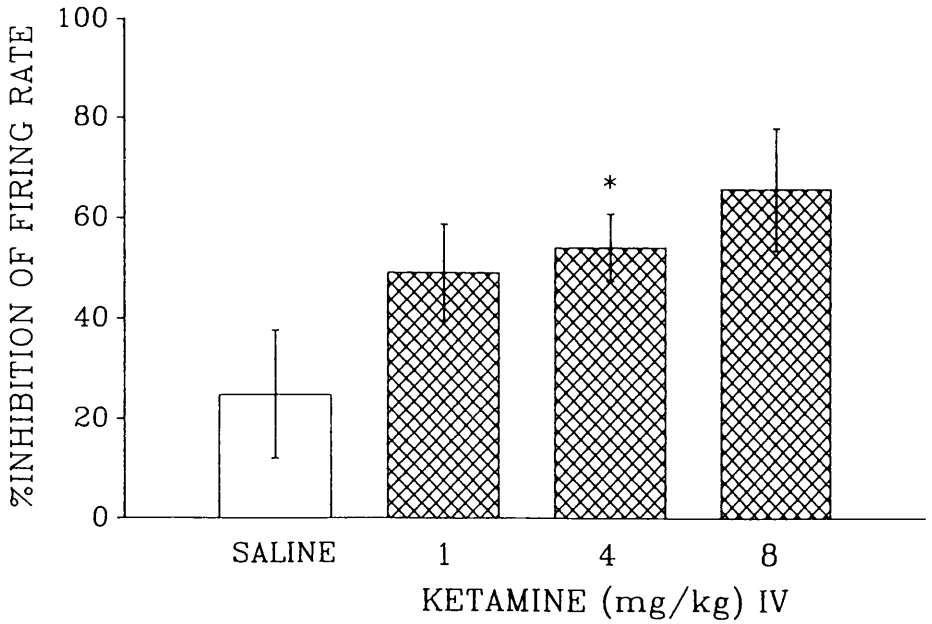

Figure 5.8 Inhibition of the neurone firing rate (Hz) produced by intravenous ketamine over a population of cells (n ranges from 4 to 13). Saline alone produced a small inhibition in the rate of firing. *p=0.03 compared to the inhibition produced by saline, Mann-Whitney U-test.

5.2.8 Inability of MK801 to alter electrically evoked 'wind up'

MK801 (0.5-1 mg/kg) administered intravenously 10 minutes prior to electrical stimulation had very little effect on the C fibre evoked responses of the neurones with the top dose of 1mg/kg causing a non-significant increase to 119% ± 12 (n=5) of control. In addition, the ability of the neurones to 'wind up' showed no alteration with either the 0.5mg/kg (n=5) or 1mg/kg dose of MK801 (Figure 5.9). The neurones were located within deep, intermediate and superficial regions. Interestingly, two neurones that did not display 'wind up' normally and were located around the start of the deeper laminae showed increased responses and characteristics similar to 'wind up' following the administration of 0.5 mg/kg MK801.

The electrically evoked A fibre responses of these neurones were also unaltered by intravenous MK801. The top dose of 1mg/kg resulted in a non-significant increase to 105% ± 14 (n=5) of control.

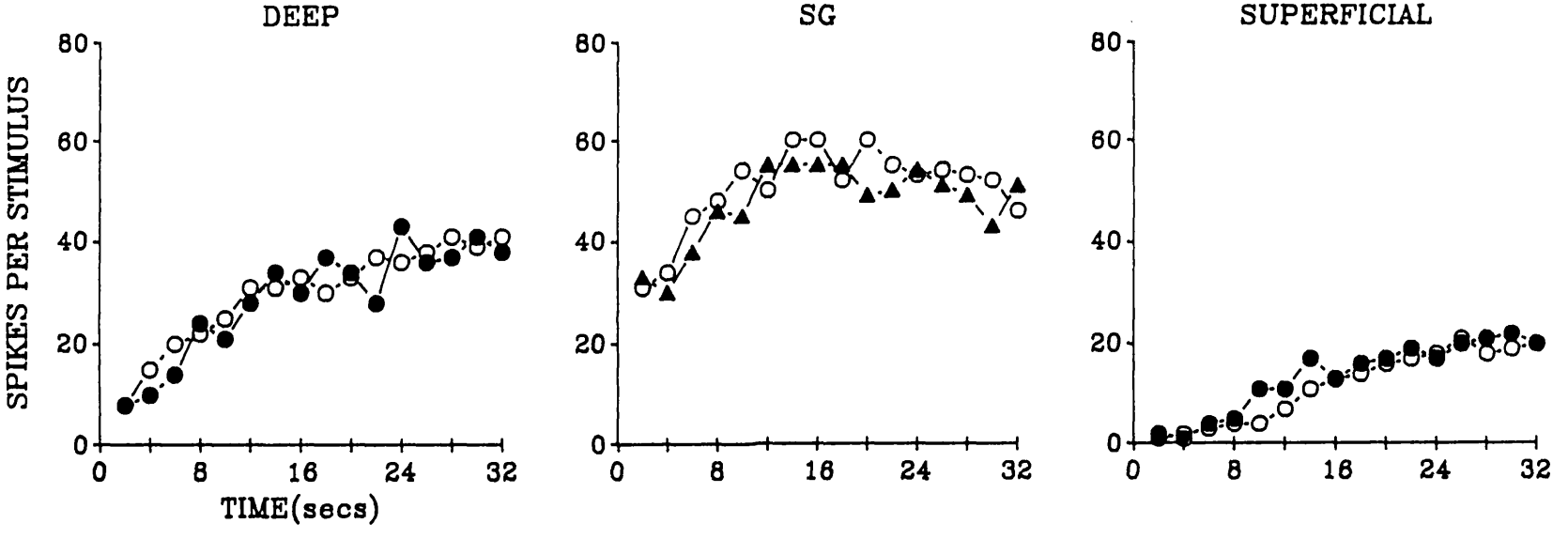

Figure 5.9 Intravenous MK801 (0.5-1mg/kg) administered 10 minutes prior to electrical stimulation of the receptive field has no effect on the C fibre stimulated 'wind up' of neurones located within different regions of the dorsal horn. The examples are from single cells located within the deep (>500μm), substantia gelatinosa (SG; 250-500μm) and superficial (0-250μm) regions.

O-O Control ▲-▲ 0.5 mg/kg MK801 ●-● 1 mg/kg MK801.

5.2.9 Inhibition of the formalin response by MK801

Intravenous MK801 (0.1 - 1mg/kg) administered during the second phase of the formalin response resulted in an inhibition of the rate of firing of 11/13 neurones, with the top dose of 1 mg/kg producing a reduction of 64% (p=0.004) (Figures 5.10 and 5.11). However, the lowest dose of 0.1 mg/kg caused a small (19%) but consistent and significant (p=0.0001) increase in the rate of firing of the neurones (Figure 5.11). Saline had a transient (2.7 ± 0.5 mins; n=6) inhibitory effect (30 ± 6% inhibition; n=6) (Figure 5.11). The effect of MK801 was of long duration, outlasting the formalin response since no recovery was seen at one hour when the second phase would be expected to have ended.

MK801 (0.5-1mg/kg) when administered intravenously as a 30 minute pretreatment caused a dose related inhibition of the second phase of the response and this reached statistical significance with the top dose of 1 mg/kg (Figure 5.12). There was a small, non-significant reduction in the first peak. The second peak of the response was more sensitive to inhibition by MK801 with 1mg/kg producing 95% (p=0.008) inhibition of this phase compared to 41% inhibition of the first peak (Figure 5.12). It should be noted that 2/8 neurones appeared insensitive to 1mg/kg MK801 and have been excluded from the above results.

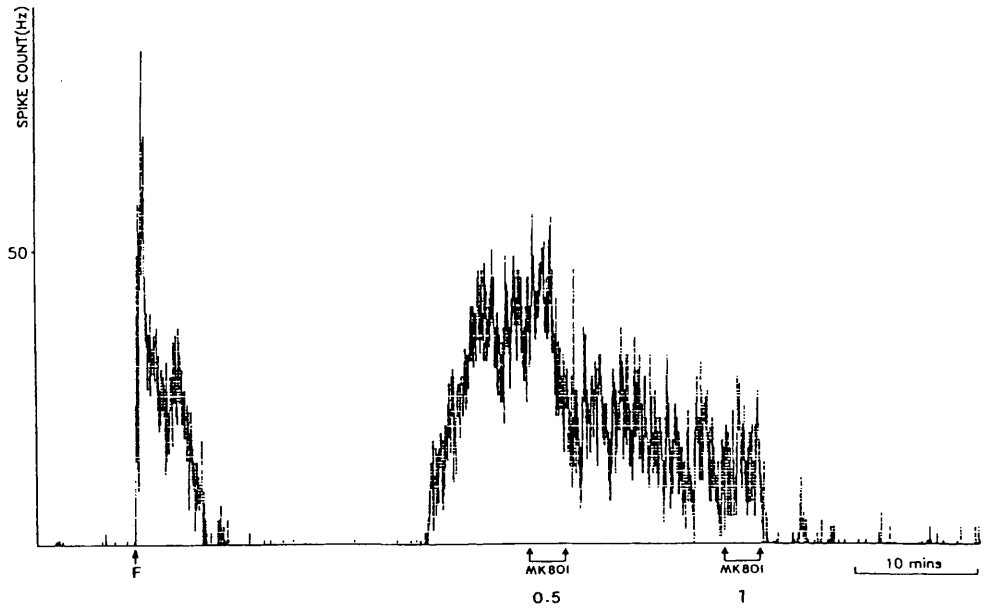

Figure 5.10 Ratemeter recording of the firing (Hz) of a single dorsal horn neurone responding to the administration of formalin into the receptive field. Intravenous administration of MK801 (0.5 and 1 mg/kg) as indicated, inhibited the firing of the neurone. This inhibition had a rapid onset and was of long duration, outlasting the formalin response.

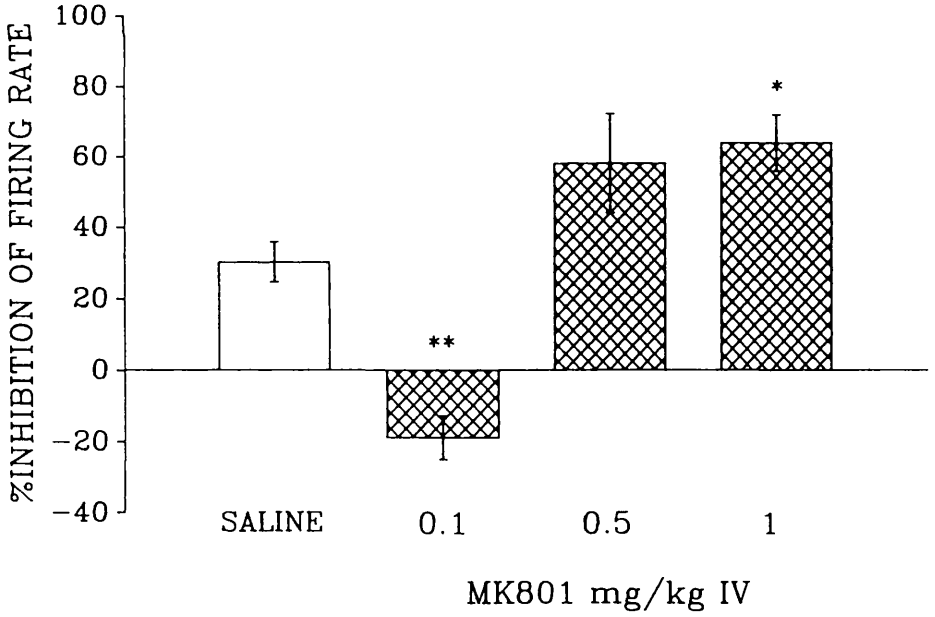

Figure 5.11 Inhibition of the neurone firing rate (Hz) over a population of cells (n ranges from 5 to 9) produced by intravenous administration of MK801 during the second peak of formalin induced activity. Saline alone produced a small inhibition in the rate of firing. The lowest dose of 0.1 mg/kg MK801 caused a small but consistent increase in the rate of firing. *p=0.004 **p=0.0001 compared to the inhibition produced by saline, Mann-Whitney U-test.

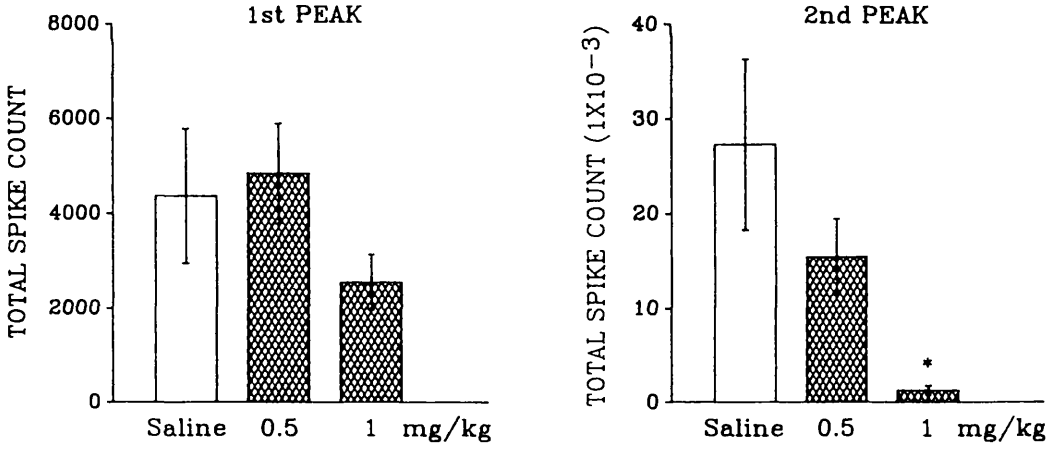

Figure 5.12 Intravenous administration of MK801 30 minutes prior to formalin injection results in a small inhibition of the first peak at the highest dose of 1mg/kg and a dose-related inhibition of the second peak. n ranges from 5 to 6. *p=0.008, Mann-Whitney U-test.

5.2.10 Peripheral inhibition of the first peak with lignocaine has no effect on the second peak of the formalin response

Subcutaneous injection of 2% lignocaine directly into the receptive field of the cell immediately prior to administration of formalin into the same site completely inhibited the first peak of the formalin response but had no effect on the size of the second peak (Figure 5.13). The latency to the arrival of the second phase was unaltered by the pretreatment with lignocaine: Control: 35 ± 13 mins; lignocaine: 30 ± 14 mins; n=10. The local anaesthetic block of formalin induced activity lasts about 15 minutes (Dickenson & Sullivan, 1987c) and so would have worn off by the time the second peak began. Therefore abolition of the first phase responses of the neurones has no effect on second phase activity of the same cells.

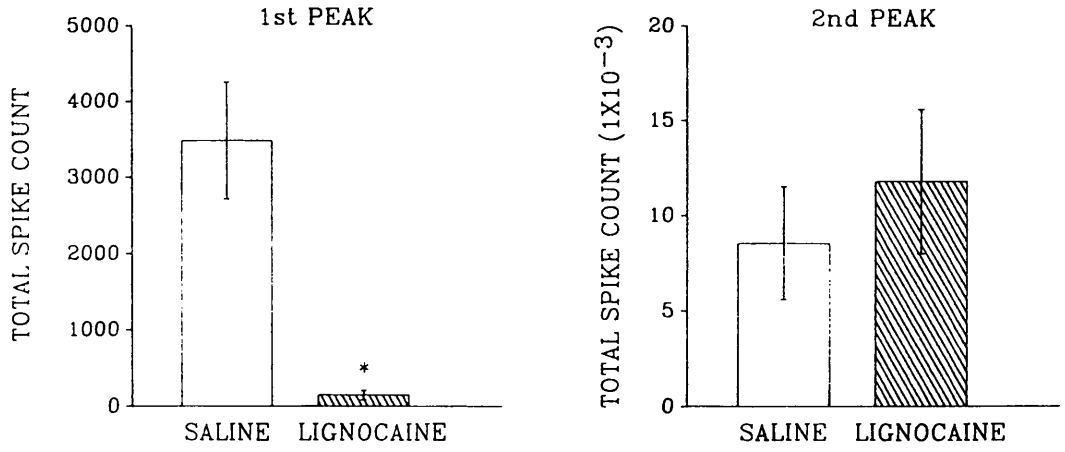

Figure 5.13 Injection of 2% lignocaine (50µl) directly into the site of receptive field immediately prior to formalin injection completely inhibited the first peak of the response but did not alter the size of the second peak. n=10 for both groups. *p=0.0004, Mann-Whitney U-test.

5.2.11 Inability of peripherally administered DGG to alter the formalin response

DGG (250μg) was injected directly into the receptive field ten minutes prior to formalin administration. This treatment was unable to alter the biphasic response of the dorsal horn neurones to formalin. Neither the size of the first and second peaks (Figure 5.14) nor the latency to arrival of the second peak (control: 28.5 ± 6.9 ; DGG: 20.5 ± 3.4 minutes) were statistically significant from controls.

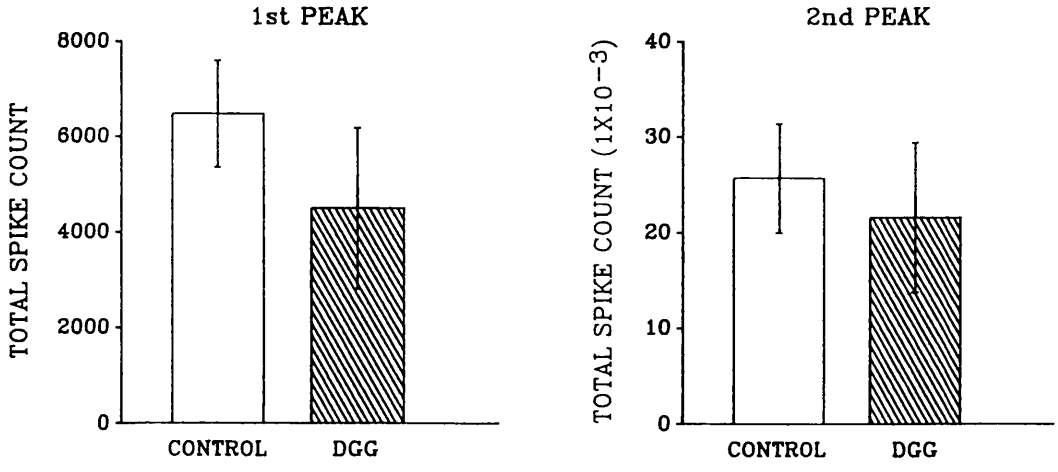

Figure 5.14 DGG (250μg) injected directly into the receptive field as a 10 minute pretreatment did not alter either peak of the formalin response. n=5 for both groups.

5.3 Discussion

Excitatory amino acid receptors are present within the the spinal cord (Greenamyre et al., 1984; Jansen et al., 1990) and within the afferent nerve where they are axonally transported towards the peripheral terminal (Lewis et al., 1987). However only the receptors located within the spinal cord appear to be involved in the response of the dorsal horn neurones to formalin administered into the receptive field. Antagonism of both NMDA and non-NMDA receptors by intrathecal DGG rapidly reduced the acute A and C fibre evoked activity of the dorsal horn neurones and this is in contrast to the actions of opioids which selectively reduce the C fibre evoked responses (Dickenson and Sullivan, 1986; Sullivan et al., 1989). Intrathecal DGG also inhibited both phases of the formalin evoked activity whilst administration of DGG directly into the receptive field prior to formalin had no effect on either peak of the response. Thus the neuronal activity elicited by formalin can be inhibited by antagonism of spinal, but not peripheral, excitatory amino acid receptors. This confirms previous *in vivo* studies where the non-selective inhibition of A and C fibre responses by intrathecal DGG was seen following stimulation of both the receptive field and the dorsal root in the rat (Dickenson and Sullivan, 1990; Schouenborg and Sjölund, 1986).

Similarly in behavioural studies in mice intrathecal administration of DGG results in a reduction in the short duration responses elicited in the tail-flick test and in response to pinch (Raigorodsky and Urca, 1990b). Unfortunately the doses of DGG that produced antinociception also resulted in motor impairment and this would obscure the results obtained in these behavioural studies. However vocalization elicited by electrical stimulation of the tail is not dependent on intact motor function and this was also reduced by DGG (Raigorodsky and Urca, 1990b). Thus DGG appears antinociceptive against the short duration responses of dorsal horn neurones and behavioural tests, and can also reduce the more prolonged firing of dorsal horn neurones elicited by formalin. However the parallel reductions in A fibre responses would imply severe tactile difficulties as well.

The ability of DGG to influence the formalin response is rapidly reduced once the formalin has been injected. Thus the top dose administered during the ongoing activity of the second peak is about 20% less effective than when applied prior to formalin, and administration of DGG 3 minutes after formalin injection results in a 32% inhibition of the second peak compared to a 98% reduction when the same dose was applied prior to formalin. This is remarkably similar to the results observed with opioid inhibition of the formalin response (Dickenson & Sullivan, 1987a) where pretreatment was considerably more effective than post-treatment. This loss of effectiveness with DGG cannot be attributed to the latency of onset since the C fibre evoked response was clearly reduced 10 minutes following administration and maximal effects are seen at 20 minutes followed by a plateau (Dickenson & Sullivan, 1990). DGG would therefore be exerting its maximal effect prior to the arrival of the formalin evoked second peak even when administered during the first peak.

The NMDA selective antagonist AP5 and the NMDA channel blockers ketamine and MK801 (Davies and Watkins, 1982; Anis et al., 1983; Wong et al., 1986; Collingridge and Lester, 1989) had no effect on the overall responses of the dorsal horn neurones to electrically evoked C fibre inputs although both AP5 and ketamine reduced the ability of these neurones to 'wind up' (Dickenson and Sullivan, 1987b, 1990; Davies and Lodge, 1987). Following repetitive stimulation of the receptive field, dorsal horn neurones often respond with increased firing to each constant peripheral stimulus and this amplifying response is termed 'wind up' (Mendell, 1966). Interestingly 'wind up' has also been observed in humans where repetitive constant heat stimulation results in increased pain ratings (Price et al., 1977). Intravenous ketamine had no effect on the initial responses of the neurones to electrical stimulation but reduced the ability of neurones located within the deeper laminae (500-1000μm) to 'wind up', confirming results obtained by Davies and Lodge (1987). Furthermore neurones located in the intermediate laminae (250-500μm), presumed to be substantia gelatinosa neurones, displayed increased 'wind up' with ketamine whilst superficially located neurones (<250μm) which less frequently display 'wind up' were not altered by ketamine. This is very similar to results obtained with AP5 where intermediate cells increased their

'wind up' but superficial neurones were unchanged (Dickenson and Sullivan, 1990). Intravenous administration of MK801 had no effect on either the C fibre evoked responses of the dorsal horn neurones or their ability to 'wind up'. This is unexpected since both ketamine and MK801 are believed to be NMDA channel blockers, although MK801 has also been shown to interact with other receptor systems (Lodge and Johnson, 1990). This difference in effect on 'wind up' is difficult to explain, but may relate to the use-dependent nature of the block produced by these antagonists.

Remarkably, despite the inability of the NMDA antagonists AP5, ketamine and MK801 to reduce the initial responses to C fibre evoked inputs they markedly reduced the second peak of formalin evoked activity. The first peak of the response appeared less sensitive to inhibition by NMDA antagonists since both AP5 and MK801 when administered as a pretreatment had only a small inhibitory effect on the first peak at doses that significantly reduce the second peak. The very short duration of action of ketamine prohibited any pretreatment studies with this compound. MK801 was approximately ten times more potent than ketamine and had a duration of action that was much longer and this is comparable with other *in vivo* studies (Davies et al., 1988). These effects of systemically administered ketamine and MK801 occurred at doses that have been shown to be NMDA blocking and subanaesthetic *in vivo* (Anis et al., 1983; Davies and Lodge, 1987; Headley et al., 1987; Davies et al., 1988). Interestingly, the lowest dose of MK801 administered during the second peak activity caused an increase in the rate of firing of the neurones. This may result from the disinhibition of the neurones recorded i.e MK801 inhibits NMDA induced inhibitory influences on the cell, prior to the inhibition of the cell itself. Low dose excitation prior to inhibition with higher doses has been observed with another class of drugs, the opioids, where low doses of mu agonists are known to cause excitations prior to their inhibitory effects (Dickenson et al., 1987). Whilst the actions of AP5 are likely to result from a direct spinal action, this cannot be assumed with ketamine and MK801 since they were administered intravenously. It is unlikely that their effects result from actions in the periphery since DGG injected into the receptive field did not influence the response to formalin. However it is possible that they may exert some of their effect by influencing

descending systems since the animals used were intact. With AP5, ketamine and MK801, a small proportion of cells were not influenced by these antagonists, and were not included in the overall results. However, there is no reason to imagine that all dorsal horn nociceptive neurones would be equally influenced by NMDA mediated events, since some cells may lack any contact with NMDA receptors. Thus, the short duration first peak and more prolonged second peak of dorsal horn neuronal activity elicited by formalin can both be inhibited by a non-selective excitatory amino acid antagonist. By contrast, antagonists selective for the NMDA receptor only reduce the activity in the second peak. It is therefore tempting to suggest that the first peak response is mediated mainly via non-NMDA receptors whilst the second peak relies on both non-NMDA and, to a large extent NMDA receptors.

AP5 and ketamine both *in vitro* and *in vivo* reduce dorsal root evoked motoneurone activity and ventral root depolarizations in the cat, rat, hamster and mouse. However, in contrast to DGG, AP5 and ketamine are more effective at reducing the polysynaptic component of the depolarization response compared to the monosynaptic component (Tang and Schroeder, 1973; Lodge and Anis, 1984; Evans et al., 1982; Long et al., 1988; Brugger at al., 1988; Bagust et al., 1989; Sjölund et al., 1990; Thompson et al., 1990). Similarly the activity of dorsal horn neurones *in vivo* and *in vitro* is reduced by DGG, ketamine and AP5 but whilst DGG reduces the monosynaptic activity, AP5 and ketamine inhibit the polysynaptic responses (Davies and Watkins, 1983; King et al., 1988). In addition AP5 is unable to alter the afferent fibre compound action potential (Raigorodsky and Urca, 1990a) and must therefore be exerting its effects after the arrival of peripheral inputs. Non-NMDA receptors may be located on postsynaptic sites that do not differentiate between A and C fibre inputs, perhaps on the cell bodies of the spinothalamic tract neurones themselves. However, NMDA receptors are more likely to be present on interneurones forming part of the pathway for the transmission of C fibre inputs. Indeed, binding studies have shown NMDA receptors localized within the interneurone rich, substantia gelatinosa region (Beal, 1983; Monaghan and Cotman, 1985; Todd and Lewis, 1986; Jansen et al., 1990).

The role of excitatory amino acids in the transmission of nociception within the dorsal horn has only recently become the subject of investigation. However *in vitro*, *in vivo* and behavioural studies now implicate the involvement of these amino acids in nociceptive events. Using an *in vitro* neonatal rat spinal cord with the tail attached Dray and Perkins (1987) demonstrated the inhibition by NMDA antagonists, of ventral root depolarizations elicited by capsaicin and bradykinin administered to the tail. However the responses elicited by electrical stimulation of the tail were unaffected and heat evoked responses were only partially reduced. By contrast, both AP5 and ketamine reduce the C fibre mediated 'wind up' of dorsal horn neurones *in vivo* (Dickenson and Sullivan, 1987b, 1990; Davies and Lodge, 1987) and ventral horn neurones *in vitro* (Thompson et al., 1990). In addition the responses of dorsal horn neurones *in vivo* to noxious heat and pinch are reduced by AP5 and ketamine whilst the responses to touch are unaltered (Conseiller et al., 1972; Kitahata et al., 1973; Lei and Wilcox, 1990; Raigorodsky and Urca, 1990a) although one study in rats and cats failed to show any effect of ketamine (Headley et al., 1987). Interestingly, intrathecal application of NMDA and AMPA augments the responses of these neurones to peripheral noxious heat (Lei and Wilcox, 1990). Thus *in vitro* and *in vivo* studies have demonstrated the ability of DGG to reduce both A and C fibre evoked responses whilst NMDA antagonists selectively reduce responses to noxious stimuli.

Behavioural studies in the rat and mouse indicate that NMDA antagonists are antinociceptive in the tail-flick, hotplate, paw pressure,writhing and formalin tests as well as reducing NMDA induced biting and hyperalgesia (Ryder et al., 1978; Fratta et al., 1980; Cahusac et al., 1984; Aanonsen and Wilcox, 1986; Post and Arweström, 1986; Raigorodsky and Urca, 1987, 1990b; Takahashi et al., 1987; Yaksh, 1989). However, the antagonists also induced motor paralysis which could complicate the results obtained in these studies. Vocalization in response to electrical stimulation of the tail negates motor problems and AP5 has also been shown to be antinociceptive in this test (Cahusac et al., 1984; Raigorodsky and Urca, 1990a, b). So despite the motor problems associated with excitatory amino acid antagonists, both AP5 and DGG produce antinociception in behavioural studies in rodents.

A recent study by Skilling and colleagues (1988) supports the hypothesis that excitatory amino acids are involved in nociceptive responses at the spinal level. Using *in vivo* microdialysis techniques in conscious rats they have demonstrated release of both glutamate and aspartate within the lumbar dorsal horn following formalin administration into the paw. Release occurred during the second peak of the response and was associated with periods of intense biting and licking of the injected paw by the animal.

The dissociative anaesthetic ketamine has been studied as an analgesic for many years but has only recently been identified as an NMDA channel blocker (Anis at al., 1983). Consequently the analgesic properties of this NMDA antagonist (which occur at lower doses than its anaesthetic effects) have been assessed in humans since ketamine was already clinically in use as an anaesthetic. Ketamine as been administered intramuscularly, intravenously, and subcutaneously and is an effective analgesic in experimental (Bovill and Dundee, 1971; Sadove et al., 1971; Clements and Nimmo, 1981; Grant et al., 1981; Maurset et al., 1989), and postoperative pain (Sadove et al., 1971; Maurset et al., 1989). The duration of action of ketamine is short being between 10-100 minutes depending on the route of administration. Ketamine infusion, both intravenous and subcutaneous, has also been effective against cancer pain (Mizuno et al., 1990; Oshima et al., 1990). The effectiveness of intrathecally administered ketamine (Bion, 1984) implies the site of action for its nociceptive effects is within the spinal cord. Thus the involvement of excitatory amino acids in nociception appears to extend from rodents to the human.

The NMDA receptor is unique in that it is both ligand and voltage gated and this results from a resting block of the ion channel by magnesium (MacDermott and Dale, 1987). Thus depolarization of the neurone is required in order for the magnesium ions to leave the channel and allow receptor activated ion flux to occur. This raises the possibility that the firing of dorsal horn neurones during the second peak of the formalin response is dependent on activity occurring in the first peak perhaps by relieving the magnesium block and allowing participation of the NMDA receptors. However the blockade of peripheral inputs during the first peak by pretreatment of the receptive field

with a local anaesthetic prevents the first peak of activity in the dorsal horn neurones but has no effect on the generation of the second peak. Thus the second peak of the response appears to be independent of the neuronal activity occurring in the first peak. It is extremely unlikely that axonal transport, as opposed to neuronal activity, is responsible for changes occurring between the first and second peak since the time span involved is so short; at least 24 hours seems necessary for effects of transport block on chemical nociception (Fitzgerald et al., 1984). However there is evidence that formalin stimulated substance P release, which occurs over a similar time frame as the dorsal horn neuronal activity and would be expected to increase central excitability, involves both neuronal activity and axonal transport (Kantner et al., 1986).

Thus, it would appear that the prolonged chemical nociceptive stimulus evoked by subcutaneous formalin produces a peripheral afferent drive that can activate central spinal NMDA receptors. The unique voltage and ligand gated properties of NMDA receptors have lead to their implication in a variety of excitability changes in CNS function including epilepsy (Fagg et al., 1986) and long term potentiation (LTP) (Bliss and Lynch, 1984). These characteristics may serve to amplify and/or prolong the neuronal activity produced by the afferent barrage in response to formalin, particularly during the second phase of the response.

Activity in the first peak of the formalin response is not a prerequisite for the second phase and so seems unlikely to prime spinal events for the NMDA involvement in the second phase. Glutamate and substance P have been shown to coexist in fine afferent fibres (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988). Thus the simultaneous release of glutamate and/or aspartate (Westlund et al., 1989) together with peptides may result in depolarization via the non-NMDA and/or peptide receptors (Urbán and Randic, 1984), perhaps removing the magnesium block of the NMDA receptor complex. This could allow the NMDA receptor to be activated by prolonged peripheral inputs such as formalin.

The involvement of spinal NMDA receptors in the amplification of dorsal horn neurone responses to peripheral inputs, as observed in 'wind up' and the formalin

response, makes this receptor a very attractive candidate for other hypersensitivity states resulting from peripheral disease or injury. In addition, opioids are less effective at inhibiting both the 'wind up' of neurones and their responses to formalin once the response has begun (Dickenson and Sullivan, 1986; Dickenson and Sullivan, 1987a). Thus the problems associated with the treatment of some pain states may arise from mechanisms such as these.

CHAPTER 6:

THE ROLE OF NITRIC OXIDE DURING THE FORMALIN RESPONSE: SOME SPINAL AND PERIPHERAL ASPECTS.

6.1 Introduction

Endothelium derived relaxing factor (EDRF, Furchgott and Zawadski, 1980; Furchgott, 1984) is now believed to be the simple chemical molecule nitric oxide (Ignarro et al., 1987; Palmer et al., 1987). The inflammatory mediators bradykinin, substance P and histamine can produce vascular relaxation indirectly by stimulating the production of nitric oxide in endothelium cells and this subsequently stimulates guanylate cyclase within the smooth muscle (Ignarro et al., 1986; Palmer et al., 1987; Lückhoff et al., 1988; Sakuma et al., 1988; Toda et al., 1990). However, far from being confined to the vasculartur, nitric oxide synthesis, release and stimulation of guanylate cyclase has now been shown to occur in several, seemingly diverse cells and tissues including the brain.

The synthesis of nitric oxide from an amino acid precursor L-arginine appears to involve a enzymatic hydroxylation/oxidation reaction. The enzyme involved in this conversion is believed to be present in the cell cytosol, to produce citrulline as a byproduct and require NADPH and calcium ions (magnesium ions in the macrophage) (Marletta, 1989). This enzymatic synthesis of nitric oxide has now been shown to occur in many cells including endothelium cells (Palmer et al., 1988; Sakuma et al., 1988; Schmidt et al., 1988; Palmer and Moncada, 1989), macrophages (Hibbs et al., 1988; Marletta et al., 1988; Stuehr et al., 1989) and neutrophils (Rimele et al., 1988; McCall et al., 1989). The pathway for synthesis of nitric oxide appears to be very similar in these cell types.

Bradykinin is known to activate C fibres and to be a peripheral mediator of chemical nociception (Reeh, 1986; Martin et al., 1987; Steranka et al., 1988; Haley et al., 1989). The ability of bradykinin and histamine to generate nitric oxide and thus produce local vascular effects presents a potential role for this mediator in peripheral inflammatory events and of particular interest is the possibility that nitric oxide may be involved in the actions of bradykinin during nociceptive events.

An exciting new development has been the discovery of nitric oxide production

within the central nervous system (Garthwaite et al., 1988; Bredt and Snyder, 1989; Garthwaite et al., 1989; Knowles et al., 1989; Pou et al., 1990). Moreover, it has been proposed that nitric oxide may be released from neurones following receptor activation (Garthwaite et al., 1988; Dickie et al., 1990). The presence of an endogenous activator of guanylate cyclase within the central nervous system has been known for some time (Deguchi, 1977; Deguchi et al., 1978), and was thought to be L-arginine (Deguchi and Yoshioka, 1982). As early as 1977 the direct stimulation of guanylate cyclase by nitric oxide in mouse cortex was demonstrated (Miki et al., 1977) but it was not until a decade later that it was realized the endogenous activator, EDRF and nitric oxide were likely to be the same molecule (Garthwaite et al. 1988).

Guanylate cyclase is present in most tissues including the brain (Drummond, 1984) and is stimulated following receptor activation by the excitatory amino acid glutamate in the cerebellum (Garthwaite and Garthwaite, 1987). Moreover, this stimulation of guanylate cyclase has recently been shown to occur via the generation of nitric oxide following activation of the N-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptor (Garthwaite et al., 1988; Bredt and Snyder, 1989; Garthwaite et al., 1989; East and Garthwaite, 1990).

The activation of dorsal horn neurones within the spinal cord following noxious peripheral stimulation *in vivo* has been shown to involve excitatory amino acid receptors, including the NMDA receptor (Davies and Lodge, 1987; Dickenson and Sullivan, 1987b; Haley et al., 1990). This raises the intriguing possibility that activation of the NMDA receptor within the spinal cord following peripheral noxious stimulation may result in the generation or release of nitric oxide. The recent development of inhibitors of nitric oxide synthesis (Rees et al., 1990) allows us to determine whether nitric oxide has a role in the peripheral generation or spinal processing of nociceptive information.

6.2 Results

6.2.1 Effect of systemically administered L-NAME on electrically evoked A and C fibre responses.

The nitric oxide inhibitor N-nitro-L-arginine methyl ester (L-NAME) administered intravenously (10-100 mg/kg) was unable to inhibit the electrically evoked C fibre responses of the neurones. The top dose of 100 mg/kg resulted in a mean of 109% ± 19 (n=6) of control responses after 40 minutes (Figure 6.1). The electrically evoked A fibre responses were significantly inhibited by the top dose of L-NAME 40 minutes after administration of the drug with a response that was 79% ± 8 (n=6; $p<0.05$) of control (Figure 6.1).

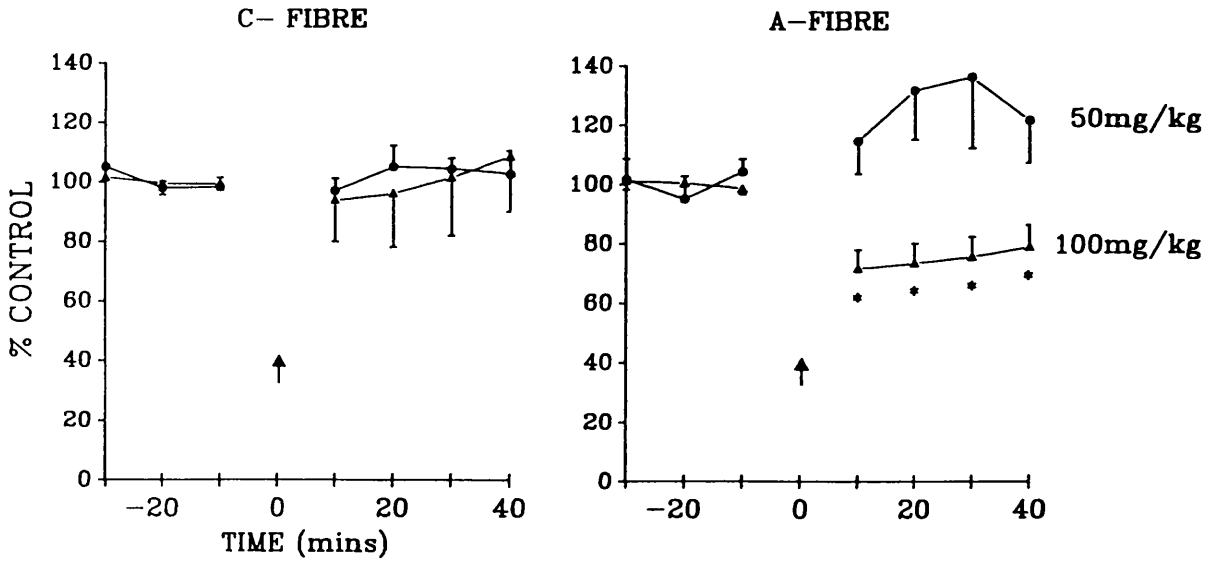

Figure 6.1 The effect of intravenous L-NAME (50-100 mg/kg, shown by arrow) on the electrically evoked A and C fibre responses over a population of dorsal horn neurones. n ranges from 4 to 6. *$p< 0.05$. Student's t-test.

6.2.2 Pretreatment with intravenous L-NAME inhibits the formalin response.

Intravenous administration of L-NAME (10-100 mg/kg) 40 minutes prior to the injection of formalin resulted in an inhibition of the second peak of the subsequent response with little change in the first peak (Figure 6.2). The highest dose used (100 mg/kg) produced a 74% inhibition in the size of the second peak but only a 45% inhibition of the first peak activity. The latency to arrival of the second peak was increased with increasing doses of L-NAME although this did not reach statistical significance at any dose. Control: 28.5 ± 6.9 minutes (n=5); 10 mg/kg: 32.2 ± 7.5 (n=5); 50 mg/kg: 42.1 ± 7.3 (n=5); 100 mg/kg: 40.7 ± 6.8 (n=6).

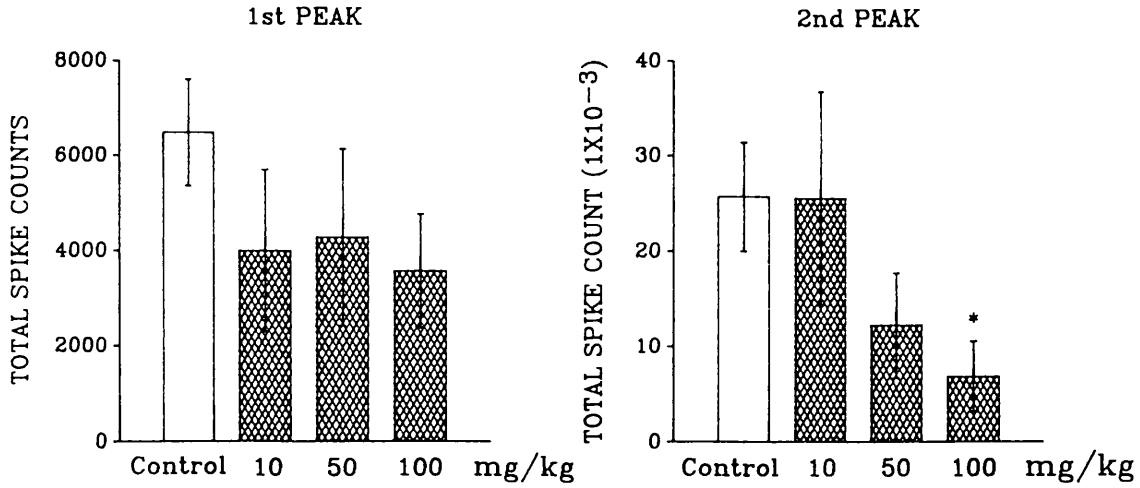

Figure 6.2 Inhibition of the second peak, but not the first peak, of the formalin response by intravenous L-NAME (10-100 mg/kg) administered as a 40 minute pretreatment. n ranges from 5 to 6. *p=0.018 Mann-Whitney U test.

6.2.3 Peripheral (s.c.) administration of L-NAME inhibits the formalin response

Injection of the top dose of L-NAME (1500 μg in 50 μl) into the receptive field produced a small peak of firing in the dorsal horn neurones that was not significantly different in either size or duration from that produced by saline: Saline: 2298 ± 698 counts, 2.7 ± 0.6 minutes, n=5 ; L-NAME: 1223 ± 410 counts, 3.5 ± 1.0 minutes, n=7.

Administration of L-NAME (500 and 1500 μg in 50μl) directly into the site of formalin injection as a 10 minute pretreatment inhibited the first phase of the formalin response (57% inhibition with 1500 μg) although this reduction failed to reach statistical significance. The second peak is significantly inhibited with the 1500 μg dose producing a 63% inhibition compared to controls (Figure 6.3). Injection of the top dose of L-NAME into the contralateral paw 10 minutes prior to formalin injection had no significant effect on the subsequent response indicating the inhibition is unlikely to result from leakage of L-NAME into the systemic circulation (Figure 6.3). The latency to arrival of the second peak is unaltered even at the highest dose tried: Control: 29.6 ± 3.6 minutes (n=7); 1500μg L-NAME: 31.5 ± 3.7 minutes (n=7).

By contrast to pretreatment, administration of 1500 μg L-NAME into the receptive field 10 minutes after the injection of formalin has no effect on the size (Control: 19,455 ± 4942 spike counts, n=12; L-NAME: 17,692 ± 3402, n=7) or time of arrival (Control: 29.6 ± 3.6 minutes; L-NAME: 27.6 ± 8.8 minutes) of the second peak of the response. Thus the ability of peripheral L-NAME to inhibit the second phase of the formalin response may be dependent on its time of administration.

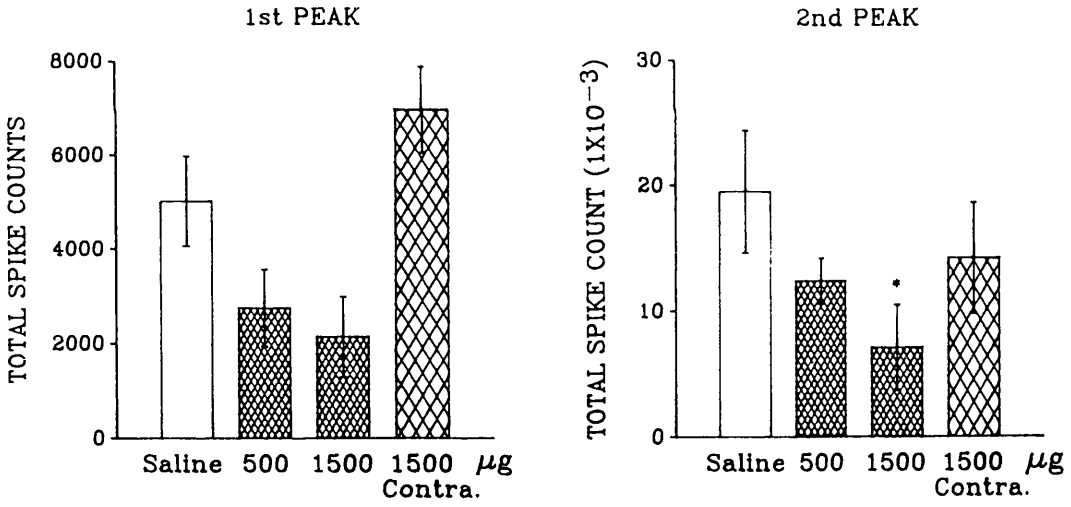

Figure 6.3 Peripheral administration of L-NAME (500 and 1500 μg in 50μl), 10 minutes prior to the injection of formalin into the same site, resulted in a reduction of the first and second peaks of the formalin response. Administration of 1500μg L-NAME into the contralateral (Contra.) paw had no significant effect on either peak of the response. n ranges from 7 to 12. *$p<0.05$ compared to controls. Mann-Whitney U test.

6.2.4 Effect of peripheral L-NAME on the electrically evoked A and C fibre responses of the neurones

Injection of 1500 μg NAME directly into the site of electrical stimulation within the receptive field resulted in an initial inhibition of both the A and C fibre evoked responses of the dorsal horn neurones. This reduction was statistically significant and had a rapid onset with maximal inhibitions observed at the first stimulation, 10 minutes following administration of the drug. The effect was transient and both the A and C fibre evoked responses which were inhibited by 35% and 12% respectively had returned to control levels by the 20 minute stimulation (Figure 6.4). Thus the reduction of the second peak of the formalin response is unlikely to result from depression of the C or A fibre activity.

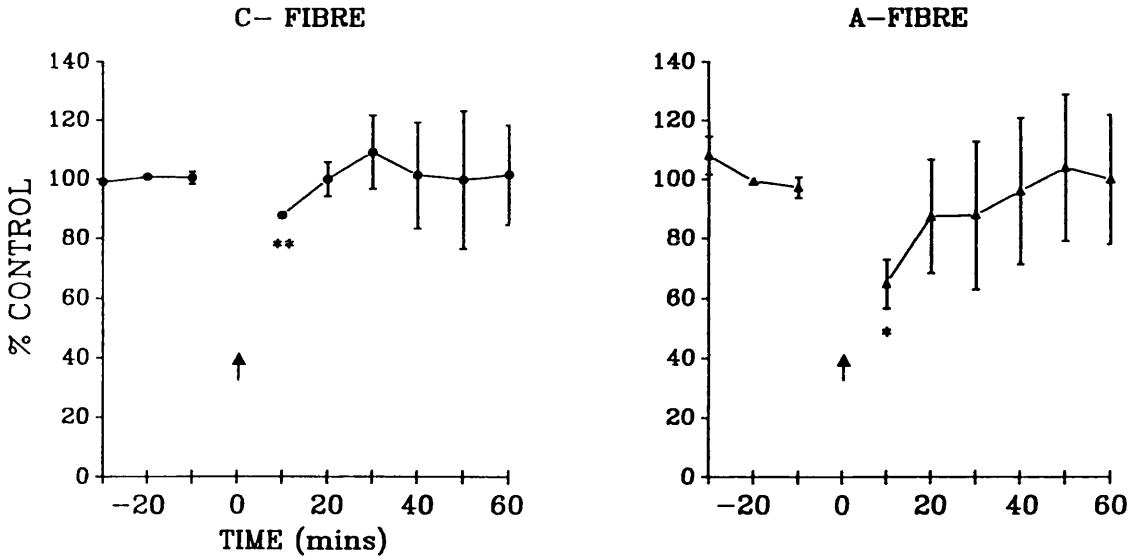

Figure 6.4 The effect of peripherally administered 1500 μg L-NAME (arrow) on the electrically evoked A and C fibre responses of the dorsal horn neurones as a percentage of the pre-drug control values. n=4 for both graphs. *$p<0.05$ **$p<0.025$. Student's t-test.

6.2.5 Intrathecal L-NAME Inhibits electrically evoked A and C fibre responses

L-NAME administered directly onto the surface of the spinal cord in a volume of 50 µl inhibited both the A and C fibre evoked responses of the neurones to a similar extent. The top dose of 1500 µg produced inhibitions of 24.1% and 30.0% for the A and C fibre evoked responses respectively, 30 minutes following intrathecal administration (Figure 6.5). The initial C fibre responses of the neurones were reduced by L-NAME but any 'wind up' elicited by repetitive stimulation appeared unaltered (Figure 6.8).

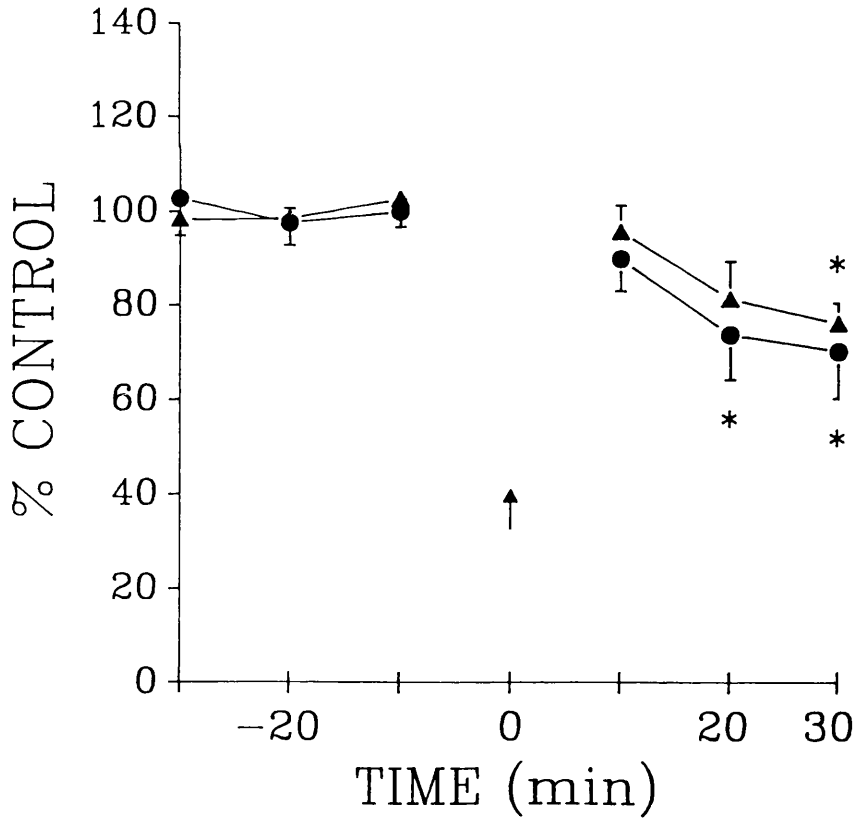

Figure 6.5 Intrathecal administration of L-NAME (1500 µg) reduces both the A fibre (▲-▲) and C (●-●) fibre responses of the neurones. n=7. *p<0.025 Student's t-test.

6.2.6 Intrathecal L-NAME inhibits the formalin response

Intrathecal administration of L-NAME 30 minutes prior to formalin injection into the peripheral receptive field resulted in a dose-related inhibition of both the first and second peaks of the formalin response (Figure 6.6). The top dose of 1500 μg L-NAME produced inhibitions of the first and second peak of 71% and 69% respectively.

The latency to arrival of the second peak was unaltered by either dose of L-NAME. Control: 28.5 ± 6.9 minutes (n=5); 500 μg L-NAME: 34 ± 6.1 minutes (n=6); 1500 μg L-NAME: 26.8 ± 8.7 minutes (n=5).

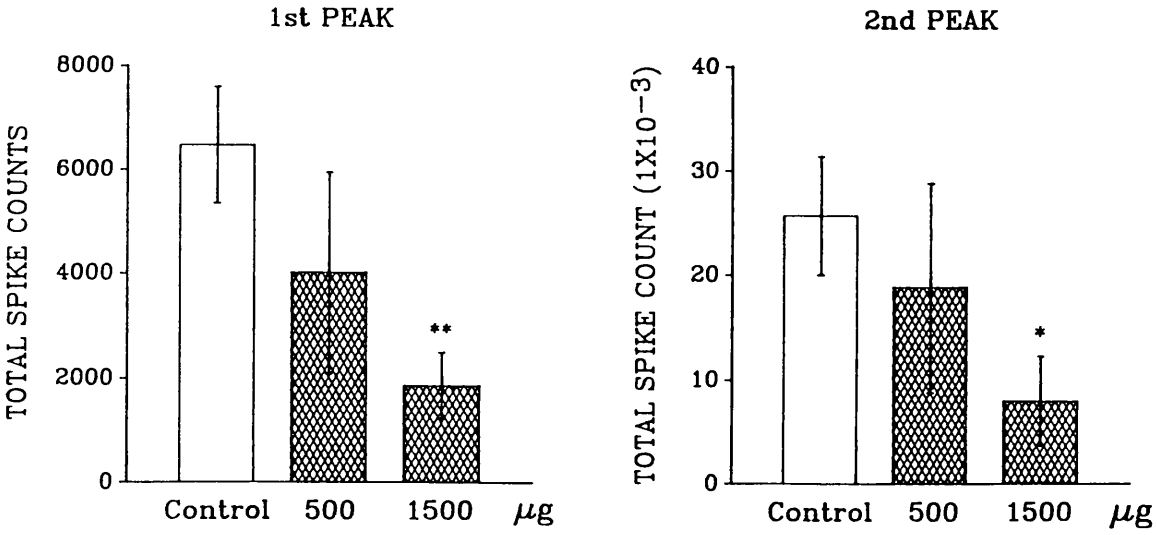

Figure 6.6 Inhibition of the formalin response by intrathecal L-NAME administered as a 30 minute pretreatment. n ranges from 5 to 6. *p=0.03 **p=0.008 Mann-Whitney U test.

6.2.7 L-Arginine inhibits electrically evoked A and C fibre responses and is unable to reverse the effects of L-NAME

Intrathecal administration of 4500 μg L-arginine in a volume of 50 μl reduced the electrically evoked A and C fibre responses of the neurones by 24 % and 67 % (p=0.02) respectively (Figure 6.7). It reduced both the initial responses of the neurones to electrical stimulation and any 'wind up' displayed by the neurones (Figure 6.8).

Intrathecal coadministration of 1500 μg L-NAME and 4500μg L-arginine in a total volume of 50 μl did not significantly alter the C fibre response although a 34% reduction in the response was observed which is very similar to that produced by 1500 μg L-NAME alone. The initial responses of the neurones were reduced by coadministration but the 'wind up' of the neurones appeared unaltered (Figure 6.8). The A fibre evoked response was not altered following coadministration although it was inhibited by both L-NAME and L-arginine when administered alone (Figure 6.7).

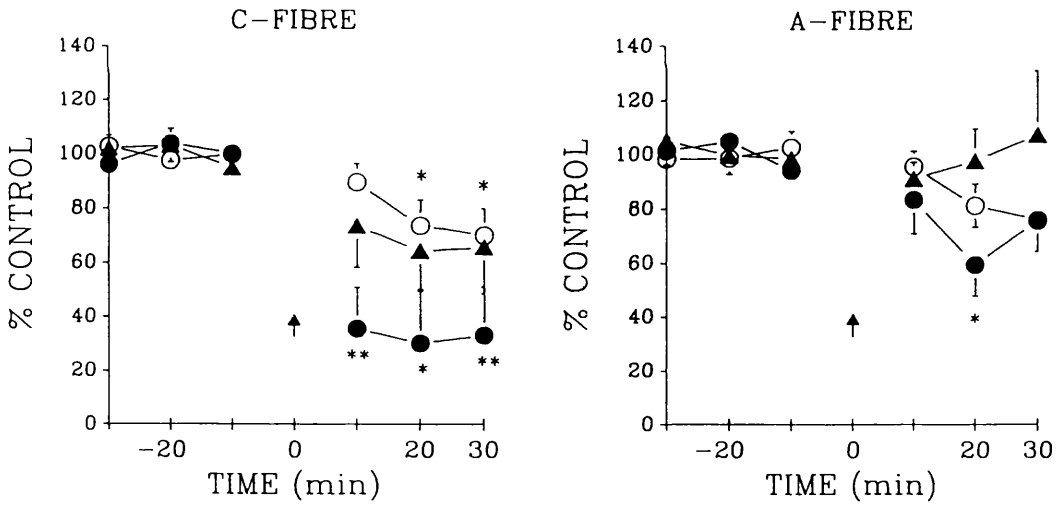

Figure 6.7 Intrathecal administration of 4500 μg L-arginine (●-●) or 1500μg L-NAME (o-o) alone, as indicated by the arrows, inhibits the A and C fibre evoked responses of the neurones whilst coadministration of 4500 μg L-arginine and 1500 μg L-NAME (▲-▲) has no significant effect. n ranges from 6 to 7. *$p<0.05$ **$p<0.025$ Mann-Whitney U test.

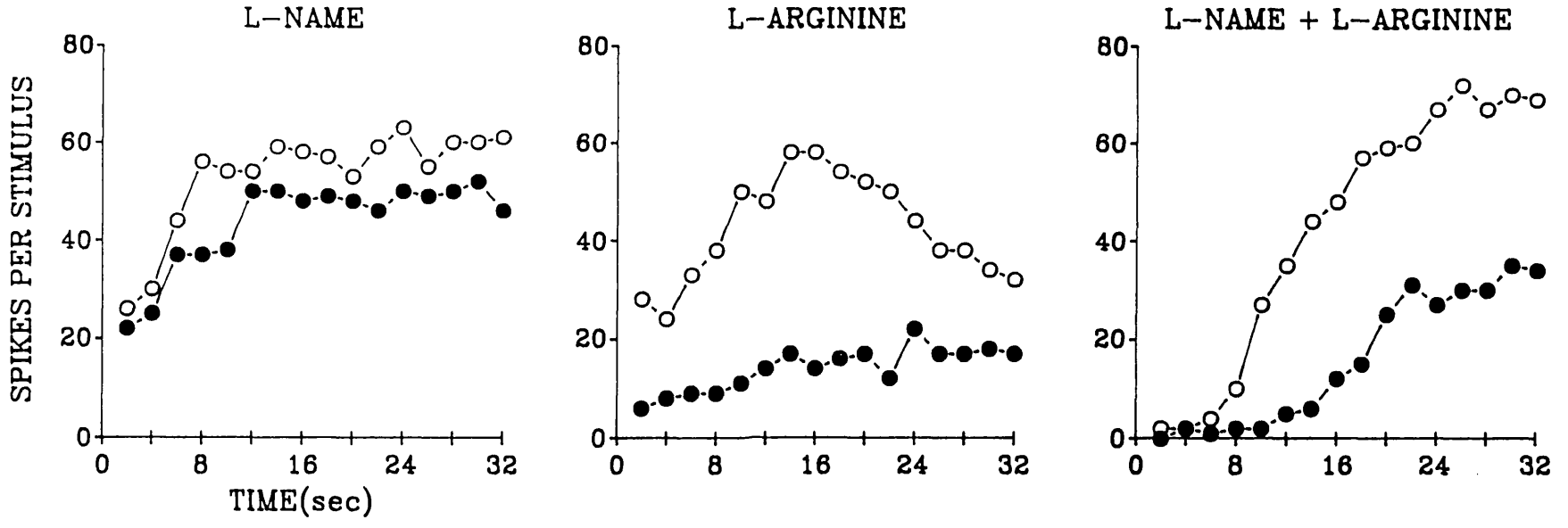

Figure 6.8 Examples of the responses of three dorsal horn neurones to repetitive C fibre strength electrical stimulation of the receptive field. All these neurones increase their responses to the constant peripheral stimulus i.e. they display 'wind up'. Intrathecal administration of L-NAME (1500 μg), L-arginine (4500 μg) or coadministration of both reduced the responses of single dorsal horn neurones without blocking the 'wind up' of the neurones. Control: o-o Treatment: ●-●

6.2.8 Intrathecal L-arginine inhibits the formalin response and is unable to reverse the effects of intrathecal L-NAME

Intrathecal administration of 4500 μg L-arginine 30 minutes prior to formalin administration inhibits the first and second peaks of the response by 65% (p=0.052) and 67% (p=0.082) respectively (Figure 6.9). Intrathecal coadministration of 4500 μg L-arginine and 1500 μg L-NAME in a total volume of 50 μl, 30 minutes prior to formalin, inhibited the first and second peaks of the formalin response by 58% (p=0.074) and 61% respectively although this reduction was not statistically significant (Figure 6.9). The latency to arrival of the second peak was not significantly altered by either treatment (Control: 28.5 ± 6.9 minutes, n=5; L-arginine: 36.1 ± 5.7 minutes, n=6; L-NAME + L-arginine: 34.5 ± 6.4 minutes, n=7).

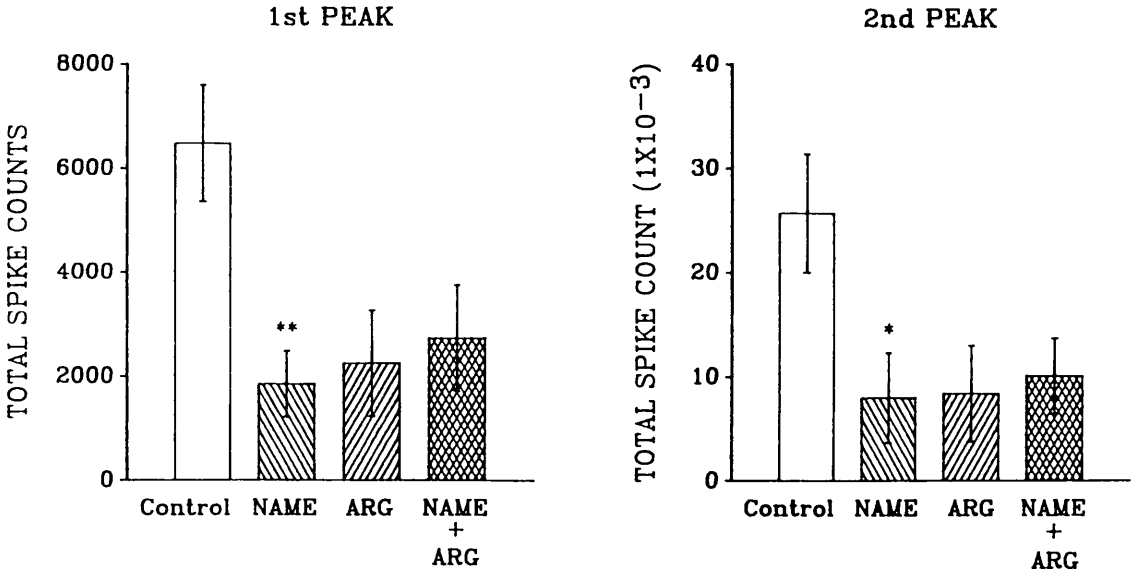

Figure 6.9 Inhibition of both the first and second peaks of the formalin response by intrathecal L-arginine (ARG, 4500 μg), L-NAME (NAME, 1500 μg) and the coadministration of L-arginine + L-NAME 30 minutes prior to formalin administration. n ranges from 5 to 7.

6.3 Discussion

N-nitro-L-arginine methyl ester (L-NAME) is a putative nitric oxide inhibitor (Moore et al.,1990; Rees et al., 1990) being more potent *in vivo* and *in vitro* than the widely used L-N^G-monomethyl arginine (L-NMMA; Gardiner et al., 1990a; Rees et al., 1990). Intravenous, peripheral or intrathecal administration of L-NAME inhibited the second peak of formalin induced activity in dorsal horn neurones. However the effects of L-NAME on the first peak of the response and the electrically evoked A and C fibre responses were more varied.

Intravenous administration of L-NAME had no effect on electrically evoked C fibre responses of the neurones and only a small inhibitory effect on A fibre responses. However, a marked inhibition of the second peak of the formalin response was observed with no significant reduction in the first peak of the response. Using a different nitric oxide inhibitor L-N^G-nitro arginine (L-NOARG) a recent behavioural study in mice has observed preferential inhibition of the second peak of formalin induced nociception although higher doses also reduced the first peak of the response (Hart et al., 1990). L-NOARG administered intraperitoneally is also antinociceptive in the hotplate and abdominal constriction tests in mice (Hart et al., 1990) although L-NMMA administered by the same route does not reduce either prostaglandin E_2 or carrageenan induced hyperalgesia (Duarte et al., 1990).

The inhibition of formalin evoked dorsal horn neuronal activity by L-NAME is unlikely to result from any non-specific events such as changes in blood pressure elicited by the compound since this could be expected to influence both peaks of the response. Although blood pressure has not been monitored in these experiments, previous studies using L-NAME and L-NMMA have demonstrated an increase in basal blood pressure in several species following intravenous and oral administration (Aisaka et al., 1989; Rees et al., 1989; Whittle et al., 1989; Gardiner et al., 1990a,b; Thiemermann and Vane, 1990). This is presumed to be a peripheral vascular effect although the possibility of a central component of action has not been ruled out in these *in vivo* studies. In addition,

L-NMMA has been shown to increase basal ateriole tone in the human and would therefore be expected to increase blood pressure if given systemically in man (Vallance et al., 1989). Hypotension in the cat has been shown to inhibit dorsal horn neuronal activity (Kitahata, 1975) so it is unlikely that the observed neuronal inhibitions result from the predicted hypertensive effects of the agent.

The antinociceptive actions of intravenous L-NAME in formalin evoked dorsal horn neuronal activity may result from either peripheral or central mechanisms of action. Administration of L-NAME directly into the receptive field of the neurones prior to formalin resulted in a significant inhibition of the second peak of the response and whilst the first peak appeared to be inhibited this did not reach statistical significance. This inhibition is unlikely to result from leakage into the systemic circulation as injection of the top dose (1500 μg) of L-NAME into the contralateral paw had no significant effect on either peak of the response. This is also confirmed in the intravenous experiments where much higher doses of L-NAME were required to produce inhibitions of the formalin response than were administered into the receptive field.

The mechanism by which L-NAME is inhibiting the formalin response can only be speculated upon. It is possible that nitric oxide is involved in the activation of C fibres by algogenic substances such as bradykinin but the ability of L-NAME to inhibit vasodilatation could account for the peripheral antinociception observed. The transient inhibition of both the A and C fibre evoked responses following injection of the top dose of L-NAME into the receptive area may account for the inhibition of the first peak. However the responses of the neurones to electrical stimulation had returned to control levels within twenty minutes indicating that the second peak reduction is unlikely to result from this transient inhibition of the A and C fibres.

The second peak of the formalin response is known to result from the development of inflammation in the periphery (Hunskaar and Hole, 1987) and one of the mediators involved is bradykinin (Haley et al., 1989; Shibata et al., 1989). Nitric oxide produces vasodilatation (Ignarro et al., 1987; Palmer et al., 1987) and inhibitors of its synthesis cause vasoconstriction *in vitro* (Sakuma et al., 1988; Rees et al., 1989; Crawley et al., 1990; Moore et al., 1990; Schmidt et al., 1990) and *in vivo* (Vallance et al., 1989;

Gardiner et al., 1990a,b). Bradykinin is formed via cleavage from a larger precursor normally circulating in the plasma; it causes vasodilatation and stimulates cGMP production within vascular smooth muscle which can be inhibited by L-NMMA (Boulanger et al., 1990). Thus the ability of inflammatory mediators such as bradykinin to cause local vasodilatation and presumably plasma extravasation are likely to be inhibited by L-NAME. This could prevent bradykinin and possibly other mediators gaining access to the C fibres. Thus the responses of dorsal horn neurones during the second, inflammatory peak of the formalin response would be reduced.

The antinociceptive effect of peripherally administered L-NAME in the formalin response is in contrast to a recent study investigating the effects of a nitric oxide inhibitor on peripheral hyperalgesia. Injection of the nitric oxide precursor L-arginine directly into the paw reduced the intensity of the hyperalgesia induced by carrageenan but not PGE_2 and this effect was abolished by L-NMMA (Duarte et al., 1990). Furthermore the antinociception induced by peripheral administration of acetylcholine was blocked by L-NMMA implying an antinociceptive role for nitric oxide rather than a nociceptive one. However the antinociception produced by acetylcholine in this study utilizing inflammatory hyperalgesia is unexpected since acetylcholine is known to stimulate C fibres and elicit pain when administered into the periphery (Yaksh and Hammond, 1982).

Intrathecal administration of L-NAME resulted in an inhibition of both peaks of the formalin evoked dorsal horn neuronal activity and a small but significant inhibition of the electrically evoked A and C fibre responses of the neurones. The inhibition of the electrically evoked activity had a slightly delayed onset, appearing to plateau at about 30 minutes. Coadministration of L-NAME with a three times higher dose of L-arginine was unable to reverse the effects of the inhibitor. In addition L-arginine alone produced a significant inhibition in the A and C fibre evoked responses and both the first and second peaks of the formalin response. In agreement with this antinociceptive action of L-arginine, analgesia has been observed in humans upon systemic administration of L-arginine (Takagi et al., 1990) and peripheral administration appears antinociceptive in carrageenan hyperalgesia in rats (Duarte et al., 1990).

The mechanism via which the inhibition produced by L-NAME occurs is not known but one theory may be proposed based on recent work by Garthwaite and colleagues (1988) and Bredt and Snyder (1989). These workers have demonstrated that activation of excitatory amino acid receptors and in particular the NMDA receptor leads to nitric oxide synthesis in the cerebellum. NMDA receptor activation results in calcium ion influx in central neurones (Mayer and Westbrook, 1987) and the generation of nitric oxide is known to be dependent on calcium ions in both endothelial cells (Lückhoff et al., 1988) and the cerebellum (Garthwaite et al., 1988). The involvement of spinal NMDA receptors in the responses of dorsal horn neurones to formalin has been described in the preceding chapter and, by analogy, it is tempting to speculate that a similar mechanism to that found in the cerebellum may be operating in the spinal cord. The inhibition of the formalin response by intrathecal L-NAME implies the generation of nitric oxide within the spinal cord may be involved in the responses of the dorsal horn neurones to this nociceptive stimulus. However whether this antinociception is occurring via inhibition of the effects of NMDA receptor activation within the dorsal horn is not known. Whilst L-NAME inhibits the electrically evoked C fibre activity of the neurones it has no effect on the 'wind up' displayed by these neurones and this is in contrast to the inhibitions produced by NMDA antagonists. Whereas NMDA antagonists reduce the 'wind up' of neurones without influencing the initial responses L-NAME reduced the initial responses but was less effective at inhibiting 'wind up'. Opioids exhibit a similar profile of inhibition, reducing the initial responses of a cell but failing to alter any 'wind up' (Dickenson and Sullivan, 1986; Dickenson and Sullivan, 1987b).

The source of nitric oxide within the central nervous system has not been determined. However it has been postulated that in the cerebellum nitric oxide may be released from neurones (Garthwaite et al., 1988; Dickie et al., 1990). Within the spinal cord release from neurones may occur but it is possible the source could be vascular since the spinal artery can produce EDRF in response to acetylcholine (Shirai et al., 1990). As the half life of nitric oxide is only a few seconds (Ignarro et al., 1987; Palmer et al., 1987) the generating cell would need to be close to the site of action of nitric oxide

and this would favour neuronal release or generation.

Nitric oxide is known to stimulate guanylate cyclase , resulting in the generation of cGMP in endothelium (Furchgott, 1984; Marletta, 1989; Boulanger et al., 1990) and the brain (Miki et al., 1977; Garthwaite et al., 1988; Bredt and Snyder, 1989; Knowles et al., 1989). The actions of this second messenger are not as well characterised as cAMP (Drummond, 1984) and the changes that may occur as a result of its production within cells are therefore hard to predict. However intrathecal administration of dibutyryl cyclic guanosine monophosphate (DBcGMP), a stable cGMP analogue, produces antinociception in the rat tail-flick test and inhibits the activity of ascending axons resulting from peripheral C fibre activation (Jurna, 1984). The antinociceptive actions of L-arginine may therefore result from the stimulation of cGMP production within the spinal cord. L-NAME appears to inhibit the effects of L-arginine implying the inhibitions produced by the precursor are via the generation of nitric oxide and this then presents a confusing picture regarding the mechanism of action of L-NAME. The doses of drugs used in these experiments are extremely high but similar to those used in behavioural and *in vivo* studies. In addition, the selectivity of L-NAME has not yet been evaluated and whilst both L-NAME and L-NMMA have been shown to inhibit the synthesis of nitric oxide from its precursor possible actions of these compounds in other systems have not been fully investigated.

Thus whilst the responses of dorsal horn neurones to peripheral formalin administration can be modulated by a putative inhibitor of nitric oxide synthesis they can also be reduced by a precursor of nitric oxide synthesis. These apparently contradictory results are in agreement with behavioural studies in both rodents and humans (Duarte et al., 1990; Hart et al., 1990; Takagi et al., 1990). The antinociceptive effects of L-NAME occur both in the periphery where it may be acting on the vasculature, and spinally where it could be preventing events occurring at the synapse following NMDA receptor activation. Thus nitric oxide may be involved, in some complex way, in nociceptive events both in the periphery and within the spinal cord.

CHAPTER 7:

PERIPHERAL OPIOID MODULATION OF THE FORMALIN RESPONSE

7.1 Introduction

Opioid receptors, following synthesis in the cell bodies of dorsal root ganglion cells, are transported along the projecting axons towards both the central and peripheral nerve terminals (Young et al, 1980; Laduron, 1984). The ability of opioids to inhibit nociceptive events at the spinal cord level, in addition to their supraspinal effects, is now well established (Yaksh and Noueihed, 1985) and it is presumed that this action is at least partly via activation of receptors located on the presynaptic terminals of these bipolar neurones (Lamotte et al., 1976; Besson and Chaouch, 1987).

More recently attention has also focussed on the role of the opioid receptors located on the peripheral terminals of afferent neurones. Whilst the effect of mu agonists on afferent fibre axons under normal conditions remains controversial (Jurna and Grossman, 1977; Senami et al., 1986) there is increasing evidence supporting a peripheral antinociceptive site of action for opioids in inflammatory states. Studies utilizing *in vivo* models of inflammation have demonstrated that mu, delta and kappa receptor subtypes are effective at producing antinociception via a peripheral mechanism (Ferreira and Nakamura, 1979b, Ferreira et al., 1982; Russell et al., 1987; Hargreaves et al., 1988; Stein et al., 1989; Levine and Taiwo, 1989). One exception is a behavioural study with formalin where kappa but not mu agonists possessed peripheral antinociceptive actions (Abbott, 1988). Studies investigating the peripheral release of substance P and bradykinin have demonstrated the ability of mu and delta agonists to inhibit this release but differ in the effects observed with kappa agonists (Inoki et al., 1980; Brodin et al., 1983; Smith and Buchan, 1984; Lembeck and Donnerer, 1985; Yaksh, 1988; Yonehara et al., 1988). Although the profile of opioid agonist inhibition in these peripheral studies differs widely, this is mirrored by the variety of nociceptive models used in the studies.

This study was performed in collaboration with Steven Ketchum from The Sandoz Institute for Medical Research. The aim was to investigate the ability of opioids to inhibit this electrophysiological model of inflammatory nociception via a peripheral mode

of action, using selective agonists at the opioid receptor subtypes.

7.2 Results

7.2.1 Inability of peripheral mu and delta opioid agonists to inhibit the formalin response

Neither the mu agonist morphine nor the delta agonist DSTBULET, administered as a pretreatment directly into the site of formalin injection, had any significant effect on either peak of the response. Morphine (100μg) at a dose slightly lower than would produce systemic effects (Le Bars et al., 1983) did not influence either the first or the second peak (Figure 7.1) and did not alter the latency to the second peak (control: 33.6 ± 5.9 ; morphine: 35.2 ± 5.7 minutes).

DSTBULET (100μg) had no significant effect on either the first or second peaks of the response (Figure 7.2). However, this compound resulted in a small, but significant, increase in latency to the second peak (control: 20.1 ± 1.8 ; DSTBULET: 34.2 ± 4.5 minutes, p=0.02). It is possible that DSTBULET may be degraded following injection into the receptive field and that higher doses of this peptide could inhibit the formalin response, however this has not been investigated further.

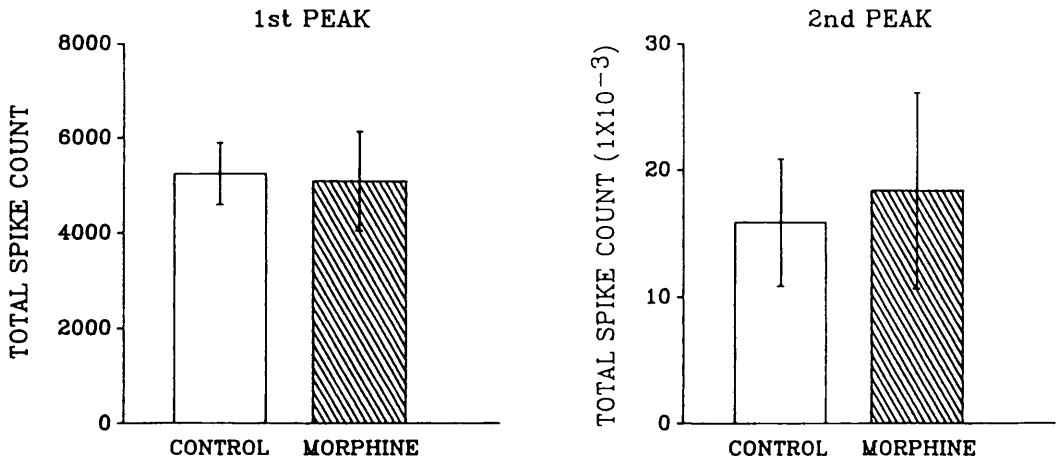

Figure 7.1 The mu receptor agonist morphine (100μg) injected directly into the peripheral receptive field as a 10 minute pretreatment had no effect on either the first or second peak of the formalin response. n ranges from 10 to 11.

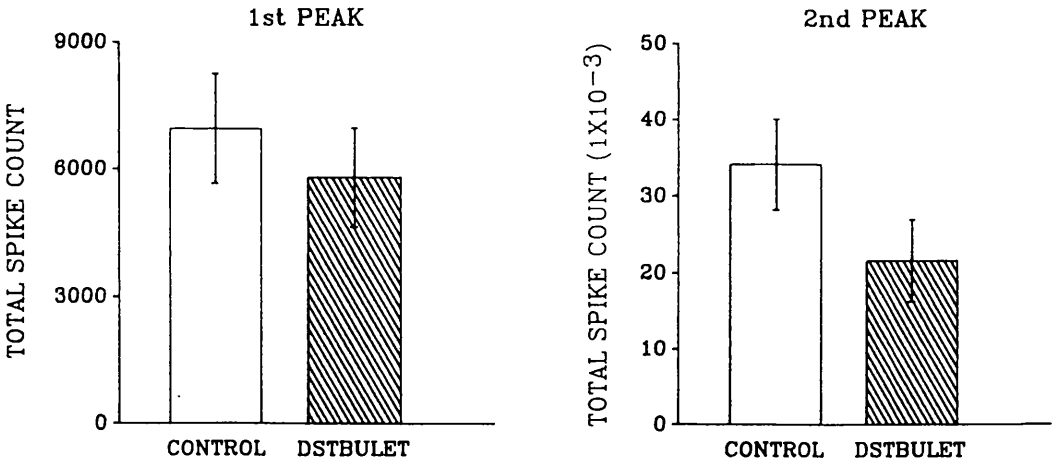

Figure 7.2 The delta receptor agonist DSTBULET (100μg) injected directly into the peripheral receptive field as a 10 minute pretreatment had no significant effect on either the first or second peak of the formalin response. n ranges from 13 to 14.

7.2.2 Peripheral (s.c.) administration of U50488H inhibits the formalin response

Injection of the kappa agonist U50488H alone into the receptive field resulted in a peak of activity that was smaller and of shorter duration than that produced by H_2O, although this difference was not statistically significant. The mean count for H_2O alone was 3669 ± 2032 (n=6) compared to 489 ± 163 (n=5) for 100μg U50488H alone. The duration of the H_2O peak was 4.10 ± 1.21 min whilst the U50488H peak was 1.74 ± 0.17 min.

Administration of U50488H directly into the receptive field 10 minutes prior to formalin resulted in a dose-related reduction of both the first and second peaks of the response (Figures 7.3 and 7.4). Both the phases of the response were inhibited to a similar extent with the top dose of 100μg U50488H producing 87% and 88% inhibitions of the first and second peaks respectively. 100μg U50488H had no significant effect on the latency to the arrival of the second peak of the formalin response (Control: 20.2 ± 2.8 ; U50488H: 29.3 ± 8.1 minutes).

This inhibition of formalin evoked activity appears to be the result of a local, peripheral action of U50488H since administration of 100μg of the drug into the contralateral paw 10 minutes prior to formalin had no influence upon the subsequent formalin response (Figure 7.4).

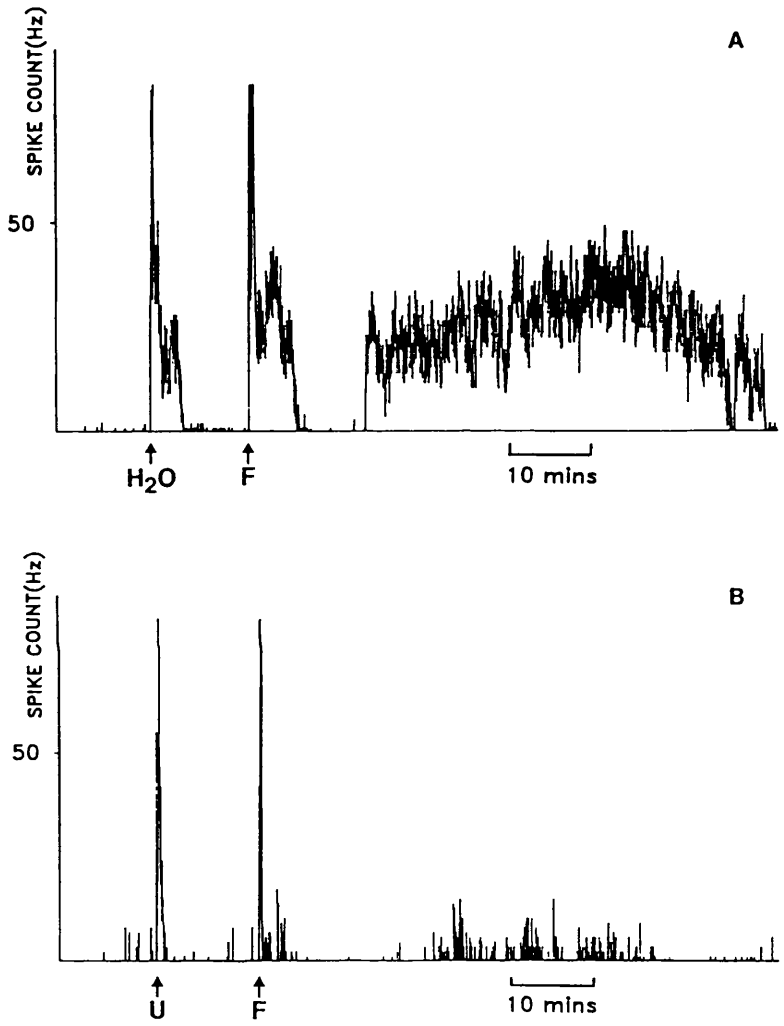

Figure 7.3 Ratemeter recordings showing the rate of firing (Hz) of single dorsal horn neurones in response to subcutaneous formalin (F). In control experiments H_2O was administered into the toe 10 minutes prior to formalin (A). Pretreatment with 100μg U50488H (U) inhibited both the first and second peak of the subsequent formalin response (B).

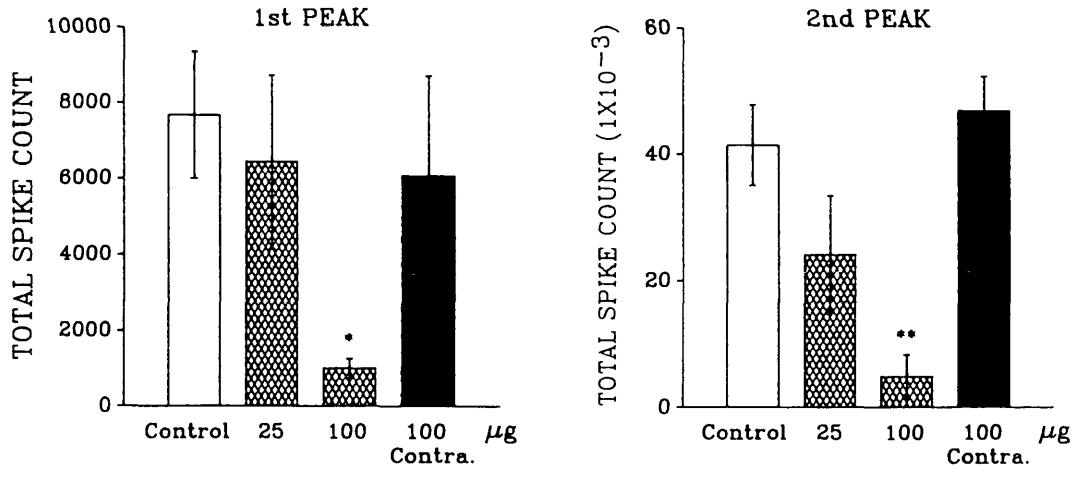

Figure 7.4 Inhibition of both the first and second peaks of the formalin response with U50488H injected into the receptive field 10 minutes prior to formalin. Injection of the top dose of U50488H into the contralateral paw (Contra.) had no effect on the subsequent formalin response. n ranges from 3 to 7. *p=0.01 **p=0.006, Mann Whitney U-test.

7.2.3 Reversal of the kappa opioid inhibition with naloxone

Naloxone (100μg) injected directly into the plantar region of the paw 12 minutes prior to formalin (2 minutes prior to distilled water or U50488H) reversed the effects of U50488H on both peaks of the response whilst having no influence on either peak when administered alone (Figure 7.5). Intraplantar naloxone alone did not alter the latency to arrival of the second peak (control: 28.1 ± 5.5 ; naloxone: 24.6 ± 3.8 minutes).

Naloxone administered i.p. as a 20 minute pretreatment partially, but non-significantly, reversed the effects of peripherally administered 100 μg U50488H without influencing the response when administered alone (Figure 7.6). The lack of statistical significance probably results from the very large standard errors present on both the treatment and control groups due to the inherent variability in neuronal activity.

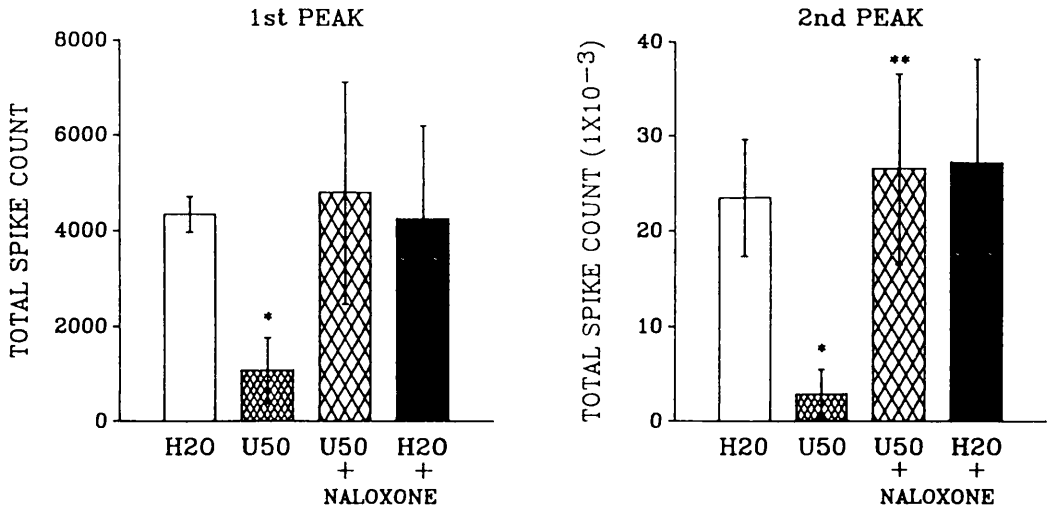

Figure 7.5 Reversal by intraplantar naloxone of the U50488H (U50) inhibition of the first and second peaks of the formalin response. 100μg naloxone was administered into the plantar region of the paw 2 minutes prior to U50488H administration into the toe. Whilst naloxone completely reversed the effect of 100μg U50488H (U50+NALOXONE) it had no effect on the formalin response when administered by itself (H2O+NALOXONE). n ranges from 4 to 9. *p≤0.05 compared to control group **p=0.03 compared to U50488H alone group, Mann Whitney U-test.

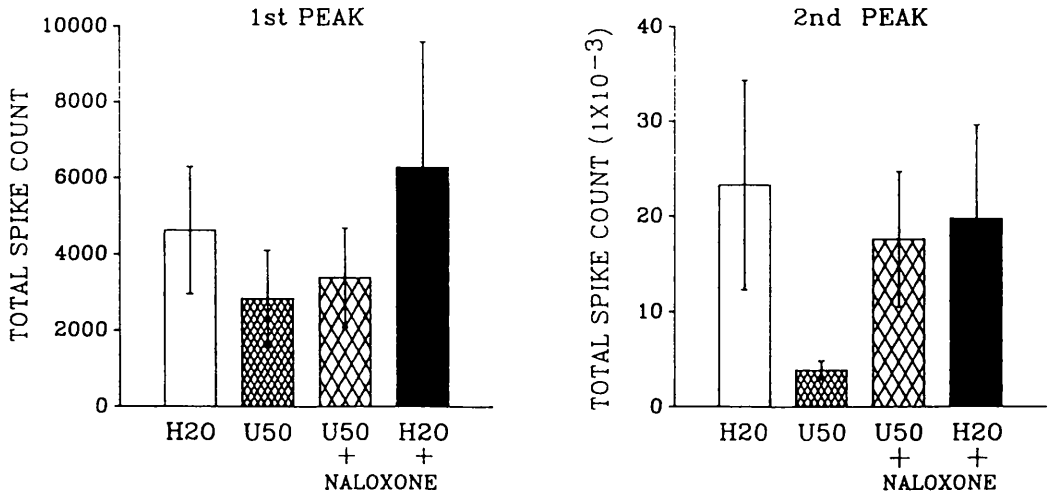

Figure 7.6 The effect of 10mg/kg i.p. naloxone, administered 20 minutes prior to formalin, on the inhibition produced by administration of 100μg U50488H directly into the receptive field, 10 minutes prior to the chemical stimulus. See above legend for abbreviations

7.2.4 Lack of effect of U50488H on electrically evoked A and C fibre neuronal responses

The electrically evoked A and C fibre responses of the neurones were not inhibited by injection of 100μg U50488H into the receptive field (and area of stimulation) (Figure 7.7) despite having a profound inhibitory effect on the formalin response at this dose. 100μg U50488H resulted in C fibre and A fibre responses that were respectively 119 ± 9 % and 101 ± 9 % of the control response 40 minutes after injection. Distilled water injected into this area was also ineffective in altering either C or A fibre evoked responses which were 96 ± 8 % and 84.2 ± 14 % of control respectively (Figure 7.7).

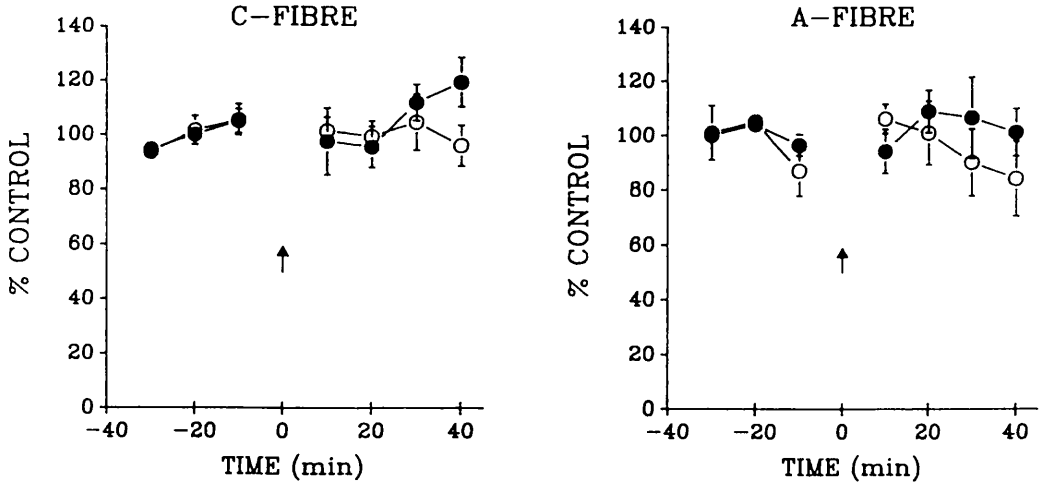

Figure 7.7 No inhibition of the electrically evoked A and C fibre activity of dorsal horn neurones was seen with either H_2O (O-O) or 100μg U50488H (●-●) when injected directly into the site of stimulation within the receptive field (arrow) (n=6).

7.2.5 U50488H inhibits responses to exogenous peripheral bradykinin

U50488H (100μg) administered directly into the site of bradykinin injection, as a ten minute pretreatment, produced an 83% inhibition in the response of dorsal horn neurones to the subsequent injection of bradykinin (10μg in 10μl) (Figure 7.8). However this reduction was not statistically significant due to the presence of large standard errors on the control group, thus reflecting the variability in the responses of neurones to bradykinin. A rapid loss in the response to exogenous bradykinin was observed within two or three injections and this may mask the effects of U50488H on all except the initial bradykinin response (Figure 7.8).

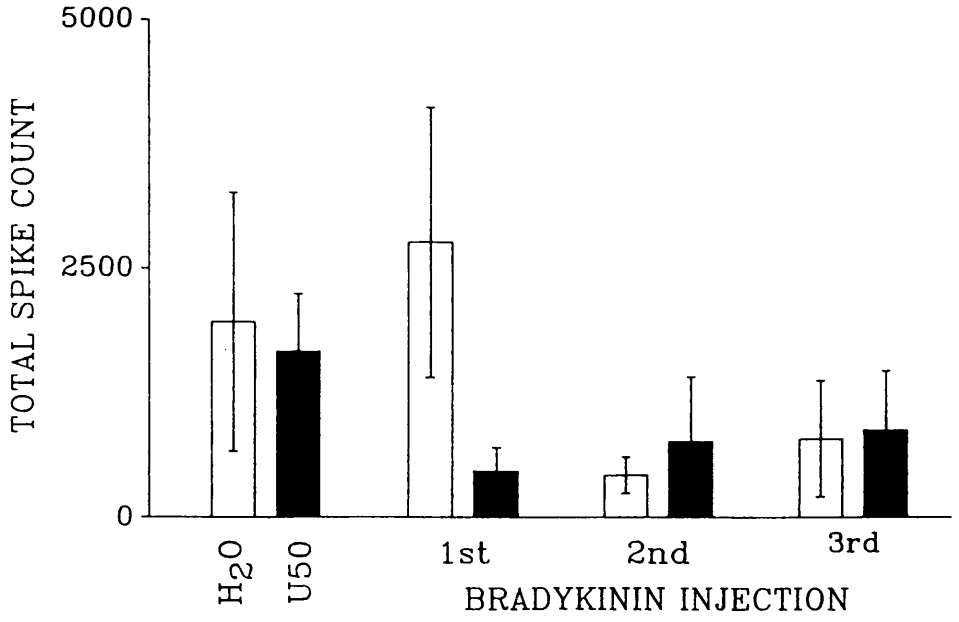

Figure 7.8 U50488H (100μg) administered directly into the receptive field 10 minutes prior to bradykinin inhibited the response of the neurones to peripheral injection of bradykinin (10μg). n is 5 for both control and U50488H groups.

7.2.6 Inability of U50488H to inhibit the plasma extravasation evoked by formalin

Injection of formalin into a toe on the rat hindpaw resulted in plasma extravasation in the area local to the injection site and in distinct zones on the glabrous side of the hindpaw, measured as Evans blue accumulation in the skin. Evans blue extravasation was also observed in the muscle beneath the skin but this was not quantified. Pretreatment with 100μg U50488H injected into the toe 10 minutes prior to formalin did not alter the amount of Evans blue that had accumulated in the skin two hours later. Control paw: 114 ± 18 ng dye/mg tissue, U50488H paw: 110 ± 15 ng/mg (n=6) (Figure 7.9). Interestingly, U50488H alone produced significantly greater Evans blue accumulation than was seen with H_2O alone. H_2O: 49 ± 5 ng/mg , U50488H: 69 ± 9 ng/mg (n=4; $p<0.05$) (Figure 7.9). The Evans blue accumulation produced by formalin (with either H_2O or U50488H pretreatment) was significantly greater than that produced by either H_2O or U50488H alone.

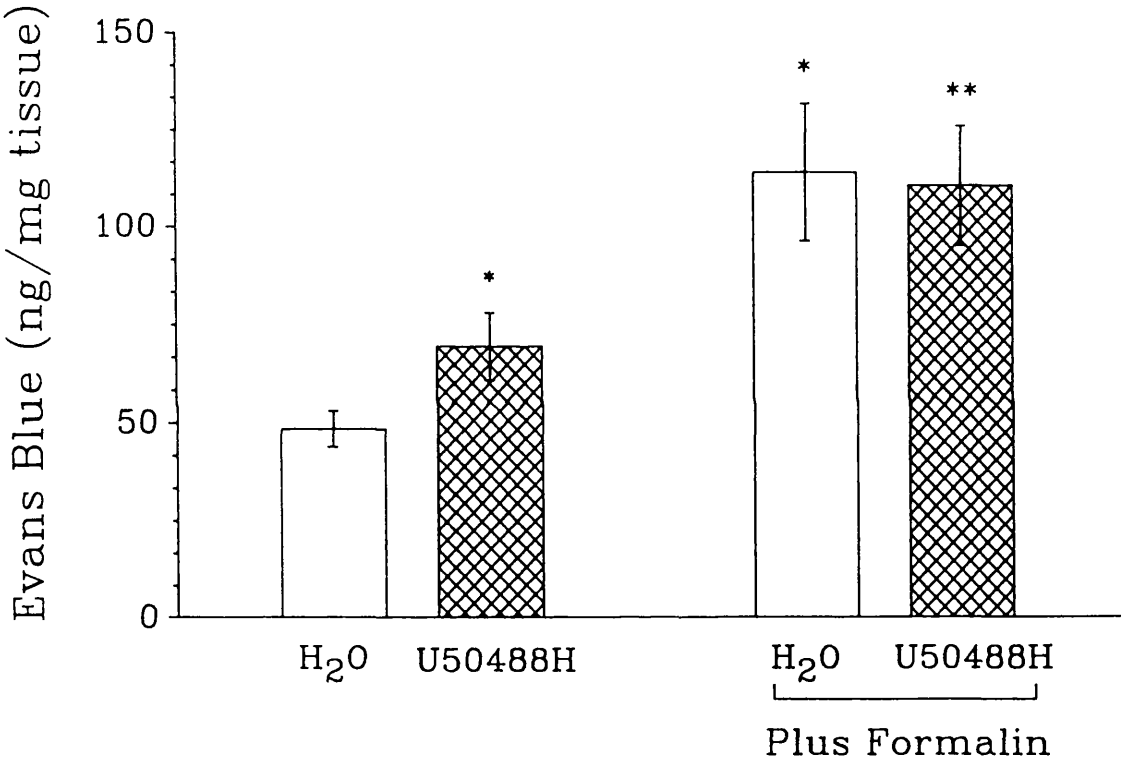

Figure 7.9 Plasma extravasation measured using Evans blue leakage into the skin. 100μg U50488H injected into a toe resulted in a greater amount of extravasation than distilled H_2O alone, but had no effect on the formalin evoked extravasation. (n ranges from 4 to 6). *$p< 0.05$ compared to H_2O alone; **$p< 0.05$ compared to U50488H alone, Student t-test

7.3 Discussion

When administered peripherally the kappa agonist U50488H inhibited both peaks of the formalin response whilst neither the mu agonist morphine nor the delta agonist DSTBULET were able to produce any significant alteration. This inhibition of formalin evoked activity appears to be the result of a local, peripheral action of U50488H since administration of the drug into the contralateral, non-inflamed paw 10 min prior to formalin had no influence upon the subsequent formalin response. It is possible that systemic leakage of U50488H may occur in the formalin inflamed tissue but not in normal tissue. However it should be noted that U50488H is able to inhibit the first peak of the formalin response which occurs prior to the generation of inflammation. In addition the systemic dose required to inhibit thermal, mechanical and electrically evoked C fibre responses of dorsal horn neurones is about 4mg/kg (Parsons and Headley, 1989; Sullivan and Dickenson, 1991), ten times the peripheral dose administered. As the formalin response and electrically evoked activity appear equally sensitive to intrathecal U50488H it is unlikely that U50488H is exerting any actions resulting from leakage into the systemic circulation (Sullivan and Dickenson, 1991).

The ability of naloxone to reverse the inhibitions produced by U50488H indicates that the effects result from an opioid action of this compound and are not the result of non-opioid effects which have been reported in other circumstances (Hayes et al., 1988). Naloxone alone, administered either via intraperitoneal or intraplantar routes had no effect on either phase of the formalin response at doses that were able to either fully or partially reverse the effects of peripheral U50488H. Thus, neither peripheral nor central endogenous opioid systems appear to be activated over the duration of this response, confirming previous findings in behavioural studies with formalin in the rat (North, 1977; Kocher, 1988). In addition, no peripheral effects of naloxone were observed in a model of unilateral arthritis (Stein et al 1989).

The emergence of endogenous opioid systems in models of prolonged inflammation has been proposed by several groups to explain both the *hyperalgesic*

effects of high doses of systemic naloxone and the *analgesic* effects of low doses of naloxone (Kayser and Guilbaud, 1981). It is not possible to determine from these studies if the site of action of the opioid antagonist is central or peripheral. However, only *analgesic* effects of opioid antagonists have been directly attributed to a peripheral mode of action although this effect may not result from antagonism of an endogenous opioid system (Rios and Jacob, 1982, 1983; Ferreira and Nakamura, 1979b, c).

The lack of effect of i.p. naloxone on the formalin response may result from the dose used. However a large range of lower doses have also proved to be ineffective in inhibiting the formalin response in the rat although analgesia with naloxone has been observed in the mouse (Kocher, 1988; Vaccarino et al., 1988). The antinociceptive effect of naloxone appears to be dependent on both the presence of an inflammatory state (Kayser and Guilbaud, 1981) and the duration of the inflammation as the analgesia is barely detectable at 1 hour (Kayser et al., 1988). The short time course of the formalin response may therefore explain the lack of effect of this opioid antagonist when administered alone.

The inability of peripherally administered U50488H to inhibit the electrically evoked A and C fibre responses of these cells tends to confirm that the effects are the result of a specific action of the compound rather than a local anaesthetic action. Furthermore, this also implies that the mode of action of U50488H must result from events occurring prior to peripheral nerve conduction since the electrical stimulus will activate axons and terminals.

The inability of U50488H to alter the formalin induced plasma extravasation in the paw indicates that its antinociceptive actions are unlikely to result from inhibition of mediator invasion from the plasma. Furthermore, it cannot be blocking axonal reflex induced release of vasoactive mediators or their subsequent vascular actions. Previous studies investigating the ability of opioids to inhibit axon reflex mediated events such as substance P or bradykinin release, plasma extravasation and oedema formation, all demonstrate the ability of mu agonists to inhibit these events but differ in their findings with kappa agonists. Using antidromic nerve stimulation to elicit either mediator release or plasma extravasation, some groups have observed inhibitions with the mixed mu and

kappa agonist EKC (Smith & Buchan, 1984; Russell et al., 1985; Yonehara et al, 1988) whilst another group reported no effect with the selective kappa agonist U50488H (Yaksh, 1988). However U50488H was able to inhibit the plasma extravasation elicited by carrageenan, a model of inflammation with a large neurogenic component (Russell et al., 1985). U50488H administration into the periphery in the absence of formalin resulted in a significantly greater amount of plasma extravasation than seen with the vehicle alone. This may result from direct excitation of the peripheral nerve terminals prior to inhibition as seen in cultured dorsal root ganglion cells (Crain & Shen, 1990) or perhaps is more likely to result from indirect mechanisms such as mast cell degranulation (Liu et al., 1983; Casale et al., 1984).

The mechanism by which peripheral U50488H is inhibiting the formalin evoked activity in these spinal neurones is unclear but it could be blocking the action of peripheral mediators on the C fibre terminals. Evidence for this possible mode of action was observed in the bradykinin experiments where peripheral U50488H was able to inhibit the activity elicited by exogenous administration of bradykinin into the receptive field. This implies U50488H is effective in modulating the actions of a peripheral mediator without necessarily inhibiting its generation or release.

Additional evidence for a direct action of U50488H on the nerve terminals was observed in the electrophysiological experiments where the formalin evoked first peak of neuronal activity was inhibited to the same extent as the second peak. Since it is generally felt that the first few minutes of activity evoked with formalin are the result of a direct action of the chemical on the free nerve endings (Hunskaar and Hole, 1987; Haley et al., 1989; Shibata et al., 1989), inhibition of this phase is presumably more likely to result from a direct action of the opioid on these endings rather than via an indirect mechanism such as inhibition of the axon reflex. The inhibition by mu and kappa opioid agonists of the spontaneous activity in unmyelinated afferent fibres resulting from inflammation in the knee joints of cats has also been attributed to a direct action on the nerves (Russell et al., 1987).

Very little is known about the transduction mechanisms involved in the transformation of pain producing events such as heat, pinch and inflammation into action

potentials carried by the peripheral nerve. The action of the inflammatory mediator bradykinin on dog polymodal receptors has been shown to be calcium dependent although this is likely to result from an indirect mechanism (Sato et al., 1989). Peripheral hyperalgesia produced by a number of agents has been postulated to involve calcium at the peripheral terminal and local administration of calcium channel blockers appeared to reduce this hyperalgesia (Ferreira and Nakamura, 1979a). Interestingly, activation of the kappa receptor has been shown to inhibit the influx of calcium into dorsal root ganglion cells via the closure of calcium conducting ion channels (North, 1986). If this mechanism was operating at the peripheral terminal it may in some way interfere with the transduction processes in peripheral nerve endings.

The inability of peripherally administered mu, delta or kappa opioid agonists to alter nociception following acute, non-inflammatory, short duration stimuli such as hotplate, tail-immersion or paw pressure tests has been demonstrated by several groups (Smith et al., 1982; Abbott, 1988; Levine & Taiwo, 1989; Stein et al., 1989) and is confirmed in this study by the inability of peripheral U50488H to alter the electrically evoked A and C fibre responses of the neurones. In addition, peripheral bradykinin release following pinching or heating of the skin is not blocked by morphine (Inoki et al., 1980). However mu, delta and kappa agonists do appear effective at inhibiting acetic acid induced writhing in mice (Bentley at al., 1981; Smith et al., 1982). These results and previous findings (Abbott, 1988) indicate that the short duration (about 60 minutes) inflammatory response to formalin is sensitive to inhibition by kappa but not mu or delta opioid agonists administered peripherally. Several groups have shown that longer duration nociceptive responses (ranging from a few days with carrageenan inflammation to weeks with Freund's Adjuvant inflammation) are inhibited by agonists at mu, delta and kappa receptors (Ferreira & Nakamura, 1979b, Ferreira et al., 1982; Russell et al., 1987; Hargreaves et al., 1988; Stein et al., 1989).

These apparent alterations in the profile of peripheral opioid inhibitions with duration of inflammation are intriguing and perhaps suggest that changes over time occur in the proportions of opioid receptor subtypes present or active at the peripheral

terminals. The idea of changes occurring in spinal opioid receptor populations following chronic inflammatory conditions has been proposed to explain the increased agonist sensitivity of these animals to systemic mu, delta and kappa opioid agonists; however, no alteration in spinal opioid receptor binding was observed (Cesselin et al., 1980; Iadorola et al., 1988; Millan et al., 1988; Delay-Goyet et al., 1989). In the absence of any increase in the binding capacity of spinal opioid receptors it is possible that the explanation for the increased sensitivities to these ligands lies in the emergence of peripheral sites of action during prolonged inflammatory events. The rapid onset of the formalin response (within a few seconds) and the ability of U50488H to inhibit all phases of activity suggest the receptor mediating this inhibitory action must already be present in an active form. It is possible that the mu and delta receptors mediating inhibition in more prolonged inflammatory states take time to either become active or perhaps to be synthesized and transported along the axon (Young et al., 1980; Laduron, 1984) to reach their peripheral site of action. However, the ability of both mu and delta opioid agonists to inhibit antidromic stimulated plasma extravasation and release of mediators in the absence of an inflammatory state, argues for the presence of active forms of these receptors as well. Perhaps the variable profile of opioid inhibition simply reflects the different peripheral stimuli present. Events not involving mediators such as electrical, thermal or mechanical stimulation may be resistant to inhibition compared to chemical or peptide mediated activation of afferent fibres. Thus whilst peripheral opioid receptors may always be present in an active form, both the subtype proportions and their effectiveness may depend on the nature of the peripheral stimulus.

As previously discussed, it is uncertain whether or not these peripheral opioid receptors have a physiological role during nociceptive events. If such a role exists the endogenous ligand and its origin are as yet unknown, however it could arrive from the systemic circulation or from the primary afferent neurones themselves. The levels of circulating ß-endorphin in rats have been shown to increase following the induction of arthritis in these animals (Millan et al., 1988) and access to the afferent terminals from the circulation would be aided by the formalin stimulus and by other inflammatory stimuli such as carrageenan (Russell et al., 1985; Hargreaves et al., 1988) since they

induce vasodilatation and plasma extravasation. The inability of the kappa agonist to inhibit this formalin induced extravasation would result in an access route that was unhindered by its own mechanism of action. However the presence of dynorphin gene products within sensory afferent fibres and dorsal root ganglion cells in both guinea pigs and mice (Weihe et al., 1985; Sweetnam et al., 1986) suggests that the endogenous kappa opioid ligand may be present locally, within the afferent fibres themselves. Once released they could then act on the afferent terminals to inhibit activation of the fibres.

The profile of inhibition of the formalin response by peripherally administered opioids differs markedly from that seen with spinally applied opioids as described in the previous chapter. Following intrathecal administration , mu and delta agonists are very potent at inhibiting both phases of the formalin response (Dickenson and Sullivan, 1987a; Sullivan et al., 1989) whereas kappa agonists are less effective (Sullivan and Dickenson, 1991). By contrast peripheral administration of the kappa agonist inhibits both peaks of the response whereas the mu and delta agonists are ineffective.

CHAPTER 8:

CONCLUSIONS

This *in vivo* study in the rat has attempted to elucidate some of the mechanisms involved in prolonged nociceptive responses, compared to those underlying responses to more acute stimuli. Electrophysiological recordings of the activity of convergent neurones located in the lumbar dorsal horn of the spinal cord was used as a measure of nociception. Many of these neurones form part of the ascending spinothalamic tract and their responses correlate well with the behaviour observed in conscious animals following noxious stimuli (Dubner et al., 1981).

The response elicited in these neurones following peripheral administration of a formalin solution is a useful model for comparing the mechanisms underlying both acute and more prolonged activation. Formalin injection into the receptive field results in a very distinctive biphasic profile of neuronal firing. The initial phase of activity is of short duration, being complete within five to ten minutes. Following a period of silence the neurone suddenly begins firing again, usually about 25 minutes following the formalin injection. This second peak of activity is more prolonged, usually lasting for about 40 minutes. Both the biphasic profile of firing and the time course of the response are very similar to the nociceptive behaviour observed in the conscious animal (Dubuisson and Dennis, 1977). The distinct separation of the first and second peak allows discrete investigation of the mechanisms underlying acute and prolonged events elicited by the same stimulus. Direct comparison of these peaks may therefore reveal alterations in the basis of the nociceptive responses of these cells. In addition, the duration of the formalin response (about 60 minutes) is ideal for electrophysiological experiments as it allows the entire response to be monitored. Interestingly there is no relationship between the electrically evoked properties of the neurones (e.g. stimulation threshold or size of the response elicited) and the size of the neuronal responses to formalin. Thus complex events may be occurring within the spinal cord which result in responses that are not directly related to the afferent input.

Excitatory amino acids appear to play a crucial role in the responses of dorsal horn neurones to nociceptive stimuli and may form part of an amplification mechanism within the spinal cord. The non-selective excitatory amino acid receptor antagonist DGG

clearly reduced both the electrically evoked A and C fibre responses and the activity evoked by formalin. By contrast the selective NMDA antagonists had no overall effect on either the A or C fibre evoked responses or the first peak of the formalin response. However both the C fibre mediated 'wind up' of these neurones and the second peak of the formalin response were reduced by NMDA antagonists. This confirms the findings of previous studies where selective NMDA antagonists reduced the 'wind up' but not the initial responses of the neurones to C fibre inputs (Davies and Lodge, 1987; Dickenson and Sullivan, 1987b). Furthermore the selective reduction of the formalin response second peak by NMDA antagonists implies the involvement of an amplification mechanism similar to 'wind up' in the prolonged responses of these neurones to this chemical stimulus. This augmentation via NMDA receptor involvement of a non-NMDA mediated response appears very similar to the mechanisms underlying long term potentiation (LTP) in the hippocampus. LTP is a long duration enhancement of synaptic transmission which results from a high frequency stimulation of the afferent inputs into the hippocampus. The induction of LTP is frequency dependent and blockade of the NMDA receptor prevents the induction of this enhanced state without altering normal synaptic transmission (Bliss and Lynch, 1988).

Thus within the dorsal horn of the spinal cord a shift of emphasis is apparent as the peripheral inputs become more prolonged. The involvement of non-NMDA receptors in the initial short duration first peak, persists during the prolonged phase but the range of receptors the transmitter (glutamate and/or aspartate) can activate expands to include the NMDA receptor. The involvement of the NMDA receptor may result from an increase in the underlying state of depolarization of the neurones leading to a removal of the tonic magnesium block normally associated with this receptor (MacDermott and Dale, 1987). This depolarization could arise from the continual activation of glutamate/aspartate receptors and/or the actions of peptides such as substance P which cause slow long lasting depolarizations in these neurones (Urbán and Randic, 1984). Substance P often coexists with glutamate in the primary afferent fibres (Battaglia and Rustioni, 1988; DeBaisi and Rustioni, 1988) and both are released following formalin activation (Kantner et al., 1986; Skilling et al., 1988). Recently substance P has been

shown to potentiate currents evoked by NMDA, but not AMPA or kainate, in isolated dorsal horn neurones. These two separate systems could therefore act in concert to augment afferent nociceptive inputs within the spinal cord (Randic et al., 1990) and selective antagonists at substance P receptors may therefore prove to be antinociceptive in prolonged nociceptive events. Thus during the prolonged phase of the response a, perhaps small, afferent input may be impinging upon an amplification system within the spinal cord. Such mechanisms may underlie central post-injury hypersensitivity events (Woolf, 1983).

Changes such as these may be of relatively short duration compared to the induction of alterations in gene transcription. Noxious stimulation, including formalin, *in vivo* in both conscious and anaesthetized animals elicits the generation of c-fos within the dorsal horn (see Fitzgerald, 1990). C-fos is a protein marker for gene transcription and is also expressed following central plasticity events such as long term potentiation in the hippocampus which also involves NMDA receptor activation (Bliss and Lynch, 1988; Wisden et al., 1990).

Numerous studies have demonstrated the ability of opioids to inhibit the C fibre evoked responses of dorsal horn neurones. However opioid reduction of the C fibre mediated 'wind up' of these neurones is less effective and the 'wind up' usually overcomes the inhibition (Dickenson and Sullivan, 1986). Interestingly the ability of intrathecal opioids to inhibit the formalin response is reduced if they are administered after the injection of formalin, again suggesting the involvement of mechanisms similar to 'wind up' during the prolonged second peak of the response. Thus alterations occur within the spinal cord following a prolonged noxious stimulus that may reduce the effectiveness of opioids suggesting that the pre-emptive use of opioids may be therapeutically useful. The involvement of the excitatory amino acids in both acute and more prolonged nociceptive events indicates a potential for the development of therapeutically useful analgesics. Clearly the use of either non-selective antagonists or non-NMDA antagonists is likely to be disastrous since both A and C fibre evoked responses are reduced. This would be undesirable for the patient and could lead to death. Alternatively the development of selective NMDA antagonists may be clinically

useful since only the nociceptive inputs would be reduced. However problems are known to exist with currently available antagonists as they produce paralysis in animals and amnesia and psychomimetic effects in man at doses which are antinociceptive (White et al., 1982). However intrathecal administration of these antagonists may be clinically useful and indeed, intrathecal ketamine (an NMDA antagonist) has been successfully used in man (Bion, 1984).

The dorsal horn neuronal activity evoked by formalin arises from events occurring in the periphery since it can be blocked by administration of a local anaesthetic into the site of formalin injection either during the second peak (Dickenson and Sullivan, 1987c) or the first peak. Bradykinin has been implicated as a peripheral mediator of nociception since it is known to activate C fibres. This study presents evidence for the peripheral involvement of bradykinin in prolonged nociceptive events since selective desensitization of the receptive field with bradykinin and peripheral administration of a bradykinin B2 receptor antagonist both reduced the second peak of the formalin response. Interestingly the first peak of the formalin response was unaltered by either treatment indicating a fundamental difference in the mechanisms of peripheral generation for each peak. A B1 antagonist was ineffective on both peaks. The response was never completely abolished suggesting other mediators are also involved in the generation of the response. These findings confirm the inflammatory basis of the formalin response second peak, but not the first peak, and implicate bradykinin, acting via B2 receptors, as a major mediator during this phase of the response. By contrast bradykinin does not appear to be involved in the spinal events occurring following formalin injection since antagonists at the B2 and B1 receptor had little effect.

Thus a fundamental difference is evident in the mechanisms responsible for the generation of short duration and more prolonged responses as represented by the first and second peaks of the formalin response. The activation of peripheral afferent fibres shifts away from the direct stimulation seen with pinch or the initial effects of formalin, to an inflammatory state where chemical mediators such as bradykinin are generated and these act on the afferent fibres. The development of stable bradykinin B2 receptor

antagonists may therefore represent a useful approach to the clinical treatment of pain arising from peripheral inflammatory mechanisms.

Another interesting possible direction in the development of analgesics could be the use of peripheral opioids. Local peripheral administration of a kappa, but not mu or delta, agonist inhibits both peaks of the formalin response in a naloxone reversible manner. This profile of inhibition markedly differs from that seen spinally where mu and delta agonists are extremely effective at inhibiting the formalin response but kappa agonists are less potent (Dickenson and Sullivan, 1987a; Sullivan et al., 1989; Sullivan and Dickenson, 1991). Thus the development of kappa agonists that cannot cross the blood brain barrier may provide analgesic agents which are devoid of many side effects but effective in inflammatory states. A major unwanted effect of opioids is constipation and this mainly results from a peripheral action of the compounds (see Simpkins et al., 1988). However kappa receptor agonists appear to be less likely to cause constipation (see Hayes et al., 1990) and a peripherally restricted kappa agonist may therefore be therapeutically very useful.

Finally the recent development of nitric oxide inhibitors had raised much interest in this molecule and it is found to be active in many systems within the body. Administration of a nitric oxide inhibitor systemically, peripherally (local to the site of formalin injection) and intrathecally reduced the second peak of the formalin response. However intrathecal administration of the nitric oxide precursor also reduced the response to formalin. Thus the involvement of nitric oxide in nociception appears to be complex and more investigation is required to determine the therapeutic potential of interfering with this system. It should be noted however that the nitric oxide precursor L-arginine has recently been administered to man as a systemic infusion and appeared to be antinociceptive (Takagi et al., 1990).

One interesting facet of the formalin response is its finite duration, subsiding after about 60 minutes and investigation into the mechanisms underlying the termination of the response may be of interest. The activation of descending inhibitory controls is one possible mechanism, however it is unlikely that this involves the activation of endogenous opioid systems since naloxone appears to have no effect on the size or

duration of the formalin response at doses that reverse opioid effects. However 5HT, noradrenaline or GABA may be involved in the termination of the formalin response. The generation of markers of gene expression within the spinal cord (e.g. c-fos) within 60 minutes following formalin administration has been interpreted as a possible mechanism for maintaining prolonged neuronal activity (Presley et al., 1990). However it is equally possible that changes arising from alterations in genetic expression may act to suppress prolonged nociception and may possibly be responsible for the termination of the formalin response.

The presence of a myriad of peptides in both the afferent fibres and within the spinal cord provides a wealth of future research directions. However the lack of selective antagonists for many of these compounds restricts, at present, investigation into their involvement within nociceptive transmission. However recent findings with a couple of these peptides have proved very interesting and indicate how some of these peptides may have subtle effects which could have important therapeutic implications. Cholecystokinin (CCK) and FLFQPQRF-NH2 are both peptides that attenuate the spinal antinociceptive effects of mu, but not delta, opioid agonists (see Magnuson et al., 1990). It is therefore possible that the loss of effectiveness of opioid agonists observed when they are administered after formalin injection may in part result from the involvement of peptides such as these. In addition the selective attenuation of the mu opioid effects suggest the development of selective delta opioid agonists may prove to be clinically useful. Thus understanding the interactive role of transmitters and modulatory peptides in nociception could be crucial in the future development of effective analgesics.

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