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NEUROPHYSIOLOGICAL AND NEUROCHEMICAL BASES OF MODULATION OF NOCICEPTIVE REFLEXES EVOKED BY HIGH INTENSITY, LOW FREQUENCY ACTIVATION OF SENSORY FIBRES IN THE RAT

BY

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This thesis is dedicated to the loving spirits of my father and mother
Arturo Augusto Romita and Antonia Maria Cihoratic Romita

ABSTRACT

The objective of this thesis was to elucidate the neurophysiological and neurochemical bases for the modulation of sensory transmission in the spinal cord evoked by the activation of primary afferents in the lightly anaesthetized rat. Effects of prolonged, intense (20 X threshold), low frequency (4 Hz) stimulation of meridian and non-meridian sites on the thermally evoked nociceptive withdrawal reflexes of the tail and limbs were studied. Threshold was the minimum current required to elicit muscle contraction.

Intense stimulation applied to meridian sites inhibited tail withdrawal. This inhibition persisted beyond one hour after the end of stimulation. Stimulation of non-meridian sites produced a smaller inhibition; this occurred during the conditioning only. Thus, a brief inhibition or both the brief and a persistent, post-stimulation inhibition were produced by stimulation of non-meridian or meridian sites, respectively. Little effect was evoked on limb withdrawal reflexes.

Expression of the post-stimulation effect required 20 X threshold stimulation with long pulse durations ($\geq$ 2 ms), low frequency of stimulation (2 Hz - 6 Hz) and long train durations (20 or 40 min). The brief effect could be evoked at 10 X threshold with short pulse durations ($\leq$ 2 ms) at higher frequencies of stimulation (8 Hz) and with short train durations (10 min).

Stimulation of meridain sites evoked both the brief and the post-stimulation effects in chronic spinal transected rats (7-14 days): in acutely spinal transected rats ($\leq$ 48 h) the brief effect was evoked only. The return of the post-stimulation effect was coincident with the return of bladder function.

Both the brief and post-stimulation inhibition were blocked by the competitive NMDA receptor antagonist 5-amino-2-phosphonovaleric acid (APV).

The wide spectrum opiate receptor antagonist naloxone, or μ-opiate antagonist ß-

funaltrexamine, attenuated both the brief and persistent inhibition. The δ- and κ-antagonists, TIPP[ψ] and nor-binaltorphimine, attenuated the inhibition during the stimulation. Both drugs blocked the post-stimulation effect and even facilitated withdrawal. In chronically spinal transected rats, naloxone blocked the inhibition.

These data suggest that intense, low frequency activation of primary afferents arising from meridian but not non-meridian sites produces both brief and persistent inhibition of the tail withdrawal reflex. Limb withdrawal reflexes are only minimally inhibited by this activation. It is suggested that the persistent antinociception may be due to long-term plastic changes in inhibitory mechanisms within the CNS because these effects persist long after the end of stimulation and presumably after synaptic inputs from these fibres have ceased. It is also suggested that inhibitory mechanisms are provoked by prolonged activation of high threshold fibres, are dependent on the parameters of stimulation, are extrasegmental in nature and differentially modulate tail vs. limb nociceptive reflexes. Activation of spinal NMDA receptors appears critical for the expression of the persistent antinociception. The inhibition is also differentially mediated by activation of multiple opiate receptors: μ-, κ- and to a lesser degree δ-receptors mediate the brief effect, while the persistent antinociception is dependent on activation of δ- and κ-receptors and to a lesser degree μ-receptors. Data from spinal animals suggest that the mechanisms mediating the inhibitory effects include both spinal and supraspinal components.

RÉSUMÉ

L'objectif de la présente thèse consistait à élucider les bases neurophysiologiques et neurochimiques de la modulation de la transmission sensorielle dans la moelle épinière suscitée par l'activation des afférents primaires chez le rat légèrement anesthésié. Les effets des stimulations électriques prolongées intenses (20 X de seuil) à hautes fréquences (4 Hz) appliquées aux sites méridiens et non méridiens des reflex de retrait nociceptifs (invoqués thermiquement) de la queue et des membres furent étudiés.

Des stimulations intenses appliqués aux sites méridiens ont inhibé le retrait de la queue. Cette inhibition a duré plus d'une heure après la fin de la stimulation. La stimulation des sites non méridiens a aussi produit cette inhibition, mais elle fut d'une plus petite magnitude et n'eut lieu que pendant le conditionnement. Donc, une brève inhibition fut provoquée par la stimulation des sites non méridiens, alors que des inhibitions post-stimulation, une brève et une persistante, furent produites par la stimulation des sites méridiens. Les reflex de retrait des membres furent très peu affectés.

L'effet post-stimulation ne s'est manifesté qu'à un seuil de stimulation de 20 X, et comportait des impulsions de longues durées ($\geq$ 2 ms), une basse fréquence de stimulation (2-6 Hz) et une longue période de stimulation (20 ou 40 min.). L'effet bref a été provoqué à un seuil de stimulation de 10 X avec des impulsions de courtes durées ($\leq$ 2 ms), une fréquences de stimulation plus haute (8 Hz) et une courte période de stimulation (10 min.).

Chez le rat dont la moelle épinière fut sectionnée de façon intense ($\leq$ 48 h), la stimulation des sites méridien a produit une légère inhibition brève du retrait de la queue; aucun effet post-stimulation ne fut produit. Chez les animaux dont la moelle épinière fut sectionnée de façon chronique (7 ou 14 jours), la réponse produite a été plus importante, et une brève inhibition post-

stimulation a été induite. Le retour de cette action post-stimulation a coïncidé avec le retour du fonctionnement de la vessie..

Les inhibitions, brève et post-stimulation, furent toutes deux bloquées par l'acide 5-amino-2-phosphonovalérique (APV), un antagoniste de récepteur compétitif.

La naloxone, un antagoniste de récepteur opiacé au large spectre d'activité, ou la β-funaltrexamine, un antagoniste compétitif de récepteur μ-opiacé, ont atténué toutes deux les inhibitions brèves et de longue durée. La nor-binaltorphimine ou le TIPP[ψ], des antagonistes de récepteur δ et κ, ont atténué les inhibitions durant la période de stimulation. Les deux antagonistes ont bloqué l'effet post-stimulation, et ont même facilité le reflex de retrait. . Chez les rats dont la moelle épinière fut sectionnée de façon chronique, la naloxone a bloqué complètement l'inhibition suscitée.

Ces données suggèrent que l'activation intense à basse fréquence des afférents primaires provenant des sites méridiens (mais non des sites non méridiens) produit des effets inhibiteurs brefs et persistants sur le reflex de retrait de la queue. Les reflex de retrait des membres ne sont inhibés que de façon imperceptible par cette activation. On suggère que l'antinociception persistante pourrait être due à des changements plastiques à long terme des mécanismes inhibiteurs dans le SNC, parce que ces effets se prolongent après la fin du stimulus sur les afférents primaires et, ainsi, probablement après que le flux synaptique de ces fibres a cessé. De plus, ces mécanismes, provoqués par l'activation prolongée des fibres à haut seuil et dépendants des paramètres de stimulation, pourraient être de nature extrasegmentaire et moduler par action différentielle les reflex nociceptifs de la queue par rapport à. ceux des membres. L'activation des récepteurs spinaux du NMDA semble déterminante pour l'expression de l'antinociception persistante. L'inhibition est aussi modifiée de façon différentielle par l'activation de plusieurs récepteurs opiacés, c'est-à-dire

les récepteurs μ, κ, et à un degré moindre, δ. Ils modifient l'antinociception brève alors que l'antinociception persistante est dépendante de l'activation des récepteurs opiacés δ, κ, et à un degré moindre, μ. Les données provenant des animaux spinaux suggèrent que les mécanismes modifiant les effets d'inhibition comprennent des composantes spinales et supraspinales.

PREFACE

Excerpt from *Guidelines Concerning Thesis Preparation*, Faculty of Graduate Studies and Research, McGill University.

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

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The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers.
Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

CHAPTER ONE

ROMITA, V.V., YASHPAL, K., HUI-CHAN, C.W.Y. AND J.L. HENRY. INTENSE PERIPHERAL ELECTRICAL STIMULATION EVOKES BRIEF AND PERSISTENT INHIBITION OF THE NOCICEPTIVE TAIL WITHDRAWAL REFLEX IN THE RAT
(Submitted to the Journal of Physiology)

CHAPTER TWO

ROMITA, V.V., SUK, A. AND J.L. HENRY. PARAMETRIC STUDIES ON ELECTROACUPUNCTURE-LIKE STIMULATION IN A RAT MODEL: EFFECTS OF INTENSITY, FREQUENCY AND DURATION OF STIMULATION ON EVOKED ANTINOCICEPTION
(Submitted to the Journal of Neuroscience)

CHAPTER THREE

ROMITA, V.V. AND J.L. HENRY. INTENSE PERIPHERAL ELECTRICAL STIMULATION DIFFERENTIALLY INHIBITS TAIL VS. LIMB WITHDRAWAL REFLEXES IN THE RAT
(Submitted to Brain Research)

CHAPTER FOUR

ROMITA, V.V. AND J.L. HENRY. NMDA RECEPTOR INVOLVEMENT IN SPINAL INHIBITORY CONTROLS OF NOCICEPTION IN THE RAT
(Submitted to NeuroReport)

CHAPTER FIVE

ROMITA, V.V. AND J.L. HENRY. SPINAL μ-, δ- AND κ-OPIATE RECEPTORS MEDIATE INHIBITION OF THE NOCICEPTIVE TAIL WITHDRAWAL REFLEX EVOKED BY INTENSE PERIPHERAL ELECTRICAL STIMULATION
(To be submitted to the European Journal of Pharmacology)

The thesis and manuscripts presented herein were prepared under the guidance of my thesis supervisor, Dr. J.L. Henry.

In Chapter one, Dr. K. Yashpal participated in some of the initial control experiments and Dr. C.W.Y. Chan assisted in reviewing the manuscript. As I was the major contributor, I wrote and prepared the manuscript in its entirety.

In Chapter two, under my direct supervision, Mr. A. Suk performed some of the experiments on the effect of frequency and pulse duration of electrical stimulation on nociceptive withdrawal reflexes, as part of a undergraduate research course (Physiology 552-411D). As I was the main contributor to the project I wrote and prepared the manuscript in its entirety.

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PART I

INTRODUCTION, RATIONALE AND OBJECTIVES

I. INTRODUCTION

Acupuncture-Analgesia and Diffuse Noxious Inhibitory Controls: Variations on a Theme?

Dating back several centuries B.C., several methods have been employed for the alleviation of pain. For effective pain relief these treatments were often painful in themselves. These forms of treatment, generally referred to as counter-irritation, are believed to activate somatosensory mechanisms within the central nervous system which produce a generalized analgesia. In 1937, Duncker was among the first to study this phenomenon. His original experiments demonstrated that if a conditioning or "active" pain was stronger than a passive or "test" pain, the active pain decreased the distant and simultaneous "passive" pain. This decrease was found to be in proportion of the relative intensity of the conditioning or "active" pain. The stronger the "active" pain, the more the "passive" pain would be diminished (Duncker, 1937). A similar relationship between the intensity of the conditioning pain and the degree of relief from pain produced was also noted by Hardy et al (Hardy et al., 1940). In these experiments a sphygmomanometer cuff was placed above the elbow and slowly inflated to pressures that were just tolerable and maintained over a period of 30 min, after which the cuff was completely deflated. The results of these experiments were striking in that as the pain in the arm became more intense with increasing pressure, the test pain produced by application of a thermal heat stimulus to the skin on the forehead was decreased in parallel (Hardy et al., 1940). Similar analgesic effects against pain elicited by cutaneous application of capsaicin or subcutaneous

application of concentrated sodium chloride were produced by several counter-irritants, such as heat or cold stimulation, electrical stimulation or strong mechanical tactile stimulation (Gammon, Starr, 1941). Another example of pain relief produced by counter-irritation is demonstrated by the ability of induced cold pain applied to the tibia to increase tooth pain threshold to electric shock (Parsons, Goetzl, 1945). More recent psychophysical experiments have confirmed these earlier studies by demonstrating that pain provoked by immersion of the hand in ice water decreased the pain sensation produced by a brief noxious thermal stimulation applied to the face (Talbot et al., 1987,1989).

Within the last two decades experiments were undertaken to define the physiological characteristics of this phenomenon. In 1979 Le Bars and coworkers recorded extracellularly the activity of convergent dorsal horn cells receiving both low and high threshold inputs in the rat. They found that the responses of these cells to C fibre input were inhibited by noxious electrical, thermal, mechanical or chemical stimuli applied to remote sites outside the respective receptive fields of theses convergent cells (Le Bars et al., 1979b) and this inhibition outlasted the stimulation, but only briefly. Non-convergent cells, on the other hand, remained unaffected by conditioning stimulation (Le Bars et al., 1979a). Le Bars et al postulated that activation of high threshold fibres by the noxious conditioning stimulation produced a diffuse inhibition of nociceptive processing throughout the spinal cord and remaining central nervous system. This group coined the term "diffuse noxious inhibitory controls" or, as it is often affectionately referred to, "DNIC". To determine whether the same process occurred in the human, subjects were asked to immerse their hands in a hot water bath for a period of two min ($\geq$ 45°C) and effects of this conditioning stimulus was then observed on the nociceptive RIII reflex in the

biceps femoris muscle of the contralateral leg elicited by high intensity stimulation of the sural nerve (Willer et al., 1984; Le Bars et al., 1992). As expected, this noxious thermal stimulation depressed the reflex and the depression outlasted the conditioning stimulus by a few min. Subsequent experiments further elaborated on the neurophysiological characteristics of DNIC.

1) DNIC is evoked by stimulation from anywhere on the body and only at intensities which activate high threshold fibres (Bouhassira et al., 1987; Le Bars et al., 1979b).

2) These inhibitory effects are extrasegmental in nature in that it modulates nociceptive processing throughout the central nervous system (Bouhassira et al., 1987; Le Bars et al., 1979b,1992; Willer et al., 1984).

3) The extent of inhibition was directly related to the intensity of the conditioning stimulation. In experiments where the tail was immersed in hot water, the inhibition of responses of trigeminal convergent cells to C fibre input were depressed in a fashion linearly related to the temperature of the hot water bath (Villanueva, Le Bars, 1985). In man, a similar relationship between the depression of the nociceptive RIII reflex and temperature of the noxious thermal conditioning stimulation applied to the hand was also demonstrated; greater depression of this reflex was evoked with higher temperatures (Willer et al., 1984).

4) DNIC appears to be frequency dependent; inhibition produced with continuous electrical percutaneous electrical stimulation of Aδ fibres was found to be less effective when applied at 8 Hz than at lower frequencies of stimulation (Bouhassira et al., 1987).

5) DNIC is subserved by spinal-supraspinal-spinal pathways in both animals and man (Le Bars et al., 1992; Roby-Brami et al., 1987; Villanueva et al., 1986a,b). The first line of evidence to demonstrate this was that complete spinal transection at the cervical level attenuated

by 80 % the inhibition evoked by noxious cutaneous stimulation (Godfrey, Morgan, 1978), suggesting that a supraspinal loop was important for triggering DNIC. The ascending component of this loop was suggested to be the anterolateral quadrant, because cervical hemicordotomy greatly attenuated DNIC when noxious stimuli were applied to segments contralateral and below the lesion but not ipsilateral or above the lesion, while bilateral cordotomy blocked DNIC irrespective of the side of the conditioning stimulation (Villanueva et al., 1986a). Therefore, the lateral spinothalamic tract and the spinoreticular tracts were implicated in the mediation of DNIC. However, lesioning of the right lateral thalamus did not effect DNIC. Therefore, it was concluded that the ascending component mediating DNIC was the spinoreticular tract (Villanueva et al., 1986a). The descending component of DNIC is suggested to be the dorsolateral funiculus because a cervical lesion to this pathway abolished the DNIC on sensory neurones ipsilateral to and below the lesion but not in neurones contralateral to the lesion (Villanueva et al., 1986b). However, the requirement for this hardwiring in the central nervous system for the expression of DNIC has been challenged by many investigators (Calvino, 1990; Gerhart et al., 1981; Ness, Gebhart, 1991a,b; Morgan et al., 1994; Pitcher et al., 1995; Pubols et al., 1988; Yezierski, Schwartz, 1986).

6) DNIC results in the extrasegmental release of opioid peptides, in particular enkephalin (Le Bars et al., 1987a,b).

7) DNIC can be blocked by intravenous administration of naloxone in man and animals (De Broucker et al., 1990; Le Bars et al., 1981).

Analgesia evoked by intense, low frequency acupuncture-like stimulation is thought to be a variation of that evoked by counter-irritation (Bing et al., 1990,1991a,b; Fox, Melzack,

1976; Melzack, 1975) and the underlying mechanisms may share some similarities with DNIC. There are many similarities as well as differences in the analgesia and/or antinociception produced by acupuncture-like stimulation which may suggest that some but not all of the neurophysiological components underlying the acupuncture evoked effects may be similar to those which evoke DNIC.

Some of the similarities between these two types of stimulation-evoked analgesia and/or antinociception are listed as follows.

1) Consistent analgesia and/or antinociception is evoked upon stimulation of high threshold sensory fibres (Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Holmgren, 1975; Levine et al., 1976; Mann, 1974; Melzack, 1975).

2) Like DNIC, intense acupuncture-like stimulation evokes a generalized extrasegmental analgesia in man (Andersson et al., 1973; Andersson, Holmgren, 1975; Chapman et al., 1975, 1977; Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Huang et al., 1978; Melzack, 1975; Melzack, Bentley, 1983; Melzack et al., 1980; Sjölund. Eriksson, 1979) and in animal studies (Kawakita, Funakoshi, 1982; Toda, Ichioka, 1978)

3) The antinociception/analgesia evoked by acupuncture-like effects may be dependent on the frequency of the stimulation (Andersson et al., 1973; Andersson, Holmgren, 1975; Chung et al., 1984b; Thomas, Lundberg, 1994).

4) Some experimental studies suggest that mechanisms underlying acupuncture-like antinociception require an intact spinal cord (Pomeranz et al., 1977; Pomeranz, Cheng, 1979). However, in other studies acupuncture-evoked antinociception has been elicited in spinal transected animals (Chung et al., 1983,1984b; Paik et al., 1981).

5) Like DNIC, the acupuncture-evoked analgesia/antinociception is sensitive to naloxone (Chung et al., 1984a,b; Hayes et al., 1978; Lee, Beitz, 1992; Sjölund, Eriksson, 1979; Willer et al., 1982)

Although, there may be many similarities, fundamental differences exist in the properties underlying the analgesia/antinociception produced by DNIC and acupuncture-like stimulation.

1) Unlike the effects produced by DNIC, analgesia/antinociception evoked by acupuncture-like stimulation requires an induction time to be fully expressed, requiring prolonged stimulation (Chapman et al., 1977; Ernst, Lee, 1987; Holmgren, 1975; Lee et al., 1973), while effects elicited by DNIC appear to be immediate (Bouhassira et al., 1987; Le Bars et al., 1979b).

2) The analgesia/antinociception evoked by acupuncture-like stimulation outlasts the duration of conditioning by tens of min (Andersson et al., 1973; Andersson, Holmgren, 1975; Fox, Melzack, 1976; Mannheimer, Carlsson, 1979), to hours or even days (Fox, Melzack, 1976; Mannheimer, Carlsson, 1979; Melzack, 1975). On the other hand, the analgesic/antinociceptive effects produced by DNIC persist only for a few min at most (Bouhassira et al., 1987; Le Bars et al., 1979b).

3) Although, controversial there is evidence to suggest that stimulation of meridian sites or acupuncture points produces greater analgesic/antinociceptive effects than stimulation of non-meridian sites (Anderson et al., 1974; Chan, Fung, 1975; Stewart et al., 1977; Toda, Ichioka, 1978). However, in DNIC-induced analgesia/antinociception, the effect is independent of the site of stimulation (Le Bars et al., 1979b).

I felt that it was important to compare the similarities and differences between the effects elicited by intense acupuncture-like stimulation and those produced by brief activation of high threshold fibres for the purpose of solidifying in the reader's mind the concept that other possible extrasegmental mechanisms may be contributing to the modulation of nociception than those neurophysiological mechanisms proposed for DNIC.

II. RATIONALE AND OBJECTIVES

During the last five years of the study of the mechanisms modulating nociception, what I have found most intriguing was not that stimulation of high threshold fibres could inhibit nociceptive processes but rather that the prolonged stimulation of these fibres, as occurs with some forms of acupuncture, elicited an affect which persisted long after the end of stimulation. Yet in the literature, little emphasis has been placed on development of a hypothesis which may attempt to explain this long-lasting phenomenon. In preparation of this thesis, I have come across some of the earlier concepts on nociception, particularly those concerned with persistent pain which I think may be worthwhile expressing here. Both Goldscheider (1894) and Livingston (1943) believed that the intensity of injury and central summation were critical variables in determining long-lasting pain (Goldscheider, 1894; Livingston, 1943). Today chronic pain is thought of in terms of "central sensitization", "central plasticity" or "wind up". What remains unchanged since the turn of the century, is the concept that intensity of the stimulation or the type of input and central summation are critical factors for the induction of central plasticity or sensitization, considered to be the neurophysiological bases of chronic, long-lasting pain (Chapman et al., 1994; Dickenson, Sullivan, 1987; King, Lopez-Garcia, 1993; Thompson et al., 1990; Thompson et al., 1994; Xu et al., 1992). What I find striking is that the long-lasting analgesia/antinociception produced by intense acupuncture-like stimulation, either in the clinical or experimental setting, also requires activation of high threshold fibres and appears to be dependent on the frequency and the duration (*vide supra*) of the stimulation. Thus, the type of input and at least some form of central summation are important in the activation of

long-lasting modulation of nociception. Therefore, the cellular basis for the two types of plasticity (i.e persistent pain/nociception vs. persistent analgesia/antinociception) may be similar. The assumption made here is that the persistent antinociception evoked by prolonged, intense acupuncture-like stimulation is manifested by long-lasting plastic changes in inhibitory mechanisms of the central nervous system.

The purpose of this thesis is to unravel some of the physiological mechanisms by which prolonged activation of high threshold fibres elicits a long-lasting modulation of sensory input. I have chosen to use intense, electroacupuncture-like stimulation in the lightly anaesthetized rat as a model to study these mechanisms. Nociceptive withdrawal reflexes were used to monitor the level of activity in nociceptive pathways. These reflexes were provoked by application of a brief noxious cutaneous thermal stimulus.

This experimental paradigm will allow me to meet **my objective which is to elucidate the neurophysiological and neurochemical bases for the antinociception evoked by prolonged activation of high threshold primary afferents.**

In Chapter one the first objective was to determine if prolonged, intense, low frequency electrical stimulation applied to meridian points could evoke inhibition of the tail withdrawal reflex that was long-lasting, in the lightly anaesthetized rat. As other studies have alluded to the possibility that frequency of stimulation may be an important variable in determining the outcome of the stimulation and as central summation may be involved in the evoked effects, the frequency of stimulation was varied to determine what effect this would have on the evoked response. Once the frequency that evoked optimal effects was determined, the next step was to

stimulate non-meridian sites. Effects of meridian vs non-meridian stimulation are not well established in the literature, as assessed in Chapter one. Furthermore, the DNIC hypothesis enunciated above predicts that stimulation of these two types of sites should not evoke different responses. Therefore, this hypothesis was tested by stimulating non-meridian sites. Finally, stimulation was applied to acute or chronic spinalized rats to determine the relative contributions of supraspinal vs. spinal mechanisms in the evoked response.

Chapter two has as the main objective to determine the optimal parameters of stimulation to produce the greatest and most prolonged effects. This parametric study will help elucidate some of the peripheral and central neurophysiological mechanisms contributing to the evoked response. Studies on DNIC or acupuncture-evoked analgesia/antinociception have indicated a dependence on the intensity of stimulation, suggesting that recruitment of high threshold fibres is necessary for the expression of the evoked antinociception. Only one study has directly examined the effects of varying the intensity of stimulation on the evoked response in relation to the evoked long-lasting antinociception. Thus, experiments were performed to study the effect of varying the intensity of stimulation on the evoked response. Effect of varying the frequency was examined. To my knowledge no study has attempted to examine the effect of varying the pulse duration or of varying the duration of the train of stimulation during high intensity, low frequency electroacupuncture-like stimulation. Therefore, these parameters which are dictated by biophysical properties for recruitment of nerve fibres and central summation mechanisms, respectively, were also varied and the effects on evoked responses were determined.

In Chapter three, the emphasis was to examine the effects of local vs. remote

extrasegmental stimulation on modulation of different nociceptive reflexes. Clinical and experimental data suggest that intense electroacupuncture-like stimulation produces generalized antinociception. Most animal models developed have studied only segmental or local effects of the stimulation. Furthermore the DNIC hypothesis predicts that the antinociceptive effects elicited by intense stimulation should be extrasegmental in nature. Therefore, stimulation was applied to meridian or non-meridian sites, either locally or remotely from the site of the test stimulus. The DNIC hypothesis also predicts that the inhibition evoked by high intensity stimulation should be uniform, and therefore the effects of the conditioning stimulation were tested on limb withdrawal reflexes in addition to the tail withdrawal reflex.

For Chapter four, as high threshold fibres are involved in nociception and antinociception, and excitatory amino acids are implicated in the transmission of sensory information and in central nervous system plasticity, the involvement of the NMDA receptor in the evoked response was investigated.

Finally, in Chapter five as many antinociceptive effects appear to be mediated by opioid peptides, the relative contributions of μ-, δ- and κ-opiate receptor activation to the evoked responses were investigated.

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PART II

HISTORICAL BACKGROUND AND LITERATURE REVIEW

I. FIGHTING FIRE WITH FIRE, PAIN WITH PAIN: A BRIEF HISTORY OF THE USE OF NOXIOUS STIMULATION FOR THE TREATMENT OF PAIN IN MAN

In an attempt to understand how activation of sensory inputs can modulate the transmission of sensory information pertaining to tissue damaging or potentially tissue damaging noxious stimulation occurring at peripheral sites, a review of the practices of ancient or present folk medicine for the treatment of pain may provide some interesting preliminary insights on how pain relief might be achieved. Below, I briefly describe some of these practices which have been reviewed in detail elsewhere (Brockbank, 1954; Kane, Taub, 1975; Melzack, Wall, 1988; Taub, 1975).

The basis for the development of some of these practices lie in religious or otherwise cultural beliefs that pain or disease may be the result of either evil spirits invading the body or the result of deserving punishment delivered by "higher powers" for sins committed. The rational treatment of chronic pain, as derived from these early beliefs, was to provide antidotes which would drive these spirits away or prove repentance for sins. Therefore, some of these treatments were designed to be discomforting and painful in nature. The effect of such treatments was often relief from the chronic pain. Thus the "cure" reinforced the original belief and the rationale that provided the initial impetus for the aversive type of treatment applied. The pain relief evoked by these methods is often referred to as counter-irritation or hyper-stimulation analgesia and has proven to be reliable enough to have survived thousands of years of evolution in thought and practices among various cultures world-wide.

Cupping:

Cupping, considered to be one of the oldest forms of counter-irritation techniques, has been in practice since the fourth century BC in Greece and Rome and was widely used in ancient India and China to treat a variety of ailments including muscular aches, headache and arthritic pains. Cupping was either wet or dry. Dry cupping consisted of glass cups heated up by coal or flaming alcohol and then inverted and pressed firmly over the painful area. As cooling occurred, the air in the cup contracted and produced a vacuum so that the skin was sucked up into the cup. The suction was so great that removal of the cups was often difficult and blue wheals resulted in the skin from extravasation of blood from small vessels.

A far more gruesome but often effective practice was that of wet cupping used primarily as a means of extracting body fluids, for example as in the case of congestive heart failure. The method consisted of placing the hot cups over scarified tissue produced by gadgets consisting of sharp blades which simultaneously cut into the skin. In addition to removing blood and fluids, this method was also used to produce pain, local irritation and inflammation all to fight disease and severe, chronic pain. Cupping is still practiced today in some mediterranean countries and in the far East.

Blisters, Setons and Issues:

In the early half of the nineteenth century, painful blisters or running sores made by threading strands of twine or silk through the skin were the vogue of the times to treat a variety of painful conditions. Blisters were commonly induced on the scalp, behind the ears, on the neck and on the soles of the feet. The usual blistering agents consisted of tartar emetic, silver

nitrate and cantharides powder, all powerful irritants. In some cases ammonia, mustard, turpentine or dilute mineral acids were used. These blisters were then punctured and the detached skin was pealed off. The exposed wound was then dressed with caustic agents to prevent healing. With respect to setons or issues, the thread was coated with chemical irritants and drawn back and forth to keep the wounds open and pus flowing. From the twelfth to the nineteenth centuries setons were often used to treat gout, spinal pain or hip-joint pain.

Cautery and Moxibustion:

Another ancient method to cause the discharge of bodily fluids for the treatment of various disease states or relief of pain was cauterization. The method required a metal rod to be heated over a flame until red hot and then it was placed over a painful area such as the hollow of the foot for the treatment of gout. Records from the twelfth century show sciatica was treated by cauterizations above the hip over the joint. To produce generalized pain relief the hot iron was placed over the tibia three finger breadths below the knee and above the ankle. In severe cases of hip pain the hot iron was placed between the trochanter major and the tuberosity of the ischium.

In China, moxibustion achieved the same effect as that of cauterization. The practice consisted of applying several cones of moxa made from the leaves of the mugwort plant (*Artemisia latifolia*) and setting the tip of the cone on fire and allowing it to burn slowly until it approached or reached the skin. This painful practice was used to treat several diseases and was often used to treat pain. Moxibustion was first introduced to the west in France and then in England in the early 1800's, where it became popular for the treatment of gout. Today

variations of acupuncture-moxibustion are still practiced in China.

Electrical Counter-irritation:

Records show that marine or fresh water electric fish may have been used for therapeutic reasons dating back well before the birth of Christ, especially the *Torpedo marmorata* (torpedo ray, electric ray), the *Malopterurus electricus* (electric or Nile catfish) or the *Gymnotus electricus* (electric eel). In the times of the emperor Tiberius, it was recommended that the electric torpedo was to be placed onto the painful area until numbness ensued to effectively treat gout, headache or even chronic and unbearable pain. In these early records there was no mention of the onset time or lasting properties of the evoked analgesia except that relief occurred gradually. In many non-Western cultures electric fish are still used for the treatment of ailments and pain.

In the late 1850's two American physicians, Francis and Garratt, were the first to report the relief of dental pain using electrical stimulation. Brief descriptions indicate that the intensity of the stimulation required, for the stimulatin to be effective, was painful at the lower intensities used and at higher intensities, the sensation experienced by patients were "disquieting, tremulous but painless". Historically this is an important finding because it is the first direct and documented indication that intensity of the stimulus is important in the effective production of analgesia.

Another American by the name of Oliver experimented with electrical stimulation to produce surgically useful anaesthesia. In 1858 Oliver in association with professor Hamilton used electrical stimulation to produce analgesia for the removal of an ulcer from the leg of a

patient. Electrodes were wrapped distal and proximal to the site of the wound and the intensity of the stimulation was gradually increased until the patient described the sensation as severe pressure. At no time during removal of the ulcer did the patient complain of pain. The important aspect of this account was that the electrical analgesia increased with time of stimulation and that there was a deficit lag in the onset and in the decline of the evoked "anaesthetic" effect. In addition to intensity being an important factor in producing analgesia, these early accounts begin to indicate that there is an induction period required for the development of pain control and that the induced analgesic effects persisted after the termination of the stimulation.

The last account out of many that I would like to mention here is that of the practices undertaken for surgical amputations at St. Francis Hospital in Hartford CT. at the beginning of the nineteenth century. Here, practitioners found that by varying the intensity, frequency and pulse durations of electrical shocks applied to the patient, maximum paraesthesia could be achieved. The parameters of stimulation recommended to evoke optimal effects were intensities in the range of 40 mA, 10 ms pulse widths and frequencies in the range of 100 Hz.

After perusing these historical accounts one begins to appreciate that the evoked effects are dependent not only the intensity of the stimulation, but in addition the frequency, the pulse width and the duration of the train of the stimulation. The obvious question that comes to mind is what peripheral and central nervous system mechanisms are triggered by these specific parameters of stimulation. The answer to this question will be pursued throughout the remaining of my thesis.

II. CLINICAL AND EXPERIMENTAL MODELS OF ACUPUNCTURE ANALGESIA

Acupuncture: classical theory:

The classical theoretical basis of traditional acupuncture was derived from the *Huang Ti Nei Ching* (The Yellow Emperor's Manual of Corporal Medicine) written between 1000-2000 years B.C. This ancient medical manuscript was a compilation of all knowledge of acupuncture which had accumulated in the preceding centuries.

Traditional acupuncture is based upon concepts of "energetic medicine", made up of two opposite components or polarities, the *yin* and the *yang*. For perfect health these two life forces must be in harmonious balance. The *ying chhi* travels within blood vessels while the *yang* or the *wei-chhi* travels outside blood vessels over 12 major channels or meridians. Each is linked to a particular organ or body function. On each meridian there exist several acupuncture points at which the "flow of energy" can be influenced by the insertion and manipulation of needles. Therefore, during disease states it was believed that needling of these acupuncture points could either strengthen energy that was deficient or weaken energy that was in excess and restore normal health in a given organ or throughout the body. A review of the metaphysical concepts underlying ancient acupuncture practices can be found in Ulett's "*Principles and Practice of Physiologic Acupuncture* (Ulett, 1982).

Acupuncture: contemporary theory:

Acupuncture has been and is used in experimental and clinical settings for the attenuation of pain. It consists of placing needles into acupuncture or meridian points and manually twirling

the needles at a low frequency (Fox, Melzack, 1976; Mann, 1974; Lee et al., 1973; Levine et al., 1976). Electroacupuncture is a slight variation of this technique in that the needles are stimulated electrically instead of manually. The stimulation is usually intense and applied at a low frequency in the range of 1 to 4 Hz (Andersson et al., 1973; Andersson, Holmgren, 1975; Holmgren, 1975; Krause et al., 1987; Lee et al., 1973; Longobardi et al., 1989; Noling et al., 1978; Oliveri et al., 1986). In some cases acupuncture stimulation may be applied transcutaneously and is referred to in the literature as acupuncture-like TENS (Eriksson, Sjölund, 1976; Eriksson et al., 1979; Fox, Melzack, 1976; Melzack, 1975; Sjölund, Eriksson, 1979). With acupuncture-like TENS the stimulation parameters may be varied and applied at an intense level where an internal high frequency train of pulses ranging form 70 to 100 Hz is given at low frequency (i.e. 1-4 Hz) (Eriksson, Sjölund, 1976; Eriksson et al., 1979; Sjölund, Eriksson, 1979); this stimulation is believed to be tolerated better by the patient. This form of stimulation is not to be confused with high frequency, low intensity transcutaneous stimulation or conventional TENS (Chan, Tsang, 1987; Guieu et al., 1991; Lundeberg, 1984).

Low frequency, high intensity stimulation produces a generalized analgesia with a gradual onset and a prolonged aftereffect (Andersson et al., 1973; Andersson, Holmgren, 1975; Chapman et al., 1975; Chapman et al., 1977; Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Huang et al., 1978; Melzack, 1975; Freeman et al., 1983). This analgesia evoked by high intensity electrical stimulation is thought to be a variation of that evoked by counter-irritation (Bing et al., 1990,1991a,b; Fox, Melzack, 1976; Melzack, 1975). The underlying mechanisms may share some similarities with diffuse noxious inhibitory controls (DNIC) served by a spinal-brain stem-spinal loop (Bouhassira et al., 1990,1992; Le Bars et al., 1979a,b; Villanueva et al.,

1986a,b; Willer et al., 1979,1984,1990). These neurophysiological mechanisms will be elaborated in a subsequent section of this thesis.

Human experimental and clinical studies:

Ever since the introduction of acupuncture to the western world, several studies have been published on the relative efficacy of acupuncture stimulation in the elevation of pain threshold. Some have shown acupuncture stimulation to produce only mild effects at best (Co et al., 1979; Gaw et al., 1975; Godfrey, Morgan, 1978; Johnson et al., 1991; Lee et al., 1975; Melzack, Katz, 1984; Mendelson et al., 1983; Richardson, Vincent, 1986) while others have shown acupuncture stimulation to produce profound analgesia that is reported to be effective enough even for open heart surgery (Hollinger et al., 1979). Several clinical reports have documented effective analgesia for the treatment of various chronic pain syndromes in up to 80 % of the patients treated. However, many of these studies were poorly controlled and therefore the value of these reports remains difficult to assess (Richardson, Vincent, 1986). In addition, the magnitude and the duration of the evoked pain relief is quite variable and it remains relatively unclear from the majority of clinical trials published as to whether acupuncture stimulation can produce benefit over prolonged periods, an issue of clinical significance and of obvious importance for the treatment of chronic pain. In the following pages I shall review some of the controlled studies in the literature on acupuncture evoked analgesia and attempt to provide a rationale for these discrepancies which I believe to be due to qualitative differences in the type of acupuncture stimulation. The effectiveness of acupuncture stimulation appears to be related to the intensity, frequency, duration and site of stimulation.

Intensity:

In reviewing the literature both experimental and clinical studies show that with low frequency acupuncture, stimulation is usually applied at an intensity just tolerable to the patient and/or subject. For example, Mann concluded from a study involving 100 experiments that aversive stimulation of acupuncture points either by manual twirling of needles or by electrical stimulation evoked mild to strong analgesic effects. In cases where mild effects were elicited a stronger analgesia could be produced by increasing the intensity of the acupuncture stimulation to unacceptably high levels (Mann, 1974). Intense stimulation has been used in other experimental studies as well. For example, continuous manual needling of *hoku (LI 4)* meridian points located in the interosseous muscle between the 1st and 2nd metacarpals and needling of the temporal mandibular joint area produced a tolerable but averse sensation. In 80% of the patients, stimulation elicited sufficient analgesia to allow successful dental treatment including deep gingival scaling and curettage, and tooth extractions including removal of an impacted tooth along with the underlying bone. The evoked analgesia could be attained by about 3 min after the insertion of needles suggesting an induction time for expression of the effect. The dental procedures could only be performed during stimulation and normal sensation returned almost immediately after the end of stimulation, suggesting that this kind of acupuncture stimulation does not elicit long lasting effects (Lee et al., 1973).

Low frequency electroacupuncture of the *hoku* point has also been shown to increase pain threshold in experimental dental studies. In these studies the threshold current intensity applied to the dental pulp to evoke pain was monitored in volunteers. In one study by Ernst and Lee stimulation of acupuncture points at intensities just above pain threshold with 0.8 ms rectangular

pulses at 2 Hz for a period of one hour produced a gradual analgesia (Ernst, Lee, 1987). Similar results were reported by Chapman and co-workers, where 80 min of electroacupuncture stimulation at 2 Hz at intensities just submaximal to the subjects' tolerance level increased the dental pain threshold by 187 %. The increase in pain threshold was maximum after 20 min of stimulation, again suggesting an induction period (Chapman et al., 1977). Similar stimulation produced analgesia equal to or better than that produced by nitrous oxide (Chapman et al., 1975). Although none of these studies compared the effects of low intensity stimulation one experimental dental study has shown that the increase in dental pain is a function of the intensity of the acupuncture stimulation. Little or no effect was elicited with low intensity stimulation below or above perception threhsold. However, stimulation at pain threshold or above produced a 3 to 4 fold increase in dental pain threhsold (Holmgren, 1975).

Most of the studies cited so far have not reported either the lack or presence of a post-stimulation analgesia. However, long-lasting aftereffects have been produced by intense stimulation. One of the first reliable experimental studies demonstrating a post-stimulation analgesia induced by electroacupuncture stimulation was preformed by Andersson and co-workers (Andersson et al., 1973; Andersson, Holmgren, 1975; Holmgren, 1975). In their experimental paradigm the *hoku* meridian points and cheek were stimulated using both surface and needle electrodes. The intensity of the electrical stimulation ranged from 15-30 mA and was described as just tolerable to the subject. Seventy-five min of stimulation at 2 Hz evoked a gradual increase in tooth pain threshold. After the stimulation was turned off the effect gradually declined over the following 15 to 20 min. In more recent experimental studies, 10 min of intense low frequency electrical stimulation of auricular acupuncture points increased

cutaneous pain threshold and this analgesic effect persisted from 10 to 20 min after the end of stimulation (Krause et al., 1987; Longobardi et al., 1989; Noling et al., 1978; Oliveri et al., 1986).

Long-lasting pain relief has also been achieved with intense acupuncture stimulation in the clinic. For example, patients suffering from chronic low back pain experienced long-term relief after stimulation of acupuncture points either by strong manual twirling of needles or by intense low frequency transcutaneous electrical stimulation. The stimulation was considered by the patients to be painful but not unbearable (Fox, Melzack, 1976). In both cases the evoked analgesia persisted for several hours and some patients experienced relief for a few days (Fox, Melzack, 1976). In a subsequent study 20 min of intense stimulation applied to painful trigger points produced long-lasting pain relief in patients suffering form phantom limb pain, peripheral nerve injury, shoulder arm pain and lower back pain. In most cases the stimulation produced a mean decrease in pain ranging from 60 to 75% and this relief again persisted for several hours to even days (Melzack, 1975). The trigger points correlated with existing meridian or acupuncture points.

Some studies suggest that reliable pain relief can be achieved only with high intensity stimulation. For example, both conventional high frequency TENS-like stimulation at 2 to 3 times the perception threshold (12- 30 mA) or acupuncture-like TENS stimulation at 2 Hz with an internal frequency of 70 Hz applied at 3 to 5 times perception threshold produced pain relief in chronic pain patients. However, low frequency acupuncture-like TENS appeared to be more effective in patients in which high frequency stimulation produced inadequate analgesia (Eriksson et al., 1979). Furthermore, acupuncture-like stimulation at three to five times perception

perception threshold, which was perceived as just tolerable, produced analgesia in chronic pain patients resistant to conventional TENS stimulation (Eriksson, Sjölund, 1976; Sjölund, Eriksson, 1979). To support further this contention Melzack et al., have demonstrated that painful ice massage applied to *hoku* meridian points decreased dental pain by 50 % or more in the majority of patients (Melzack, Bentley, 1983; Melzack et al., 1980a) and this form of stimulation was comparable to that of intense acupuncture-like TENS in the treatment of chronic low back pain (Melzack et al., 1980b). In other studies, 1 Hz electrical stimulation applied transcutaneously to auricular points at or above pain threshold elevated experimental cutaneous pain (Krause et al., 1987; Longobardi et al., 1989; Noling et al., 1978; Oliveri et al., 1986). However, when stimulation was applied at intensities well below pain threshold little effect was produced on experimental cutaneous pain (Johnson et al., 1991) or in chronic pain patients (Melzack, Katz, 1984).

These experimental and clinical studies demonstrate clearly the importance of the intensity of the stimulation in producing a reliable and long-lasting analgesia, and it has been suggested that the evoked analgesia is mediated by activation of high threshold sensory afferents, perhaps C fibres (Andersson et al., 1973; Andersson, Holmgren, 1975; Fox, Melzack, 1976; Melzack, 1975). Future studies need to be performed to elucidate further the effect of varying the intensity of stimulation on the analgesic response produced during and after the end of stimulation and to investigate the peripheral and central mechanisms contributing to these effects.

Frequency:

There is some evidence to suggest that the analgesia evoked by acupuncture stimulation is dependent on the frequency of stimulation. For example, prolonged acupuncture stimulation at 2 Hz evoked a gradual increase in tooth pain threshold which slowly decayed over the next 15 to 20 min following the end of the stimulation (Andersson et al., 1973; Andersson, Holmgren, 1975). Interestingly, when the stimulation was applied at 10 Hz tooth pain threshold increased abruptly but the post-stimulation effect ended within by 5 to 10 min after the end of the stimulation. When the same stimulation was applied at 100 Hz only a mild elevation in pain threshold was produced during the stimulation and no post-stimulation effect was observed. The authors suggested that the increase in toot pain threshold with low frequency stimulation is mediated via activation of high threshold primary afferents while the effects produced by 100 Hz stimulation may resemble the effects thought to be mediated by activation of large diameter, low threshold primary afferents (Loeser et al., 1975; Long, 1976; Meyer, Fields, 1972; Shealy, Maurer, 1974; Wall, Sweet, 1967).

A recent clinical study showed that both intense low frequency acupuncture and high frequency acupuncture produce relief in chronic pain patients. However, 2 Hz stimulation was shown to provide better long-term relief than 80 Hz stimulation (Thomas, Lundberg, 1994), suggesting that over time low frequency stimulation produced better long-lasting effects. In some cases high frequency stimulation produced better post-stimulation analgesia than low frequency stimulation. For example, intense transcutaneous stimulation just below pain threshold applied over the wrists of patients with rheumatoid arthritis produced greater pain relief with 70 Hz or with 3 Hz with an internal train of 70 Hz. Single pulses given at 3 Hz produced the least improvement. The post-stimulation analgesia experienced by these patients also differed in

duration. In this study a high frequency of stimulation produced a post-stimulation effect lasting up to 28 hours. In comparison, 3 Hz stimulation produced an analgesia lasting only 4 hours (Mannheimer, Carlsson, 1979). The different results obtained in this study may be due to the intensity of stimulation. The analgesia evoked with higher frequencies may resemble that elicited by conventional TENS (Mannheimer, Carlsson, 1979). Furthermore, it in unclear whether meridian points were stimulated. Therefore, the analgesia produced here may be unrelated to acupuncture-evoked effects.

In recent years there has not been any systematic attempt to study the effect of frequency either within the low range or high range of stimulation in humans. This is unfortunate because little is known therefore about maximizing the analgesic effects produced the type of stimulation described. In addition such studies can reveal important information about the intrinsic neurophysiological mechanisms mediating the evoked responses.

Train duration:

In reviewing the literature it was curious to see that the duration of acupuncture-like treatment with low frequency, high intensity stimulation was quite varied. For example, in some studies high intensity stimulation was applied for only 10 minutes (Fox, Melzack, 1976; Mannheimer, Carlsson, 1979) in other studies the duration of treatment was 20 min (Melzack, 1975) to 40 min (Research Group of Acupuncture Anesthesia, 1973; Sjölund, Eriksson, 1979) and yet in other studies this stimulation was given for 75 to 80 minutes (Andersson et al., 1973; Andersson, Holmgren, 1975; Chapman et al., 1975; Chapman et al., 1976; Chapman et al., 1977). Interestingly, the post-stimulation analgesia produced during these treatments appeared

to be different when comparisons were made between effects elicited by relatively short durations of stimulation versus long durations of stimulation. For example, 10 to 30 minutes of stimulation elicited effects lasting several hours or even days (Fox, Melzack, 1976; Mannheimer, Carlsson, 1979; Melzack, 1975) while 40 minutes of stimulation evoked a post-stimulation analgesia that persisted for 30 minutes (Research Group of Acupuncture Anesthesia, 1973). When the stimulation was applied for 75 minutes, the duration of the post-stimulation effect was relatively short-lived, lasting 15 minutes (Andersson et al., 1973; Andersson, Holmgren, 1975). Many factors, could give rise to the varied durations of the post-stimulation analgesia such as the relative intensities used in the stimulation, the location of the stimulation as well as the frequency of stimulation. To my knowledge no study has systematically evaluated the effect of the duration of the train of stimulation on the evoked analgesia in humans.

Meridian vs non-meridian stimulation:

A few clinical trials have demonstrated no difference in effect of meridian versus non-meridian point stimulation (Co et al., 1979; Gaw et al., 1975; Godfrey, Morgan, 1978; Lee et al., 1975). However, there are a number of factors which, when taken into account, render these results inconclusive. First, in these studies, there is no mention of which acupuncture points were used or where the chosen non-meridian points were situated, except for the vague mention that these non-meridian points lie just outside the meridian points. Therefore, the question arises as to whether these points truly lie outside the receptive field of the meridian points? Second, the intensity of acupuncture stimulation by rotation of needles was not mentioned except that the "teh chi" sensation was achieved, which is characterized by a feeling

of heaviness, numbness and/or soreness (Co et al., 1979; Gaw et al., 1975; Godfrey, Morgan, 1978; Lee et al., 1975). Therefore it is not clear if the stimulation was applied at an intensity sufficient to evoke a true and reliable physiologically-evoked increase in pain threshold (*vida supra*). Third, there is no mention of the time course of the effect and at best there is only a vague description of the magnitude of pain relief. A closer analysis of Gaw et al's (Gaw et al., 1975) results shows only an 8-14% improvement in pain scores. Lee et al (Lee et al., 1975) reported 65% of patients experienced little or no pain relief and 35% of the patients experienced 50 to 75% pain relief. A number of socio-psychological factors could have contributed to this marginal improvement; 1) the natural course of the pain pathology, 2) the patients expectation and desire to experience relief of pain and 3) the increased attention given to the patient which may increase the pain threshold. These are all factors contributing to a placebo effect.

To address the question of the effect of placebo in acupuncture treatments, Mendelson et al., (Mendelson et al., 1983), in a double blind crossover study, treated chronic back pain sufferers with non-aversive manual acupuncture of meridian points and compared the effects versus placebo which involved the insertion of needles into non-meridian points pre-treated with lidocaine. They found that during the first phase of the study, the treated group experienced a 40 % decrease in pain scores versus 26 % for the placebo. During the second phase of the study the group of patients previously treated with acupuncture was subsequently treated with placebo resulting in a 40 % decrease in pain scores. On the other hand, the previously treated placebo group which subsequently received acupuncture showed only a 19 % decrease. Therefore, in the second phase of the cross-over study the placebo was more effective than the active treatment and overall the effect of acupuncture treatment was not significantly different

from placebo. The placebo component of low intensity acupuncture produces a significant bias and therefore may be the underlying factor giving rise to the results obtained by others (Co et al., 1979; Gaw et al., 1975; Godfrey, Morgan, 1978; Lee et al., 1975).

Support favouring meridian point stimulation over non-meridian point stimulation comes from two experimental pain studies. In one case, strong but tolerable stimulation of non-meridian points did not affect pain detection threshold to thermal cutaneous stimuli, but did increase pain tolerance levels in human subjects. However, stimulation of meridian points effectively increased both pain measures, and the evoked effects were superior to those produced by non-meridian point stimulation (Stewart et al., 1977). In a second study intense electroacupuncture increased cutaneous pain threshold measured by immersion of a hand in ice water. This study also showed that acupuncture stimulation produced greater effects than stimulation of non-meridian points or placebo controls receiving no stimulation (Anderson et al., 1974).

Reliable data regarding the effects of meridian vs. non-meridian stimulation on evoked analgesia is desperatlely needed. Furthermore, we still remain in the "dark" with respect to the differences in peripheral and central mechanisms underlying the evoked responses elicited by stimulation of meridian and non-meridian points.

III. ANIMAL MODELS OF ACUPUNCTURE AND STIMULATION-EVOKED ANTINOCICEPTION

In search of the appropriate animal model

Since the early 1970's several animal models have been developed to explore the physiological bases for stimulation-evoked antinociception. However, much of the data published is difficult tc interpret because of the wide range of stimulus parameters used. In addition, many experiments were performed on unanaesthetized as well as anaesthetized preparations, further complicating the interpretation of the data.

As for acupuncture, the animal models developed fall into two major categories: 1) experiments performed in unanaesthetized animals with low or high intensity stimulation at either low or high frequency, or 2) experiments performed in anaesthetized or decerebrated preparations. As this review proceeds it will become apparent that many of the animal models developed for the study of acupuncture-induced antinociception do not yield analgesic effects resembeling those evoked by intense, low frequency stimulation in humans (Andersson et al., 1973; Andersson, Holmgren, 1975; Chapman et al., 1975; Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Huang et al., 1978; Melzack, 1975; Sjölund, Eriksson, 1979).

The unanaesthetized preparation:

In the awake animal, several groups have demonstrated that stimulation of meridian or acupuncture points with low intensity, low frequency stimulation produced an antinociception that outlasts the stimulation. For example, 10 to 30 min of stimulation of meridian points in the

hindlimb with pulses given at 2 Hz, 3 Hz or 2 and 15 Hz inhibited the withdrawal response evoked by a noxious thermal test stimulus applied to either the snout or the tail in rabbits (Han et al., 1983; Han, Xie, 1984; McLennan et al., 1977) or in rats (Chen, Han, 1992a,b; Yaksh, 1981; Zhou et al., 1993). In some experiments inhibition was produced by 100 Hz (Wang et al., 1990a,b) and even 2 KHz or 5 KHz stimulation (Lin et al., 1992). In most cases the inhibition was maximum during the stimulation and persisted for at least 10 min. In awake mice, 6 Hz stimulation of forelimb meridian points at intensities below threshold for Aδ fibres increased vocalization threshold during the stimulation. This effect peaked at 20 min after the stimulation and remained elevated for up to two hours (Pomeranz, Chiu, 1976). In the same paradigm when stimulation was applied at an intensity just below that required to produce vocalization, 0.2 Hz, 4 Hz or 200 Hz (Cheng, Pomeranz, 1979; Pomeranz, Paley, 1979) increased vocalization threshold, the magnitude of which appeared to be greater with increasing frequency.

The data from the majority of studies cited suggest that evoked responses are relatively independent of the intensity or the frequency of the stimulation in that antinociceptive effects during the stimulation and/or long-lasting aftereffects are always produced despite different parameters of stimulation. However, this does not appear to be the case in the human. As one can begin to appreciate it is difficult to draw conclusions from these animal experiments in relation to the effects produced in the human with intense, low frequency acupuncture-like stimulation. In the unanaesthetized preparation, it remains relatively unclear as to what extent stress contributed to the evoked responses vs. responses evoked directly by activation of peripheral inputs. This concern becomes acute in light of the fact that the stress of restraint

(Calcagnetti et al., 1990; Calcagnetti, Holtzman, 1990) or even of mild shock can elicit a long-lasting antinociception (Przewlocka et al., 1990). Furthermore, the parameters of stimulation in these studies do not resemble those that produce a reliable long-lasting analgesia in the human.

The anaesthetized or decerebrated preparation:

In anaesthetized preparations both low and high intensity electroacupuncture are reported to produce antinociception. In the anaesthetized cat, responses of Lamina V dorsal horn neurones to noxious mechanical stimuli were tested for the effect produced by 30 min of low intensity, low frequency electroacupuncture of meridian points in the hindlimb. In 37 of 50 cells, responses were depressed by about 20 % of the control during the conditioning stimulation. This inhibitory effect quickly decayed when the conditioning stimulus ended and the responses returned to control values within five to ten min. Clearly, the effects produced by this form of stimulation were minor and short lasting. Again the effects did not resemble acupuncture analgesia in the humans. Other, electrophysiological experiments in decerebrated cats, 100 Hz stimulation of hindlimb meridian points at varying intensities produced negative dorsal root potentials (Chan, Fung, 1975; Fung, Chan, 1976), suppressed cutaneous (Chan, Fung, 1975) and sural polysynaptic reflexes (Fung, Chan, 1976) and increased excitability of primary afferent terminals (Fung, Chan, 1976); these effects persisted up to 800 ms after the end of a brief, 20 ms train in the decerebrated cat (Chan, Fung, 1975; Clement-Jones et al., 1980; Fung, Chan, 1976). In this experimental paradigm, these responses are difficult to compare with effects produced by intense, low frequency stimulation because 100 Hz stimulation may recruite

different central mechanisms altogether.

In lightly anaesthetized rats, 45 Hz stimulation of forelimb meridian points at low or high intensities for 15 min inhibited the jaw-opening reflex evoked by noxious electrical stimulation of the tooth pulp (Toda, Ichioka, 1978). At high intensities the inhibition was about 60 % and, in addition, a post-stimulation effect lasting 15 to 20 min was evoked. In lightly anaesthetized rats, stimulation with 4 Hz electroacupuncture applied with slow increments of current from 10 to 25 times threshold produced mild inhibition and a post-stimulation effect lasting 10 min (Pomeranz, Nguyen, 1987).

Although, these models are not ideal, one can begin to see that to attempt to study the analgesic phenomena produced by acupuncture-like stimulation, animal models must be developed in which the parameters of stimulation closely resemble those of electroacupunture in humans. These models must be free of stress and therefore necessitate the use of either decerebration or some form of anaesthesia.

Such models have been developed, although with limitations (Chung et al., 1983,1984a,b; Kawakita, Funakoshi, 1982; Taylor et al., 1990). Interestingly, as in the human studies the effects produced by conditioning stimulation in animal models that adhere to the criteria above also appear to be dictated by the intensity, frequency, duration and site of the stimulation.

Stimulation-evoked antinociception: dependence on parameters of stimulation

Intensity:

Unlike the unanesthetized preparation, in the anesthetized and decerebrated animals the

evoked antinociception appears to be dependent on the intensity of the stimulation. One of the more reliable animal studies on acupuncture-evoked antinociception was performed by Kawakita and Funakoshi. They demonstrated that 5 Hz stimulation of hind limb meridian points or the common peroneal nerve at an intensity sufficient to recruit Aδ and possibly C fibres depressed the nociceptive jaw-opening reflex (Kawakita, Funakoshi, 1982). The maximum inhibition occurred at the end of the 15 min of the stimulation and the inhibition persisted for at least another 15 min. On the other hand, stimulation at lower intensities sufficient to recruit only Aß fibres only produced a mild depression of the jaw-opening reflex at 10 min after the onset of the stimulation, ar^{-1}, when the stimulation was turned off, no lingering effects were seen.

Similar intensity-dependent effects were produced in decerebrated animal models of acupuncture. For example, in the decerebrated (Paik et al., 1981) and/or spinalized cat (Chung et al., 1983; Paik et al., 1981) the flexion reflex elicited by stimulation of C fibres in the sural nerve depressed up to 50 % of the pre-conditioning control values by electrical stimulation of the *zusanli* (ST-36) meridian point in the hindlimb (Paik et al., 1981) or by direct stimulation of the common peroneal nerve (Chung et al., 1983; Paik et al., 1981). This inhibition persisted for up to 1 hour beyond the stimulation (Chung et al., 1983; Paik et al., 1981). The stimulation parameters used were 20 Volts, 2 ms pulses given at 2 Hz. The duration of the stimulation ranged from 15 min up to 1 hour (Chung et al., 1983; Paik et al., 1981). It appears from these studies that the antinociceptive effects evoked by intense, low frequency stimulation of either acupuncture points or by direct stimulation of peripheral nerve are comparable. Therefore, other animal models of stimulation-evoked antinociception elicited by direct stimulation of peripheral nerves may be adequate models for the study of acupuncture-evoked antinociception.

In primates the antinociception evoked by peripheral nerve stimulation was also found to be dependent on the intensity of the stimulation (Chung et al., 1984b). In decerebrated or decerebrated and spinalized monkeys, five minutes of electrical stimulation of Aß fibres in the tibial nerve inhibited C fibre-evoked responses of spinothalamic tract cells in the lumbosacral spinal cord, but the inhibition was minor and did not persist after the end of stimulation. In contrast, when stimulation was applied at an intensity which recruited Aδ and C fibres, a progressively increasing inhibition of these cells was noted. Also, at this intensity, a post-stimulation effect lasting 50 to 100 seconds was produced (Chung et al., 1984b). Furthermore, prolonged conditioning stimulation of tibial nerve C fibres at 2 Hz inhibited responses of spinothalamic cells to test stimulation of C fibres in the sural nerve, or to application of a noxious heat stimulus to the cutaneous receptive field (Chung et al., 1984a). In this experimental paradigm transcutaneous electrical stimulation produced inhibition of the C fibre-evoked responses , but only when the current exceeded the Aδ fibre threshold (Han et al., 1980).

Finally, in another study not directly related to acupuncture, low frequency repetitive stimulation of C fibres in the common peroneal nerve produced long-lasting inhibition of the sural-gastrocnemius reflex and application of natural noxious conditioning stimuli also produced this long-lasting inhibition in decerebrated and spinalized rabbit (Clarke et al., 1989; Taylor et al., 1990); non-noxious stimulation had no effect.

Taken together these studies suggest that high intensity stimulation of small diameter primary afferents produces long-lasting antinociceptive effect.

Frequency:

The frequency of the stimulation also appears to be an important parameter for the expression of antinociceptive effects elicited by intense electrical stimulation of the peripheral nerve. In decerebrated and spinalized primates, 5 min of stimulation of the tibial nerve at an intensity 10 times the threshold of Aδ fibres produced a frequency- dependent inhibition of spinothalamic tract cells. Within the range of 0.5 Hz to 20 Hz stimulation the inhibition was mild to strong; 0.5 Hz stimulation produced the weakest effect while 20 Hz stimulation evoked the greatest inhibition (Chung et al., 1984b). Furthermore, intense acupuncture-like TENS applied with an internal 85 Hz train of pulses given at 3 Hz was more effective in producing inhibition of spinothalamic tract cells than a train of pulses given at 85 Hz. Finally, the inhibition of the sural-gastrocnemius reflex evoked by stimulation of C fibres in the common peroneal nerve in the decerebrated and spinalized rabbit was found to be a frequency dependent phenomenon (Taylor et al., 1990). The optimum frequency to elicit this inhibition was found to be 5 Hz with an internal frequency of 70 to 100 Hz (Taylor et al., 1990). When the frequency of stimulation was applied at 0.5 Hz, facilitation instead of inhibition occurred (Taylor et al., 1990).

Train Duration:

It is interesting that 5 minutes of high intensity stimulation of Aδ and C fibres in peripheral nerves elicits a post-stimulation inhibition of primate spinothalamic tract cells that lasts only 50 to 100 seconds (Chung et al., 1984b), Yet, when the stimulation is maintained for 15 minutes the inhibition of these cells remains for up to 30 minutes (Chung et al., 1984a). In addition, in the decerebrated and spinalized rabbit, the depression of the sural-gastrocnemius

reflex evoked by intense, low frequency stimulation of the common peroneal nerve is also a function of the duration of the conditioning stimulation. Longer durations of stimulation produced greater depression of the reflex (Taylor et al., 1990). Taken together, the data suggest that duration may have a critical role in determining the outcome of the evoked antinociception.

Meridian vs. non-meridian:

Data from lightly anaesthetized rats suggest that depression of the nociceptive jaw-opening reflex evoked by 45 Hz stimulation in the forelimb is greater with stimulation of meridian vs. non-meridian points (Toda, Ichioka, 1978). At low intensities of stimulation, no difference in effect was produced when either point was stimulated. However, at intensities which recruit Aδ fibres, stimulation of meridian points produced a greater depression of the jaw-opening reflex than stimulation of non-meridian points (Toda, Ichioka, 1978). In another experiment, 100 Hz stimulation of hindlimb meridian points at varying intensities produced an inhibition of cutaneous polysynaptic reflexes while stimulation of non-meridian points produced either negligible effects or facilitated these reflexes in the anaesthetized rat (Chan, Fung, 1975).

IV. NEUROCHEMICAL BASES OF ACUPUNCTURE-EVOKED ANALGESIA OR ANTINOCICEPTION

Neurochemicals from primary afferents

Little is known of the role of neurochemicals released from primary afferent terminals within the spinal cord in the mediation of the diffuse inhibitory control over nociceptive processing, evoked by brief noxious somatic stimulation or by brief or prolonged intense electrical stimulation of peripheral nerve or by intense electroacupuncture-like stimulation in the human.

Chemicals implicated in synaptic transmission of sensory information in the spinal cord and released from primary afferents include excitatory amino acids (Radhakrishnan, Henry, 1993; Salt, Hill, 1983; Shen et al., 1975; Watkins, Evans, 1981; Yashpal et al., 1991) and neuropeptides (Cridland, Henry, 1988; Henry, 1976; Radhakrishnan, Henry, 1991; Xu et al., 1990; Yashpal et al., 1995). These likely mediate the inputs which activate the mechanisms which bring about the antinociceptive response evoked by activation of high threshold primary afferents. For example, in adult rats intrathecally administered capsaicin depolarized small diameter primary afferents releasing neurosubstrates resulting in nociception (Gamse, 1982; Hayes, Tyers, 1980) and antinociception (Dickenson et al., 1990; Nagy et al., 1981; Sun, Larson, 1993). Furthermore, depletion of C fibres by capsaicin treatment in neonatal rats attenuated nociception (Hara et al., 1984; Masters, Komisaruk, 1991) and antinociception (Masters, Komisaruk, 1991; Yashpal et al., 1995) provoked by noxious cutaneous stimulation.

Below I review some evidence which implicates excitatory amino acids in the mediation

of the sensory inputs to the spinal cord. In addition, this review includes opioids and their receptors, which have been implicated in modulation of nociceptive mechanisms in the spinal cord.

Excitatory amino acids:

Glutamate is likely to mediate synaptic transmission between primary afferents and second order dorsal horn neurones in the spinal cord. It is contained in the terminals of primary afferents (Battaglia, Rustioni, 1988; De Biasi, Rustioni, 1988,1990; Maxwell et al., 1990a,b; Merighi et al., 1991; Phend et al., 1994) including those of small diameter inputs (Battaglia, Rustioni, 1988; De Biasi, Rustioni, 1988; Phend et al., 1994), and it is released upon stimulation of low (Jeftinija et al., 1991; Kangrga, Randic, 1991a) or high threshold afferents (Kangrga, Randic, 1991a; Paleckova et al., 1992). Autoradiographic evidence strongly implicates glutamate in transmission of sensory information based on anatomical localization of receptors. Labelled glutamate binds to receptors selective for N-methyl-D-aspartate (NMDA) (Collingridge, Lester, 1989; Mayer, Westbrook, 1987; Nicoll et al., 1990) throughout the spinal gray matter; the highest density was found in the dorsal horn in both cat (Mitchell, Anderson, 1991) and rat (Greenamyre et al., 1984; Henley et al., 1993). In the human spinal cord, MK-801, a non-competitive NMDA antagonist densely occupied NMDA receptors in the substantia gelatinosa of the dorsal horn (Shaw et al., 1991).

Electrophysiological experiments further support glutamate's role in mediation of synaptic transmission of sensory information. For example, iontophoretic application of glutamate excite superficial and deep dorsal horn cells (Aanonsen et al., 1990; Dickenson, Sullivan, 1987;

Gerber, Randic, 1989; Schneider, Perl, 1985). This excitatory action of glutamate on dorsal horn cells (Schneider, Perl, 1988) is via generation of inward currents (Yoshimura et al., 1991) through activation of NMDA receptors (Jahr, 1992). NMDA receptor antagonists are demonstrated to attenuate dorsal horn neurone responses to applied glutamate and to high threshold inputs evoked electrically or by noxious cutaneous stimuli. For example, the dissociative anesthetic phencyclidine (PCP), a non-competitive antagonist of the NMDA receptor (Anis et al., 1983), blocked responses of spinothalamic tract cells to iontophoretically applied glutamate, to noxious mechanical stimuli and to electrical Aδ and C fibre excitatory inputs in primates (Willcockson et al., 1986). Dorsal horn cell responses to noxious thermal cutaneous stimulation in unanesthetized, decerebrated cats (Radhakrishnan, Henry, 1993) are blocked by ketamine, a dissociative anesthetic similar to PCP (Anis et al., 1983), and by 2-amino-5-phosphonovaleric acid (APV) a competitive antagonist of the NMDA receptor (Aanonsen, Wilcox, 1986). Activation of the NMDA receptor appears to be involved in the synaptic events elicited by noxious and non-noxious cutaneous mechanical stimulation (King, Lopez-Garcia, 1993) and is suggested to have critical importance in the development of "windup" in the spinal cord (Savola et al., 1991; Dickenson, Sullivan, 1987; Thompson et al., 1990; King, Lopez-Garcia, 1993; Thompson et al., 1994). In *in vitro* experiments, ventral root potential responses to electrical C fibre input were antagonized by APV (Thompson et al., 1994) in the isolated spinal cord of the rat.

Data from behavioural paradigms also implicate excitatory amino acids in nociceptive sensory pathways. Intrathecal administration of NMDA into the rat lumbar spinal cord produced central experimental pain disorders such as irritability, circling, biting and excessive grooming

resulting in loss of hair, and skin ulcerations from autotomy (Zochodne et al., 1994). In other experiments, acute administration of NMDA to the spinal cord elicited thermal hyperalgesia (Malmberg, Yaksh, 1993b; Meller et al., 1992; Mjellem-Joly et al., 1992; Raigorodsky, Urca, 1987) and APV blocked facilitation of the thermally elicited tail withdrawal reflex evoked by noxious cutaneous input (Yashpal et al., 1991). Furthermore, spinal administration of NMDA potentiated the formalin-evoked behavioural nociceptive responses (Coderre, Melzack, 1992a). When selective NMDA antagonists were given spinally, the evoked nociceptive behavioural responses to formalin injection were blocked (Coderre, Melzack, 1992b; Vaccarino et al., 1993). Finally, in several chronic pain models, NMDA receptor activation has also been implicated in thermal (Mao et al., 1992a,b; Meller et al., 1994; Tal, Bennett, 1994) or mechanical (Neugebauer et al., 1993) hyperalgesia induced by sustained activation of C fibres.

Although the evidence implicates the NMDA receptor in processing of nociceptive information, there is also evidence to suggest spinal activation of these receptors provoke inhibition of nociceptive pathways. Spinal administration of NMDA (Mjellem-Joly et al., 1992; Raigorodsky, Urca, 1987) elicit both pronociceptive and antinociceptive responses. A low dose of NMDA given by lumbar puncture prior to subcutaneous formalin injection of the hindpaw decreased the nociceptive behaviour of the late phase in mice (Mjellem-Joly et al., 1992) and the same dose of NMDA produced potentiation of morphine-elicited antinociception in intact rats but not in acute ($\leq$ 26 h) spinal rats (Advokat et al., 1994). In addition, intrathecal administration of higher doses of NMDA produced facilitation of the thermally elicited tail withdrawal reflex in rats followed by inhibition of the reflex lasting for several minutes (Raigorodsky, Urca, 1987). This pronociceptive effect followed by the lasting antinociceptive

effect was also produced in chronically ($\geq$ 5 days) spinalized animals (Raigorodsky, Urca, 1987), implicating spinal mechanisms in the NMDA-evoked antinociception.

Opioid modulation of sensory transmission:

Since the discovery of multiple opiate receptors and opioids in the central nervous system, the use of specific antagonist has contributed to the abundant information concerning their roles in the modulation of the transmission of sensory information (Basbaum, Fields, 1984; Millan, 1986). In the next few pages I shall focus on the relative contribution of each of the opioid peptides in modulation of nociception and review their involvement in mediation of acupuncture-evoked analgesia or antinociception.

Enkephalins:

Laminae I and II of the dorsal horn are shown to be immunoreactive for met- and leu-enkephalin-like material in the rat (Bresnahan et al., 1984; Hökfelt et al., 1977; Miller, Seybold, 1987) the cat (Glazer, Basbaum, 1983; Miller, Seybold, 1987) the primate (LaMotte, De Lanerolle, 1983) and in the human spinal cord (Przewlocki et al., 1983). The spinal source for these opioid peptides appears to be interneurones because rhizotomy or spinal section does not significantly alter the levels of enkephalin-like immunoreactivity. However, in addition to intrinsic spinal neurones, enkephalinergic fibres originating in the brain stem nuclei such as the raphe project to the spinal cord (Hökfelt et al., 1977).

The enkephalins bind to both μ- and δ- receptors but have a greater affinity for the latter (Millan, 1986).

Dynorphin:

Laminae I and V of the dorsal horn, the dorsal roots and the dorsal ganglia have all been shown to contain dynorphin A (1-17), dynorphin A (1-8) and dynorphin B (1-13) immunoreactive-like-material (Basbaum et al., 1986; Botticelli et al., 1981; Miller, Seybold, 1987; Przewlocki et al., 1983). At the level of the sacral spinal cord dynorphin is thought to originate in part from primary afferents (Basbaum et al., 1986), but in the remaining spinal cord the source also appears to be spinal interneurones because spinal transection at mid-thoracic level or dorsal rhizotomy does not alter dynorphin immunoreactivity (Basbaum et al., 1986).

Dynorphin is selective for the kappa receptor (Millan, 1986).

ß-Endorphin:

Fibres of passage containing ß-endorphin immunoreactive-like material have been shown to course down the dorsolateral and lateral funiculi terminating in the region of the central canal of the spinal cord (Tsou et al., 1986). Evidence suggest that ß-endorphin exerts its action via binding to the μ- and δ-receptor (Hong et al., 1993).

Role of μ-opiate receptors:

Evidence for the role of μ-opiate receptors in sensory transmission is supported by anatomical, electrophysiological and behavioural data linking this receptor to modulation of sensory processing in the CNS. In the dorsal horn 70-90 % of opiate receptors are of the μ-subtype (Stevens et al., 1991). Autoradiographic evidence demonstrated radio-labelled DAMGO, a μ-opiate receptor agonist, binding in the spinal cord, with up to 74 % of the total

binding occurring in laminae I and II of the dorsal horn (Besse et al., 1991). This finding was further supported by dense binding of labelled FK-33-824, another highly selective μ-agonist, in superficial dorsal horn (Gouarderes et al., 1991). In both these studies dorsal rhizotomy progressively decreased binding of labelled agonist by at least 70 %, suggesting that μ-receptors are predominately pre-synaptic on primary afferent terminals (Besse et al., 1992; Gouarderes et al., 1991).

In *in vitro* release studies, μ-receptor agonists have been shown to inhibit the release of substance P from primary afferent terminals (Bourgoin et al., 1994) and inhibit the release of aspartate and glutamate elicited by high intensity stimulation of primary afferents (Kangrga, Randic, 1991b), further supporting pre-synaptic modulation of sensory transmission in the spinal cord. In rats made polyarthritic with injection of Freunds adjuvant, spinal perfusion with DAMGO produced a naloxone-reversible inhibition of the release of CGRP-like material and naloxone by itself produced an even greater release of this peptide in polyarthritic rats (Collin et al., 1993), therefore implicating μ-receptor modulation of sensory transmission in chronic pain states.

In addition to pre-synaptic modulation, there is also evidence to suggest post-synaptic modulation of sensory transmission via activation of the μ-receptor. In the *in vitro* slice preparation of the substantia gelatinosa of the spinal trigeminal nucleus pars caudalis in the guinea pig and rat, met-enkephalin evoked a naloxone-reversible hyperpolarization manifested by increased potassium conductance in the majority of neurones (Grudt, Williams, 1994). Furthermore, in patch clamp studies simultaneous or prior application of μ-agonist DAGO depressed responses evoked by NMDA in disassociated cells from the superficial dorsal horn

of the rat (Rusin, Randic, 1991) suggesting direct modulation of the NMDA receptor ion channel.

With respect to more global actions of μ-receptor activation on nociception in the dorsal horn, μ agonists produced depression of C fibre-evoked activity in the lumbar spinal cord of the rat (Villanueva et al., 1991) and depressed responses of wide dynamic range neurones to application of noxious cutaneous stimulation in the cat (Omote et al., 1991). Low doses of morphine potentiated lignocaine-evoked depression of responses of neurones to single C fibre inputs or lignocaine-evoked depression of "windup" elicited by repetitive C fibre stimulation (Fraser et al., 1992); in the rat higher doses of morphine applied alone depressed "windup" in dorsal horn neurones when pre-administered prior to stimulation (Chapman et al., 1994). In addition, the flexor reflex evoked by noxious pinch is depressed by systemic administration of μ-agonists morphine and fentanyl (Herrero, Headley, 1991) in rats.

Several behavioural studies show that μ-opiate receptor agonists produced antinociceptive effects. For example, intrathecal administration of DAMGO produced antinociceptive effects in both the tail flick test and hot plate test (Stewart, Hammond, 1993; Suh et al., 1994). Morphine or DAGO was effective in blocking the tail withdrawal reflex evoked by immersion of the tail in 53°C water (Nagasaka, Yaksh, 1995). Intrathecal morphine or fentanyl produced antinociception in the hot plate test and in the acetic acid induced writhing test (Furst, 1991). When morphine was given intrathecally, it dose dependently decreased both the early and late phases of nociceptive behaviour evoked by injection of formalin in the hind paw of the rat (Malmberg, Yaksh, 1993a).

Role of δ-opiate receptors:

Of the total population of opiate receptors in the spinal cord, 7-28 % are found to be of the δ-receptor subtype (Stevens et al., 1991). Labelled DTLET, a δ-receptor agonist, binds in rat spinal cord and about 20 % of the total binding occurs in Laminae I and II of the dorsal horn (Besse et al., 1991); binding of the δ-antagonist ^{3}H-naltrindole is predominantly in the substantia gelatinosa (Drower et al., 1993). These receptors are located mainly pre-synaptically on primary afferents because dorsal rhizotomy decreased the binding of labelled δ-agonist by up to 60 % (Besse et al., 1992).

In an immunohistochemistry study, antisera raised against a cloned δ-opiate receptor, DOR-1, bound to a dense plexus within the superficial dorsal horn. This immunoreactivity was greatly decreased after dorsal rhizotomy, suggesting a presynaptic localization. Furthermore, a population of small diameter primary afferents which were DOR-1 immunoreactive were also immunoreactive for CGRP. These DOR-1 and CGRP immunoreactive fibres were opposed to terminals containing immunoreactive enkephalin-like material, suggesting enkephalin from intraspinal sources may be the natural ligand for the receptor encoded for DOR-1 and that this opioid may regulate the release of CGRP from the primary afferent terminals (Dado et al., 1993). Pre-synaptic δ-receptor activation has been shown to inhibit the release of CGRP from primary afferent terminals in *in vitro* slice preparations (Bourgoin et al., 1994) and δ-agonists depressed the constant release of substance P in the intact spinal cord of the rat (Collin et al., 1991). This effect was reversed by the δ-antagonist naltrindole and, in addition, the antagonist alone enhanced the release of substance P (Collin et al., 1991), suggesting a tonic opioid mediated inhibition of primary afferents.

Electrophysiological data show that δ-agonists depressed C fibre-evoked activity in the dorsal horn of the rat (Villanueva et al., 1991) and depressed the activity of wide dynamic range neurones elicited by noxious cutaneous stimuli in the cat lumbar spinal cord (Omote et al., 1991).

Several algesiometric tests have implicated activation of the δ-opiate in antinociception. For example, in the nociceptive tail withdrawal reflex evoked by noxious thermal stimulation δ_1-receptor agonists such as DPDPE (Drower et al., 1991; Nagasaka, Yaksh, 1995; Shah et al., 1994; Sofuoglu et al., 1991; Stewart, Hammond, 1993; Suh et al., 1994), [D-Ala_2]-Deltorphin II (Improta, Broccardo, 1992) or DADL (Nagasaka, Yaksh, 1995) given intrathecally produced antinociception and the δ_2-agonist DSLET also dose dependently inhibited this reflex (Shah et al., 1994; Sofuoglu et al., 1991) in rats. Furthermore activation of δ-receptors produces rather long-lasting antinociception (Improta, Broccardo, 1992). In the hot plate test DPDPE was effective in producing antinociception (Drower et al., 1991; Stewart, Hammond, 1993; Suh et al., 1994), however, agonists selective for the δ_2-receptor do not appear to induce antinociception in this paradigm (Stewart, Hammond, 1993).

Role of κ-opiate receptors:

κ-opiate receptors are sparse in the adult rat spinal cord (Sullivan, Dickenson, 1991) making up only 3-5 % of all opiate receptors in the dorsal horn (Stevens et al., 1991). Nonetheless, these receptors are implicated in sensory transmission because selective κ-receptor ligands bind in the spinal cord with up to 10 % of the total binding occurring in Laminae I and II (Besse et al., 1991) of the dorsal horn. These receptors are found to be predominately located

post-synaptically as demonstrated by dorsal rhizotomy experiments (Besse et al., 1990), suggesting that the κ-receptor may modulate synaptic transmission of sensory information in the dorsal horn at a post-synaptic site.

In addition to probable post-synaptic modulation of sensory transmission via activation of the κ-receptor, a presynaptic action has also been suggested. For example, selective κ-ligands inhibit the release CGRP-like material from primary afferent terminals (Bourgoin et al., 1994).

Electrophysiological data have supported the role of the κ-receptor in modulation of sensory transmission. For example, systemic administration of the κ-agonist U50,488H depressed the flexor withdrawal reflex elicited by noxious pinch (Herrero, Headley, 1991) and intrathecal administration depressed the C fibre-evoked spinal flexor reflex (Hernandez et al., 1993) in rats. Systemically applied κ-opioids have been shown to suppress the firing of dorsal horn neurones elicited by noxious thermal cutaneous stimulation (Headley et al., 1984)

When κ-receptor agonists bremazocine, ethylketocyclazocine or pentazocine were given to mice and rats a long-lasting antinociception was produced in the acetic acid writhing test, but these agonists were ineffective in the hot plate test (Furst, 1991). Intrathecal administration of U50,488H (Nagasaka, Yaksh, 1995) or ketocyclazocine (Goodchild et al., 1991) depressed the nociceptive tail withdrawal reflex evoked by noxious thermal stimulation in rats. Subcutaneous administration of various κ-agonists also produced antinociception in the thermally evoked tail withdrawal test at moderate heat intensities and depressed the withdrawal reflex in response to pressure; these antinociceptive effects were effectively blocked by selective κ-antagonists (Millan, 1989). However, κ-agonists had no effect on vocalization threshold to noxious electrical stimulation (Millan, 1989). κ-agonists also appear to be effective in chemical

nociception. In rats, intrathecal administration of the κ-agonists U50,488H had little effect on the early phase of the nociceptive behaviour induced by formalin injection in the rat hindpaw, but the 2nd late phase was depressed in a dose dependent manner (Malmberg, Yaksh, 1993a).

Opioids and electroacupuncture analgesia:

Several lines of evidence suggest that at the spinal level opioids mediate the analgesic or antinociceptive effects evoked by intense, low frequency stimulation, both in the human and animal models of stimulation-evoked antinociception.

Low frequency acupuncture in the human elicited an increase in ß-endorphin but not enkephalin in cerebrospinal fluid (CSF) (Clement-Jones et al., 1980). In another study, acupuncture-like TENS stimulation at 3 Hz for 30 min increased CSF levels of dynorphin-like material. This increase was found to be related to the segmental level of stimulation (Sjölund et al., 1977). In an animal model of acupuncture, manual stimulation of hindlimb meridian or non-meridian points produced an extrasegmental increase in levels of met-enkephalin-like immunoreactivity in CSF as well (Bing et al., 1991a).

In humans, systemic administration of naloxone blocked the analgesia evoked by intense acupuncture-like TENS (Sjölund, Eriksson, 1979) in 6 out of 10 patients while the analgesia produced by conventional high frequency TENS appeared to be naloxone insensitive (Sjölund, Eriksson, 1979). A similar naloxone blockade of the inhibition of the blink reflex elicited by intense electroacupuncture has also been demonstrated (Willer et al., 1982).

The antinociceptive effects produced by intense stimulation in animal models of acupuncture are also susceptible to naloxone. For example, in the decerebrated and spinalized

primate, naloxone had no effect on the evoked inhibition on spinothalamic cells during intense, low frequency conditioning stimulation of the tibial nerve. The post-stimulation effect was found to be naloxone sensitive (Chung et al., 1984a,b). However, in other paradigms naloxone was found to be more effective in blocking the evoked antinociception. For example, in the decerebrated and spinalized cat recruitment of Aδ and C fibres in the common tibial or peroneal nerve evoked a long-lasting depression of the flexion reflex recorded electrophysiologically from the ventral roots (Chung et al., 1983) and in the rabbit prolonged intense electrical stimulation applied to the toes depressed the sural-gastrocnemius reflex (Taylor et al., 1990). In both studies the evoked inhibition was completely reversed by naloxone (Chung et al., 1983; Taylor et al., 1990). In a more recent study by Lee and Beitz (1992), 4 Hz and 100 Hz stimulation of acupuncture points in the hindlimb attenuated the expression of the *c-fos* oncogene in the spinal cord induced by noxious mechanical stimulation. Suppression of *c-fos* expression by 4 Hz stimulation was completely reversed by prior administration of naloxone. Suppression of c-*fos* expression by 100 Hz stimulation was only partially antagonized by naloxone (Lee, Beitz, 1992).

Therefore, it is likely that prolonged activation of high threshold primary afferents evokes the release of substances within the CNS which in turn provoke inhibitory mechanisms via release of endogenous opioids and activation of opiate receptors to negatively modulate nociception within the spinal cord.

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PART III

RESEARCH PAPERS

CHAPTER ONE

INTENSE PERIPHERAL ELECTRICAL STIMULATION EVOKES BRIEF AND PERSISTANT INHIBITION OF THE NOCICEPTIVE TAIL WITHDRAWAL REFLEX IN THE RAT

1. In a study of modulation of nociception by sensory inputs, electrical stimulation was applied to specific sites in the hindlimb and effects on the nociceptive tail withdrawal reflex were monitored in the lightly anaesthetized rat.

2. Stimulation was applied to previously defined sites in the hindlimb, meridian points *femur-futu* (ST-32), *fengshi* (GB-31) and *zusanli* (ST-36). It consisted of a 4 Hz train of 2 ms square pulses given for 20 min at 20 times the threshold intensity required for muscle twitch. Tail withdrawal was provoked by application of a noxious heat stimulus applied to the tip of the tail.

3. During stimulation the latency of the withdrawal increased to approximately 70 % of the maximal possible inhibition. Following stimulation the inhibition persisted for greater than one hour.

4. Stimulation at 2 or 6 Hz elicited similar effects, but stimulation at 8 Hz evoked inhibition only during the stimulation.

5. When stimulation was applied to sites away from defined meridian points inhibition of the tail withdrawal occurred only during stimulation.

6. In acutely transected animals ($\leq$ 48 h) stimulation of meridian points elicited a small, brief increase in latency but only during stimulation. At 7 and 14 days after spinal transection, this response during stimulation was greater in magnitude, and a brief post-stimulation increase was

also observed. The return of this latter effect was coincident with the return of bladder function.

7. These data suggest that high intensity, low frequency electrical stimulation of hindlimb meridian points in the lightly anaesthetized rat produces both brief and persistent inhibitory effects on the nociceptive tail withdrawal reflex. These effects appear to be elicited by different mechanisms. The persistent effect may represent a plastic change in central inhibitory mechanisms. Data from spinal animals indicate a major participation of supraspinal structures, but that spinal mechanisms are also capable of sustaining both types of effect.

Key Words: nociception, pain, electrical stimulation, spinal cord

Both high and low threshold sensory inputs are thought to modify central mechanisms of nociception. For example, light vibration (Lundeberg *et al.* 1984; Woolf, 1989) and aversive stimulation (Macdonald, 1989; Melzack, 1989) have both been used to alleviate pain in humans. In animal studies, vibration applied just outside the receptive field depresses activity of wide dynamic range neurones in the cat dorsal horn (Salter & Henry, 1990). High intensity thermal stimulation applied to the same dermatome in the rat facilitates a nociceptive withdrawal reflex (Cridland & Henry, 1988; Clarke *et al.* 1992). In contrast to this evoked facilitation, an inhibition has been observed when a high intensity stimulus is applied at a different dermatomal level (Pitcher *et al.* 1995).

In human patients and subjects, high intensity electrical stimulation has been shown to attenuate chronic and experimental pain (Andersson *et al.* 1973; Andersson & Holmgren, 1975; Melzack, 1975; Fox & Melzack, 1976; Sjölund & Eriksson, 1979). However, attempts to elicit a parallel effect of electrical stimulation in experimental animals have generally led to an antinociception which may be attributed to stress. In awake mice, low frequency electrical stimulation at intensities subthreshold to produce vocalization evokes a naloxone-reversible, post-stimulation antinociception which lasts up to 2 h (Pomeranz & Chiu, 1976; Pomeranz & Paley, 1979). This effect may have been related to stress because the intense stimulus was applied to awake, restrained animals, and stress-induced antinociception appears to be opioid mediated. For example, rats in a novel environment, which is presumed to be mildly stressful, exhibit a potentiated morphine-induced antinociception (Calcagnetti *et al.* 1990) and restrained rats

subjected to a non-aversive foot-shock show a naloxone-sensitive antinociception in anticipation of a painful stimulus (Przewlocka *et al.* 1990). Some animal studies have avoided the effects of stress; in the anaesthetized cat (Pomeranz & Cheng, 1979) and rat (Pomeranz & Nguyen, 1987), low frequency yet low intensity electrical stimulation produces antinociception, but this effect is brief and thereby does not resemble analgesia produced in humans by high intensity, low frequency electrical stimulation. A significant aspect of the analgesia reported in human patients and subjects given high intensity electrical stimulation is the prolonged nature of the analgesic effect, lasting several minutes, to hours to even days (Melzack, 1975; Fox & Melzack, 1976; Sjölund & Eriksson, 1979). The closest model that reflects a prolonged antinociceptive effect has been achieved in several animal studies by high intensity, low frequency electrical stimulation applied directly to peripheral nerves. This produces an inhibition of the flexion reflex in decerebrated and spinalized cats which lasts 10-60 min (Chung *et al.* 1983), an inhibition of the sural-gastrocnemius reflex in the decerebrated and spinalized rabbit (Taylor *et al.* 1990), and an inhibition of spinothalamic tract cells in anaesthetized, decerebrated and spinalized monkeys (Chung *et al.* 1984). However, these animal models are all restricted to segmental mechanisms of stimulation-evoked antinociception, as opposed to the more generalized analgesia which characterizes the analgesia-induced in humans by high intensity, low frequency electrical stimulation (Melzack, 1975; Fox & Melzack, 1976; Sjölund & Eriksson, 1979). This type of stimulation in humans is often referred to as electroacupuncture.

One feature which characterizes electroacupuncture in humans is the use of meridian points as specific sites for insertion of the stimulating needles. Analgesia is evoked by stimulation at

meridian points but not by stimulation at other, so-called non-meridian points (Anderson *et al.* 1974; Stewart *et al.* 1977). Similar meridian points have been reported in experimental animals (Pomeranz & Cheng, 1979; Bing *et al.* 1990; Bing *et al.* 1991), based on points in humans. However, animal studies have generally failed to compare meridian vs. non-meridian point stimulation or have reported that stimulation of meridian and non-meridian points achieves similar levels of antinociception (Bing *et al.* 1991).

Our objective in the present study was to investigate the effects of high intensity, low frequency electrical stimulation of peripheral sites on nociceptive mechanisms in the rat. After exploring a number of possible models, the one in which a persistent antinociception could be produced reliably and in which stress could be eliminated, was the lightly anaesthetized rat (Fung *et al.* 1975; Toda & Ichioka, 1978; Kawakita & Funakoshi, 1982; Sjölund, 1985). We used a high intensity, low frequency train of electrical stimuli, as these parameters resemble those used clinically for alleviation of pain in humans (Andersson *et al.* 1973; Melzack, 1975; Fox & Melzack, 1976; Sjölund & Eriksson, 1979). These parameters are very different from low intensity, high frequency transcutaneous electrical nerve stimulation (TENS or so-called "conventional TENS"), which appears to be based on activation of large diameter afferents (Salter & Henry, 1990). In addition, as some studies have implicated supraspinal structures in acupuncture-induced antinociception (Shen *et al.* 1975; Wang *et al.* 1990), a series of experiments was done to include a similar study in spinal transected rats. Preliminary versions of the data have been presented in abstract form (Romita *et al.* 1992; Romita *et al.* 1993).

METHODS

In all cases, the guidelines described in The Care and Use of Experimental Animals, of the Canadian Council of Animal Care, Vols. I and II, were strictly followed. Moreover, the experimental protocols were reviewed and approved by the McGill University Animal Care Committee.

Animal preparation

Male Sprague Dawley rats (300-400 g) were lightly anaesthetized for the duration of the experiment with an intraperitoneal injection of a freshly prepared mixture of sodium pentobarbital (20 mg/kg, Abbott Laboratories Ltd.) and chloral hydrate (120 mg/kg, Fisher Scientific) in 50% propylene glycol and 30% physiological saline (0.9% NaCl). This provides a level of anaesthesia sufficient to prevent any overt sign of discomfort to the rat during experimentation, yet a stable response is obtained in the tail withdrawal test for about 1 hour. To maintain this light state of anaesthesia throughout the duration of the experiment, subsequent injections of the mixture were given. Thus, an injection of ½ the initial dose of anaesthetic was given 35.5 min after the first injection; this injection was timed to occur 1.5 min prior to the beginning of electrical stimulation. Subsequent injections of ⅓, ¼ and 1/6 the initial dose were given at 30 min intervals.

Tail withdrawal reflex

The level of nociception was determined as the latency of tail withdrawal from a noxious radiant heat stimulus. A portion of the tip of the tail was blackened to facilitate the absorption of heat. This portion was positioned above a focused projector bulb to elicit the tail withdrawal reflex. This withdrawal exposed the light beam to a photodetector which stopped a timer giving reaction time measured to 0.01 s. The intensity of the bulb was set so that the baseline reaction time was 4-6 s. The timer was turned off automatically if a tail withdrawal did not occur within 12 s, the cut-off time.

At each sample time two readings were taken, separated by 40-50 s, at two different sites rostral and caudal within the 2 cm blackened segment of the tail. Thus, the tail withdrawal latency was never measured twice from the same site within a 3 to 5 min period. The average of the two readings was calculated and the value expressed as a percent of the

maximum possible inhibition (MPI) according to the formula

$$MPI = \frac{(\text{POST-TREATMENT LATENCY - PRE-TREATMENT LATENCY})}{(\text{CUT-OFF TIME - PRE-TREATMENT LATENCY})} \times 100$$

To measure the latency of the tail withdrawal during the period of the electrical stimulation, the stimulator was temporarily turned off, just long enough for the reading to be taken; this was necessary because the stimulus was above the threshold to elicit a direct contraction of muscles (*vide infra*).

Electrical Stimulation

Previous studies have defined meridian points in the hindlimb of the rat (Bing *et al.*1990; Bing *et al.*1991) and cat (Pomeranz & Cheng, 1979). Stimulation of these sites elicits an antinociceptive or analgesic effect. The present study is based on stimulation of these sites as illustrated in fig. 1. Two pairs of stainless steel insect pins were inserted in the vicinity of meridian points *femur-futu* (ST-32), *fengshi* (GB-31) and *zusanli* (ST-36) as defined by other investigators (Pomeranz & Cheng, 1979; Bing *et al.*1990; Bing *et al.*1991) or in non-meridian points in the nearby medial and lateral gastrocnemius, femur biceps and semitendinosus muscles of the hindlimb. To stimulate *femur-futu* the cathode was placed along the medial side of the knee and the anode was inserted along the lateral side of the knee so that it lay across *zusanli* (ST-36). To stimulate *fengshi*, the electrodes were inserted from the lateral aspect under the femur midway between the hip and the knee.

The needles were connected to coupled Grass stimulators (SD9 and SD5) which passed a train of monophasic square pulses 2 ms in duration. Stimulation was applied to the respective sites at 20 times the threshold for muscle contraction (20-30 mA; minimum 15 volts). Threshold was taken as the lowest intensity of stimulation which just produced muscle contraction. The duration of the train was 20 min. The frequency was usually 4 Hz. These parameters of stimulation never provoked any behavioural signs of discomfort. In control animals, electrodes were inserted into the respective areas of the hindlimb but the stimulators were not switched on.

Experimental protocol

The lightly anaesthetized rat was placed in a plastic restrainer on the apparatus used to measure tail withdrawal latency, needles were inserted and threshold determined. Before testing was started, the animals were allowed to stabilize for 30 min; 3 baseline readings of the tail withdrawal latency were then taken at 3 min intervals. Stimulation was applied starting 1 min after the last baseline reading. Latency was then recorded at 2 and 5 min after the onset of stimulation and then at 5 min intervals up to 95 min.

Spinal transection

In some animals the spinal cords were transected to determine whether the effects observed in intact rats could be elicited after spinal section. Rats were anaesthetized with chloral hydrate (300 mg/kg, i.p.; Fisher Scientific). The antibiotic, Tribrissen 24% (0.02 ml/100 g; Trimethoprim and Sulfodiazine) was injected subcutaneously 3 h prior to and 3 h after the surgery. The spinal cord was exposed at the 6th and 7th thoracic segments. After making a slit in the dorsal part of the dura mater the cord was transected and aspirated by suction approximately 2 millimetres caudal and rostral to the level of transection. Gelfoam and/or bone wax was placed into the empty vertebral column to reduce bleeding and to seal the empty vertebral cavity.

Four hours after surgery, the rats had completely recovered from the anaesthetic and produced a tail withdrawal to the noxious heat stimulus applied to the tip of the tail at a consistent latency. This was seen in all spinalized animals.

Spinalized rats were maintained on their regular diet and their bladders were voided 3 to 4 times daily for the first 5 to 8 days following surgery; after this time bladder function had returned in all animals. They were maintained for at least 14 days. Animals were selected for study on the basis of the following criteria. All animals were healthy in general appearance. Normal eating and drinking were maintained and there was no long-term weight loss. Normal voiding and defecation were sustained after the 8 day period of recovery. All animals showed normal grooming behavior. All animals were mobile despite hindlimb paralysis and hindlimb pinch produced a withdrawal but no vocalization. In fact, all animals thus selected exhibited a consistent baseline reaction time throughout the recovery period. Therefore, no animals were omitted for inconsistent responding.

For testing, each spinal animal was placed, unanaesthetized, in a plastic restrainer so that only the tail and one hindlimb protruded. The unanaesthetized rat was used because, in pilot studies, there was no sign of distress to the animals yet consistent baseline readings were observed. The anaesthetic produced inconsistent withdrawal latencies at the doses used in intact rats. The restrainer was covered with a black cloth to minimize visual stimuli. Rats which were used experimentally more than 24 hours after transection were introduced and habituated to the restrainer for 60-90 min 1 or 2 days prior to experimentation. The protocol was followed as described above for the lightly anaesthetized preparation. Some animals were used in more than one group but in no group were more than half the animals used previously. Control rats, studied at different times after spinal transection, were treated in a similar fashion as the intact control rats.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (S.E.M.) for each time point in each group of rats. In the anaesthetized rats, the net effect was obtained by subtracting the mean value in the unstimulated group from the mean value at the respective time in the stimulated group, to eliminate the effects of administration of anaesthetic. In the unanaesthetized, spinal rats no such subtraction was necessary. These values from treated rats which received electrical stimulation and from control animals which received no stimulation were analyzed using a two way analysis of variance (ANOVA). The between-subject factor was stimulation at meridian vs. non-meridian points, or stimulation with one set of parameters vs. stimulation with another set of parameters. Time was taken as the within-subject factor. Tukey's Wholly Significant Difference Test was used to make post-hoc comparisons between means. In some analyses, the mean MPI was calculated for the period of stimulation and for subsequent post-stimulation periods. For the 20 min period of stimulation the mean MPI was calculated over the first 5 readings. For the three 25 min post-stimulation periods the mean MPI was calculated over the five readings taken in each period. Paired t-tests were used to compare the means between treatment groups.

Measurement of Arterial Pressure and Heart Rate

To determine whether cardiovascular changes might have contributed to the effects seen on withdrawal latency some animals were used to determine the effects of the stimulation on arterial pressure and heart rate. Under chloral hydrate anaesthesia (300 mg/kg, i.p.; Fisher Scientific) the left femoral artery was cannulated with Intramedic PE-50 tubing 24

to 48 hours prior to the experiment. Heparin sodium salt (150 units/ml; Sigma) was used to flush the catheter twice daily to maintain patency.

During the experiment, the same protocol was followed as above. Thus, rats were lightly anaesthetized with the mixture of sodium pentobarbital and chloral hydrate. The arterial catheter was connected to a Statham pressure transducer (Gould P23-ID) and arterial pressure was monitored via a Grass model 5 D.C. preamplifier and driver amplifier. Heart rate was calculated from these recordings by counting the number of beats in a 10 s period and multiplying this value by 6 to obtain the rate in beats per min (bpm). Needles were inserted into hindlimb meridian points and threshold was determined. After a 30 min period of stabilization, three baseline readings were taken at 3 min intervals; 1 min after the last baseline reading, hindlimb meridian points were stimulated. Arterial pressure and heart rate were then recorded at 2 and 5 min after the onset of stimulation and at 5 min intervals thereafter, up to 60 min.

RESULTS

In control rats, ie. those in which stimulating electrodes were inserted into meridian points but the stimulator was not turned on, reaction time in the tail withdrawal test remained relatively constant throughout the experiment, with the exception of slight increases in latency occurring as a result of the administration of the successive doses of anaesthetic. This provided the control for the use of anaesthetic. The data from these control animals for each paradigm are illustrated in the respective figures.

Effects of electrical stimulation of meridian points in intact rats

Electrical stimulation of hindlimb meridian points applied at 20 times threshold and at 4 Hz for a duration of 20 min (n = 8), increased withdrawal latency. Readings were significantly different from those in the control group (n = 12) throughout the period of stimulation and for

the next 75 min as illustrated in Fig. 2.

The response was characterized by what appeared to be two peaks occurring during the period of stimulation as well as a third peak occurring during the post-stimulation period; two peaks were similarly observed during the period of stimulation in all subsequent groups (*vide infra*). In this series, these two peaks were observed at 10 and 20 min after the onset of stimulation and were 70.6 ± 7.45 and 68.0 ± 6.96 % of the MPI, respectively. The third peak was 48.8 ± 9.62 % of the MPI and occurred at the reading taken 10 min after the end of the stimulation.

Effects of varying the frequency of stimulation of meridian points in intact rats

Fig. 3 illustrates the effects produced at different frequencies of stimulation, which included 2, 4, 6 and 8 Hz; in all cases, the parameters were otherwise the same as those in the previous series of experiments. It is also important to point out that the group stimulated at 4 Hz was different from that in the previous series - it was run at the same time as the other groups in the present series to achieve consistency.

Stimulation at 4 (n = 7) and 6 (n = 8) Hz produced the same type of response reported from the previous series of experiments. Stimulation at 2 Hz (n = 7) produced a significant inhibition compared to the control group, but the post-stimulation effect was smaller in amplitude and shorter in duration when compared to the respective effect in the groups stimulated at 4 and 6 Hz. Stimulation at 8 Hz (n = 6) produced an effect only during the period of stimulation. It is important to note that the magnitude of the inhibition produced during stimulation was the

same at all frequencies. It was only the persistent effect, observed following the period of stimulation, which showed significant differences between the four groups.

Effects of electrical stimulation of non-meridian points in intact rats

Stimulation of non-meridian points (n = 9; see Fig. 1) also increased tail withdrawal latency, although in this case there was no post-stimulation effect. The data are presented in Fig. 4. The standard parameters of stimulation were 4 Hz, 2 ms pulses at 20 times threshold in a 20 min train. There appeared to be two peaks during the stimulation, occurring at 10 and 20 min. These peaks were 45.9 $\pm$ 7.83 and 47.3 $\pm$ 7.56 % of the MPI. This inhibition was significantly different from the control group (n = 12; $p < 0.01$). Two way ANOVA ($0.01 > p < 0.05$) and paired t-test ($p < 0.001$) comparisons indicated that the inhibition evoked by non-meridian point stimulation was significantly less than that evoked by meridian point stimulation.

Effects of electrical stimulation of meridian points in acutely spinal transected rats

The data from acutely spinal transected rats are illustrated in Fig. 5. In the 7 rats tested 4 hours after transection, stimulation of hindlimb meridian points produced a brief increase in withdrawal latency. Only one peak was observed, at 5 min after the onset of stimulation. This peak was 14.6 $\pm$ 4.66 % of the MPI and was significantly different from the control group of spinal animals (n = 18; $p < 0.01$). There was no post-stimulation effect in this group. (As there was no significant difference in mean baseline values for withdrawal latencies in the control group for acute and chronic spinal transected animals the data were pooled from both groups to yield

an "n" of 18.)

In the group tested 24 h after transection (n = 6) the duration of the antinociceptive effect evoked by electrical stimulation was more prolonged than in the group tested at 4 h (Fig. 5). Thus, at 10 and 15 min after the onset of the stimulation, the tail withdrawal latency increased to 9.36 ± 2.77 and 14.2 ± 4.58 % of the MPI, respectively ($p < 0.05$ and $p < 0.01$, respectively). There was no post-stimulation effect in this group.

Two days after spinal transection (n = 7), stimulation resulted in a more pronounced effect with respect to amplitude and duration (Fig. 5). The greatest effect in this group was 24.8 ± 8.57 % of the MPI and occurred at 15 min after the onset of the stimulation. The withdrawal latencies were significantly different ($p < 0.01$) from those in the control group throughout the period of stimulation. A significant post-stimulation effect was seen but only at the reading taken 5 min after the stimulation was turned off ($p < 0.05$). In comparison with the effects seen in rats tested 4 hours after transection, the mean increase in withdrawal latency in rats tested 2 days after transection was greater at 15 and 20 min after the onset of the stimulus ($p < 0.01$) and this effect remained significantly greater at 5 and 10 min after the end of the stimulation ($p < 0.05$). In comparison with the effects seen in rats tested 24 hours after transection, the latency in the rats tested 2 days after transection was greater only at the 20 min reading after the end of stimulation ($p < 0.05$).

Effects of electrical stimulation of meridian points in chronically spinal transected rats

The data from chronically spinal transected rats are illustrated in Fig. 6. All testing was done after bladder function had returned. In the group of rats tested 7 days after spinal transection (n = 10), stimulation produced an increase in withdrawal latency throughout the period of stimulation ($p < 0.01$). Two peaks, occurring at 2 and 15 min after the onset of the stimulation, were 15.7 ± 3.59 and 21.6 ± 6.76 % of the MPI, respectively. In contrast to the rats tested acutely after spinal transection (see Fig. 5), when the stimulation was turned off the withdrawal latency remained elevated at approximately 10 % of the MPI for the next 15 min ($p < 0.05$).

In the group tested 14 days after spinal transection (n = 8; Fig. 6), stimulation produced an increase in withdrawal latency similar to that seen in the rats tested 7 days after spinal transection. The first two peaks occurred during the stimulation at 2 and 15 min after the onset and were 14.5 ± 3.95 and 17.3 ± 4.06 % of the MPI, respectively ($p < 0.01$). The post-stimulation effect persisted for 15 min and was maximum at 17.5 ± 5.87 % of the MPI ($p < 0.01$).

Effects of electrical stimulation of meridian points on arterial pressure and heart rate

In the group of animals tested for the effects of stimulation on arterial pressure and heart rate (n = 5), the mean baseline heart rate, systolic pressure and diastolic pressure prior to stimulation were 402 ± 5.7 bpm, 126 ± 1.8 mmHg and 85 ± 4.1 mmHg, respectively. Stimulation of hindlimb meridian points applied for 20 min at 4 Hz and 2 ms failed to produce any change in arterial pressure or heart rate during or after the stimulation.

DISCUSSION

Our experiments indicate that high intensity, low frequency electrical stimulation of previously defined sites in the rat hindlimb inhibits the nociceptive tail withdrawal reflex provoked by noxious cutaneous heat. We interpret this to be an antinociceptive effect. The effect consists of a brief response during stimulation and a persistent response which lasts more than one hour after the stimulus. Neither response appears to be secondary to a change in arterial pressure. It appears that the spinal cord can sustain both types of inhibition. However, the data also indicate an important role for supraspinal structures in eliciting both antinociceptive responses in the intact rat.

The brief and persistent effects constitute different responses.

It is suggested that the brief and the persistent effects constitute two different responses because the brief effect could be elicited independently of the prolonged effect: the brief effect was elicited alone at 8 Hz, with stimulation at non-meridian points and with stimulation in acutely spinal transected rats. Suggested mechanisms underlying each response will be elaborated separately in later sections, but it is important to point out that other reports have not made this distinction between brief and persistent effects. Experimental studies have reported only a brief, non-persistent effect. Some lasting effects have been observed but, as discussed in detail below, in most cases these may be attributed to the decay of a brief response because recovery occurs relatively rapidly compared to the effect in the present study.

Features of the brief antinociception.

Previous reports of antinociception during stimulation fall into four categories. (1) In the *anaesthetized* cat (Pomeranz & Cheng, 1979) and rat (Kawakita & Funakoshi, 1982; Pomeranz & Nguyen, 1987) *low intensity*, low frequency stimulation produces antinociception which recovers within 10 min. (2) In the *unanaesthetized* rat (Wang *et al.* 1990) and rabbit (Han & Xie, 1984) *low intensity*, low frequency stimulation produces antinociception which lasts 10-20 min, although in this case the possibility remains that the effects could have been due to stress as a result of using awake animals. (3) Studies using *high intensity*, low frequency stimulation in the *unanaesthetized* mouse (Pomeranz & Paley, 1979) showed an effect lasting up to two hours, but this effect was almost certainly due to stress because the high intensity stimulus was applied to awake animals. (4) To our knowledge, only one report has used *high intensity*, low frequency stimulation in the *anaesthetized* animal (Kawakita & Funakoshi, 1982): the antinociception elicited outlasted the period of stimulation by 15 min. This persistent effect will be elaborated further below, but after a survey of the literature it is clear that most previous studies focused on a brief effect only.

The observation that stimulation at 8 Hz elicits only the brief inhibition suggests that the peripheral and/or central mechanisms mediating this response are relatively independent of frequency and follow relatively higher frequencies than those mediating the persistent inhibition. In this context, post-stimulation analgesia evoked by intense stimulation just tolerable to human subjects has been reported in an experimental pain study with 2 but not 10 Hz stimulation (Andersson & Holmgren, 1975) and the perceptual response to activation of C fibres in human peripheral nerves begins to fail at 5 Hz (Torebjörk & Hallin, 1973; Liu *et al.* 1995). Therefore,

we suggest that the antinociception observed during stimulation may be due to continuous synaptic activation.

Features of the persistent antinociception

While it is difficult to draw specific parallels, the post-stimulation antinociception reported here is unique among animal studies in that the duration of the effects resemble those of electroacupuncture-evoked analgesia in humans, which can last several hours to a few days clinically (Melzack, 1975; Fox & Melzack, 1976; Sjölund & Eriksson, 1979; Thomas & Lundberg, 1994). Animal studies on electroacupuncture in which stress was not a factor have generally failed to show long-lasting effects comparable to those produced in our paradigm. Two exceptions are notable. In one study, a persistent effect was obtained using high frequency stimulation at 10 times threshold in the anaesthetized rat (Sjölund, 1985), but this type of stimulation resembles TENS and thus is different from that used in the present study. In the other study, a long-lasting inhibitory effect of high intensity, low frequency stimulation on spinal nociceptive neurones has also been reported (Chung *et al.* 1984), but in this case stimulation was applied directly to a peripheral nerve.

Clinical reports tend to indicate that high intensity electrical stimulation at just tolerable levels evokes a long-lasting analgesia in humans. Analgesia has been reported with low intensity, high frequency stimulation but this analgesia is marginally effective in that only 10-40 % of patients experience sufficient pain relief (Loeser *et al.* 1975; Long, 1976). On the other hand, stimulation at 5-8 times the perception threshold increases tooth pain threshold (Andersson *et al.* 1973;

Chapman *et al.*1977) and stimulation at 3-5 times perception threshold, described as just tolerable, produces analgesia in chronic pain patients who otherwise lack sufficient pain relief with low intensity, high frequency stimulation (Sjölund & Eriksson, 1979). Thus, the clinical data are in general agreement with our view from the present study that low frequency, high intensity stimulation produces a generalized antinociception or analgesia including a prolonged aftereffect.

Mechanisms underlying the persistent antinociception.

Our data indicate that the frequency of stimulation is an important factor in triggering the spinal mechanisms mediating the persistent inhibition. Few reports have systematically studied the optimal frequency for electroacupuncture. Usually, a frequency of 2-6 Hz is used, and most studies have used only one frequency within this low range (Chen & Han, 1992; Pomeranz & Cheng, 1979). Some have compared effects of 15 Hz vs. 100 Hz (Han *et al.*1991) or even 2 kHz vs. 5 kHz (Lin *et al.*1992). In our experiments the antinociception elicited by 2 Hz stimulation lasted only 15 min. Thus, it is possible that at such a low frequency, optimal temporal summation of central mechanisms is not achieved. Frequencies of 4-6 Hz appear to be necessary to produce the maximum persistent effect. As discussed above, the lack of a response at 8 Hz may be due to the failure of the synapses of small diameter afferents to follow this frequency and/or inability of central mechanisms to evoke the post-stimulation inhibition at this higher frequency of stimulation. Therefore, we propose that activation of small diameter afferent fibres for 20 min at 2 to 6 Hz triggers mechanisms which evoke a plastic change giving rise to the persistent depression of the tail withdrawal reflex.

Significance of stimulation at meridian vs non-meridian points.

The selection of points for stimulation was based upon sites reported in the literature (Pomeranz & Cheng, 1979; Bing *et al.* 1990; Bing *et al.* 1991). The persistent antinociception in our study was dependent on stimulation of meridian points. Clinical studies indicate that meridian point stimulation is important (Anderson *et al.* 1974; Stewart *et al.* 1977) although few such studies actually compare data from meridian vs. non-meridian points. In animal studies, high intensity stimulation of meridian points but not of non-meridian points in the rat produces a persistent inhibition of the jaw-opening reflex (Toda & Ichioka, 1978).

The importance of meridian points has not always been supported by data from animal experiments (Bing *et al.* 1991), possibly due to the fact that most studies have focused on the antinociception which occurs during the stimulation. Some studies have reported a difference between stimulation of meridian vs. non-meridian points in the rat (Takeshige *et al.* 1992) or mouse (Pomeranz & Chiu, 1976), but stimulation was only of large diameter afferents, the effects produced were only brief in duration and the experiments were done on awake animals. Thus, the antinociception studied was not similar to the persistent effect we are reporting here.

The literature on human pain may be considered to support the importance of meridian points. In a study on pain rating upon immersion of one hand in ice-water, electroacupuncture produced analgesia only from meridian points (Anderson *et al.* 1974). In another study, only meridian point stimulation increased tolerance to thermal pain (Stewart *et al.* 1977). Unfortunately, in neither of these studies were details of the stimulation provided and the duration of the pain

relief was not mentioned. There are clinical reports on chronic pain in humans in which non-meridian points produce the same relief as meridian points (Gaw *et al.* 1975; Lee *et al.* 1975; Godfrey & Morgan, 1978). However, in none of these studies were the parameters of stimulation reported, so it is not clear that high threshold afferents were activated, details of what constituted meridian or non-meridian points were not mentioned and the duration of the effect was not reported (in most cases assessment was done immediately following stimulation). On the basis of our results, showing a brief antinociceptive effect which is independent of stimulation of meridian points, it would be predictable that there would be no difference between meridian point and non-meridian point stimulation in clinical studies which do not report persistent analgesia. Therefore, support for a difference between meridian point vs. non-meridian point stimulation may come from only those clinical and basic studies in which a persistent rather than just a brief antinociceptive effect is elicited by high intensity stimulation.

Spinal mechanisms

We interpret our data from spinal transected animals to indicate that there is a supraspinal component to both the brief and persistent responses. However, as both effects can also be produced in the spinal rat, it appears that the spinal cord is capable of sustaining at least a component of each response. Data from our acutely transected animals are in agreement with the finding that noxious stimulation depresses activity of dorsal horn neurones for several minutes in intact rats, yet shortly after spinal transection this stimulation produces a weak inhibition (Cadden *et al.* 1983). In addition, electrical stimulation of the tail evokes a brief antinociception in the acutely spinal rat (Woolf *et al.* 1977). Several studies have shown that

high intensity stimulation applied either cutaneously or to peripheral nerves produces segmental inhibition of spinal nociceptive reflexes, lasting 10 min to 1 hour (Chung *et al.* 1983; Taylor *et al.* 1990). High intensity stimuli also inhibit convergent dorsal horn neurones (Cadden *et al.* 1983; Chung *et al.* 1984) in acutely spinal transected animals and noxious stimulation produces an extrasegmental antinociception in spinal animals (Ness & Gebhart, 1991a; Ness & Gebhart, 1991b; Pitcher *et al.* 1995). Thus, although supraspinal structures appear to have a dominant role in eliciting antinociception induced by activation of Aδ and C fibres (Cadden *et al.* 1983), there is sufficient evidence to suggest that intraspinal mechanisms may also provide an important contribution to inhibition of nociceptive mechanisms.

The persistent antinociception occurred in our chronically but not acutely spinal animals. The temporal appearance of this persistent response was coincident with the return of bladder function. Our data thus indicate that intraspinal mechanisms contributing to the antinociceptive effects evoked by high intensity electrical stimulation of hindlimb meridian points may be derived in part from a spinal source and may be acting with or independently from supraspinal structures. After spinal transection these mechanisms may require a period of recovery to be expressed, indicating adaptive changes in spinal analgesic mechanisms.

Summary and conclusions

The data presented suggest that high intensity, low frequency electrical stimulation elicits two antinociceptive responses, one which occurs during the stimulation and is dependent on continuous synaptic activation and the other which can persist for more than one hour after the

end of afferent stimulation. It is also suggested that the persistent antinociception is evoked by activation of high threshold, small diameter sensory afferents which trigger long-term changes in spinal and supraspinal mechanisms. This persistent response appears to be dependent upon central temporal summation because 2 Hz stimulation evokes a limited post-stimulation effect, while 4 or 6 Hz stimulation evokes a persistent antinociception lasting greater than one hour after the termination of the stimulation. In our model, the post-stimulation antinociception is evoked only from meridian points. In addition to supraspinal mechanisms, spinal mechanisms appear to be able to produce antinociception in response to prolonged activation of high threshold sensory inputs.

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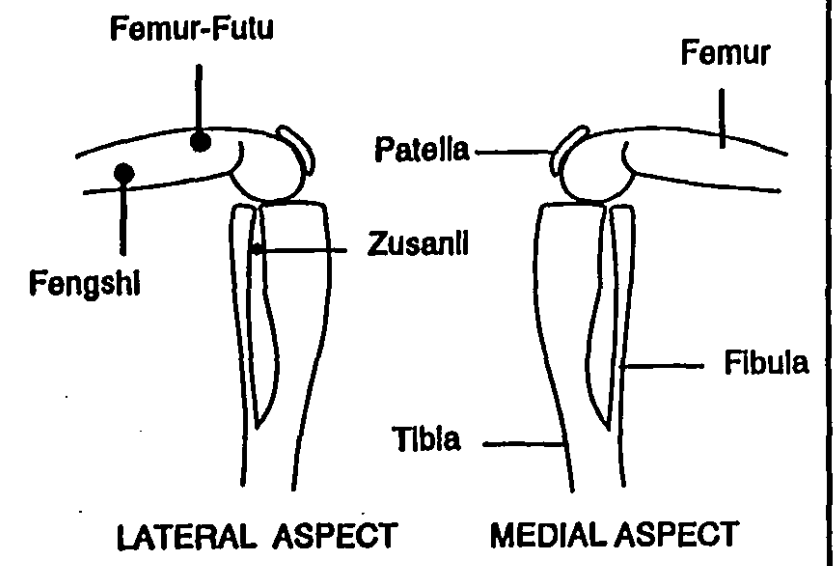
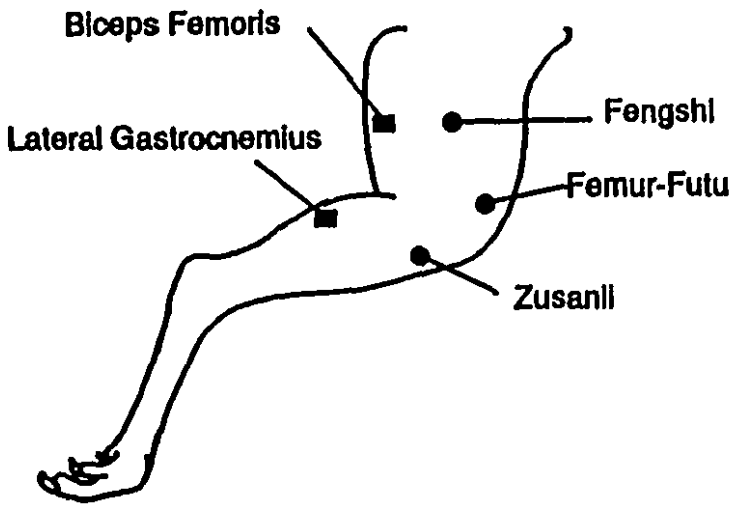
Fig. 1. Schematic illustration of hindlimb meridian and non-meridian points stimulated in the rat as well as notable meridian points which were avoided in placing electrodes for stimulation at non-meridian points. ● = meridian point. ■, non-meridian point. Upper left, meridian points stimulated in relation to bony structures. Lower left, points stimulated as visualized from lateral aspect. Right, non-meridian points stimulated as well as meridian points avoided in this stimulation; the dotted line represents a middle line along the dorsal aspect of the hindlimb. The points were derived from the literature (Pomeranz & Cheng, 1979; Bing *et al.* 1990; Bing *et al.* 1991).


Femur-Futu
Fengshi
Zusanli
LATERAL ASPECT
Femur
Patella
Fibula
Tibia
MEDIAL ASPECT


Biceps Femoris
Lateral Gastrocnemius
Fengshi
Femur-Futu
Zusanli

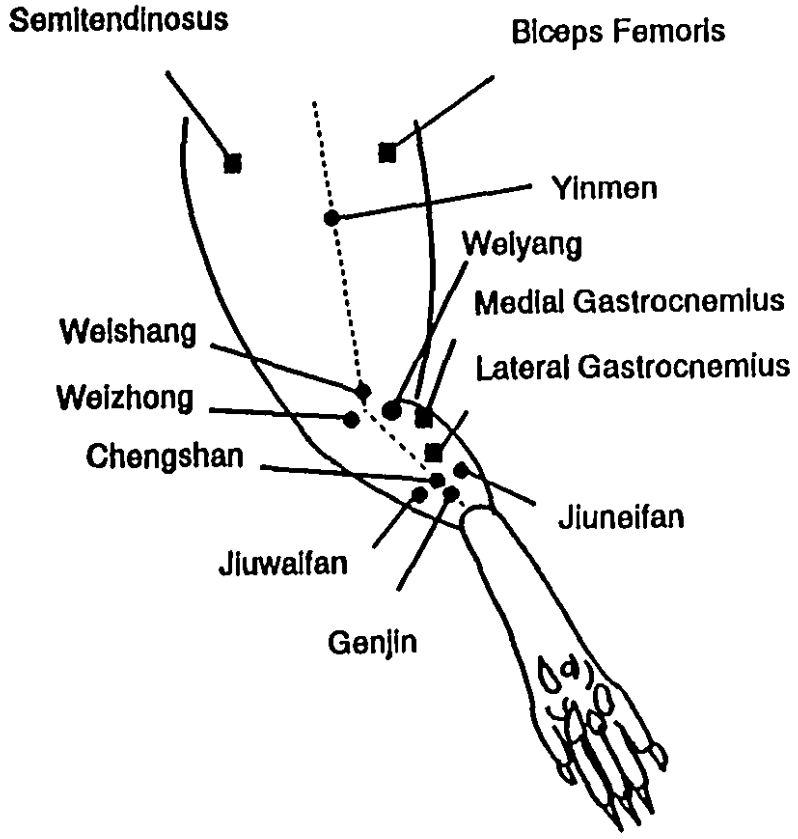

Semitendinosus
Biceps Femoris
Yinmen
Weiyang
Medial Gastrocnemius
Weishang
Lateral Gastrocnemius
Weizhong
Chengshan
Jiuneifan
Jiuwaifan
Genjin

Fig. 2. Effect on tail withdrawal latency of high intensity (at 20 times threshold), low frequency (4 Hz) electrical stimulation for 20 min (duration of stimulation indicated by hatched area). Threshold was that which just elicited muscle twitch by direct stimulation. Stimulation was of hindlimb meridian points *femur-futu* (ST-32), *fengshi* (GB-31) and *zusanli* (ST-36). Dotted lines indicate administration of anaesthetic. Tail withdrawal is expressed as a % of the maximum possible inhibition (MPI). ▵, stimulated group (n = 8); ○, needles only (n = 12); ▲, difference between the two groups. * $p < 0.05$; ** $p < 0.01$.

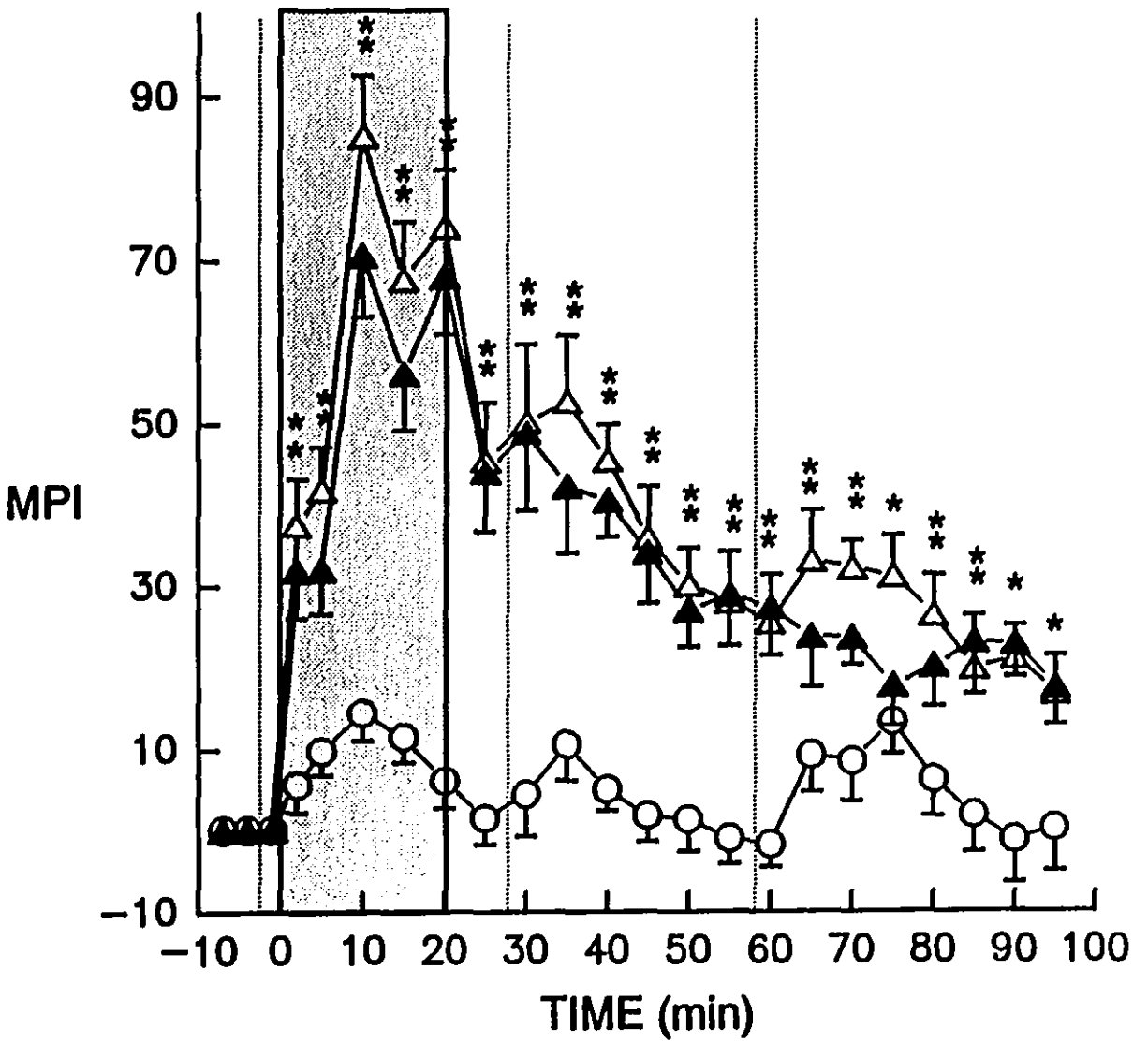

MPI
90
70
50
30
10
−10
−10 0 10 20 30 40 50 60 70 80 90 100
TIME (min)

Fig. 3. Effect on tail withdrawal latency of high intensity stimulation (at 20 times threshold) at 2 (n = 7), 4 (n = 7), 6 (n = 8) or 8 (n = 6) Hz for 20 min. The mean net effect on tail withdrawal latency is expressed as a % of the maximum possible effect calculated for the first 20 min period of stimulation and subsequent 25 min periods after the stimulation. The open bar represents period 1 (0 - 20 min) during the stimulation, the closed bar represents period 2 (25 - 45 min) after the stimulation, the cross hatched bar represents period 3 (50 - 70 min), and the diagonal filled bar represents the final period of observation, period 4 (75 - 95 min).

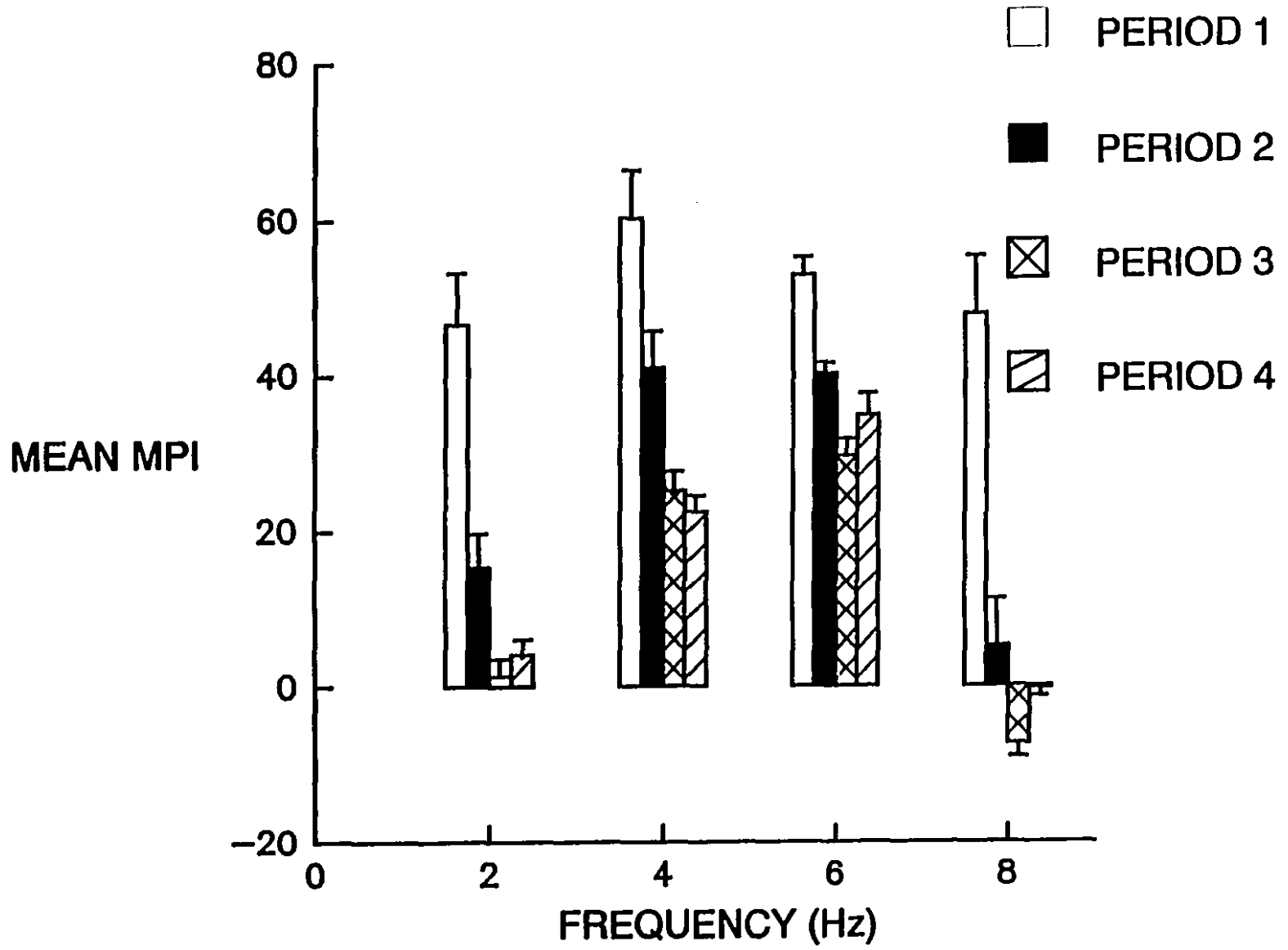

PERIOD 1
PERIOD 2
PERIOD 3
PERIOD 4
MEAN MPI
80
60
40
20
0
−20
0
2
4
6
8
FREQUENCY (Hz)

Fig. 4. Effects of stimulation of non-meridian points in the hindlimb. Stimulation parameters were the same as in fig. 2. Electrodes were inserted in the gastrocnemius muscles and biceps of the hindlimb. ▵, stimulated group (n = 9); ○, needles only (n = 12); ▲, difference between the two groups.

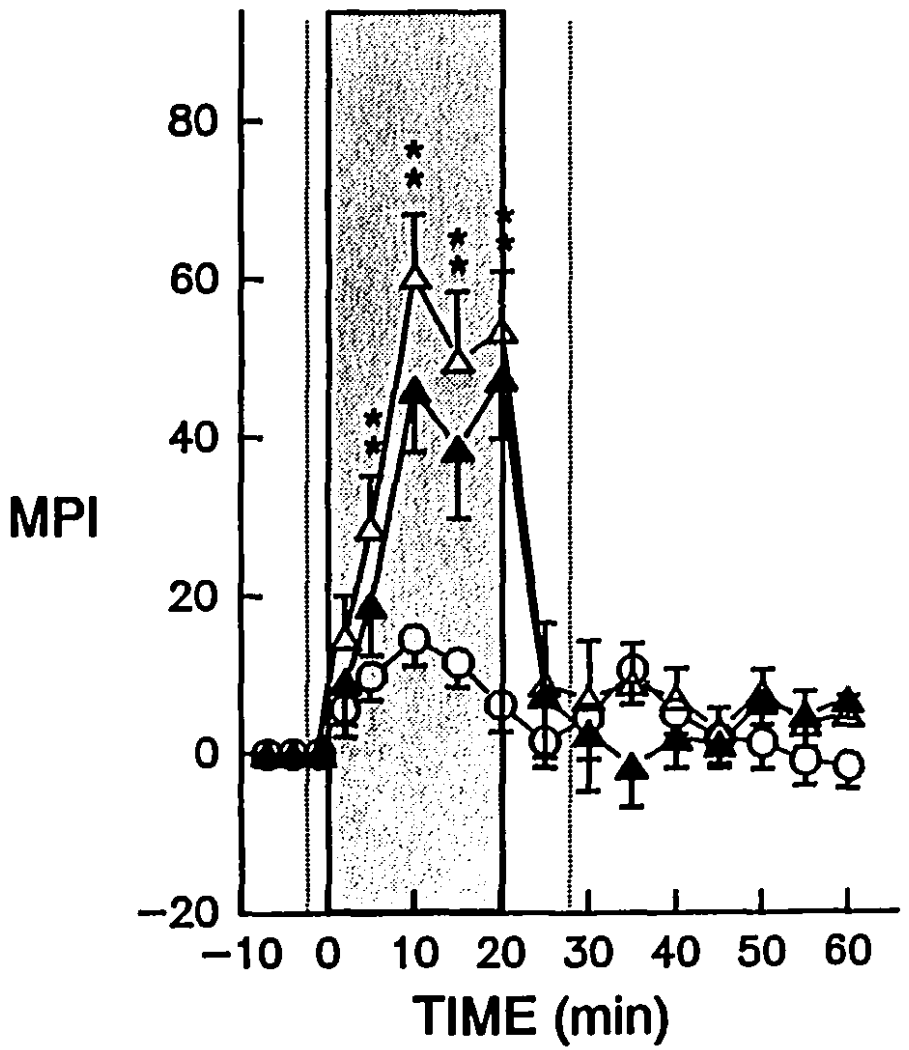

80
60
40
20
0
-20
MPI
-10
0
10
20
30
40
50
60
TIME (min)

Fig. 5. Effects of acute spinal transection on response to stimulation of meridian points in the hindlimb. Transection was at the T6/T7 spinal level. ▲, stimulated 4 hours after transection (n = 7); ▽, stimulated 24 hours after transection (n = 6); ■, stimulated 2 days after transection; ○, needles only (n = 18). Details are as in the legend to Fig. 2.

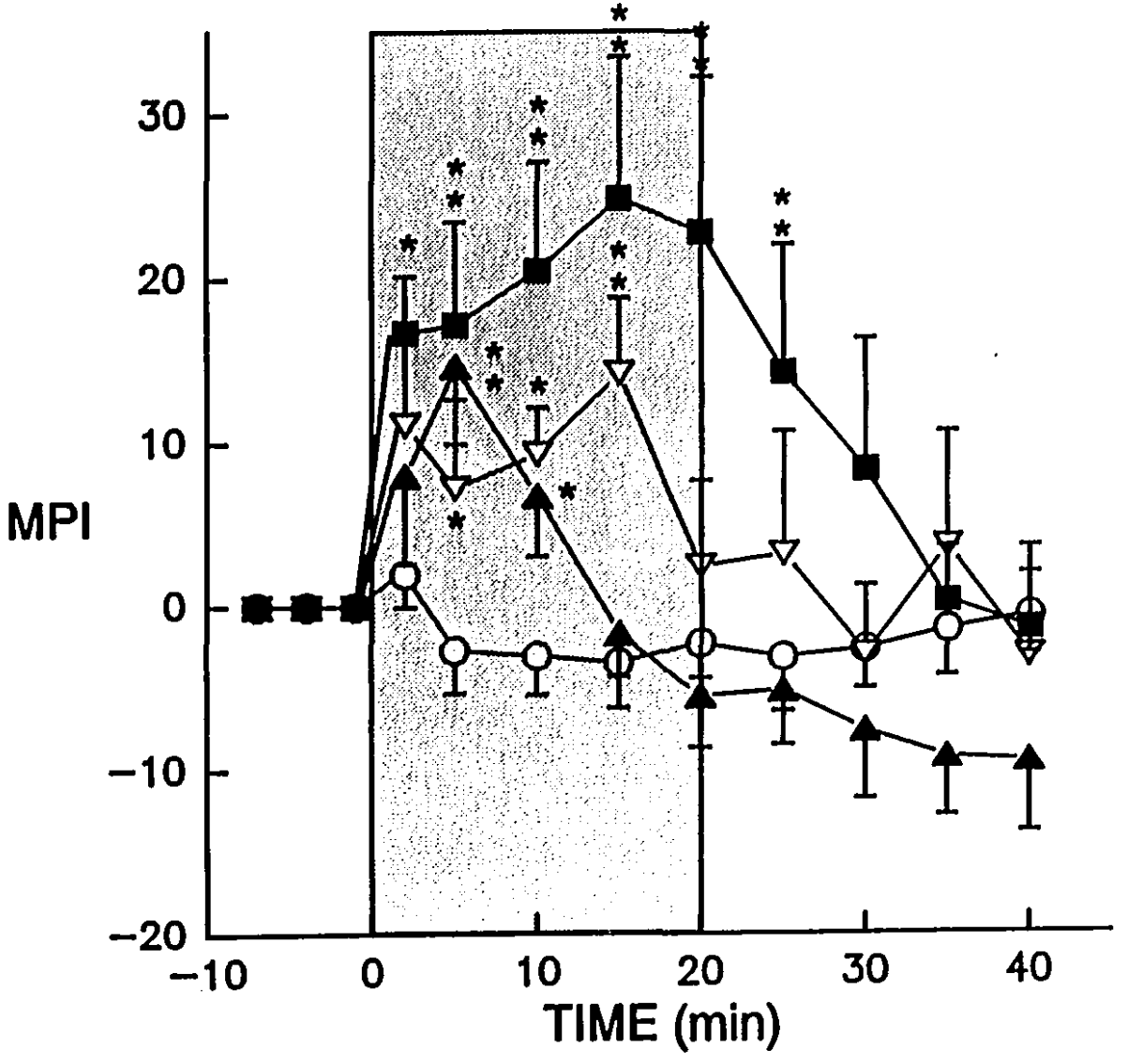
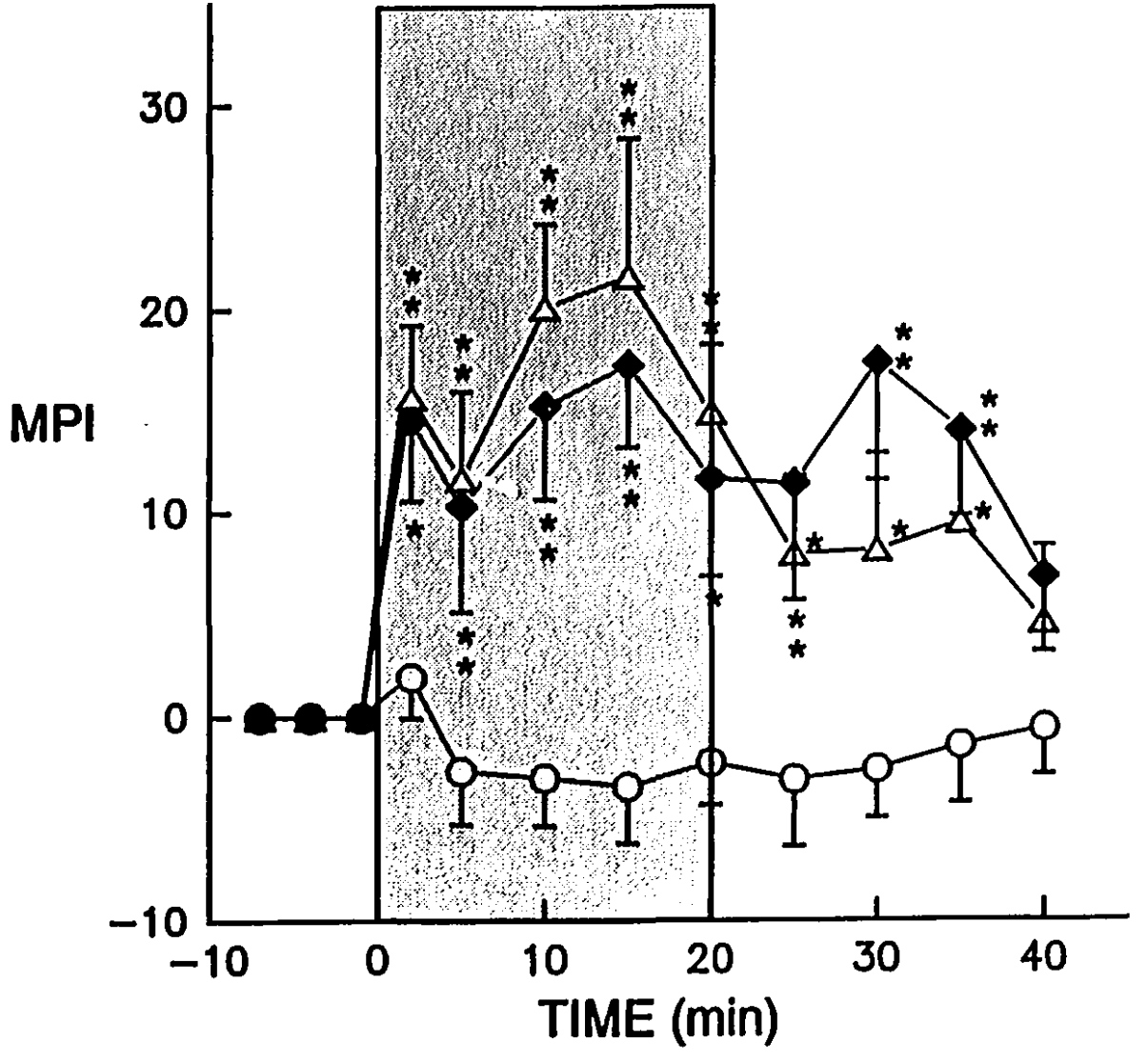

30
20
10
0
−10
−20
MPI
−10
0
10
20
30
40
TIME (min)

Fig. 6. Effects of chronic spinal transection on response to stimulation of meridian points in the hindlimb. Transection was at the T6/T7 spinal level. ▵, stimulated 7 days (n = 10) and ◆ 14 days (n = 8) after transection; ○, needles only (n = 18). Details are as in the legend to Fig. 2.


MPI
30
20
10
0
−10
−10
0
10
20
30
40
TIME (min)

CHAPTER TWO

PARAMETRIC STUDIES ON ELECTROACUPUNCTURE-LIKE STIMULATION IN A RAT MODEL: EFFECTS OF INTENSITY, FREQUENCY AND DURATION OF STIMULATION ON EVOKED ANTINOCICEPTION

We have found that electroacupuncture-like stimulation of defined sites in the hindlimb of the rat inhibits a nociceptive withdrawal reflex. The lightly anaesthetized rat was used and tail withdrawal from a noxious radiant heat stimulus was the nociceptive reflex. Standard stimulation of hindlimb meridian points *femur-futu* (ST-32), *fengshi* (GB-31) and *zusanli* (ST-36) consisted of a 2 ms square voltage pulse at 4 Hz for a duration of 20 min, applied at 20 times the threshold to evoke muscle twitch. This produced two types of inhibition of the reflex (n = 7); one was an increase in the latency of up to 80% during the stimulation, termed the brief antinociception, and the other was a post-stimulation increase of up to 60% lasting greater than one hour, termed the persistent antinociception. When the stimulus intensity was reduced to 10 times threshold (n = 7) the latency during stimulation increased up to 50% but the persistent response did not occur. Stimulation at threshold produced neither effect (n = 12). When the train duration was altered, 10 min of stimulation produced only the brief effect (n = 9), while 40 min of stimulation produced both effects, although the persistent effect lasted only 20 min (n = 9). Stimulation at 6 Hz produced responses similar to those at 4 Hz, while stimulation at 2 Hz produced smaller effects. At 8 Hz only the brief antinociception was elicited. With a pulse duration of 0.2 ms the brief response was observed but the persistent response was markedly attenuated, while 5 ms (n = 8) produced responses similar to those with 2 ms. These data suggest that high intensity, low frequency electrical stimulation of meridian points in the rat hindlimb produces both brief and persistent antinociceptive effects on the tail withdrawal reflex, and both effects are dependent upon the parameters of stimulation. The persistence of the latter effect beyond the period of stimulation suggests events occurring after direct synaptic activity, possibly mediated via plastic changes at spinal and/or supraspinal levels.

Key words: nociception, pain, analgesia, spinal reflex, acupuncture, C fiber, Aδ fiber, electrical stimulation

Analgesia in human subjects and patients can be achieved by a number of therapeutic measures, including acupuncture. Despite the widespread use of acupuncture clinically, other than anecdotal information little is known about the optimal parameters which produce pain relief. It is generally accepted that acupuncture activates deep receptors (Chiang et al.1973) although it is controversial whether this stimulus recruits large and small diameter afferents (Melzack, 1975; Fox, Melzack, 1976; Mannheimer, Carlsson, 1979; Sjölund, Eriksson, 1979; Kawakita, Funakoshi, 1982) or large diameter afferents only (Levine et al.1976; Pomeranz, Cheng, 1979; Pomeranz, Nguyen, 1987). Electroacupuncture usually consists of high intensity, low frequency electrical stimulation (Andersson et al.1973; Andersson, Holmgren, 1975; Holmgren, 1975; Melzack, 1975; Fox, Melzack, 1976; Eriksson et al.1979; Mannheimer, Carlsson, 1979; Sjölund, Eriksson, 1979) and is distinct from TENS, which consists of low intensity, high frequency stimulation (Lundeberg, 1984; Chan, Tsang, 1987; Guieu et al.1991).

Yet, even with electroacupuncture a variety of stimulation parameters has been used. For example, within this range of parameters biphasic square pulses of variable duration have been used (Andersson et al.1973; Andersson, Holmgren, 1975) as have brief monophasic pulses given in a 70 ms train (Eriksson et al.1979; Sjölund, Eriksson, 1979). Frequencies of 2 (Andersson, Holmgren, 1975; Holmgren, 1975; Sjölund, Eriksson, 1979) or 4 Hz (Andersson, Holmgren, 1975) have been applied and intensities have been used up to those which are described as being just tolerable to the patient (Andersson et al.1973; Andersson, Holmgren, 1975; Melzack, 1975; Fox, Melzack, 1976; Eriksson et al.1979; Mannheimer, Carlsson, 1979; Sjölund, Eriksson, 1979). While acupuncture-evoked effects appear to be manifested via activation of small diameter, high threshold primary sensory afferents, possibly C fibers (Andersson, Holmgren,

1975; Kawakita, Funakoshi, 1982; Chung et al.1984a; Chung et al.1984b), some reports suggest that activation of large diameter primary afferents alone evokes analgesic and antinociceptive effects (Levine et al.1976; Pomeranz, Cheng, 1979; Han et al.1983; Han, Xie, 1984; Pomeranz, Nguyen, 1987).

Attempts have been made to develop animal models of acupuncture (Pomeranz, Cheng, 1979; Kawakita, Funakoshi, 1982; Bing et al.1991a; Bing et al.1991b) and there are reports of antinociceptive effects in animals when specific sites are stimulated which resemble meridian points in humans (Ulett, 1982; Pomeranz, 1987). We have recently stimulated some of these points in the lightly anesthetized rat and found that high intensity, low frequency electrical stimulation elicits an antinociception which persists more than one hour (Romita et al.1992; Romita et al.1993). In the present study we have used this animal model to determine the optimal parameters of electrical stimulation to achieve lasting effects.

Materials and Methods

In all cases, the guidelines described in The Care and Use of Experimental Animals, of the Canadian Council of Animal Care, Vols. I and II, Second Edition, were strictly followed. The experimental protocols were also reviewed and approved by the McGill University Animal Care Committee.

Animal preparation

Male Sprague Dawley rats (300-400 g) were lightly anesthetized for the duration of the

experiment with an intraperitoneal injection of a freshly prepared mixture of sodium pentobarbital (20 mg/kg, Abbott Laboratories Ltd., Montreal, Canada) and chloral hydrate (120 mg/kg, Fisher Scientific, Montreal, Canada) in 50% propylene glycol (Baker Chemical Co., Phillipsburg, NJ. U.S.A.) and 30% physiological saline (0.9% NaCl). This provides a level of anesthesia sufficient to prevent any overt sign of discomfort to the rat during experimentation, yet a stable withdrawal response is obtained in the tail flick test for about 1 hour. To maintain this light state of anesthesia throughout the full duration of the experiment, subsequent injections of the mixture were given: an injection of ⅓ the initial dose of anesthetic 35.5 min after the first injection, timed to occur 1.5 min prior to the beginning of electrical stimulation, and subsequent injections of ⅓, ¼ and 1/6 the initial dose at 30 min intervals thereafter.

Tail withdrawal test

The lightly anesthetized rat was placed in a plastic restrainer on the apparatus used to measure tail withdrawal latency (Isabel et al.1981), needles were inserted and threshold determined as described below. Before testing was started, the animals were allowed to stabilize for 30 min, after which 3 baseline readings of the tail withdrawal latency were taken at 3 min intervals. Stimulation was then applied starting 1 min after the last baseline reading and the latency was recorded at 2 and 5 min after the onset of stimulation, and at 5 min intervals for 75 min after the end of stimulation.

Nociception was determined as the latency of the tail withdrawal from a noxious radiant heat stimulus. To measure this latency a portion of the tip of the tail was blackened to facilitate the absorption of heat. This portion was then positioned above a focused projector bulb to

provoke the withdrawal reflex. Flick of the tail exposed the light beam to a photodetector which in turn stopped a timer giving reaction time measured to 0.01 s (Isabel et al.1981). The intensity of the bulb was set so that the baseline reaction time was 4-6 s. Trials were terminated automatically if a withdrawal did not occur within 12 s, the cut-off time.

At each sample time two readings were taken, separated by 40-50 s, at two different sites within the 2 cm blackened segment of the tail. Thus, the withdrawal latency was never measured twice from the same site within a 3-5 min interval. The average of the two readings was calculated and the value expressed as a percent of the maximum possible inhibition (MPI) according to the formula (Yaksh, Rudy, 1977)

$$\text{MPI} = \frac{(\text{POST-TREATMENT LATENCY} - \text{PRE-TREATMENT LATENCY})}{(\text{CUT-OFF TIME} - \text{PRE-TREATMENT LATENCY})} \times 100$$

To measure the latency during the period of the electrical stimulation, the stimulator was temporarily turned off long enough for the reading to be taken; this was necessary because the stimulus was above the threshold to elicit a direct contraction of muscles.

Electrical stimulation

Two pairs of stainless steel insect pins were inserted into meridian points *femur-futu* (ST-32) and *fengshi* (GB-31) of the hindlimb as defined by others (Pomeranz, Cheng, 1979; Ulett, 1982; Pomeranz, 1987; Bing et al.1991a; Bing et al.1991b). In the case of the electrodes inserted to stimulate *femur-futu* the cathode was placed along the medial side of the knee and the anode was

inserted along the lateral side of the knee so that it lay across *zusanli* (ST-36). To stimulate *fengshi* the electrodes were inserted under the femur midway between the hip and the knee. In the control groups, electrodes were inserted into the respective areas of the hindlimb but no electrical stimulation was applied. Fig. 1 illustrates the relative positions of the points stimulated.

Threshold was taken as the lowest intensity which just produced muscle contraction. The needles were connected to coupled Grass stimulators (SD9 and SD5) which passed a train of monophasic square wave pulses. The standard parameters were 2 ms square pulses applied at 4 Hz for a duration of 20 min and applied at 20 times threshold.

Four series of experiments were done in which the parameters of electrical stimulation were varied to determine the profile of the evoked responses.

i) Intensity of stimulation. Stimulation was applied at 1, 10 or 20 times the threshold as defined above.

ii) Duration of the train of stimulation. The duration of the train was 10 or 40 min. Comparison was made with the group stimulated above for 20 min.

iii) Frequency of the train of stimulation. The frequency of stimulation was 2, 6 or 8 Hz. Comparison was made with the group stimulated with these parameters at 4 Hz, above.

iv) Duration of the square wave pulse. In the last series of experiments the duration of the pulse was 0.2 or 5 ms. The intensity was 20-30 mA at 20 times the threshold obtained with a 2 ms pulse or 40-60 mA at 20 times the threshold obtained with a 0.2 ms pulse. Comparison was made with the group stimulated with a 2 ms pulse.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (S.E.M.). Results are expressed as the mean MPI at each sample time and as net effect, obtained by subtracting the mean MPI at each point in the control group from the respective MPI in the test group, to eliminate the influence of supplemental doses of anesthetic. Data from treated and control animals were analyzed using two way analysis of variance (ANOVA). Stimulation vs. no stimulation was taken as the between subject factor for otherwise similarly treated groups and time was taken as the within subject factor. Tukey's Wholly Significant Difference Test was used to make post hoc comparisons between means. The same method of statistical analysis was used to compare two groups of rats treated differently (i.e. stimulation at 10 times threshold vs 20 times threshold). In some analyses, one way repeated measures analysis of variance, pair-wise multiple comparisons (Student-Newman-Keuls method) and paired Students *t*-test for single pair-wise comparisons were used to compare the mean MPI calculated over the period of stimulation and for subsequent post-stimulation periods, between treatment groups.

RESULTS

In control rats, ie. those in which stimulating electrodes were inserted into meridian points but the stimulator was not turned on, withdrawal reaction time remained relatively constant throughout the experiment, with the exception of transient increases in latency occurring as a result of the administration of the successive doses of anesthetic. The data from these control animals for each paradigm are illustrated in the respective figures.

i) Effects of intensity of stimulation

The intensity at which the stimulus was applied was important in distinguishing between the brief and the persistent antinociceptive responses, as described below.

20 times threshold: The intensity of the stimulus was 20-30 mA (minimum of 15 V). This increased tail withdrawal latency (n = 7). The data are illustrated in figure 2. The response was characterized by an increase in withdrawal latency during the period of stimulation as well as for more than one hour after the stimulus was turned off. During the stimulation the maximum net increase was 73.6 $\pm$ 5.56 %. After the stimulation was turned off the net latency remained significantly elevated for more than one hour. The withdrawal response was significantly different from that in the control group (n = 20) at each time point from the beginning of stimulation to the end of the experiment ($p < 0.01$).

10 times threshold: When the same stimulus was applied at 10 times threshold (n = 7), the latency increased to peaks of 50.2 $\pm$ 14.0 % and 50.2 $\pm$ 12.1 % of the MPI, respectively, at 10 and 20 min after the onset of the stimulation. The data are illustrated in figure 3A. These effects were significantly different ($p < 0.01$) from the group of control rats (n = 12). However, tail withdrawal latency returned to control values within 10 min after the stimulation was turned off. Thus, the inhibition seen in the group stimulated at 20 times threshold (figure 2) did not occur.

Threshold: Stimulation at threshold (n = 12) had no effect at any time compared to the control group (n = 12), as illustrated in figure 3B.

ii) Effects of duration of the train of stimulation

10 min: The standard stimulation applied for only 10 min (n = 9) increased withdrawal latency to a maximum of 50.0 ± 8.3 % of the MPI at 10 min after the onset of stimulation (figure 4). The tail withdrawal latency remained significantly elevated throughout the 10 min period of the stimulation and for the first 2 readings after the stimulation was turned off ($p < 0.01$).

40 min: When applied for 40 min (n = 9) an increase in latency also occurred (figure 5). The readings between the beginning of the stimulation and 40 min after the end of stimulation were significant ($p < 0.01$).

iii) Effects of frequency of stimulation

2 Hz: Stimulation at 2 Hz (n = 7) increased withdrawal latency (figure 6) throughout the period of stimulation (maximum 60.3 ± 12.07 % of the MPI) and for 15 min after the stimulation was turned off ($p < 0.01$).

4 Hz: The data are illustrated in figure 2 (n = 7).

6 Hz: Stimulation at 6 Hz (n = 8; figure 7) increased the latency throughout the period of stimulation (maximum 78.5 ± 4.19 %) and for greater than one hour after the stimulus was turned off ($p < 0.01$).

8 Hz: 8 Hz (n = 6) increased the latency to a maximum of 62.3 ± 9.39 % of the MPI. The

inhibition occurred at all times during the stimulation but, other than that at 5 min after the stimulation was turned off, no post-stimulation inhibition was observed (figure 8).

Statistical comparisons of the effects of stimulation at the different frequencies were made by dividing the time during and following the stimulation into four periods: 1) the 5 observations during the 20 min period of stimulation (0 - 20 min), 2) the first 25 min period after the stimulation was turned off (25 - 45 min), 3) the subsequent 25 min period (50 - 70 min) and 4) the final 25 min period (75 - 95 min). The mean net effect during each of these periods was compared among the 4 groups stimulated at either 2, 4, 6 or 8 Hz. Statistical comparisons were also made against the unstimulated control group. Table I summarizes the statistical correlations between groups stimulated at different frequencies for time periods 1 through 4.

iv) Effects of duration of the pulse

The pulse duration was set at 0.2-5 ms and applied either at 10 times or at 20 times threshold. The data obtained in the 4 periods just described are illustrated in figure 9.

Pulse duration 0.2 ms. Stimulation with a 0.2 ms pulse at 20 times threshold (approximately 40 mA; $n = 7$) increased withdrawal latency to a maximum of 85.06 ± 2.56 % of the MPI during the stimulation and a brief post-stimulation inhibition was seen. This post-stimulation response peaked at 43.56 ± 11.07 % of the MPI at 10 min and ended 15 min after the stimulation was turned off. The inhibition evoked during and after the stimulation was significant when compared to the control group ($n = 20$; $p < 0.01$).

Statistical analysis did not show any significant difference between the response evoked

during stimulation with 0.2 ms pulses at 20 times threshold and that with 2 ms pulses, also at 20 times threshold (approximately 20 mA). However, while the post-stimulation period was significantly different ($p < 0.001$) from that in the control group ($n = 20$) it was significantly less ($p < 0.001$) than that evoked by stimulation with 2 or 5 ms pulses.

With a pulse duration of 0.2 ms ($n = 8$) stimulation at 10 times threshold (approximately 20 mA) increased the latency to a mean of 36.2 ± 5.73 % of the MPI during stimulation ($p < 0.01$). No post-stimulation effect was observed. The magnitude of the effect in period 1 was also significantly less than that evoked in the other group ($p < 0.05$).

Pulse duration: 2 and 5 ms. The data obtained with stimulation of hindlimb meridian points with a 2 ms square voltage pulse are described above. Stimulation with a 5 ms pulse ($n = 8$) increased the latency in period 1 to a mean of 55.1 ± 8.17 % of the MPI and for the remaining 3 periods ($p < 0.01$). These effects were not statistically different from the antinociceptive effects elicited when stimulation was applied with 2 ms pulses.

DISCUSSION

The data indicate that conditioning electrical stimulation of hindlimb meridian points in the lightly anesthetized rat evokes an inhibition of the nociceptive tail withdrawal reflex. We interpret this to be an antinociceptive effect. This effect can be separated experimentally into two types of inhibition of the withdrawal reflex, a brief response seen during the stimulation period and a persistent post-stimulation response lasting greater than one hour. As the two responses appear to be different, possible mechanisms by which each is elicited are described

below.

I. Parameters of stimulation suggest the persistent antinociception may be dependent on activation of high threshold inputs and central mechanisms

Intensity. The data indicate that the persistent antinociceptive effect is elicited only by high intensity stimulation and it is suggested that recruitment of high threshold afferents is necessary for activation of the central mechanisms which produce this persistent effect. Several reports in the literature provide data to support this interpretation. Stimulation of hindlimb meridian points at intensities which recruit Aδ and C afferents, but not lower intensities, leads to a persistent depression of the jaw opening reflex elicited by electrical stimulation of the incisor pulp in the rat (Kawakita, Funakoshi, 1982). Conditioning stimuli applied at intensities sufficient to recruit C fibers in the common peroneal (Chung et al.1983) or tibial nerve (Chung et al.1983) depress the late flexion reflex and the sural-gastrocnemius reflex for 10 to 60 min in the cat. Furthermore, recruitment of unmyelinated fibres elicits a long-lasting attenuation of spinal reflexes in the spinal, decerebrated rabbit (Taylor et al.1990). In primates, electrical stimulation of the tibial nerve at a strength which recruits only Aα and Aß fibers produces a minor inhibition of lumbar spinothalamic tract cells with no post-stimulation effect (Chung et al.1984b), yet recruitment of Aδ and C fibers with higher intensities produces an inhibition lasting up to 30 min (Chung et al.1984a; Chung et al.1984b). It has also been shown that recruitment of C fibers, by application of natural visceral and cutaneous stimuli, produces a greater inhibition of longer duration than that occurring with recruitment of lower threshold

afferents (Ness, Gebhart, 1991a; Ness, Gebhart, 1991b). Thus, evidence from other paradigms supports the idea that recruitment of high threshold primary afferents leads to the persistent post-stimulation effects observed in the present study.

Frequency. The persistent post-stimulation antinociception also appears to be dependent on the frequency of stimulation and the data again implicate central mechanisms in mediation of this effect. Stimulation at 4 or 6 Hz, but not at 8 Hz, elicited the persistent antinociception. At 2 Hz, there was some persistent effect in our experiments but it was less in amplitude and duration; this is discussed below.

The observation that stimulation at 8 Hz elicits the brief inhibition alone suggests that the peripheral and/or central mechanisms mediating this response are relatively independent of frequency and follow relatively higher frequencies while those mediating the persistent inhibition appear to be dependent on lower frequencies of stimulation. The difference in responsiveness to various frequencies may be due to principles of synaptic rather than axonal transmission as C-fibres appear to be able to follow 100 Hz when stimulated electrically (Douglas, Ritchie, 1957; Iggo, 1960). Thus, the relative failure of the persistent effect at 8 Hz may be due to the inability of the central mechanisms mediating this effect to follow such a frequency. In this context, post-stimulation analgesia evoked by intense stimulation just tolerable to human subjects has been reported in an experimental pain study with 2 but not 10 Hz stimulation (Andersson, Holmgren, 1975) and the perceptual response to C fibre stimulation in human peripheral nerves begins to fail at 5 Hz (Torebjörk, Hallin, 1973; Liu et al.1995). Therefore, we suggest that the brief antinociception produced during the stimulation results from activation of different central

mechanisms than those mediating the persistent effect. It is suggested, then, that the brief antinociception observed during stimulation may be due to continuous synaptic activation as opposed to the persistent effect, which appears to be mediated via a form of central summation.

Pulse duration. The threshold stimulus required for activation of nerve fibres is determined by the duration and the amplitude of the applied current pulse and membrane resistance, and the internal resistance of a nerve fiber play critical roles in governing the required duration of the current pulse. Low threshold, large diameter fibres, with a low internal resistance and a high membrane resistance require pulse durations in the order of tens to hundreds of μs to be activated, while high threshold, small diameter fibres, such as C fibers, have high internal and low membrane resistances and therefore require long pulse durations. Pulse durations of 0.5 ms (Wall, Woolf, 1984; Thompson et al.1990; Xu et al.1990), 0.8 ms (De Koninck, Henry, 1991) or equal or greater than 1.0 ms (Mendell, 1966; Chung et al.1983; Pérez et al.1993) are generally used to activate C fibres. Short pulse durations in the range of 0.1 ms have been used to activate C afferents but intensities required are in the order of 200 to 400 times threshold for Aß activation (Hoheisel, Mense, 1990; Xian-Min, Mense, 1990). In our experiments, little or no post-stimulation antinociception was produced when the stimulation pulse was 0.2 ms in duration, yet pulse durations of 2 and 5 ms produced the persistent antinociception. These data are therefore consistent with the interpretation that recruitment of high threshold, small diameter fibers is required to activate central mechanisms which produce the persistent effect.

II. Optimal train duration suggests the persistent effect is due to central summation.

Dependence of a persistent inhibition on the duration of the stimulus has been reported elsewhere. A five min train recruiting Aδ and C fibers elicited a post-stimulation inhibition of spinothalamic tract cells lasting less than 2 min (Chung et al.1984b). However, when the same stimulus was maintained for 15 min the inhibition persisted up to 30 min (Chung et al.1984a). Thus, our data lead us to suggest that prolonged stimulation allows adequate summation of CNS mechanisms to elicit a persistent effect.

With regard to longer train durations, an effect parallel to ours has been reported (Chen, Han, 1992) whereby during continuous electroacupuncture the evoked antinociception decays gradually with time. In human studies the duration of acupuncture-like treatment with low frequency, high intensity stimulation is quite varied. For example, 10 to 30 min of stimulation elicits effects lasting several hours or even days (Melzack, 1975; Fox, Melzack, 1976; Mannheimer, Carlsson, 1979), while 40 min of stimulation evokes an analgesia that persists for 30 min (Research Group of Acupuncture Anesthesia, 1973). When the stimulation is applied for 75 min, the duration of the post-stimulation analgesia is relatively short-lived, lasting 15 min (Andersson et al.1973; Andersson, Holmgren, 1975). We show here that in the rat the duration of the stimulation has a critical role in determining the duration of the post-stimulation antinociception.

The observation that 20 min of stimulation elicited a greater and longer-lasting persistent antinociception than 10 or 40 min of stimulation is difficult to account for on the basis of peripheral mechanisms only. In fact, the persistence of the effect for greater than one hour after stimulation (and thus presumably after primary afferent synaptic inputs have ceased) leads us to the proposal that the persistent changes are due to central mechanisms of summation.

Central summation may explain how stimulation at 2 Hz produces only a post-stimulation

effect lasting 15 min, while at 4 and 6 Hz this effect lasts more than one hour. In this context, other studies have shown long-lasting effects produced by stimulation of Aδ and C fibers. For example, "wind up" can be produced optimally with low frequency stimulation (Mendell, 1966; Thompson et al.1990; King et al.1990) and long-lasting summation of ventral root potentials is evoked with 1-5 Hz stimulation of dorsal roots *in vitro* at strengths which recruit C fibers (Thompson et al.1994). Finally, Ness and Gebart have demonstrated that the magnitude and duration of the inhibition of neuronal responses to C fiber input evoked by noxious stimulation is a function of the number of preceding conditioning stimuli (Ness, Gebhart, 1991b); they suggest that central sensitization or wind up is the underlying mechanism by which the increase in madgnitude and duration of the evoked inhibition is mediated (Ness, Gebhart, 1991b).

III. Electrical stimulation for the treatment of pain

A brief attempt will be made to relate the present conclusions to approaches to the treatment of pain. The antinociception evoked in this study by high intensity, low frequency stimulation relates in some respects to the analgesia reported in patients given peripheral electrical stimulation. Analgesia which lasts several hours to days can be elicited at high intensities reported to be just tolerable to the patient (Melzack, 1975; Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Sjölund, Eriksson, 1979). Studies on chronic pain patients which did not experience pain relief with low intensity, high frequency stimulation experienced relief when the stimulus intensity was increased to just tolerable levels (Eriksson, Sjölund, 1976; Sjölund, Eriksson, 1979). In an experimental setting, electrical stimulation at five to eight times the perception threshold, evoking strong muscular contractions, increases experimental tooth pain

threshold (Andersson et al.1973; Andersson, Holmgren, 1975). Thus, in animal studies, in chronic pain patients and in human experimental pain, persistent antinociception or analgesia appears to be achieved when relatively high intensities of stimulation are used.

On the other hand, low intensity stimulation which is well below the human pain threshold usually produces a short lasting or brief analgesia. This kind of analgesia is typically achieved with high frequency, low intensity stimulation sufficient to recruit the myelinated, large diameter sensory primary afferents (Meyer, Fields, 1972; Loeser et al.1975; Long, 1976), thus being of closer resemblance to TENS, which is usually applied at higher frequencies such as 100 Hz. While some lasting effects have been reported with high frequency, low intensity electrical stimulation (Guieu et al.1991), in general the elicited analgesia is produced only during the period of stimulation with little or no post-stimulation effect (Meyer, Fields, 1972; Sjölund, Eriksson, 1979). In fact, the analgesia produced by this type of stimulation has been reported as being only marginally effective and not always reliable (Loeser et al.1975; Eriksson, Sjölund, 1976; Long, 1976; Sjölund, Eriksson, 1979) and seems to be more effective in the treatment of patients suffering acute pain than chronic pain (Levine et al.1976; Meyer, Fields, 1972).

Summary and conclusion

Our data indicate that low frequency electrical stimulation of hindlimb meridian points in the rat produces two kinds of inhibition of the tail withdrawal reflex, a brief and a persistent effect. It is suggested that these two responses are dependent on recruitment of high threshold primary afferents but distinguishable on the basis of the involvement of central summation. It is proposed that the brief antinociception is relatively independent of frequency and pulse duration

because it occurs with low and high frequencies of stimulation and it is elicited with short or long pulse durations. Finally, the brief antinociception is elicited only during the stimulation and thus appears to be dependent on continuous synaptic activation.

The persistent antinociception can be elicited only when longer pulse durations are used. The central mechanisms mediating the persistent effect also appear to differ from those mediating the brief response as the persistent antinociception can last more than one hour after the end of the stimulation, suggesting involvement of central summation. In addition, as the optimal duration of the train of stimulation is about 20 min, this suggests some type of extinction with longer periods of stimulation.

The brief antinociception in our study bears some resemblance to the analgesia evoked in human patients by low intensity stimulation, whether at high or low frequency (Wall, Sweet, 1967; Shealy et al.1970; Meyer, Fields, 1972; Loeser et al.1975; Long, 1976; Eriksson et al.1979; Sjölund, Eriksson, 1979; Guieu et al.1991). On the other hand, the persistent antinociception in our study bears some resemblance to the long-term analgesia evoked in human patients by high intensity, low frequency electrical stimulation (Andersson et al.1973; Andersson, Holmgren, 1975; Melzack, 1975; Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Sjölund, Eriksson, 1979). The clinical implication of these findings is that the specific parameters of electrical stimulation for the alleviation of pain are important to achieve optimal analgesia.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

MPI maximum possible inhibition

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TABLE 1. Statistical comparison of the net mean increase in tail flick latency elicited by high intensity stimulation of meridian points at varied frequencies during the period of stimulation and for subsequent periods after the stimulation. Paired t-test: * = $p < 0.05$, ** = $p < 0.01$, NS = not significant.

FREQUENCY	4 Hz (n = 7)				6 Hz (n = 8)				8 Hz (n = 6)				CONTROL (n = 20)			
	PERIOD				PERIOD				PERIOD				PERIOD			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
2 Hz (n = 7)	NS	**	**	**	NS	**	**	**	NS	**	**	NS	**	*	NS	N S
4 Hz (n = 7)					NS	NS	**	*	NS	**	**	**	**	**	**	**
6 Hz (n = 8)									NS	**	**	**	**	**	**	**
8 Hz (n = 6)													**	NS	NS	N S

Fig. 1. Schematic representation of meridian points *fengshi (GB-31)*, *femur-futu (ST-32)* and *zusanli (ST-36)* in relation to skeletal structures of the hindlimb. These points were selectedfrom previous reports from other investigators.

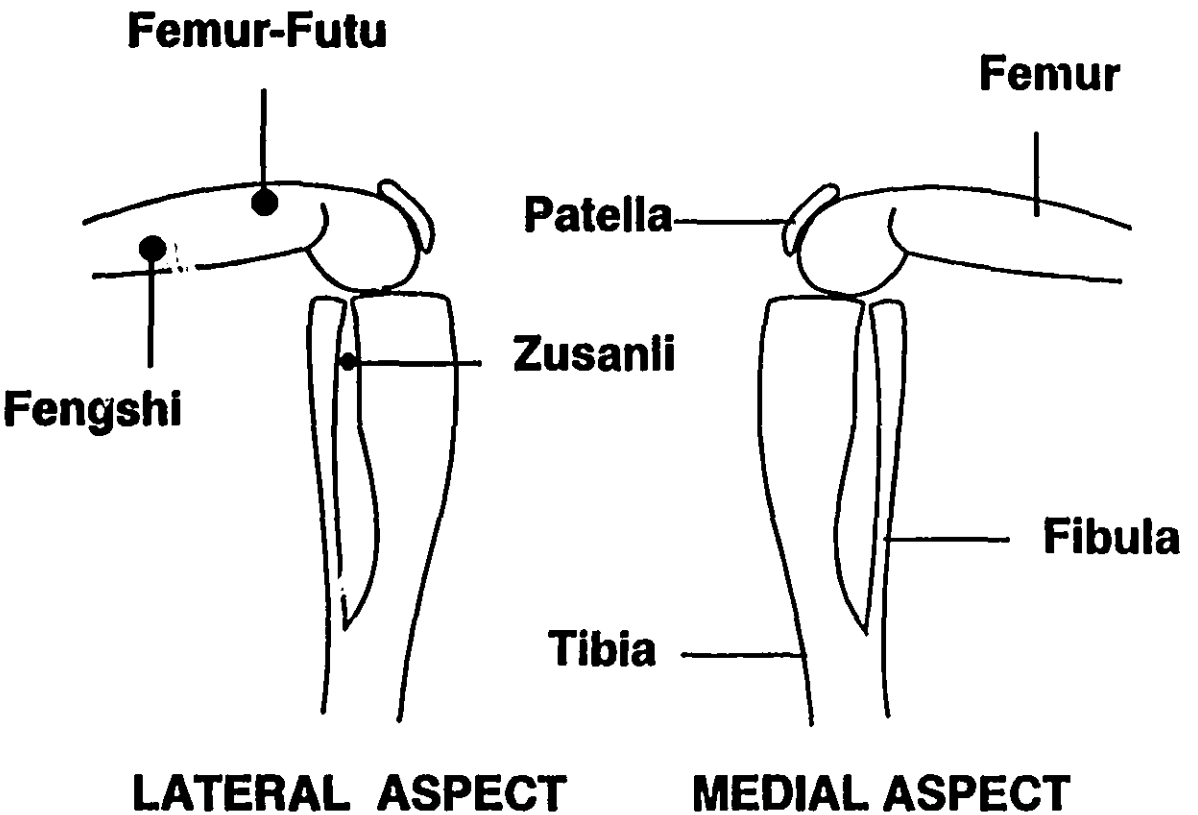

Femur-Futu
Fengshi
Zusanli
LATERAL ASPECT
Femur
Patella
Fibula
Tibia
MEDIAL ASPECT

Fig. 2. Effect on tail withdrawal latency of high intensity (20 times threshold), low frequency (4 Hz) electrical stimulation of hindlimb meridian points *fengshi* (GB-31), *femur-futu* (ST-32) and *zusanli (ST-36)* with 2 ms square wave pulses for 20 min (the duration of the train of stimulation is indicated by the hatched area). Vertical dotted lines indicate times of administration of supplemental doses of anesthetic. Tail withdrawal latency is expressed as the mean % of the maximum possible inhibition (MPI) at each sample time. ▵, stimulated group (n = 7); ○, needles only (n = 20); ▲, ▵ minus ○. Difference between the two groups: * $p < 0.05$; ** $p < 0.01$.

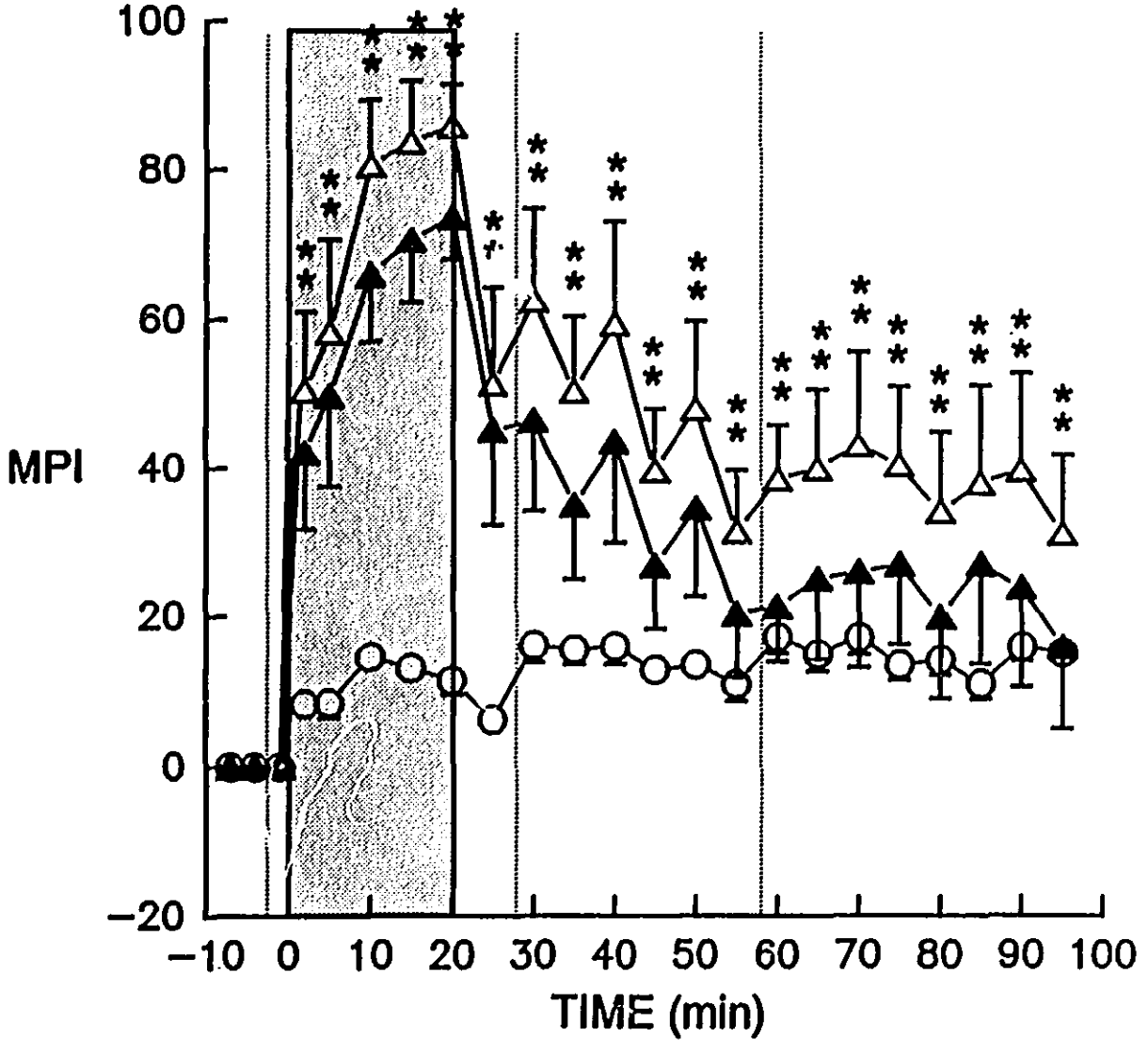

100
80
60
40
20
0
−20
MPI
−10
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

Fig. 3. Effect of varying the intensity of stimulation: stimulation at 10 times threshold is shown on the left (n = 7; ▵); stimulation at threshold is shown on the right (n = 12; ▵). Parameters were standard. ○, needles only (n = 12); ▲, ▵ minus ○. * $p < 0.05$; ** $p < 0.01$. The durations of the experiments were the same as in figure 2, but the abscissae were truncated due to the brevity of the responses. Other details are as in figure 2.

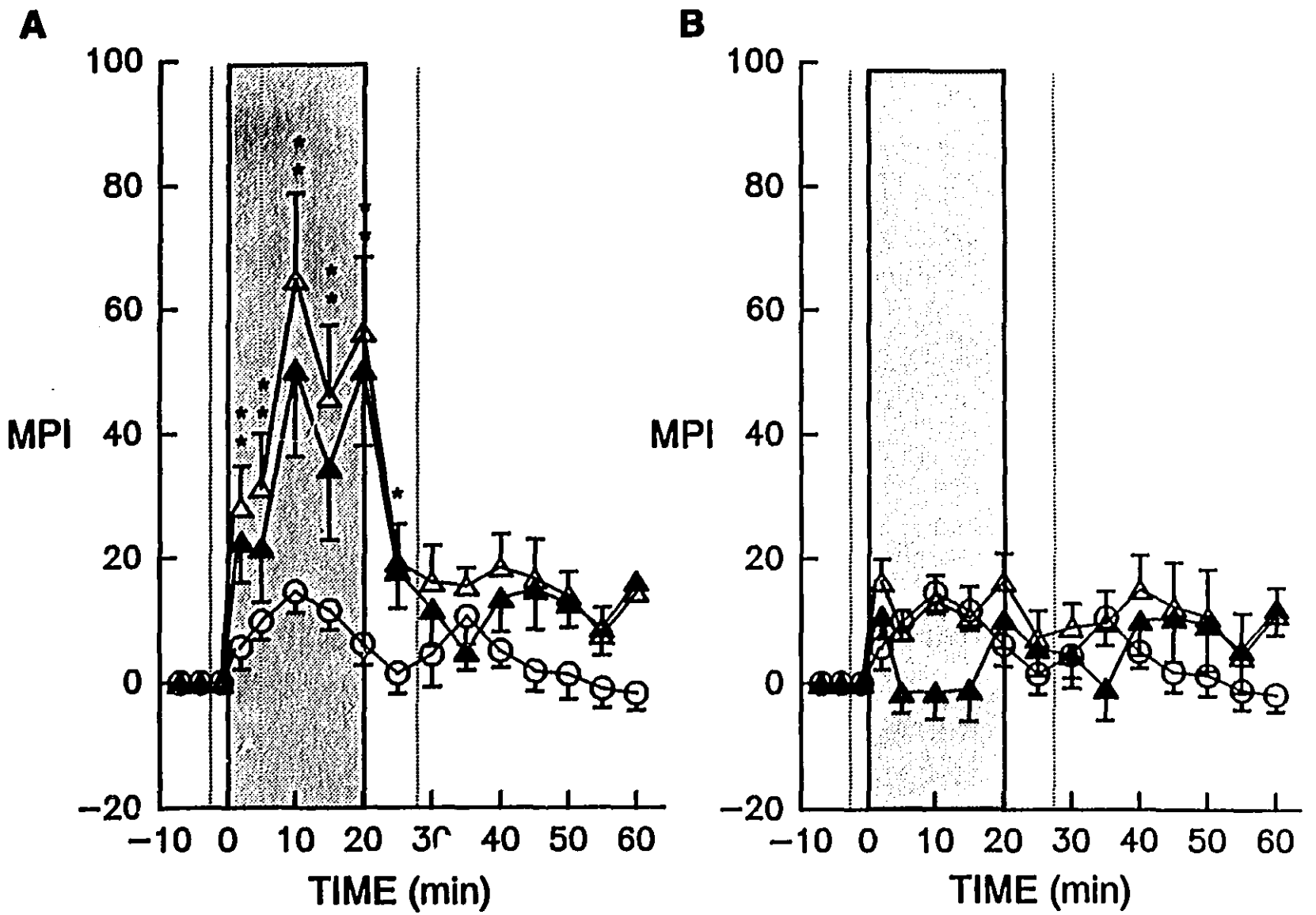

A
B
100
80
60
40
20
0
−20
MPI
−10
0
10
20
30
40
50
60
TIME (min)

Fig. 4. Effect of varying the duration of the train of stimulation: 10 min. Parameters were otherwise standard. △, stimulated group (n = 9); ○, needles only (n = 12); ▲, △ minus ○. * $p < 0.05$; ** $p < 0.01$.

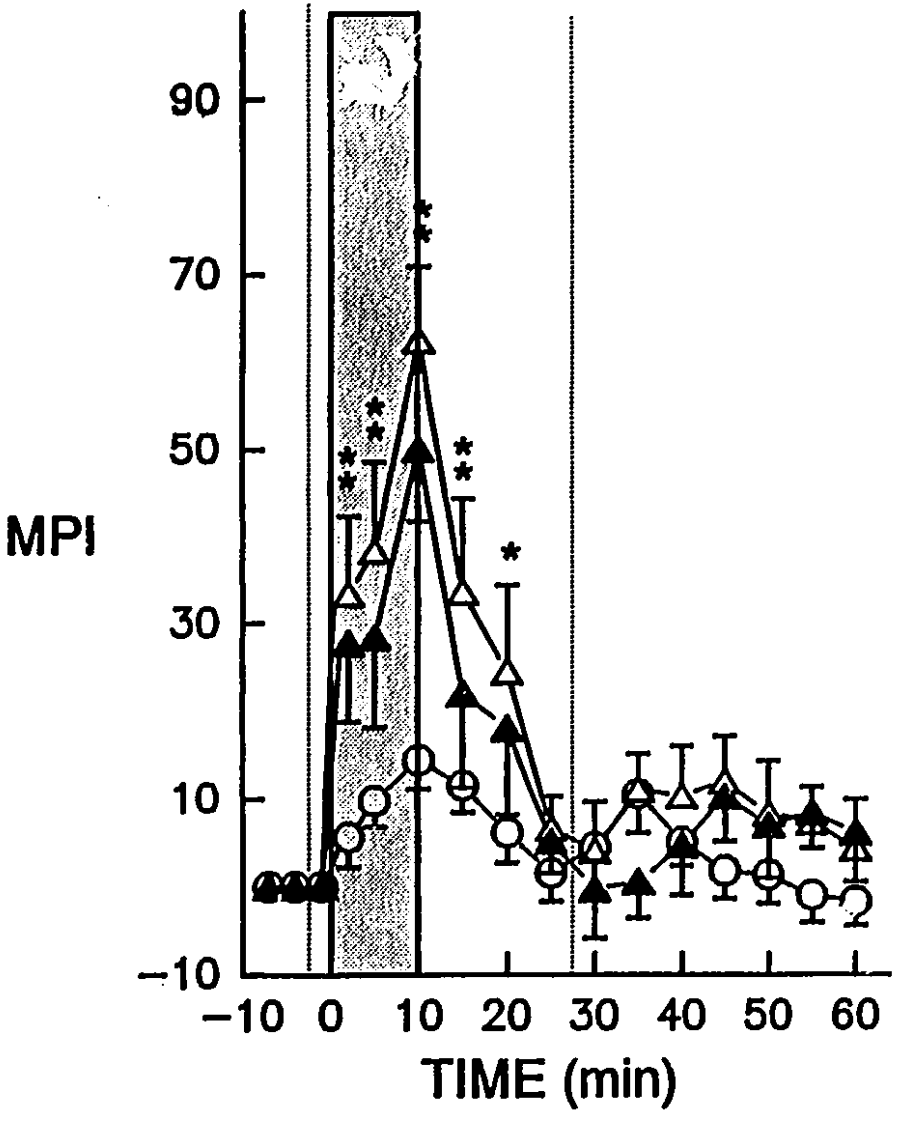

MPI
90
70
50
30
10
−10
−10 0 10 20 30 40 50 60
TIME (min)

Fig. 5. Effect of varying the duration of the train of stimulation: 40 min. Parameters were standard. △, stimulated group (n = 9); ○, needles only (n = 12); ▲, △ minus ○. ** $p <$ 0.01.

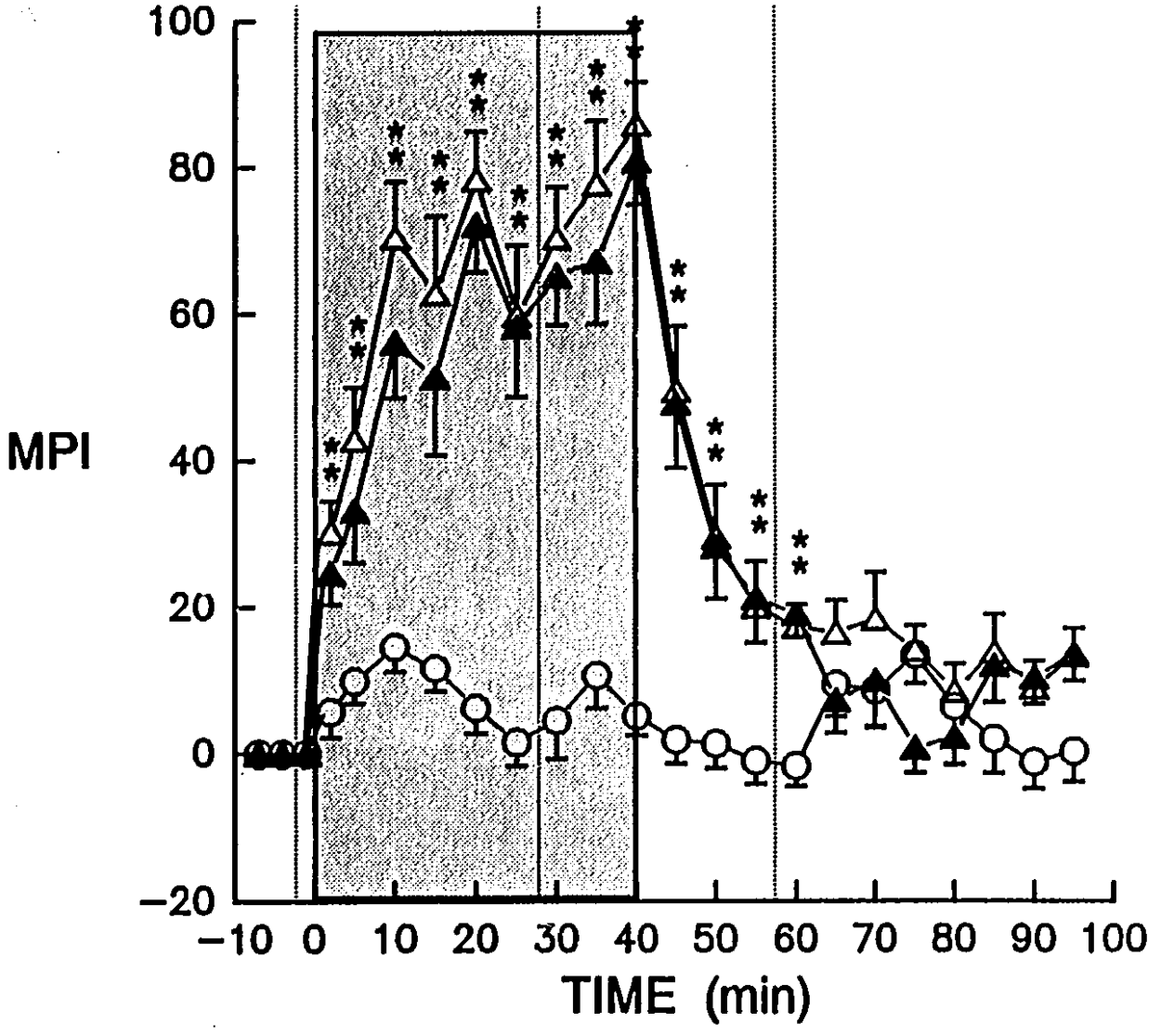

MPI
100
80
60
40
20
0
−20
−10
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

Fig. 6. Effect of varying the frequency of stimulation: 2 Hz. Parameters were standard. △, stimulated group (n = 7); ○, needles only (n = 20); ▲, △ minus ○. ** $p < 0.01$.

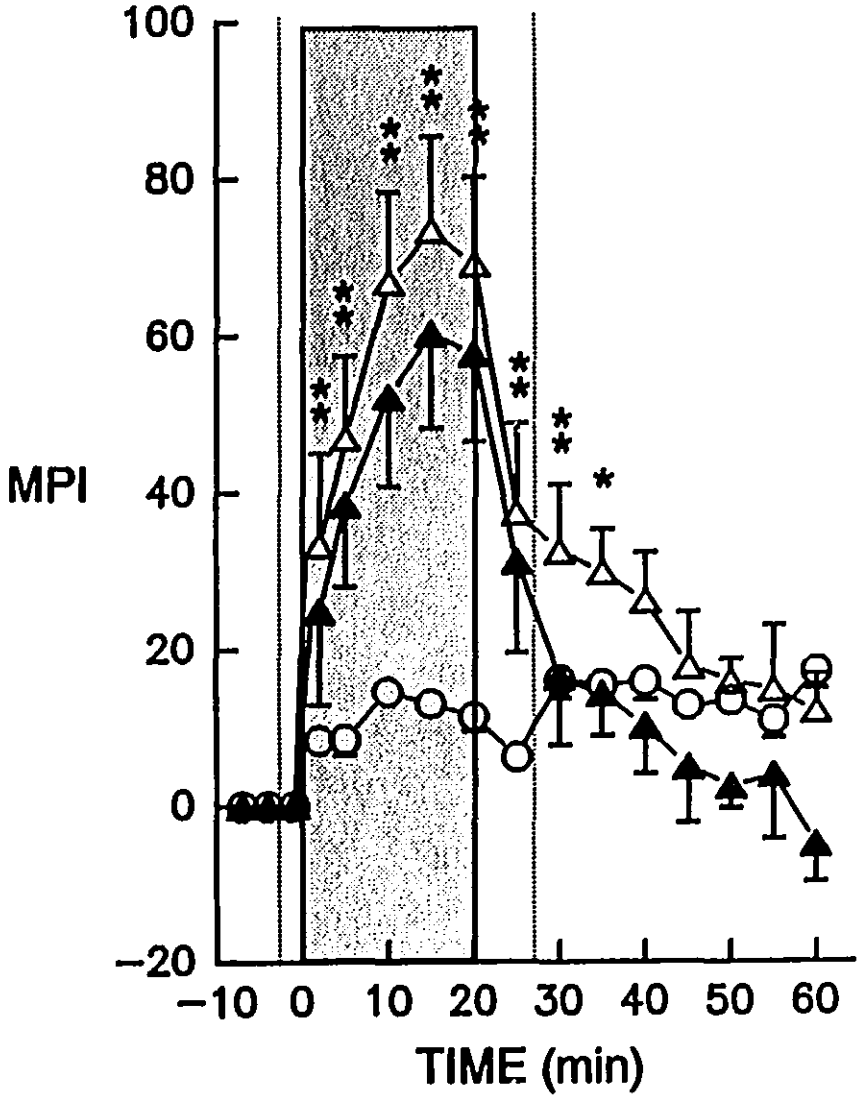

100
80
60
40
20
0
−20
MPI
−10
0
10
20
30
40
50
60
TIME (min)

Fig. 7. Effect of varying the frequency of stimulation: 6 Hz. Parameters were standard. △, stimulated group (n = 8); ○, needles only (n = 20); ▲, △ minus ○. * $p < 0.05$; ** $p < 0.01$.

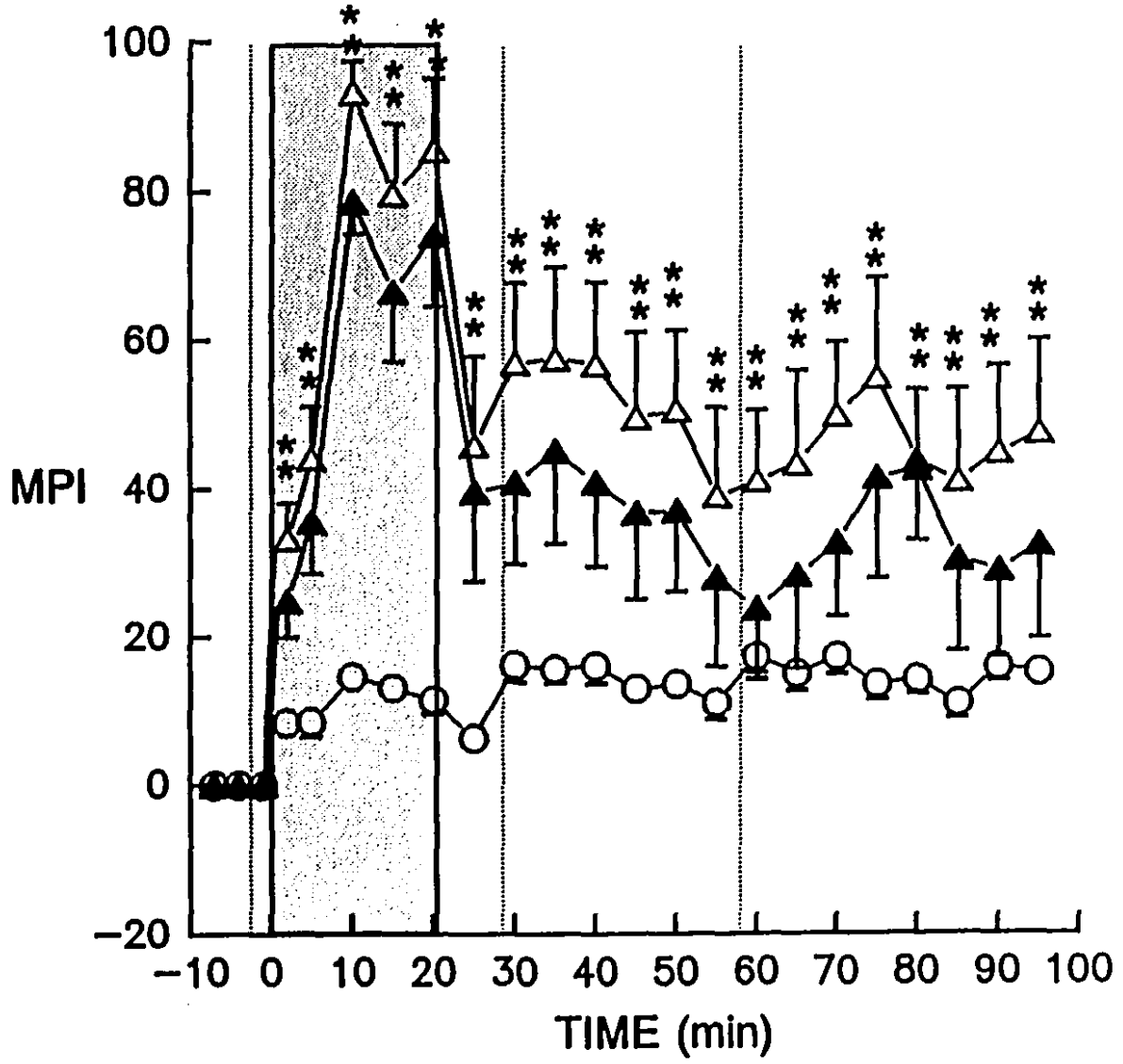

100
80
60
40
20
0
−20
MPI
−10
0
10
20
30
40
50
60
70
80
90
100
TIME (min)

Fig. 8. Effect of varying the frequency of stimulation: 8 Hz. Parameters were standard. △, stimulated group (n = 6); ○, needles only (n = 20); ▲, △ minus ○. ** $p < 0.01$.

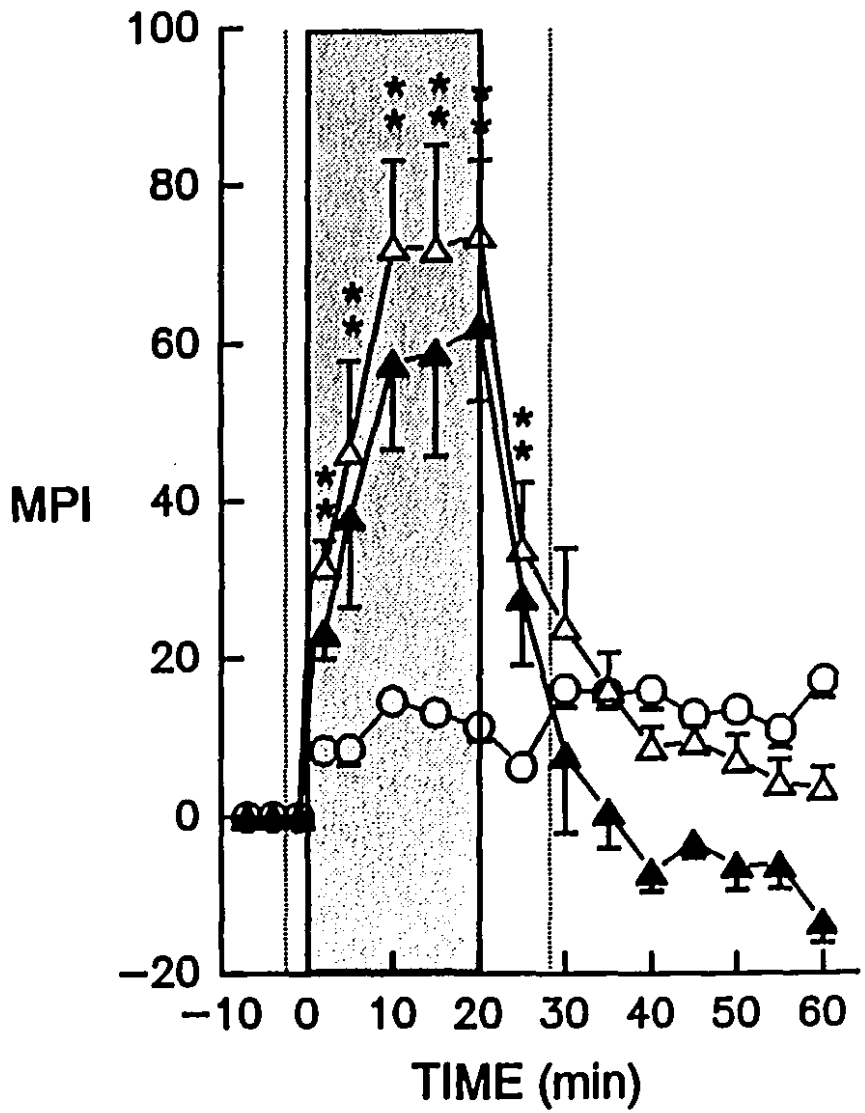
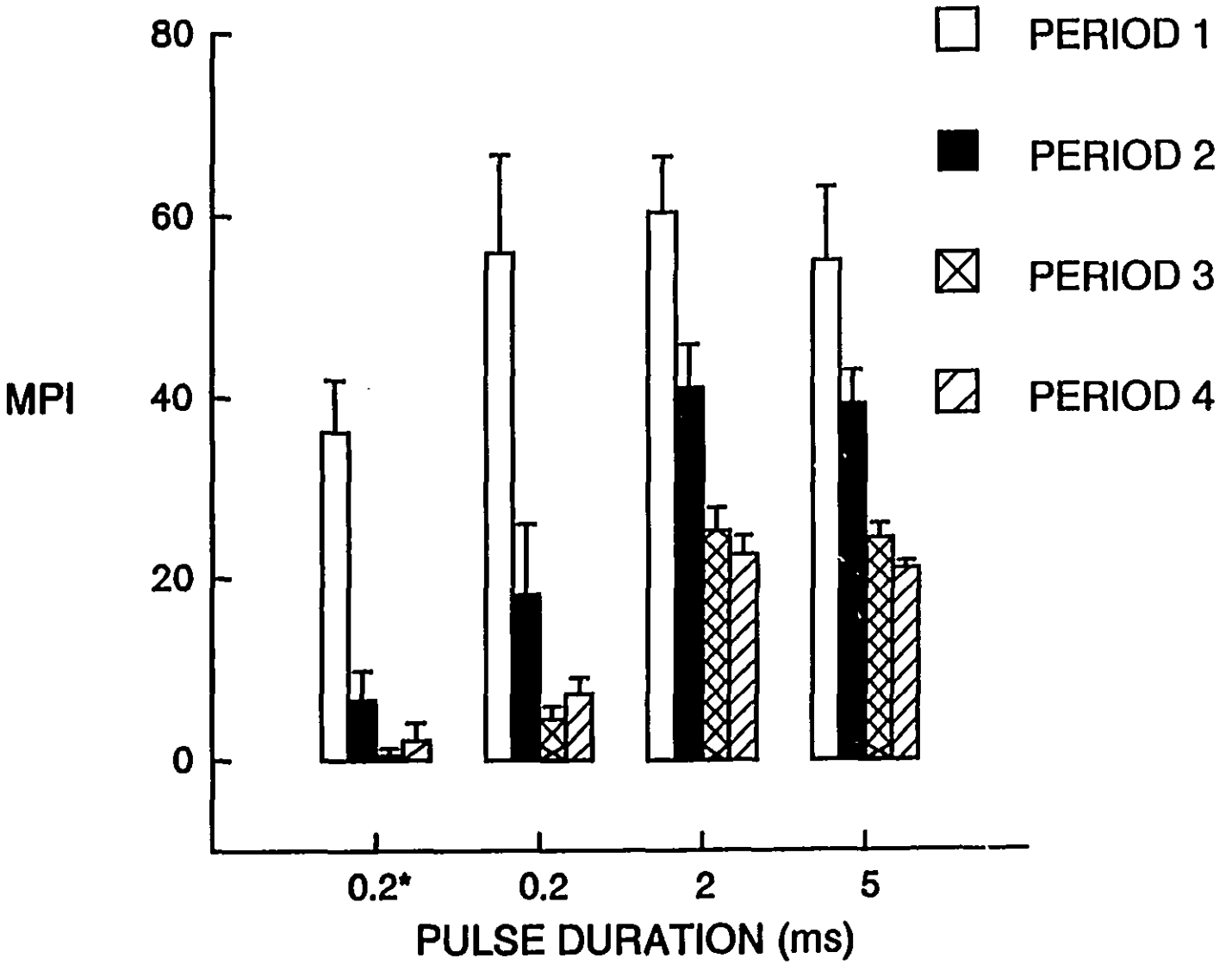

100
80
60
40
20
0
−20
MPI
−10
0
10
20
30
40
50
60
TIME (min)

Fig. 9. Effect of varying pulse duration. Electrical stimulation was with 0.2* msec pulses at 10 times threshold, with 0.2 msec pulses at 20 times threshold, with 2 msec pulses at 20 times threshold or with 5 msec square pulses at 20 times threshold. The effect is expressed as the mean % of the MPI calculated for the 20 min period of stimulation and for subsequent 25 min periods after the stimulation, as described in the text.


80
60
40
20
0
MPI
PERIOD 1
PERIOD 2
PERIOD 3
PERIOD 4
0.2*
0.2
2
5
PULSE DURATION (ms)

CHAPTER THREE

INTENSE PERIPHERAL ELECTRICAL STIMULATION DIFFERENTIALLY INHIBITS TAIL VS. LIMB WITHDRAWAL REFLEXES IN THE RAT

Abstract: In an on-going study on mechanisms by which activation of sensory afferents regulates nociception, high intensity, low frequency electrical stimulation was applied to previously defined meridian and non-meridian points of the hindlimb or forelimb, and the effects measured on the withdrawal reflex of the tail or limb in the lightly anesthetized rat. Withdrawal was evoked by application of noxious radiant heat to the tip of the tail or to the plantar surface of a hindpaw or forepaw. Parameters of conditioning electrical stimulation were 2 ms pulses at 4 Hz for 20 min at 20 X threshold (20-30 mA) where threshold was the minimum intensity which evoked muscle twitch. In experiments on tail withdrawal, stimulation applied to meridian points *fengshi* (GB-31), *femur-futu* (ST-32) and *zusanli* (ST-36) of the hindlimb or to *wai-kuan* (TH-5) and *hoku* (LI-4) of the forelimb increased the latency of the withdrawal reflex to 70-100 % of the maximum possible inhibition (MPI) during the stimulation. Inhibition persisted for more than one hour after the end of stimulation. Bilateral stimulation of hindlimb meridian points evoked a greater inhibition during the stimulation (> 95 % of the MPI); the inhibition persisted for 40 min. Stimulation of non-meridian sites in hindlimb or forelimb inhibited the withdrawal reflexes by 45%-50% of the MPI during the stimulation only. Thus, the evoked inhibition has two components, a brief effect elicited by non-meridian point stimulation and a persistent post-stimulation effect produced only upon stimulation of meridian points. Stimulation produced little effect on nociceptive limb withdrawal reflexes. The results suggest that high intensity, low frequency electrical stimulation of meridian points produced a long-lasting, extrasegmental inhibition of the tail withdrawal but not of limb withdrawal reflexes. This differential inhibition may be due to differences in neuronal circuitry and CNS modulatory control mechanisms. The persistent inhibition appears to be dependent on the site of stimulation because it is not evoked

because it is not evoked by stimulation of sites outside of meridian points.

Key words: nociception, pain, antinociception, analgesia, noxious, spinal reflex, spinal cord

1. Introduction

Activation of high threshold primary afferents can modulate transmission of nociception in the spinal cord at a remote site[8,48,49] and this is believed to be the neurophysiological basis for the analgesic effects evoked by counter-irritation practices to achieve the attenuation of pain by application of a spatially remote aversive stimulus[16,22,47]. An early study in human subjects demonstrated that application of a chemical irritant to the leg increases tooth pain threshold in response to electric shock[36]. In human subjects noxious thermal stimulation of the hand attenuates the RIII reflex in the biceps femoris muscle of the contralateral leg elicited by high intensity transcutaneous stimulation of the sural nerve[49]. Analgesia evoked by high intensity electrical stimulation has been proposed to be a variation of that evoked by counter-irritation[3-5,15,29] and the underlying mechanisms of both may share some similarities with diffuse noxious inhibitory controls (DNIC) served by a spinal-brain stem-spinal loop[6,7,45,46]. In common with DNIC, high intensity, low frequency electrical stimulation produces a generalized antinociceptive/analgesic effect, but unlike DNIC this effect requires prolonged stimulation[15,28,29] and the effect persists for hours to days[14,15,29,40]. On the other hand, the antinociception evoked by brief application of aversive stimuli is generally short lived lasting only minutes[25,33,49].

Only a few experiments have studied the effects of prolonged, high intensity, low frequency electrical stimulation on remote nociceptive mechanisms in animals. In decerebrated, spinalized animals high intensity, low frequency stimulation of peripheral nerves has been reported to produce a long-lasting inhibition of the flexion reflex[10], of the sural-gastrocnemius reflex[13,43] and of spinothalamic tract cells[11] but in all cases the inhibition was restricted to the same segment stimulated and therefore may not be derived via the same mechanisms which yield

the generalized analgesia such as that seen in response to aversive stimulation in human subjects. One study, however, has demonstrated that high intensity, low frequency electrical stimulation at Aδ- and C fiber strength applied to peripheral or to hindlimb meridian points evoked a relatively long-lasting inhibition of nociception at a remote site as measured by the jaw opening reflex in the rat[23]. In addition, we have recently found that high intensity, low frequency electrical stimulation of selected points in the hind limb of the lightly anaesthetized rat elicits brief and persistent inhibitory effects on the tail withdrawal reflex. Our objective in the present study was therefore to determine whether such stimulation elicits similar inhibition on nociceptive withdrawal reflexes of the hindlimb and forelimb and how regionally generalized the effects are on the tail withdrawal reflex.

2. Materials and methods

In all cases, the guidelines described in The Care and Use of Experimental Animals, of the Canadian Council of Animal Care, Vols. I and II, Second Edition, were strictly followed. The experimental protocols were also reviewed and approved by the McGill University Animal Care Committee.

Animal preparation

Male Sprague Dawley rats (300-400 g) were lightly anesthetized for the duration of the experiment with an intraperitoneal injection of a freshly prepared mixture of sodium pentobarbital (20 mg/kg, Abbott Laboratories Ltd.) and chloral hydrate (120 mg/kg, Fisher

Scientific) in 50% propylene glycol and 30% physiological saline (0.9% NaCl). This provides a level of anesthesia sufficient to prevent any overt sign of discomfort to the rat during experimentation, yet a stable withdrawal response is obtained for about 1 hour. To maintain this light state of anesthesia throughout the full duration of the experiment, subsequent injections of the mixture were given. Thus, an injection of ⅓ the initial dose of anesthetic was given 35.5 min after the first injection; this second injection was timed to occur 1.5 min prior to the beginning of electrical stimulation. Subsequent injections of ⅓, ¼ and 1/6 the initial dose were given at 30 min intervals.

Withdrawal reflex tests

To measure the latency of the withdrawal reflex a portion of the tip of the tail was blackened to facilitate the absorption of heat. This portion was then positioned above a focused projector bulb to provoke the tail withdrawal reflex. Withdrawal of the tail exposed the light beam to a photodetector which in turn stopped a timer giving reaction time measured to 0.01 s[20]. The intensity of the bulb was set so that the baseline reaction time was 4-6 s. Trials were terminated automatically if a tail withdrawal did not occur within 12 s, the cut-off time. The latency of the withdrawal reflex of a hindlimb or forelimb was measured in the same way, in this case applying the heat stimulus to the blackened plantar surface.

At each sample time two readings were taken, separated by 40-50 s, at two different sites within the 2 cm blackened segment of the tail. Thus, the tail withdrawal latency was never measured twice from the same site within a five min interval. The average of the two readings was calculated and the value expressed as percent of the maximum possible inhibition (MPI)

according to the formula[52]

$$MPI = \frac{(\text{POST-TREATMENT LATENCY} - \text{PRE-TREATMENT LATENCY})}{(\text{CUT-OFF TIME} - \text{PRE-TREATMENT LATENCY})} \times 100$$

To measure the latency of the withdrawal during the period of the electrical stimulation, the stimulator was turned off just long enough for the reading to be taken; this was necessary because the stimulus was above the threshold to elicit a direct contraction of muscles.

Placement of Needles

For hindlimb stimulation, two pairs of stainless steel insect pins were inserted into the vicinity of meridian points, *fengshi* (GB-31), *femur-futu* (ST-32) and *zusanli* (ST-36). To stimulate *femur-futu* the cathode was placed along the medial side of the knee and the anode was inserted along the lateral side of the knee so that it lay across *zusanli* (ST-36). To stimulate *fengshi* the electrodes were inserted under the femur midway between the hip and the knee. These meridian points were selected on the basis of what has been published in the literature[3-5,37,38,44]. To stimulate non-meridian points, the electrodes were placed in the medial and lateral gastrocnemius muscles and the biceps femoris and semitendinosus muscles. The positions of the meridian points relative to skeletal muscle are illustrated in figure 1.

For forelimb, to stimulate *Wai-kuan* (TH-5) the cathode was placed between the radius and ulna proximal to their distal ends while the anode was placed into the nearby muscle. To stimulate *Hoku* (LI-4) the cathode was placed into the interosseous muscle between the first and

second metacarpals while the anode was placed into the interosseous muscle between the third and forth metacarpals. Again meridian points were selected on the basis of what have been reported in the literature to produce antinociception in experimental animals[1,2,5,9,37,44]. Non-meridian point stimulation was achieved by placing the electrodes into surrounding muscle of the forearm. Positions of the meridian points relative to skeletal structures are illustrated in figure 1.

Electrical Stimulation

The threshold intensity which just produced muscle contraction in response to electrical stimulation was determined. The needles were connected to coupled Grass stimulators (SD9 and SD5) which passed a train of monophasic square wave pulses. The standard parameters of stimulation were 2 ms square voltage pulses at 4 Hz for a duration of 20 min and applied at 20 times threshold (20-30 mA, min 15 volts). In the control groups, electrodes were inserted into the respective areas of the hindlimb but no electrical stimulation was applied.

Experimental Protocol

The lightly anesthetized rat was placed on the apparatus used to measure tail withdrawal latency[20], needles were inserted into the respective sites and threshold determined. Before testing, animals were allowed to stabilize for 30 min. Three baseline readings of the latency of the tail withdrawal or limb withdrawal reflex were taken at 3 min intervals. Stimulation was applied 1 min after the last baseline reading and latencies of the withdrawal reflexes were recorded at 2 and 5 min after the onset of stimulation, and thereafter at 5 min intervals up to 95

min.

Statistical Analysis

Results are expressed as the net effect, obtained by subtracting the mean MPI at each time point in the control group from the respective MPI in the test group, to eliminate the influence of supplemental doses of anesthetic. All values are expressed as the mean ± standard error of the mean (S.E.M.). Data from treated rats which received electrical stimulation and control animals which received no stimulation were analyzed using two way analysis of variance (ANOVA). Stimulation vs. no stimulation was taken as the between subject factor for otherwise similarly treated groups and time was taken as the within subject factor. Tukey's Wholly Significant Difference Test was used to make post hoc comparisons between means. The same method of statistical analysis was used to compare two groups of rats treated differently (i.e. stimulation of meridian vs. stimulation of non-meridian points).

3. RESULTS

1) Effect of stimulation of the hindlimb on tail withdrawal latency

Unilateral stimulation of hindlimb meridian points. Electrical stimulation of meridian points in one hindlimb (n = 9) inhibited the tail withdrawal reflex (Fig. 2a). Inhibition was observed at the first reading taken after the beginning of the stimulation ($p < 0.01$). During stimulation the inhibition was maximum at 10 and 20 min after the onset of stimulation, at 71.6 ± 6.30 and 73.3 ± 5.52 % of the MPI, respectively. After the end of stimulation, a post-

stimulation effect remained; this effect was maximum at 47.37 ± 8.03 % of the MPI 15 min after termination of the conditioning stimulation and persisted for more than one hour ($p < 0.01$; n = 9).

Bilateral stimulation of hindlimb meridian points. Bilateral hindlimb stimulation (Fig. 2b) inhibited the tail withdrawal reflex by about 90 % of the MPI at 20 min after the onset of the stimulation (n = 10; $p < 0.01$). Two way ANOVA ($0.01 > p < 0.05$) and paired t-test ($p < 0.001$) comparisons showed that the inhibition evoked during stimulation was significantly greater during the period of stimulation than that produced by unilateral hindlimb stimulation (Fig. 2a). Bilateral stimulation also produced a post-stimulation effect which peaked at 66.8 ± 7.67 % of the MPI at 10 minutes and persisted for up to 40 minutes after the end of stimulation.

Stimulation of hindlimb non-meridian points. Unilateral stimulation of non-meridian points (n = 7) inhibited the tail withdrawal reflex but only during the stimulation. This inhibition was 48.1 ± 9.11 and 49.8 ± 8.56 % of the MPI at 10 and 20 min, respectively, after the onset of stimulation (Fig. 2c) ($p < 0.01$). Two way ANOVA ($0.01 > p < 0.05$) and paired t-test ($p < 0.001$) comparisons show the inhibition during stimulation was less than that produced by meridian point stimulation. No post-stimulation effect was evoked.

2) Effect of stimulation of the hindlimb on forelimb withdrawal latency

Unilateral stimulation of hindlimb meridian points only slightly inhibited the withdrawal of the ipsilateral (n = 6; Fig. 3a) and contralateral (n = 6; Fig. 3b) forelimb. In the ipsilateral forelimb the inhibition of the withdrawal was only statistically significant at 2 ($p < 0.01$) and 10 ($p < 0.05$) minutes during the stimulation. No post-stimulation effect was produced. In the

contralateral forelimb, the evoked effect was significant throughout the duration of the stimulation ($p < 0.01$) and at 5 min after the end of stimulation ($p < 0.05$). Thereafter, no significant difference was seen when compared to the unstimulated control group.

3) *Effect of stimulation of forelimb on tail withdrawal*

Unilateral stimulation of forelimb meridian points. Stimulation of forelimb meridian points inhibited the tail withdrawal reflex by 100 % of the MPI (n = 6) at 20 minutes (Fig. 4a). At 5 and 10 minutes after the end of stimulation the latency remained elevated at 99.0 ± 4.39 and 86.4 ± 8.23 % of the MPI, respectively. Thereafter the inhibition remained at 20-30 % of the MPI for up to one hour.

Unilateral stimulation of forelimb non-meridian points. Stimulation of forelimb muscle (n = 10), avoiding the meridian points, inhibited tail withdrawal by 44.4 ± 9.29 and 45.4 ± 9.68 % of the MPI, respectively, at 10 and 20 min after the onset of the stimulation (Fig. 4b). Two way ANOVA and paired t-tests showed that the magnitude of the evoked antinociception at this remote site was significantly different ($p < 0.01$) from that evoked by stimulation of meridian points. No post-stimulation effect was produced after the end of stimulation.

4) Effect of stimulation of the forelimb on hindlimb withdrawal latency

Unilateral stimulation of forelimb meridian points only slightly but significantly ($p < 0.01$) inhibited the withdrawal of the ipsilateral (n = 6; Fig. 5a) or contralateral (n = 6; Fig. 5b) hindlimb. In the ipsilateral hindlimb this inhibition remained significant ($p < 0.05$) at 5 min after the end of the stimulation. In the contralateral hindlimb, a slight post-stimulation inhibition

was produced and lasted up to 15 minutes after the end of stimulation ($0.01 > p < 0.05$). The evoked inhibition ipsilateral and contralateral to the stimulated forelimb were not different from each other.

4. DISCUSSION

Persistent inhibition of nociceptive mechanisms

The present study shows that prolonged, low frequency activation of high threshold, primary afferents can activate inhibitory mechanisms in the central nervous system (CNS) which attenuate the activity of nociceptive pathways mediating the tail withdrawal reflex. This inhibition can be elicited by stimulation of local or more remote peripheral sites. Furthermore, the evoked response appears to have two components: stimulation of meridian points elicited a post-stimulation inhibition, termed the persistent effect, while stimulation of meridian or non-meridian points evoked inhibition during the stimulation, termed the brief effect. The brief effect appears susceptible to spatial summation because this effect was significantly greater with bilateral than with unilateral stimulation.

Other animal studies have demonstrated a long-lasting inhibition of nociceptive pathways resulting from activation of high threshold sensory afferents. For example, Chung et al. demonstrated that prolonged conditioning stimulation of peripheral nerve at an intensity sufficient to recruit C fibers elicits a post-stimulation inhibition of spinothalamic tract cell responses to C fiber inputs evoked by electrical or noxious cutaneous thermal stimulation in decerebrated and spinalized monkeys[11]. In decerebrated and spinal transected cats[10] and rabbits[13,43], prolonged

recruitment of C fibers inhibits nociceptive reflexes for up to one hour after the end of stimulation. Prolonged, low frequency electrical activation of Aδ and possibly C fibers evokes a long-lasting inhibition of the nociceptive jaw opening reflex[23]. In humans, long-lasting stimulation evoked analgesia is also achieved by intense stimulation. For example, high intensity, low frequency stimulation is reported to produce long-lasting pain relief[1,2,14,15,29] suggested to be mediated by activation of high threshold sensory afferents[1,2,15,29].

Thus, expression of a post-stimulation inhibition appears to be dependent on activation of high threshold fibers. Aß fiber activation during a conditioning stimulus only mildly inhibits the nociceptive jaw opening reflex[23] and spinothalamic tract cell responses to C fiber inputs and no post-stimulation effect is seen in either case[12,17], while recruitment of Aδ fibers inhibits background and evoked activity in spinothalamic tract neurons[12,17] and recruitment of Aδ[12] and C fibers[12,17] elicits a post-stimulation inhibition.

The duration of this post-stimulation inhibition appears to be dependent on the duration of the conditioning stimulation. For example, short periods of high intensity, low frequency stimulation of peripheral nerve elicit short lasting effects[12,19], while prolonged periods evoke long-lasting inhibition of primate spinothalamic tract cells[11] and produce prolonged inhibition of the flexion reflex in the cat[10]. These data may be interpreted to suggest that central mechanisms involved in the persistent inhibition require central summation activated by prolonged input from high threshold afferents. Support for this suggestion comes from studies showing the magnitude and the duration of the inhibition of dorsal horn units to C fiber input evoked by noxious visceral or cutaneous stimulation is dependent on the duration and number of preceding conditioning stimulations[32,33]. Thus, on the basis of our own results and those of others, we suggest that

prolonged stimulation of high threshold afferents from specific sites in the periphery provokes a functional plastic change in inhibitory mechanisms in the CNS which is expressed as a persistent inhibition of nociceptive pathways mediating the tail withdrawal reflex lasting after activation of primary afferents has ended.

Extrasegmental inhibitory controls

Brief application of noxious or intense cutaneous stimuli has been shown to inhibit nociception in spinal segments remote from the site of the conditioning stimulation in animals[17,26,31] and in man[41,42,48,49]. Our data are consistent with this, in that stimulation of meridian points in the forelimb and hindlimb produced a persistent inhibition of the tail withdrawal reflex. Yet innocuous cutaneous stimulation[17,26] or stimulation of peripheral nerves at intensities which recruit only Aα or Aß fibers[17] produces little or no extrasegmental antinociception.

To elicit reliable pain relief in humans it is generally accepted that high intensity stimulation is required[1,2,14,15,28,29]. For example, noxious cutaneous stimulation[41,42,48,49], intense electrical stimulation applied to acupuncture points[1,2,15,28,29] or high intensity auricular stimulation[24,27,34,35] all elicit a reliable, general analgesia. It is important that some studies suggest that analgesic or antinociceptive effects produced by high intensity electroacupuncture-like stimulation is restricted segmentally. For example, stimulation of the cheeks was reported to be effective in increasing experimental tooth pain threshold but stimulation of the *hoku* meridian points in the hands produced little effect[1,2]. However, stimulation of the hand meridian points appeared to be important in prolonging the post-stimulation analgesia once elicited by

stimulation of the cheek[1,2], suggesting an extrasegmental component to the evoked increase in pain threshold. The majority of studies, however, support the notion that high intensity stimulation at a level just tolerable to the patient is what is required to evoke a reliable and extrasegmental effect. This is clearly illustrated by evidence demonstrating that high intensity, low frequency auricular stimulation at or above pain threshold increases pain threshold extrasegmentally[24,27,34,35] while low intensity, low frequency stimulation is ineffective[21].

Meridian points

In our study, the chosen meridian points lie close to joints and bone while non-meridian points are deep in muscle tissue. There is anatomical evidence to suggest that there are differences in the nociceptors arising from deep muscle vs. joint, bone, fascia and skin. It has been reported that there are fewer Aδ and C-fibers in nerves innervating muscle than joints. In addition, nociceptive inputs arising from deep skeletal muscle appear to be under tonic presynaptic inhibition[18,51]. Dorsal horn neurons receiving exclusive inputs from deep muscle nociceptors are rare in adult cats[18] and rats[51], yet in the same studies, dorsal horn cells with nociceptive inputs from the skin and from non-skeletal muscle structures from around the popliteal fossa were found[18,51]. Thus, we propose that the attenuated brief inhibition of the tail withdrawal reflex and lack of a post-stimulation effect upon stimulation of non-meridian points may be a consequence of reduced high threshold input from deep skeletal muscle and/or decreased efficacy in synaptic transmission at the central synapse formed by afferent inputs arising from deep muscle. On the other hand, intense stimulation of meridian points may activate a greater number of high threshold inputs resulting in inhibition that is greater in magnitude and

longer in duration.

Differential inhibition of nociceptive mechanisms

A second type of observation from this study is that inhibitory mechanisms differentially modulate different nociceptive pathways. Unlike the effects on tail withdrawal, the limb withdrawal reflexes were only slightly depressed. This differential effect may be a consequence of different neuronal circuitry mediating the tail and limb nociceptive reflexes and therefore may be under different control mechanisms. Intense stimulation of meridian points only moderately inhibited the limb withdrawal reflexes during the stimulation and no post-stimulation effect was evoked. While we entertain the possibility that the mechanisms underlying inhibition of nociceptive pathways in our experimental paradigm may be similar to those described for DNIC[25,26] these mechanisms do not fully explain the results obtained here. The antinociceptive effect elicited by DNIC was initially considered to be exclusive for sensory cells in the dorsal horn receiving both nociceptive and non-nociceptive inputs[26]. However, noxious stimuli can produce extrasegmental inhibition of dorsal horn cells receiving convergent sensory information and in addition inhibit cells receiving nociceptive input only[17,31-33,39]. Furthermore, not all convergent dorsal horn cells are subject to this inhibition[17,31,39,53]. If the neurons which mediate the limb withdrawal reflex[50] are influenced to a lesser degree by inhibitory mechanisms, the cutaneous nociceptive inputs from the hindpaw may still express their sensory message and elicit a limb withdrawal reflex. Yet, these same inhibitory mechanisms are effective in dampening the activity of other nociceptive neurons, perhaps those involved in the transmission of sensory information pertaining to noxious stimuli to higher brain structures. The question still remains

as to whether cutaneous nociceptive inputs arising from the tail are under different inhibitory controls than cutaneous nociceptors arising from the limbs.

Our data suggest that the central reflex pathways mediating tail withdrawal are modulated differently than those mediating limb withdrawal. In support of our evidence a recent study has demonstrated a similar differential effect elicited on the withdrawal latencies of the tail and hindlimb[30] after application of noxious stimulation. Thermal stimulation applied to the hindpaw increased the tail withdrawal latency. However, noxious thermal stimulation of one hindpaw or the tail had little effect on or produced facilitation of the withdrawal of the unconditioned hindlimb, respectively[30]. Yet noxious thermal or mechanical stimulation applied extrasegmentally inhibited the activity of nociceptive neurons in lumbar regions[30]. It was suggested that the application of the noxious stimuli evoke supraspinal mediated inhibition of nociceptive neurons and facilitation of neurons mediating the polysynaptic withdrawal reflex of the limbs. Under these conditions transmission of nociception to the brain is blocked but the activity of nociceptive pathways mediating withdrawal may be enhanced to facilitate movement of limbs away from the noxious stimulation. Therefore, the circuitry mediating the limb withdrawal may be under different control mechanisms than that mediating tail withdrawal.

In light of the data and arguments presented, we conclude that high intensity, low frequency stimulation of meridian points differentially inhibits different nociceptive reflexes (i.e. tail vs. limb) and that this differential control may be due to different neuronal circuitry and different control mechanisms arising from the central nervous system (i.e. supraspinal and spinal influences).

Summary and Conclusions

High intensity, low frequency stimulation of meridian points of the forelimb and hindlimb elicited a long-lasting, extrasegmental inhibition of the tail but not the limb nociceptive withdrawal reflexes. This differential inhibition may be a consequence of differences in neuronal circuitry, in modulatory control mechanisms intrinsic to the CNS or on neuronal inputs mediating the withdrawal reflexes.

The persistent nature of the evoked inhibition appears to be dependent on the stimulation of meridian points because stimulation of sites outside meridian points evokes a brief inhibition only. Lack of a post-stimulation effect may be a consequence of qualitative and/or quantitative differences in high threshold inputs arising from meridian vs. non-meridian points.

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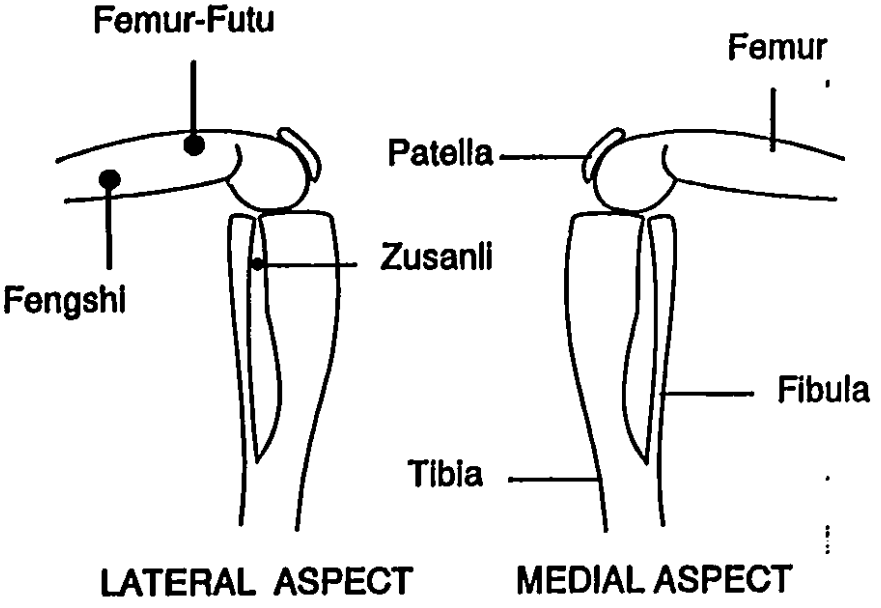
Fig. 1. Schematic illustration of hindlimb and forelimb meridian points stimulated in the rat. Left shows hindlimb meridian points stimulated in relation to bony structures. These points were used in previous publications from other laboratories[3-5,37,38,44]. Right shows forelimb meridian points stimulated in relation to bony structures. These points were also used in previous publications from other laboratories[1,2,5,9,37,44].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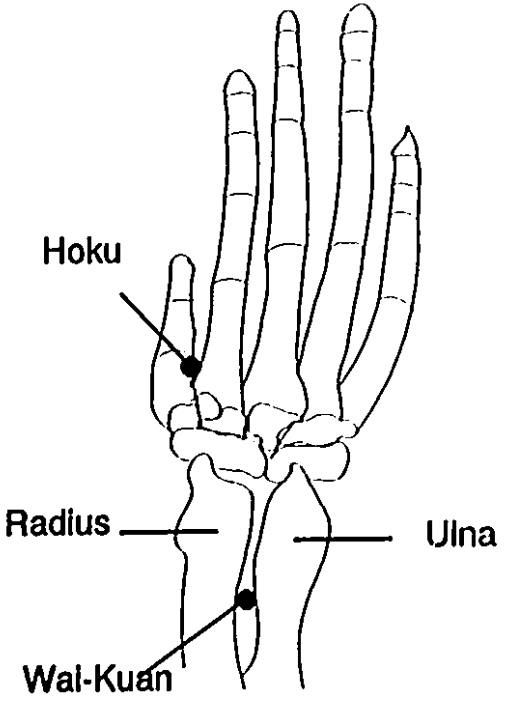
Hoku
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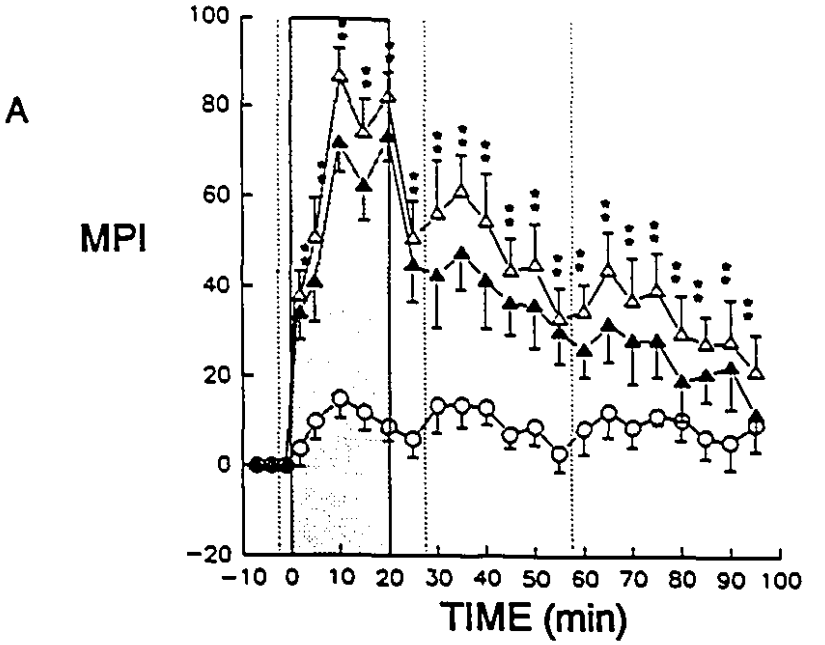
Wai-Kuan

Fig. 2. Effect on tail withdrawal latency of electrical stimulation of hindlimb meridian points. Stimulation parameters are 2 ms pulses at 4 Hz for 20 min (duration of the stimulation indicated by hatched area) at 20 X threshold. Dotted lines indicate times of administration of supplemental doses of anesthetic. a) Unilateral stimulation of the hindlimb (n = 9). b) Bilateral stimulation of the hindlimb (n = 10). c) Stimulation of non-meridian points of the hindlimb (n = 7). The control group for a,b and c had needles placed in meridian points but received no stimulation; unilateral (n = 9). △, stimulated; ○, needles only; ▲, difference between the two groups. * $p < 0.05$; ** $p < 0.01$.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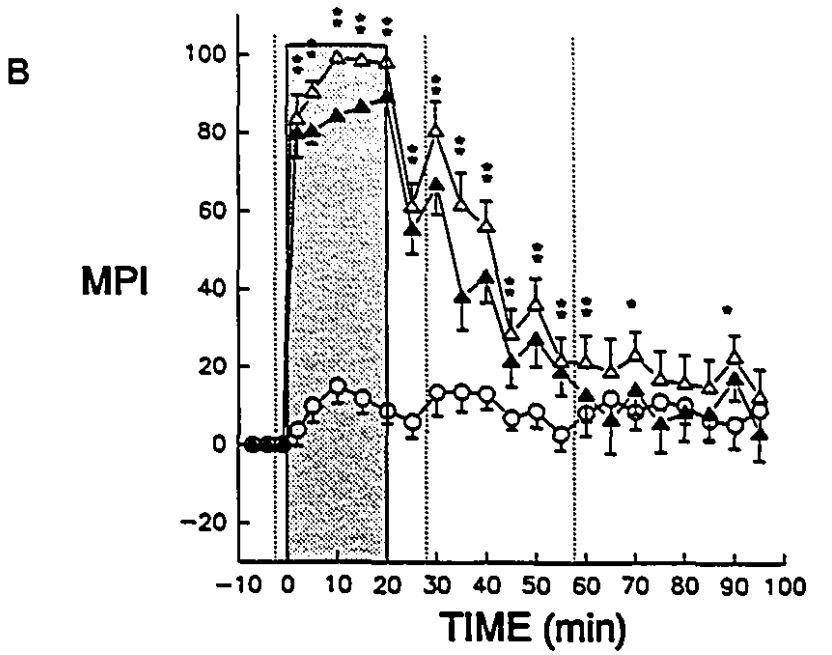
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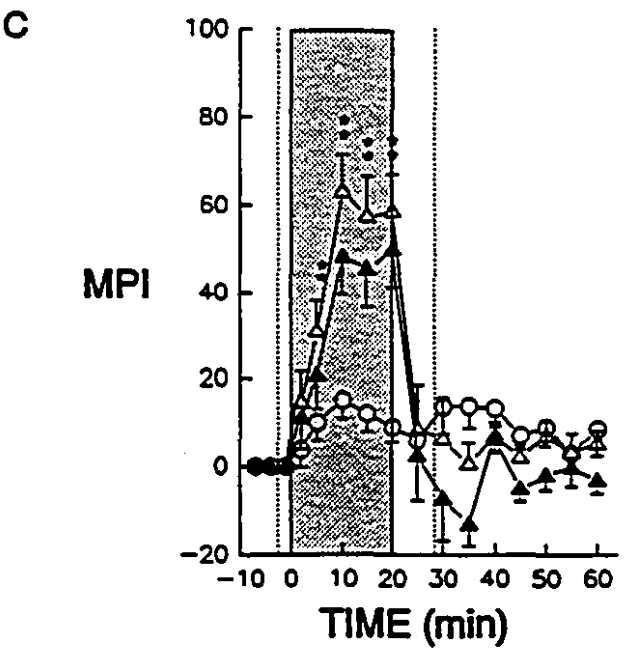
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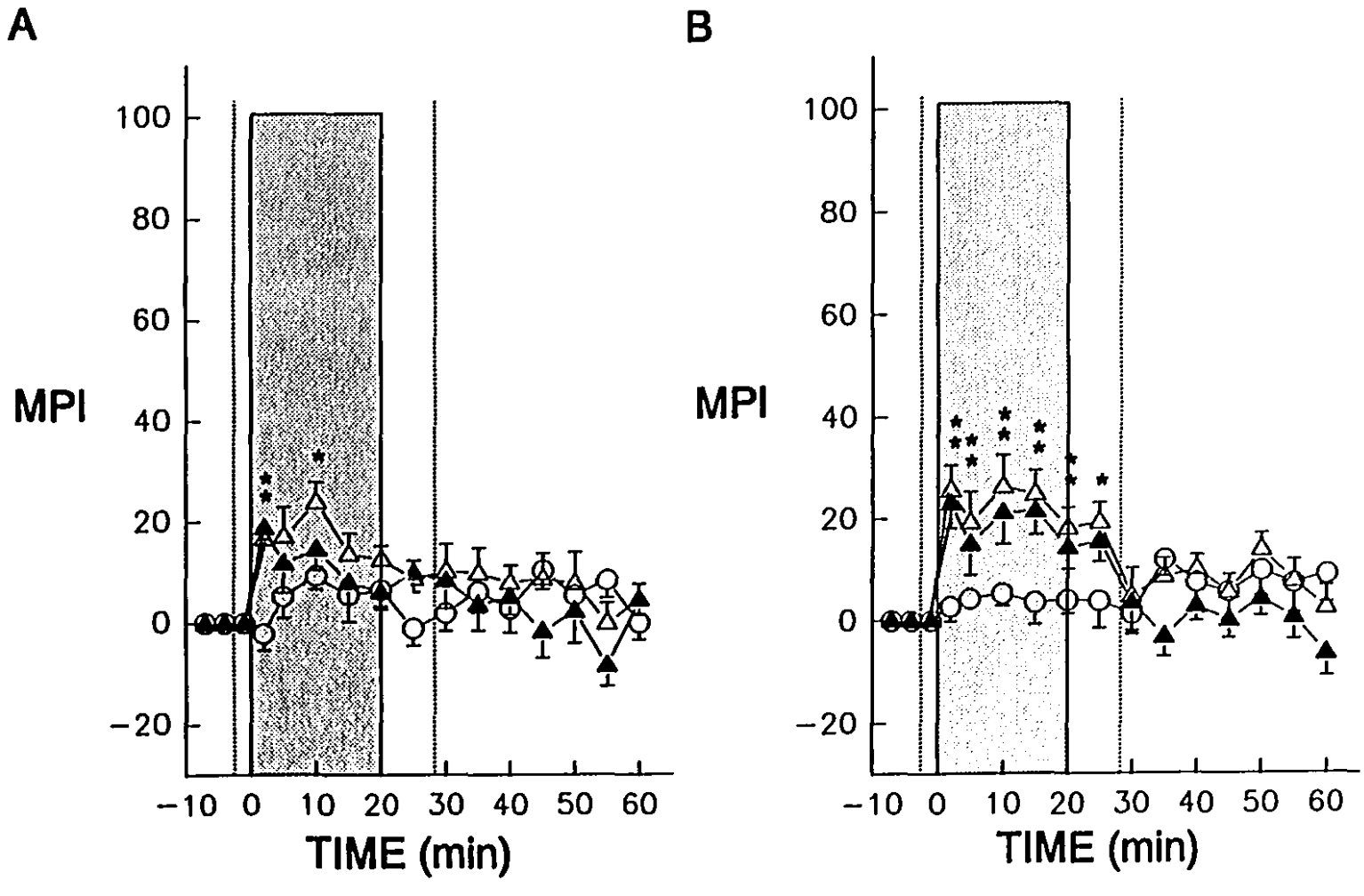
TIME (min)

Fig. 3. Effect of stimulation of hindlimb meridian points on withdrawal latency of the a) ipsilateral (n = 6) and b) contralateral forelimb (n = 6). The control group for a and b had needles placed in hindlimb meridian points but received no stimulation (n = 10). ▵, stimulated; ○, needles only; ▲, difference between the two groups. * $p < 0.05$; ** $p < 0.01$.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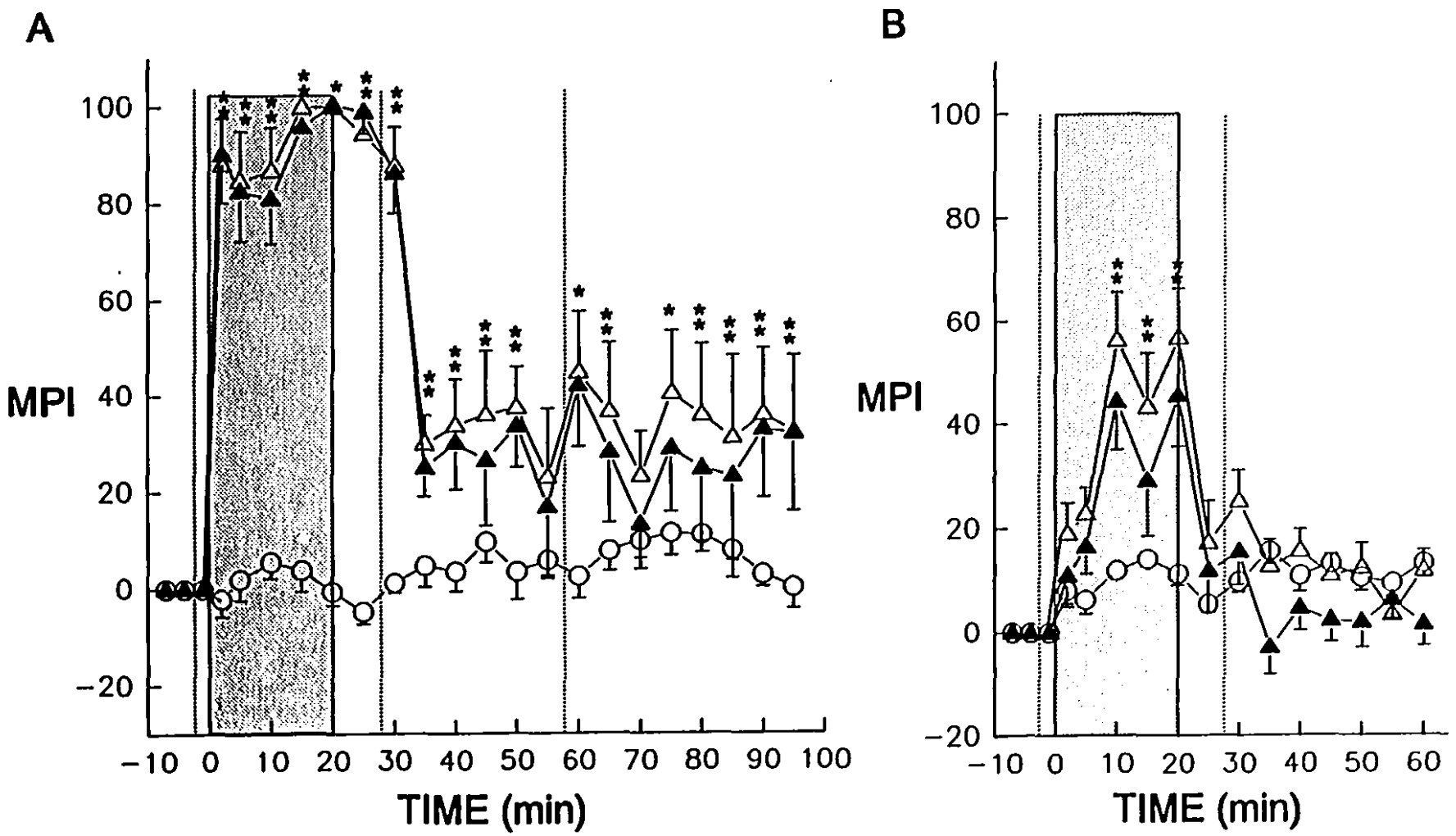
TIME (min)
TIME (min)

Fig. 4. Effect on tail withdrawal latency of electrical unilateral stimulation of a) meridian (n= 6) and b) non-meridian points (n = 10) of the forelimb. The control group had needles placed unilaterally into forelimb meridian points (n = 10). ▵, stimulated; ○, needles only; ▲, difference between the two groups. * $p < 0.05$; ** $p < 0.01$.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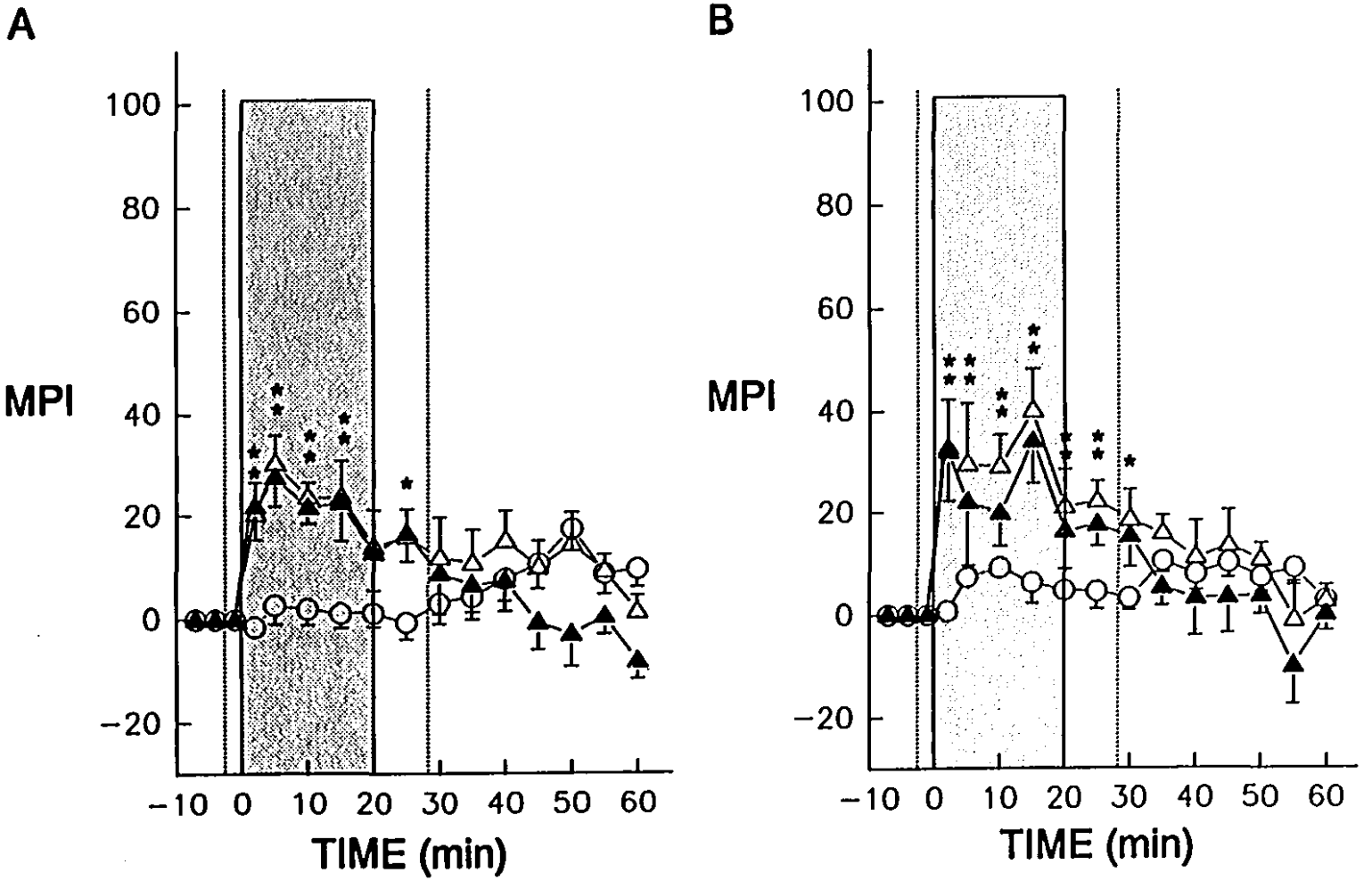
TIME (min)
TIME (min)

Fig. 5.Effect of stimulation of forelimb meridian points on the withdrawal latency of the a) ipsilateral (n = 6) an b) contralateral hindlimb (n = 6). The control group for a and b had needles placed unilaterally in forelimb meridian points but received no stimulation (n = 10). ▵, stimulated; ○, needles only; ▲, difference between the two groups. * $p < 0.05$; ** $p < 0.01$.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TIME (min)
TIME (min)

CHAPTER FOUR

NMDA RECEPTOR INVOLVEMENT IN SPINAL INHIBITORY CONTROLS OF NOCICEPTION IN THE RAT

Low frequency electrical stimulation of high threshold sensory afferents elicits a prolonged inhibition of nociceptive mechanisms in the lightly anaesthetized rat. The present study was done to determine the role of NMDA receptor activation in mediation of this inhibition. The latency of the tail withdrawal from a noxious thermal test stimulus to the tip of the tail was taken as an indication of the excitability of nociceptive pathways. Conditioning electrical stimulation at 20 times threshold for muscle twitch with 2 ms pulses at 4 Hz for 20 min applied to previously defined meridian points in the hindlimb evoked a 70 % inhibition of the withdrawal reflex in rats which received an i.th. injection of CSF (n = 11) compared to the unstimulated controls (n = 10). This inhibition lasted longer than one hour after the end of the conditioning stimulus. The competitive NMDA receptor antagonist 5-amino-2-phosphonovaleric acid (APV) blocked the inhibition during the stimulus and during the post-stimulation period. It is concluded that activation of NMDA receptors is critical for the expression of the long-term plastic changes in the central nervous system which resulted in the persistent inhibition of the nociceptive tail withdrawal reflex.

Key words: nociception, antinociception, pain, analgesia, spinal reflex, acupuncture, NMDA, antagonists , APV

Introduction

Excitatory amino acids, glutamate and aspartate, released from primary afferents are implicated in synaptic transmission of sensory information in the spinal cord. In the dorsal horn, they are contained in the terminals of small diameter primary afferents [1] and are released by stimulation of these afferents *in vivo* [2]. Glutamate binds to N-methyl-D-aspartate (NMDA) receptors throughout the rat dorsal horn [3]. *In vivo*, NMDA receptor antagonists block dorsal horn neurone responses to glutamate and attenuate inputs from high threshold afferents evoked electrically or by noxious cutaneous stimuli [4]. Intrathecal (i.th.) administration of NMDA into the rat lumbar spinal cord produces autotomy [5] and thermal hyperalgesia [6,7], and the NMDA receptor antagonist, 2-Amino-5-phosphonovaleric acid (APV), blocks thermal hyperalgesia evoked by noxious cutaneous input [8].

Evidence also indicates that activation of NMDA receptors triggers mechanisms inhibitory to nociception [7]. Recently, we have found that intense electrical stimulation of the hindlimb meridian points in the rat [9-11] provokes a long-lasting inhibition of the thermally elicited tail withdrawal reflex [12,13]. Therefore, our objective here was to determine if NMDA receptors are involved in the mediation of this long-lasting inhibition. APV was used as the NMDA receptor antagonist because it blocks dorsal horn neuron responses to iontophoretic application of NMDA [4,14].

Materials and Methods

In all cases, the guidelines described in The Care and Use of Experimental Animals, of the Canadian Council of Animal Care, Vols. I and II, Second Edition, were strictly followed.

The experimental protocols were also reviewed and approved by the McGill University Animal Care Committee. Male Sprague Dawley rats (300-400 g) were anaesthetized with chloral hydrate (300 mg/kg, i.p.; Fisher Scientific) and implanted with intrathecal catheters (Intramedic PE-10) to the 6th lumbar vertebral level. Patency of the catheter was determined by injection of 20 μl of 2% lidocaine HCl (Abbott) 1-2 days before experimentation; only animals showing reversible sensory and motor deficits after injection of lidocaine were used. For the experiment, each rat was lightly anaesthetized with an i.p. injection of a freshly prepared mixture of Na pentobarbital (20 mg/kg, Abbott Laboratories Ltd., Montreal) and chloral hydrate (120 mg/kg, Fisher Scientific, Montreal) in 50% propylene glycol (Baker Chemical Co., Phillipsburg N.J., USA) and 30% physiological saline (0.9% NaCl). The level of anaesthesia was sufficient to prevent any overt sign of discomfort to the rat during experimentation, yet stable tail withdrawal latencies were obtained. Before testing was started, the animals were allowed to stabilize for 30 min; then three baseline readings of the tail withdrawal latency were taken at 3 min intervals. Stimulation of meridian points $^{9\text{-}11}$ was started 1 min after the last baseline reading and withdrawal latency was recorded at 2 and 5 min after the onset of stimulation, and at 5 min intervals for up to 95 min. Results are expressed as the net effect, obtained by subtracting the mean MPI at each time point in the control group from the respective mean MPI in the test group, to eliminate the influence of supplemental doses of anesthetic. All values are expressed as the mean $\pm$ standard error of the mean. Data from treated rats which received electrical stimulation and control animals which received no stimulation were analyzed using two way analysis of variance. Stimulation vs. no stimulation or drug treatment vs. vehicle was taken as the between subject factor and time was taken as the within subject factor. Tukey's Wholly

Significant Difference Test was used to make post hoc comparisons between means.

Results

Effect of intrathecal injection of CSF on stimulation-evoked inhibition of the withdrawal reflex. Injection of 20 μl of CSF (n = 11) had no effect on the evoked inhibition (Fig. 1). The tail withdrawal reflex was inhibited to 70.9 ± 8.03 and 65.7 ± 7.26 % of the MPI, respectively, at 10 and 20 min after the onset of stimulation. A prolonged post-stimulation effect lasting greater than one hour was evoked. The maximum inhibition of this post-stimulation effect was 57.1 ± 9.63 % of the MPI at 10 min after the stimulation was turned off. This effect was statistically different ($p < 0.01$) when compared to the unstimulated group (n = 10) receiving 20 μl CSF.

Effect of intrathecal injection of APV on stimulation-evoked inhibition of the tail withdrawal reflex. Intrathecal injection of 2 nmoles of APV (n = 6) significantly ($p < 0.01$) blocked the evoked inhibition both during and after the stimulation (Fig. 2) when compared to the stimulated control group receiving an intrathecal injection of CSF. During the stimulation the tail withdrawal was depressed to less than 20 % of the MPI; no post-stimulation effect was produced. The 10 and 20 min readings after the onset of stimulation were significantly different ($p < 0.01$) from the unstimulated control group also receiving an intrathecal injection of 20 μl of CSF (n = 10).

Discussion

These data indicate NMDA receptor involvement in the inhibition of thermally elicited

tail withdrawal evoked by intense, low frequency conditioning electrical stimulation of peripheral sites corresponding to meridian points [9-11]. As we have found that the inhibition during the stimulation and the persistent post-stimulation effect constitute different responses [13], it is significant that both were blocked by intrathecal administration of APV.

The involvement of NMDA receptors in these responses is consistent with evidence of their involvement in the formalin-induced [15,16] and neuropathic [17,18] pain. Interestingly, at the cellular level NMDA receptors appear to be involved preferentially in the late component of the nociceptive response of dorsal horn neurons to noxious stimulation of the peripheral receptive field [4]. At a behavioral level, NMDA receptors do not appear to be involved in short term transmission of nociceptive information because NMDA receptor antagonists do not affect the tail withdrawal reflex [8] or limb withdrawal reflex [17] initiated by acute noxious stimuli. By extrapolation, then, the mechanisms underlying the antinociceptive responses in this study may be related to relatively long term changes in central nervous function.

The stimulation-induced inhibition may have been brought about by spinal and/or supraspinal mechanisms. A schematic representation of the spinal and supraspinal inhibitory mechanisms elicited by activation of the NMDA receptor is illustrated in Figure 3. In support of a spinal site, conditioning stimulation similar to that used here provokes inhibition of the tail withdrawal reflex in spinal rats [12], physiologically-induced inhibition of nociceptive mechanisms has been elicited in intact or spinal rats [19,20] and intrathecal administration of NMDA produces inhibition of a thermally elicited tail withdrawal reflex in both intact and chronically spinal rats [7]. It is also possible that the NMDA receptor step is at the spinal level in a spinal-supraspinal-spinal pathway. Extrasegmental antinociception produced by Aδ and C fiber activation is

mediated via an ascending spinoreticular tract [21] and descending fibers in the dorsolateral funiculi [22]. Intrathecal administration of NMDA has been reported to potentiate morphine-elicited antinociception in intact but not acute spinal rats [23].

Conclusion

Thus, prolonged intense peripheral electrical stimulation of meridian points evokes inhibition of the tail withdrawal reflex during the stimulation and a long-lasting, post-stimulation inhibition. This inhibition infers that activation of afferent inputs provokes a persistent decrease in excitability of nociceptive pathways mediating the tail withdrawal reflex. This antinociceptive effect appears to be dependent on the activation of the NMDA receptor complex. This may be the mechanism occurring in the spinal cord by which activation of small diameter fibers provoke diffuse antinociceptive effects [20,24,25].

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Figure 1. Stimulation-evoked inhibition of the tail withdrawal reflex in lightly anaesthetized rats pretreated with an intrathecal injection of 20 μl of CSF to the lower lumbar level, 1.5 min prior to stimulation. The tail withdrawal latency, used to monitor nociception, was expressed as a percent of the maximum possible inhibition (MPI). Baseline reaction time was set at 4-6 s. Trials were terminated automatically if the withdrawal latency did not occur within 12 s, the cut-off time. The hatched area indicates the duration of the train of stimulation. Vertical dotted lines indicate times of administration of supplemental doses of anesthetic. Two pairs of stainless steel insect pins were inserted into meridian points, *fengshi* (GB-31), *femur-futu* (ST-32) and *zusanli* (ST-36). These points were selected on the basis of what has been published in the literature [9-11,26-28]. Needles were connected to coupled Grass stimulators (SD9 and SD5) which passed a train of monophasic square wave pulses. The standard parameters were 2 ms pulses at 4 Hz for a duration of 20 min and applied at 20 times the threshold to produce muscle contraction (20 mA -30 mA). ▵, stimulated group (n = 8); ○, unstimulated control (n = 12); ▲, difference between the two groups. * $p < 0.05$; ** $p < 0.01$.

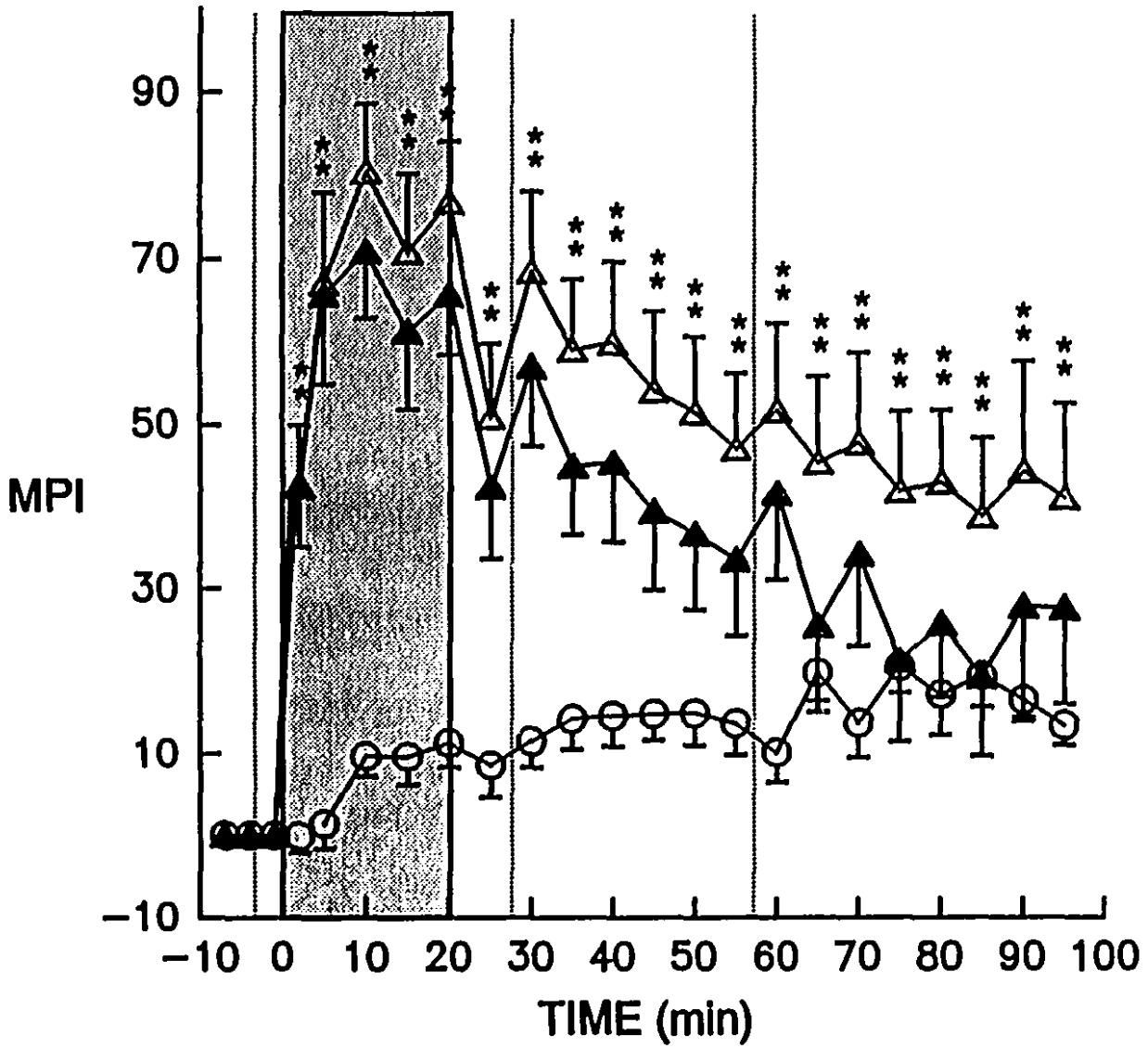
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Figure 2. Block of the stimulation-evoked inhibition of the tail withdrawal reflex by intrathecal injection of 2 nmoles of APV to the lower lumbar level, 1.5 min prior to stimulation. APV (5-amino-2-phosphonovaleric acid, Sigma, St. Louis, USA) was dissolved in artificial CSF and given at a dose of 2 nmol, a dose shown to block facilitation of the nociceptive tail withdrawal reflex by a noxious cutaneous input [8]. ▵, stimulated group (n = 6) ; ○, unstimulated control (n = 10); ▲, difference between the two groups. * $p < 0.05$; ** $p < 0.01$.

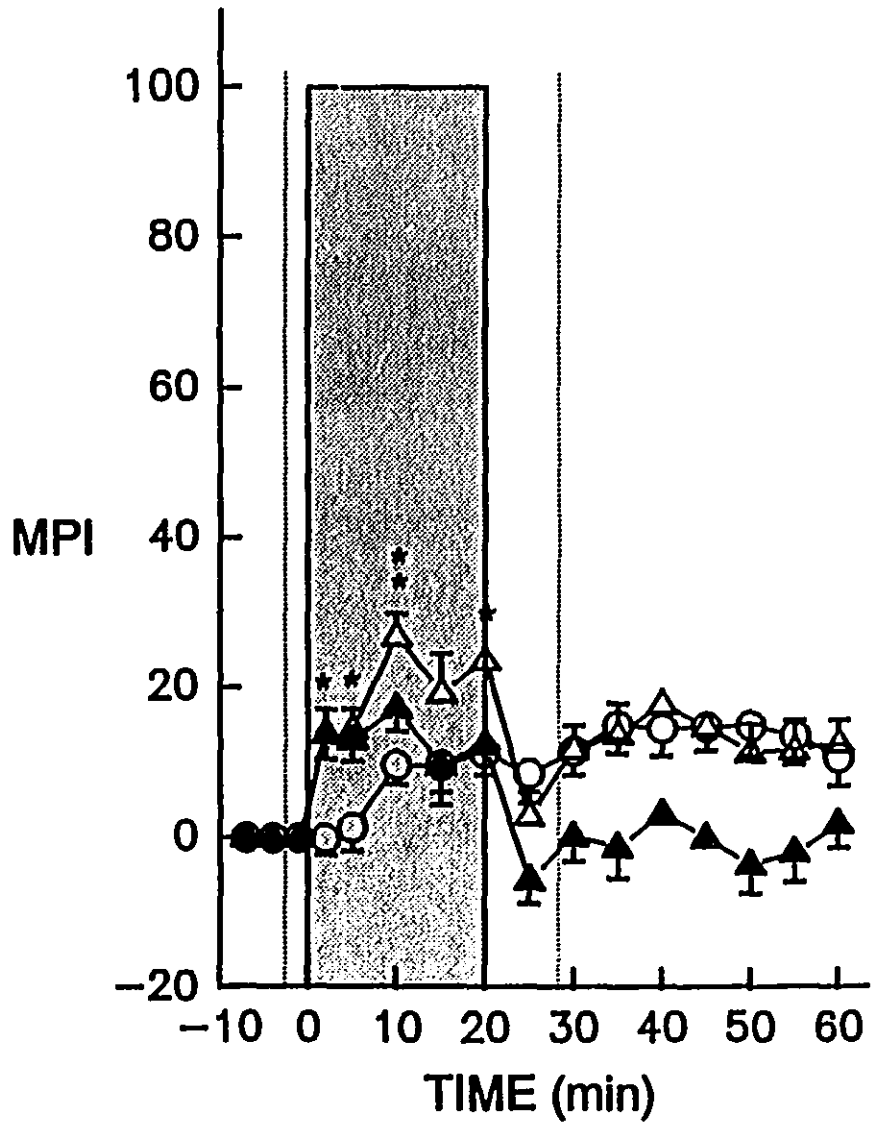
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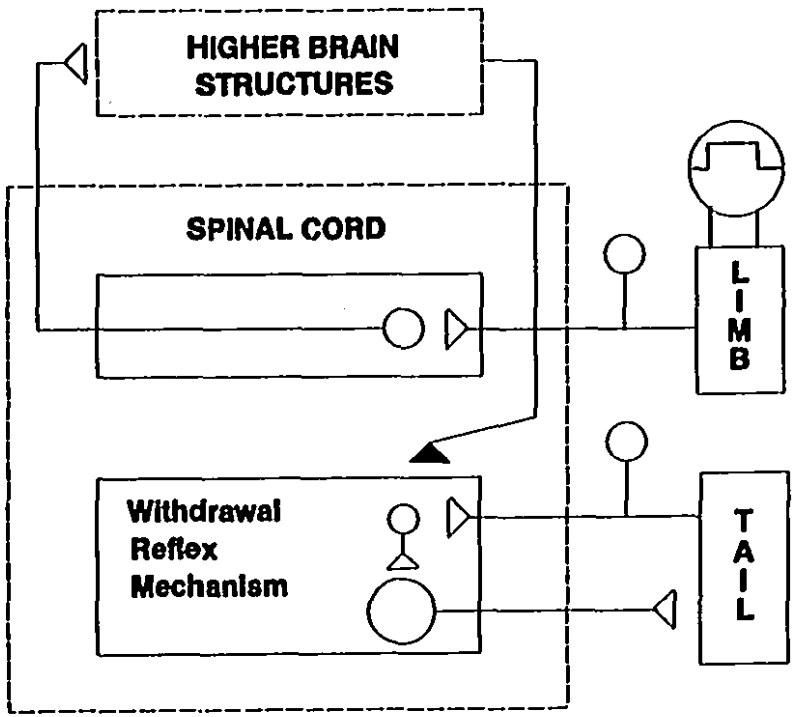
Figure 3. Schematic representation of possible neuronal circuitry mediating inhibition of the tail withdrawal reflex evoked by electrical activation of high threshold inputs arising from the limbs. Circuitry involving spino-supraspinal-spinal (**A**) and purely spinal (**B**) connections. Open and filled triangles represent excitatory and inhibitory inputs, respectively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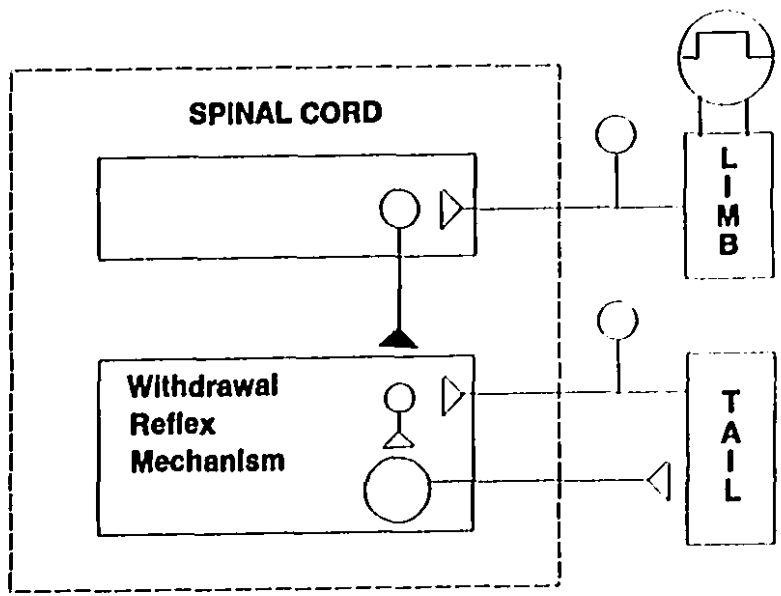
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CHAPTER FIVE

SPINAL μ-, δ- AND κ-OPIATE RECEPTORS MEDIATE INHIBITION OF THE NOCICEPTIVE TAIL WITHDRAWAL REFLEX EVOKED BY INTENSE PERIPHERAL ELECTRICAL STIMULATION IN THE RAT

Abstract

Opioids have been implicated in the antinociception evoked by prolonged activation of high threshold primary afferents. Our objective was to determine whether activation of spinal opiate receptors mediates inhibition of nociceptive processing provoked by intense peripheral electrical stimulation. Inhibition of the nociceptive tail withdrawal reflex infers decreased excitability of nociceptive pathways mediating this spinal reflex. Withdrawal was provoked by noxious thermal stimulation applied to the tip of the tail. Conditioning electrical stimulation was applied with 2 ms square pulses, at 4 Hz for 20 min at 20 times the threshold to previously defined meridian points in the hindlimb. Threshold was the minimum current required to elicit muscle twitch. Conditioning stimulation inhibited the withdrawal reflex during the stimulation and this inhibition persisted for greater than one hour after the end of the stimulation in anaesthetized intact rats (n = 8) and for up to 15 min in unanaesthetized spinal rats (n = 12). In control anaesthetized intact (n = 12) and control unanaesthetized spinal rats (n = 14) placement of electrodes with no stimulation produced no effect. In spinal rats, preadministration of naloxone (25 mg/kg; i.p.) completely blocked the evoked inhibition (n = 11). In intact animals both naloxone (n = 8) and ß-FNA (10 nmols, i.th.; n = 9) antagonized the inhibition during the stimulation and the post-stimulation inhibition by 50-60 %, respectively. Tipp[ψ] (10 nmols, i.th.; n = 7) and nor-BNI (10 nmols, i.th.; n = 13) attenuated the inhibition during the stimulation by 30 % and 56%, respectively. Both antagonists completely blocked the post-stimulation effect and even facilitated the withdrawal. The data suggest that all three types of opiate receptor differentially mediate the evoked antinociception. Spinal activation of μ-, κ- and, to a lesser extent, δ-opiate receptors

partly mediate the evoked inhibition during the stimulation, while the post-stimulation inhibition is dependent on activation of δ- and κ-receptors and to lesser extent μ-receptors.

Key words: nociception, antinociception, pain, analgesia, spinal reflex, opiate receptors, antagonists, naloxone, ß-FNA, TIPPψ, Nor-BNI, electrical stimulation, rat

1. Introduction

Although opiates have been used for the treatment of pain dating back to the times of ancient Greece and Rome (Benyhe, 1994), it was not until the early 70's that opiates such as morphine were implicated in the modulation of sensory transmission at the level of the spinal cord. Dostrovsky and Pomeranz (Dostrovsky and Pomeranz, 1973) showed that systemic application of morphine depressed the responses of dorsal horn neurons to the excitatory effects of iontophoretically applied excitatory amino acids. Furthermore, iontophoretic application of morphine depressed responses of nociceptive sensory dorsal horn neurons to noxious cutaneous thermal stimulation and this depressant effect was blocked by naloxone (Calvillo, Henry and Neuman, 1974). This was the first line of evidence implicating spinal opiate receptors in the modulation of nociception at the level of the first synapse. Since then, a number of different types of opiate receptor have been discovered in the central nervous system. The μ- (Besse, Lombard and Besson, 1991; Besse, Lombard and Besson, 1992; Gouarderes et al. 1991; Stevens et al. 1991), δ- (Besse, Lombard and Besson, 1991; Stevens et al. 1991; Besse, Lombard and Besson, 1992; Drower et al. 1993) and κ-opiate receptors (Besse et al. 1990; Besse, Lombard and Besson, 1991; Stevens et al. 1991; Sullivan and Dickenson, 1991) are found in the dorsal horn of the spinal cord and have been implicated in inhibitory mechanisms resulting in antinociception (Fields and Basbaum, 1989).

It is well documented that relatively brief noxious stimulation applied cutaneously can provoke inhibitory mechanisms in the CNS resulting in brief inhibition of responses of convergent dorsal horn neurons to C fiber input (Le Bars, Dickenson and Besson, 1979b; Le

Bars et al. 1981; Ness and Gebhart, 1991; Le Bars et al. 1992) as well as inhibition of nociceptive withdrawal reflexes in the rat (Ness and Gebhart, 1991; Morgan, Heinricher and Fields, 1994; Pitcher et al. 1995b). In addition, prolonged, intense stimulation of peripheral nerve or hindlimb meridian points produces a long-lasting inhibition of the jaw-opening reflex in the rat (Kawakita and Funakoshi, 1982). Furthermore, prolonged intense electrical stimulation applied to a peripheral nerve or applied cutaneously produces a long-lasting depression of the flexor reflex or of the sural-gastrocnemius reflex in the spinal and decerebrated cat (Chung et al. 1983) and in the spinal rabbit (Clarke, Ford and Taylor, 1989; Taylor et al. 1990). These antinociceptive effects evoked by either brief noxious cutaneous stimulation (Le Bars et al. 1981), or by prolonged, intense electrical stimulation of peripheral nerve (Chung et al. 1983) or cutaneous structures (Clarke, Ford and Taylor, 1989; Taylor et al. 1990) are antagonized by naloxone, suggesting that the evoked effect is mediated via activation of opiate receptors.

We have recently demonstrated that prolonged, intense low frequency electrical stimulation applied to specific sites in the hindlimb evokes a long-lasting inhibition of the thermally elicited tail withdrawal reflex both in intact and chronically spinalized rats (Romita et al. 1993). Systemic administration of high doses of naloxone attenuate the evoked inhibition during the stimulation and block the post-stimulation effect in the intact rat (Romita et al. 1992), while in the spinal rat this inhibition is blocked completely (Henry and Romita, 1993). The objective of the present study was to determine whether activation of spinal μ-, δ- and κ-opiate receptors are involved in the mediation of the stimulation-evoked inhibition of the tail withdrawal reflex. Preliminary data have appeared in abstract form (Romita and Henry, 1994).

2. Materials and methods

In all cases, the guidelines described in The Care and Use of Experimental Animals, of the Canadian Council of Animal Care, Vols. I and II, Second Edition, were strictly followed. The experimental protocols were also reviewed and approved by the McGill University Animal Care Committee.

2.1. Intrathecal injection

In some experiments intrathecal catheters were used to deliver drug or vehicle and therefore necessitated prior implantation of catheters using the following procedure. Male Sprague Dawley rats (300-400 g) were anaesthetized with chloral hydrate (300 mg/kg, i.p.; Fisher Scientific, Montreal). The muscle at the base of the skull was retracted until the occipital membrane was exposed. An incision was made through the membrane and the dura lying underneath. A polyethylene catheter (Intramedic PE-10; Fisher Scientific) was inserted through the incision at the atlanto-occipital junction and gently inserted under the dura until the subarachnoid tip of the catheter lay underneath the sixth lumbar vertebral. The outer portion of the catheter was fixed with dental cement to a stainless steel cranial screw embedded in the skull. The antibiotic, Tribrissen 24% (0.02 ml/100 g; Trimethoprim and Sulfodiazine) was injected subcutaneously 3 h prior to and 3 h after the surgery. Animals were left to recover for two to three weeks prior to testing provided no neurological deficit was apparent. Position and patency of the catheter were determined by intrathecal injection of 20 μl of a 2 % solution of

lidocaine HCl (Abbott Laboratories, Montreal) followed by 10 to 15 μl of artificial CSF (CSF; aqueous solution of 128.6 mM NaCl, 2.6 mM KCl, 2.0 mM $MgCl_2$ and 1.4 mM $CaCl_2$; phosphate buffered, ph 7.33) into the catheter one to two days prior to experimentation; this produced motor deficits such as dragging of the hindlimbs and sensory deficits manifested as lack of withdrawal of the hindlimb and lack of vocalization in response to a noxious pinch. Only those animals that showed reversible sensory and motor deficits after injection of the local anaesthetic were used in subsequent experiments.

In other experiments drugs were administered intrathecally by lumbar puncture at the level of the sixth lumbar vertebra.

2.2. Spinal transection

In some animals the spinal cord was transected to determine whether the effects observed in intact rats could be elicited after spinal section and whether these effects could be blocked using naloxone. Rats (300-400 g) were anaesthetized with chloral hydrate (300 mg/kg, i.p.; Fisher Scientific). Tribrissen was given as above. The spinal cord was exposed at the 6th and 7th thoracic segments. After making a slit in the dorsal part of the dura mater the cord was transected and aspirated by suction approximately 2 millimetres caudal and rostral to the level of transection. Gelfoam and/or bone wax was placed into the empty vertebral column to reduce bleeding and to seal the empty vertebral cavity. Spinalized rats were maintained on their regular diet and their bladders were voided 3 to 4 times daily for the first 8 days following surgery; after this time bladder function had returned in all animals. Animals were used experimentally

21 days after spinal transection. Animals were selected for study on the basis of the following criteria. All were healthy in general appearance. Normal eating and drinking were maintained and there was no long-term weight loss. Normal voiding and defecation were sustained after the 8 day period of maintenance. All animals showed normal grooming behaviour. All animals were mobile despite hindlimb paralysis and hindlimb pinch produced a withdrawal reaction. In fact, all animals thus selected exhibited a consistent baseline reaction time throughout the recovery period. Therefore, no animals were omitted for inconsistency of responding.

2.3. Tail withdrawal test

The tail withdrawal latency in the nociceptive withdrawal reflex was determined. To measure the withdrawal latency a portion of the tip of the tail was blackened to facilitate the absorption of heat. The blackened tip was positioned above a focused projector bulb which heated the skin surface and provoked the tail withdrawal reflex. Withdrawal of the tail exposed the light beam, thus activating a photodetector which stopped a timer measuring reaction time to 0.01 s (Isabel, Wright and Henry, 1981). The intensity of the bulb was set so that the baseline reaction time was 4-6 s. Trials were terminated automatically if the withdrawal latency did not occur within 12 s, the cut-off time.

At each sample time two readings were taken, separated by 40-50 s, at two different sites within the 2 cm blackened segment of the tail. Thus, the tail withdrawal latency was never measured twice from the same site within a three to five min interval. The average of the two readings was calculated and the value expressed as percent of the maximum possible inhibition

(MPI) according to the formula (Yaksh and Rudy, 1977)

$$MPI = \frac{(\text{POST-TREATMENT LATENCY} - \text{PRE-TREATMENT LATENCY})}{(\text{CUT-OFF TIME} - \text{PRE-TREATMENT LATENCY})} \times 100$$

To measure the withdrawal latency during stimulation, the stimulator was temporarily turned off just long enough for the reading to be taken; this was necessary because the stimulus was above the threshold to elicit a direct contraction of muscles.

2.4. Experimental protocol

At the beginning of the experiment each rat was lightly anaesthetized with an initial intraperitoneal injection of a freshly prepared mixture of sodium pentobarbital (20 mg/kg, Abbott Laboratories Ltd.) and chloral hydrate (120 mg/kg, Fisher Scientific) in 50% propylene glycol (Baker Chemical Co., Phillipsburg, N.J.) and 30% physiological saline (0.9% NaCl). To maintain a light state of anaesthesia throughout the full duration of the experiment, subsequent injections of the mixture were given. Thus, ⅓ of the initial dose of anaesthetic was given 35.5 min after the first injection; this second injection was timed to occur 1.5 min prior to the beginning of electrical stimulation. Thereafter, subsequent injections of ⅓, ¼ and 1/6 the initial dose were given at 30 min intervals. The level of anaesthesia was sufficient to prevent any overt sign of discomfort to the rat during experimentation, yet stable tail withdrawal latencies were obtained for greater than 1.5 hours after the second supplementary dose.

The lightly anaesthetized rat was placed in a plastic restrainer on the apparatus used to measure the withdrawal latency (Isabel, Wright and Henry, 1981), needles were inserted and threshold determined. Before testing was started the animals were allowed to stabilize for 30 min, then three baseline readings of the tail withdrawal latency were taken at 3 min intervals. Stimulation was then applied starting 1 min after the last baseline reading and tail withdrawal latency was recorded at 2 and 5 min after the onset of stimulation, and at 5 min intervals for up to 95 min.

In spinal transected rats experiments were performed 21 days after spinal transection. Unanaesthetized rats were placed in plastic restrainers so that only the tail and one hindlimb protruded. Animals were unanaesthetized because in pilot studies the anaesthetic produced inconsistent tail withdrawal latencies at the doses used in intact rats. The restrainer was covered with a black cloth to minimize visual stimuli. Rats were introduced and habituated to the restrainer for 60-90 min 1 or 2 days prior to experimentation. The protocol was otherwise followed as described above for the lightly anaesthetized preparation.

2.5. Placement of stimulation needles

Two pairs of stainless steel insect pins were inserted into meridian points, *fengshi* (GB-31), *femur-futu* (ST-32) and *zusanli* (ST-36). To stimulate *femur-futu* (ST-32) the cathode was placed along the medial side of the knee and the anode was inserted along the lateral side of the knee so that it lay across *zusanli* (ST-36). To stimulate *fengshi* the electrodes were placed under the femur midway between the hip and the knee. These meridian points were selected on the basis

of what has been published by other groups (Pomeranz and Cheng, 1979; Ulett, 1982; Pomeranz, 1987; Bing, Villanueva and Le Bars, 1990; Bing et al. 1991; Bing, Villanueva and Le Bars, 1991).

2.6. Parameters of electrical stimulation

Needles were connected to coupled Grass stimulators (SD9 and SD5) which passed a train of monophasic square wave pulses. The standard parameters were 2 ms pulses at 4 Hz for a duration of 20 min and applied at 20 times the threshold (20-30 mA). Threshold was the lowest intensity which just produced muscle contraction. Control groups were implanted with stimulating electrodes but received no electrical stimulation.

2.7. Drug preparation

The wide spectrum opiate antagonist, naloxone (Endo Laboratories, NJ; 25 mg/kg i.v.), was dissolved in physiological saline (0.9%) to yield 2.5 mg of antagonist/100 μl saline and administered via intraperitoneal injection 30 min prior to electrical stimulation in intact and spinal animals. The specific μ-opiate receptor antagonist ß-funaltrexamine (ß-FNA; Research Biochemicals International, Natic, MA) was dissolved in CSF to yield 10 nmoles of antagonist/10 μl of CSF. This solution was made immediately prior to administration, which occurred 24 hours prior to the experiment. H-Tyr-ticψ[CH_2NH]Phe-Phe-OH (TIPP[ψ]), a stable pseudopeptide δ-opiate receptor antagonist, was first dissolved in DMSO (dimethyl sulfoxide;

Fisher Scientific) and then diluted with artificial CSF. The final solution contained 10 nmoles of the antagonist/10μl of CSF and 7.4 % of DMSO. Nor-binaltorphimine (nor-BNI; Research Biochemicals International), a selective κ-antagonist, was dissolved in artificial CSF to yield 10 nmoles of the antagonist/10 μl of CSF. This solution was made immediately prior to the experiment.

2.8. Statistical analysis

Results are expressed either as the net mean increase in tail withdrawal latency expressed as MPI at each time point or as the net mean MPI calculated for the period of stimulation and for subsequent post-stimulation periods. The MPI was obtained by subtracting the mean MPI at each time point in the control group from the respective mean MPI in the test group, to eliminate the influence of supplemental doses of anaesthetic. The mean MPI for the period during the 20 min of stimulation (period 1) and for the three subsequent 25 minute periods following the end of stimulation (periods 2-4), was calculated by averaging the net MPI obtained from the five readings taken at each period. The mean MPI was expressed as a histogram. All values are expressed as the mean $\pm$ standard error of the mean (S.E.M.). Data from treated rats which received electrical stimulation and control animals which received no stimulation were analyzed using two way analysis of variance (ANOVA). Stimulation vs no stimulation or drug treated vs vehicle was taken as the between subject factor and time was taken as the within subject factor. Tukey's Wholly Significant Difference Test was used to make post hoc comparisons between means. One way repeated measures analysis of variance, pair-wise

multiple comparisons (Student-Newman-Keuls method) and paired Students *t*-test for single pair-wise comparisons were used to compare the net mean increase in withdrawal latency at periods 1-4, between treatment groups.

3. Results

3.1. Effect of electrical stimulation on tail withdrawal reflex in intact rats

In intact animals treated with an i.p injection of 0.9% NaCl ($n = 8$), electrical stimulation of hindlimb meridian points inhibited the withdrawal reflex by approximately 70 % of the MPI during the stimulation. After the stimulation ended this inhibition persisted for at least 1 hour. Two way ANOVA showed that this effect during the stimulation was significantly ($p \leq 0.01$) greater than the unstimulated control group ($n = 12$).

3.2. Effect of naloxone on response to electrical stimulation in intact rats

In contrast to vehicle-treated rats, the evoked inhibition in naloxone-treated rats ($n = 8$) reached a maximum of only 43.0 ± 9.99 % of the MPI during the stimulation period. A low-amplitude post-stimulation effect was observed which lasted only 15 min after the end of stimulation. Two-way ANOVA showed that both the inhibition during electrical stimulation and the persistent post-stimulation inhibition were greater than those in the unstimulated control group and less than those in the stimulated, saline-treated group ($p \leq 0.01$), respectively, by

two way analysis of variance. Figure 1 illustrates the data as histograms representing the net mean inhibition calculated for the period during the stimulation and for the following post-stimulation periods for both the vehicle and naloxone treated groups. Paired t-tests show that the attenuation produced by naloxone was significant for all periods ($0.05 \geq p \leq 0.01$). Naloxone attenuated the evoked response by 47.1 % and 41.6 % for periods 1 and 2, respectively. For periods 3 and 4 the inhibition of the effect was approximately 60 % of the control group. The % inhibition of the evoked response for each of the respective periods and by each of the opioid antagonists is summarized in Figure 7.

3.3. Effect of electrical stimulation in spinalized rats

In unanaesthetized rats, three weeks after spinal transection electrical stimulation produced a small but significant inhibition both during and after the end of stimulation ($n = 12$). The evoked inhibition reached a maximum value of 23.1 ± 4.81 and 22.0 ± 3.09 % of the MPI at 10 and 20 min after the onset of stimulation, respectively. For 15 min after the end of stimulation the inhibition remained slightly elevated at about 8 % of the MPI. Two-way ANOVA showed this effect to be statistically different ($p < 0.01$) from the unstimulated control group ($n = 14$).

3.4. Effect of naloxone on response to electrical stimulation in spinalized rats

Pretreatment with naloxone blocked the stimulation-evoked inhibition of the withdrawal

reflex (n = 11). The data are illustrated in Figure 2. A two-way ANOVA showed this antagonism to be significant ($p < 0.01$) when compared to the vehicle-treated rats (n = 12) during stimulation. Although the values of the mean inhibition for each time taken during the first 15 min after the end of stimulation were lower in the naloxone treated group than the those obtained from the vehicle-treated group, no difference was found when these two groups were compared statistically.

3.5. Effect of spinal administration of vehicle on the stimulation-evoked inhibition

Figure 3 illustrates the long-lasting inhibition of the tail withdrawal latency provoked by intense electrical stimulation in rats given vehicle intrathecally (n = 8). During the stimulation the maximum inhibition was 82.2 ± 4.17 % of the MPI. This inhibitory effect on the withdrawal reflex persisted for greater than one hour after the end of the stimulation. Two way ANOVA showed that the inhibition was significant ($p \leq 0.01$) throughout when compared to the unstimulated control group (n = 16). Both the test group and the unstimulated control group received spinal administration of CSF or CSF containing DMSO, by lumbar puncture. There was no difference in the evoked inhibition between groups treated with CSF or CSF-DMSO nor was there any difference between the two unstimulated control groups treated with vehicle. Therefore, the data were pooled. The ANOVA also failed to show any statistical difference in the evoked inhibition between rats in which vehicle was given either intraperitonealy, intrathecally or by lumbar puncture.

3.6. Effect of ß-FNA on stimulation-evoked inhibition

Spinal administration of ß-FNA 24 hours before testing attenuated the inhibition during stimulation and blocked the post-stimulation antinociception (n = 9). The withdrawal reflex was inhibited to a maximum of 48.8 ± 12.1 % of the MPI during the stimulation. Two-way ANOVA showed that this effect was significantly different ($p \leq 0.05$) when compared to the unstimulated control group (n = 8). No statistical difference was seen beyond stimulation between these two groups. By comparison, in rats treated with a spinal injection of CSF (n = 10), stimulation produced a maximum inhibition of 82.5 ± 6.25 % of the MPI during the stimulation which lasted beyond one hour after the end of stimulation. The evoked inhibition in this group of rats was statistically greater ($p \leq 0.01$) than the inhibition evoked in the ß-FNA treated group. Figure 4 illustrates the net mean inhibition elicited by stimulation for the periods 1 to 4 for both the vehicle and ß-FNA treated rats. Paired t-tests showed that the attenuation produced by the μ-receptor antagonist was significant for all periods ($p \leq 0.01$). ß-FNA produced a 47.5 % inhibition of the evoked response during period 1, an 85.2 % inhibition during period 2 and a 50 - 60 % inhibition of the evoked response during periods 3 and 4 (Figure 7).

3.7. Effect of TIPP[ψ] on stimulation-evoked inhibition

Spinal administration of TIPP[ψ] slightly attenuated the inhibition produced during the stimulation but completely blocked the post-stimulation effect (n = 7). During the stimulation

the withdrawal reflex was inhibited to a maximum of 55.97 ± 11.18 % of the MPI. This effect was statistically significant ($p \leq 0.01$) when compared to the unstimulated control group ($n = 16$) using two-way ANOVA. No significant difference for any of the post-stimulation values was seen between these two groups. The inhibition produced in rats treated with TIPP[ψ] was also found to be significantly less ($p \leq 0.01$) than that produced in the stimulated group receiving only vehicle. In the vehicle treated rats, the stimulation-evoked inhibition was about 82 % of the MPI during the stimulation and this effect persisted for more than one hour after the end of stimulation ($n = 8$) as shown in figure 3. Illustrated in figures 5 and 7 is the effect of TIPP[ψ] on the evoked response. The mean MPI during periods 1 and 2 was attenuated by 30.4% and 83.5%, respectively. During periods 3 and 4, TIPP[ψ] produced a reversal. The net mean MPI was reversed by approximately 125 % of the control. Paired t-tests showed the attenuation and reversal by TIPP[ψ] to be significant ($p \leq 0.01$).

3.8. Effect of Nor-BNI on stimulation-evoked inhibition

Spinal administration of nor-BNI also attenuated the evoked inhibition during the stimulation and blocked the post-stimulation effect ($n = 13$). The maximum inhibition was 38.38 ± 11.14 % of the MPI during the stimulation (Fig. 6). This response was found to be statistically different ($p < 0.01$) when compared to the vehicle treated unstimulated control group ($n = 16$) using two way ANOVA. The post-stimulation values were found not to be statistically different between these two groups. The mean inhibition at each time point was also found to be significantly less than the evoked inhibition in the stimulated vehicle group ($0.05 \geq p \leq 0.01$).

Figure 7 illustrates the net mean inhibition calculated for periods 1 to 4. The κ-opiate receptor antagonist decreased the mean inhibition for period 1 by approximately 56%. During period 2 the inhibition was completely blocked by 100 % and during periods 3 and 4 the treated group appeared to produce reversal (i.e. a net mean facilitation in the withdrawal reflex); responses during periods 3 and 4 were attenuated by 138% and 152 % of the control values. Paired t-tests demonstrated that the attenuation during the stimulation, and the block and reversal were statistically significant ($p < 0.01$).

4. Discussion

These data show that spinal μ-, δ- and κ-opiate receptors are implicated in the mediation of the decreased activity in nociceptive pathways provoked by prolonged intense peripheral electrical stimulation. In our paradigm this decrease was reflected by the inhibition of the thermally evoked tail withdrawal reflex which was strong during the conditioning stimulation and persisted for more than one hour after the end of the stimulation. Furthermore, it appears that the three opioid receptors differentially contribute to the evoked inhibition of this reflex. During the stimulation μ-, κ- and to a lesser extent δ-receptors mediate at least part of the inhibition, while δ-, κ- and to a lesser extent μ-receptors mediate the post-stimulation inhibition.

4.1. Naloxone antagonism implicates multiple opiate receptors in mediation of stimulation-evoked inhibition of the tail withdrawal reflex

A high dose of naloxone attenuated the evoked-inhibition in intact animals and completely blocked inhibition of the tail withdrawal in chronic spinal transected rats, suggesting that the evoked inhibition was mediated at least by activation of the μ- and probably by activation of multiple opiate receptors. *In vitro* studies show that naloxone is predominantly a μ-opioid receptor antagonist but at higher concentrations it can act as a wide spectrum antagonist at δ- and κ- receptors (Lord et al. 1977). Furthermore, in animal studies a high dose of naloxone given systemically has been used to block effects believed to be mediated via the multiple opiate receptors (Martin, 1967; Berkowitz, Finck and Ngai, 1977; Malick and Goldstein, 1978; Sawynok, Pinsky and LaBella, 1979). Although naloxone given at doses ranging from 0.1-10 mg/kg is reported to successfully block opioid mediated effects (Martin, 1967; Sawynok, Pinsky and LaBella, 1979), systemic doses in the range of 5 to 40 mg/kg were administered to block the inhibition of the tail withdrawal reflex evoked by nitrous oxide gas (Berkowitz, Finck and Ngai, 1977) or by intracerebroventricular (ICV) administration of substance P (Malick and Goldstein, 1978) in the rat. In both these studies lower doses of naloxone only produced partial block while higher doses were more effective, suggesting that in some paradigms higher doses of systemic naloxone are required for adequate opiate antagonism. In light of this evidence, we chose to give 25 mg/kg of naloxone to ensure adequate opiate block without compromising the tail withdrawal reflex; 30 mg/kg of naloxone given systemically does not affect the tail withdrawal latency (Berkowitz, Finck and Ngai, 1977). It is important to mention at this point that naloxone at the dose used in our experiment has no pharmacological interaction with benzodiazepines (Billingsley and Kubena, 1978) and/or GABA (Billingsley and Kubena, 1978; Dingledine, Iversen and Breuker, 1978) receptors. A systemic dose of 60 mg/kg of naloxone

was required to block the anticonflict activity of the benzodiazepine, chlorodiazepoxide (Billingsley and Kubena, 1978) in rats and greater than 100 mg/kg of naloxone was required to elicit convulsion activity in mice which is believed to be associated with GABAergic mechanisms (Dingledine, Iversen and Breuker, 1978). Therefore, antagonism of the stimulation-evoked inhibition of the tail withdrawal reflex by naloxone implicates activation of opiate receptors.

Naloxone blocked the evoked inhibition during the stimulation equally well as did the other three antagonists used in this study. However, naloxone did not affect the post-stimulation inhibition to the same extent as did some of the other antagonists. For example, the mean evoked inhibition during the post-stimulation periods were completely blocked and reversed by TIPP[ψ] or nor-BNI (vide infra). Therefore, even at a dose of 25 mg/kg, naloxone antagonism of the post-stimulation effect appears to be limited. This limitation may be related to the pharmacokinetic properties of naloxone when administered systemically. It has been reported that the highest concentration of labelled naloxone in the brain occurs at 25 min after subcutaneous administration. However, one hour after administration these levels are decreased to 25 % or less, suggesting that naloxone accesses the central nervous system rapidly but there is also a rapid disassociation from brain opiate receptors; the half-life of systemically administered naloxone was found to be 0.4 hours (Misra et al. 1976). Furthermore, δ- and κ-opiate receptors have 10 X and 30 X less affinity for naloxone binding, respectively, as compared to the μ-receptor (Chang, Hazum and Cuatrecasas, 1980). Therefore, at post-stimulation periods monitored over one hour after administration of naloxone, levels of the antagonist in the central nervous system (CNS) may have been inadequate for effective block of multiple opiate receptors.

Our findings are consistent with reports of naloxone antagonism of antinociceptive effects evoked by intense peripheral stimulation. For example, responses of convergent wide dynamic range neurons to C fiber input were depressed by noxious cutaneous stimulation (Le Bars, Dickenson and Besson, 1979a; Le Bars et al. 1981) and in other studies, brief acupuncture-like stimulation depressed neuron responses in the trigeminal sensory system (Bing, Villanueva and Le Bars, 1990) in rats. Both noxious cutaneous and acupuncture-like stimulation activated neurons in the subnucleus reticularis dorsalis which receive only high threshold inputs (Bing, Villanueva and Le Bars, 1991; Villanueva, Bing and Le Bars, 1994), suggesting that the evoked-depression was mediated via activation of high threshold Aδ and C fibers. Both cutaneously evoked and acupuncture evoked depression were only partially antagonized by naloxone (Le Bars et al. 1981; Bing, Villanueva and Le Bars, 1990) and in both cases noxious cutaneous or acupuncture-like stimulation evoked extrasegmental release of met-enkephalin-like immunoreactive material in the spinal cord (Le Bars et al. 1987; Bing et al. 1991). In support of our data obtained in chronically spinal transected rats, studies have shown that intense peripheral stimulation produces a naloxone reversible antinociception in spinal animals as well. For example, in the decerebrated and spinalized cat stimulation of Aδ and C fibers in the common tibial or peroneal nerve evokes a long-lasting depression of the flexion reflex recorded electrophysiologically from the ventral roots (Chung et al. 1983) and in the rabbit prolonged intense electrical stimulation applied to the toes depresses the sural-gastrocnemius reflex (Taylor et al. 1990). In both studies the evoked inhibition was completely reversed by naloxone (Chung et al. 1983; Taylor et al. 1990). It appears from our data and from those reported in the literature, that stimulation-evoked inhibition in the intact animal is only partially reversed by

naloxone while almost complete reversal is seen in spinal preparations. Not surprisingly, it appears that spinal antinociceptive mechanisms are opioid mediated while in the spinally intact preparation non-opioid mechanisms may also participate (Basbaum and Fields, 1984; Fields and Basbaum, 1989; Bowker and Abbott, 1990; Sorkin, McAdoo and Willis, 1992; Westlund et al. 1992).

4.2. ß-FNA antagonism implicates μ-opiate receptors

Twenty four hour pretreatment with spinally administered ß-FNA attenuated the evoked response. ß-FNA is a non-competitive μ-opiate receptor antagonist which binds covalently and irreversibly to the μ-receptor complex, and has little activity with δ- and κ-receptors (Tam and Liu-Chem, 1986; Ward, Portoghese and Takemori, 1982a; Ward, Portoghese and Takemori, 1982b; Ward, Loprect and James; 1986). However, δ- (Tam and Liu-Chem, 1986; Ward, Loprect and James, 1986; Mjanger and Yaksh, 1991) and κ-antagonist properties (Tam and Liu-Chem, 1986; Ward, Loprect and James, 1986) of ß-FNA have been reported but only when a high dose was used. For example, in the hot plate nociceptive test, 24 hour pretreatment with intrathecally administered ß-FNA produced a concentration-dependent rightward shift of dose response curves for antinociceptive effects produced by spinal administration of several μ-opiate receptor agonists; only at a high dose of 20 nmoles did ß-FNA show some δ- antagonistic properties (Mjanger and Yaksh, 1991). On the basis of this study and others (Chen and Han, 1992) using spinal administration of ß-FNA to antagonize antinociceptive responses, we administered 10 nmoles of ß-FNA 24 hours prior to intense electrical stimulation. Therefore,

our results suggest that intense peripheral stimulation of the hindlimb activates long-lasting inhibitory mechanisms in the CNS which depress transmission of sensory information in the spinal cord, at least in part via activation of μ-opiate receptors. It appears from the data that the contribution of the μ-receptor in the evoked-inhibition is about equal at all periods studied.

4.3. Role of μ-opiate receptors in sensory processing in the spinal cord

Evidence for the role of μ-opiate receptors in sensory transmission is supported by anatomical, electrophysiological and behavioral data linking this receptor with modulation of sensory processing in the CNS. In the dorsal horn 70-90 % of opiate receptors are of the μ-subtype (Stevens et al. 1991). Autoradiographic evidence demonstrated radiolabelled DAMGO, a μ-opiate receptor agonist, binding in the spinal cord with up to 74 % of the total binding occurring in laminae I and II of the dorsal horn (Besse, Lombard and Besson, 1991). This finding was further supported by dense binding of labelled FK-33-824 another highly selective μ-agonist in superficial dorsal horn (Gouarderes et al. 1991). In both studies dorsal rhizotomy progressively decreased binding of labelled agonist by at least 70 %, suggesting that μ-receptors are predominately pre-synaptic on primary afferent terminals (Besse, Lombard and Besson, 1992; Gouarderes et al. 1991).

In *in vitro* release studies, μ-receptor agonists have been shown to inhibit the release of substance P (Bourgoin et al. 1994) and of aspartate and glutamate (Kangrga and Randic, 1991) elicited by high intensity stimulation of primary afferents, further supporting pre-synaptic modulation of sensory transmission in the spinal cord. In rats made polyarthritic with injection

of Freund's adjuvant, spinal perfusion with DAMGO produced a naloxone-reversible inhibition of the release of CGRP like material; naloxone by itself produced an even greater release of this peptide in polyarthritic rats (Collin et al. 1993), therefore implicating μ-receptor modulation of sensory transmission in this chronic pain model.

In addition to presynaptic modulation, there is also evidence to suggest postsynaptic modulation of sensory transmission via activation of the μ-receptor. For example, in the *in vitro* slice preparation of the substantia gelatinosa of the spinal trigeminal nucleus pars caudalis in the guinea pig and rat, met-enkephalin evoked a naloxone-reversible hyperpolarization manifested by increased potassium conductance in a majority of neurons (Grudt and Williams, 1994). Furthermore, in patch clamp studies simultaneous or prior application of the μ-agonist DAGO depressed responses evoked by NMDA in disassociated cells from the superficial dorsal horn of the rat (Rusin and Randic, 1991), suggesting direct modulation of the NMDA receptor ion channel.

With respect to more global actions of μ-receptor activation on nociception in the dorsal horn, μ agonists depress C fiber evoked activity in the lumbar spinal cord of the rat (Villanueva et al. 1991) and responses of wide dynamic range neurons to application of noxious cutaneous stimulation in the cat (Omote et al. 1991). Low doses of morphine potentiate lignocaine-evoked depression of responses of neurons to single C fiber inputs or lignocaine-evoked depression of windup elicited by repetitive C fiber stimulation (Fraser, Chapman and Dickenson, 1992); in the rat higher doses of morphine applied alone depress windup in dorsal horn neurones when pre-administered prior stimulation (Chapman, Haley and Dickenson, 1994). In addition, the flexor reflex evoked by noxious pinch is depressed by systemic administration of μ-agonists morphine

and fentanyl (Herrero and Headley, 1991) in rats.

Several behavioral studies show that μ-opiate receptor agonists produce antinociceptive effects. For example, intrathecal administration of the μ-agonist DAMGO produces antinociceptive effects in both the tail flick test and hot plate test (Stewart and Hammond, 1993; Suh et al. 1994). Morphine or DAGO are effective in blocking the tail withdrawal reflex evoked by immersion in 53°C water. Intrathecal morphine or fentanyl produces antinociception in the hot plate test and in the acetic acid induced writhing test (Furst, 1991). When morphine is given intrathecally, it dose dependentiy decreases both the early and late phases of nociceptive behaviour evoked by injection of formalin in the hind paw of the rat (Malmberg and Yaksh, 1993).

In light of the evidence supported by the literature, we propose that the intense, electrical conditioning stimulation provokes the release of endogenous opioids which may depress the tail withdrawal reflex by acting on presynaptic and postsynaptic μ-opiate receptors in the spinal cord.

4.4. TIPP[ψ] implicates δ-opiate receptors

Unlike naloxone and ß-FNA, an equal dose of TIPP[ψ] inhibited the response during the stimulation to a lesser extent, yet for the post-stimulation periods this antagonism was greater and the tail withdrawal reflex was even facilitated. TIPP[ψ] is a pseudotetrapeptide reported to be a pure δ-receptor antagonist with selectivity for the δ-receptor orders of magnitude higher than other δ-antagonists, both in the CNS (Visconti et al. 1994) and in the periphery (Schiller et al. 1993). Furthermore, TIPP[ψ] has been shown to be completely stable in enzymatic

degradation studies for up to 24 hours of incubation in *in vitro* rat brain membrane preparations, suggesting that this antagonist potentially may have long-lasting action in *in vivo* studies (Schiller et al. 1993). Ten nmoles was given because doses of δ-receptor antagonists in this range have been shown to block the depression of the tail withdrawal reflex elicited by spinal administration of DPDPE, a δ_1-receptor agonist (Drower et al. 1991) or by noxious thermal cutaneous stimulation (Pitcher et al. 1995a) in the rat. The antagonism of the evoked effect by TIPP[ψ] strongly suggests that δ-opiate receptors also participate in the stimulation-evoked inhibition. In addition, there appears to be some differential action in that activation of the δ-receptors is implicated to a lesser degree in the evoked inhibition of the tail withdrawal during the period of stimulation and appears to have a greater role in meditation of the post-stimulation effect.

4.5. Role of δ-opiate receptors in sensory processing in the spinal cord

Of the total population of opiate receptors in the spinal cord, 7-28 % are of the δ-receptor subtype (Stevens et al. 1991). Labelled DTLET, a δ-receptor agonist, binds in rat spinal cord and about 20 % of the total binding occurs in laminae I and II of the dorsal horn (Besse, Lombard and Besson, 1991). Binding of the δ-antagonist ^{3}H-naltrindole has been shown in the substantia gelatinosa (Drower et al. 1993). These receptors are predominately located presynaptically on primary afferents because dorsal rhizotomy decreased the binding of labelled δ-agonist up to 60 % (Besse, Lombard and Besson, 1992).

In an immunohistochemistry study, antisera raised against a cloned δ-opioid receptor, DOR-1, bound to a dense plexus within the superficial dorsal horn. This immunoreactivity was

greatly decreased after dorsal rhizotomy, suggesting a presynaptic localization. Furthermore, a population of small diameter primary afferents which were DOR-1 immunoreactive were also immunoreactive for CGRP. These DOR-1 and CGRP immunoreactive fibres were opposed to terminals containing immunoreactive enkephalin-like material, suggesting enkephalin from intraspinal sources may be the natural ligand for the receptor encoded for DOR-1 and that this opioid may regulate the release of CGRP from the primary afferent terminals (Dado et al. 1993). Presynaptic δ-receptor activation has been shown to inhibit the release of CGRP from primary afferent terminals in *vitro* slice preparations (Bourgoin et al. 1994) and δ-agonists depress the release of substance P in the intact spinal cord of the rat (Collin et al. 1991). This effect is reversed by the δ-antagonist naltrindole and, in addition, the antagonist alone enhances the release of substance P (Collin et al. 1991), suggesting a tonic opioid mediated inhibition of primary afferents.

Electrophysiological data show that δ-agonists depress C fibre evoked activity in the dorsal horn of the rat (Villanueva et al. 1991) and depress the activity of wide dynamic range neurons elicited by noxious cutaneous stimuli in the cat lumbar spinal cord (Omote et al. 1991).

Several algesiometric tests have implicated activation of the δ-opiate in antinociception. For example, in the nociceptive tail withdrawal reflex evoked by noxious thermal stimulation δ_1-receptor agonists such as DPDPE (Drower et al. 1991; Sofuoglu, Portoghese and Takemori, 1991; Stewart and Hammond, 1993; Shah, Davis and Yoburn, 1994; Suh et al. 1994; Nagasaka and Yaksh, 1995), [D-Ala_2]-Deltorphin II (Improta and Broccardo, 1992) or DADL (Nagasaka and Yaksh, 1995) given intrathecally produce antinociception. The δ_2-agonist DSLET also dose dependently inhibits this reflex (Sofuoglu, Portoghese and Takemori, 1991; Shah, Davis and

Yoburn, 1994) in rats. Furthermore activation of δ-receptors produces long-lasting antinociception (Improta and Broccardo, 1992). In the hot plate test DPDPE was effective in producing antinociception (Drower et al. 1991; Stewart and Hammond, 1993; Suh et al. 1994). However, agonists selective for the δ_2-receptor do not appear to induce antinociception in this paradigm (Stewart and Hammond, 1993).

The evidence supported by the studies cited above leads us to suggest that the intense conditioning stimulation activates inhibitory mechanisms in the central nervous system which produce long-term depression of the tail withdrawal reflex via activation of predominantly presynaptic δ-opiate receptors.

4.6. Nor-BNI implicates κ-opiate receptors

Nor-BNI antagonized the evoked effect during the stimulation equally well as did naloxone and ß-FNA. However, for the post-stimulation periods the antagonism by nor-BNI was far more effective in that the mean inhibition evoked during the post-stimulation periods was blocked and even reversed, as was the case with TIPP [ψ]. Nor-BNI is a highly potent and selective κ-opiate receptor antagonist with little activity at the μ- and δ-receptors. This κ-antagonist has been reported to shift the dose-response curves for selective κ-agonists, ethylketocyclazocine or U50,488H, induced inhibition of spontaneous contraction in smooth muscle preparations of the guinea pig ileum (Portoghese, Lipkowski and Takemori, 1987) and also displace κ-agonist binding in *in vitro* brain slice preparations (Takemori et al. 1988). Furthermore, nor-BNI effectively blocks κ-agonist evoked antinociception in the acetic acid induced withering test in

mice (Takemori et al. 1988). This selective κ-antagonist is believed to be extremely long-acting in that systemic administration of nor-BNI produces rightward shifts in the antinociceptive dose response curves for U50,488 in the thermally elicited tail withdrawal reflex in rhesus monkeys for up to 21 days (Butelman et al. 1993) and when given ICV it effectively blocks the depression of the tail withdrawal reflex elicited by ICV injection of κ-agonists for up to 28 days (Horan et al. 1992). Therefore, antagonism by nor-BNI of the stimulation-evoked inhibition of the tail withdrawal reflex implicates κ-receptors. Furthermore, κ-receptors appear to differentially mediate the evoked-inhibition in that these receptors appear to have a greater role in mediation of the post-stimulation response.

4.7. Role of κ-opiate receptors in sensory processing in the spinal cord

κ-receptors are sparse in the adult rat spinal cord (Sullivan and Dickenson, 1991) making up only 3-5 % of all opiate receptors in the dorsal horn (Stevens et al. 1991). Nonetheless, these receptors are implicated in sensory transmission because selective labelled κ-receptor ligands bind in the spinal cord with up to 10 % of the total binding occurring in Laminae I and II (Besse, Lombard and Besson, 1991) of the dorsal horn. These receptors are found to be predominately located post-synaptically as demonstrated by dorsal rhizotomy experiments (Besse et al. 1990) suggesting that the κ-receptor may modulate synaptic transmission of sensory information in the dorsal horn at a post-synaptic site.

In addition to probable post-synaptic modulation of sensory transmission via activation of the κ-receptor, a presynaptic action has also been suggested. For example, a selective κ-ligand

inhibits the release CGRP-like material from primary afferent terminals (Bourgoin et al. 1994).

Electrophysiological data have supported the role of the κ-receptor in modulation of sensory transmission. For example, systemic administration of the κ-agonist U50,488H depresses the flexor withdrawal reflex elicited by noxious pinch (Herrero and Headley, 1991) and intrathecal administration depresses the C fiber evoked spinal flexor reflex (Hernandez et al. 1993) in rats. Systemically applied κ-opioids have been shown to suppress the firing of dorsal horn neurones elicited by noxious thermal cutaneous stimulation (Headley, Parsons and West, 1984).

When κ-receptor agonists bremazocine, ethylketocyclazocine or pentazocine were given to mice and rats a long-lasting antinociception was produced in the acetic acid writhing test, but these agonist were ineffective in the hot plate test (Furst, 1991). Intrathecal administration of U50,488H (Nagasaka and Yaksh, 1995) or ketocyclazocine (Goodchild et al. 1991) depresses the nociceptive tail withdrawal reflex evoked by noxious thermal stimulation in rats. Subcutaneous administration of various κ-agonists produces antinociception in the thermally evoked tail withdrawal test at moderate heat intensities and depresses the withdrawal reflex in response to pressure; these antinociceptive effects were effectively blocked by selective κ-antagonists (Millan, 1989). However, κ-agonists had no effect on vocalization threshold to noxious electrical stimulation (Millan, 1989). κ-agonists also appear to be effective in chemical nociception. In rats, intrathecal administration of the κ-agonists U50,488H has little effect on the early phase of the nociceptive behaviour induced by formalin injection in the rat hindpaw, but the second phase is dose-dependently depressed (Malmberg and Yaksh, 1993).

The literature is consistent with our interpretation that κ-opiate receptors participate in modulation of sensory information. This modulation may be manifested by presynaptic and post-

synaptic mechanisms and may be the physiological basis by which inhibition of the tail withdrawal reflex is expressed in our experimental paradigm.

4.8. Summary and Conclusion:

Prolonged intense, low frequency electrical stimulation of specific peripheral sites in the rat hindlimb evokes a long-lasting inhibition of the thermally evoked tail withdrawal reflex. On the basis of effective antagonism by selective μ-, δ- and κ-receptor antagonists we conclude that activation of all three opioid receptors in the spinal cord is important for the expression of the long-lasting antinociception. Furthermore, the data suggest that activation of these receptors differentially contributes to the evoked response. The antinociception elicited during the stimulation appears to be partially mediated by activation of μ-, κ- and to a lesser extent by δ-receptors. The post-stimulation antinociception appears to be dependent on δ- and κ-receptor activation while μ-receptors play a lesser role. These data may shed some light on the neurochemical mediation of the long-lasting and general analgesic effects produced by intense electrical stimulation for the clinical treatment of pain.

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Figure 1. Effect of intense electrical stimulation of hindlimb meridian points *femur-futu* (GB-31), *fengshi* (ST-32) and *zusanli* (ST-36) on tail withdrawal latency in vehicle (n = 8) and naloxone (25 mg/kg; i.p; n = 8) treated rats. Standard parameters of stimulation were 2 ms square wave pulses, at 2 Hz for 20 min at 20 X threshold (min current required to evoke muscle twitch). The net mean inhibition of the tail withdrawal was calculated for the first 20 min period of stimulation and for subsequent 25 min periods after the end of stimulation. Data are expressed as the mean % maximum possible inhibition. The open bar represents period 1 (0 - 20 min) during the stimulation, the closed bar represents period 2 (25 - 45 min) after the stimulation, the cross hatched bar represents period 3 (50 - 70 min), and the diagonal filled bar represents the final period of observation, period 4 (75 - 95 min). * $p < 0.05$; ** $p < 0.01$.

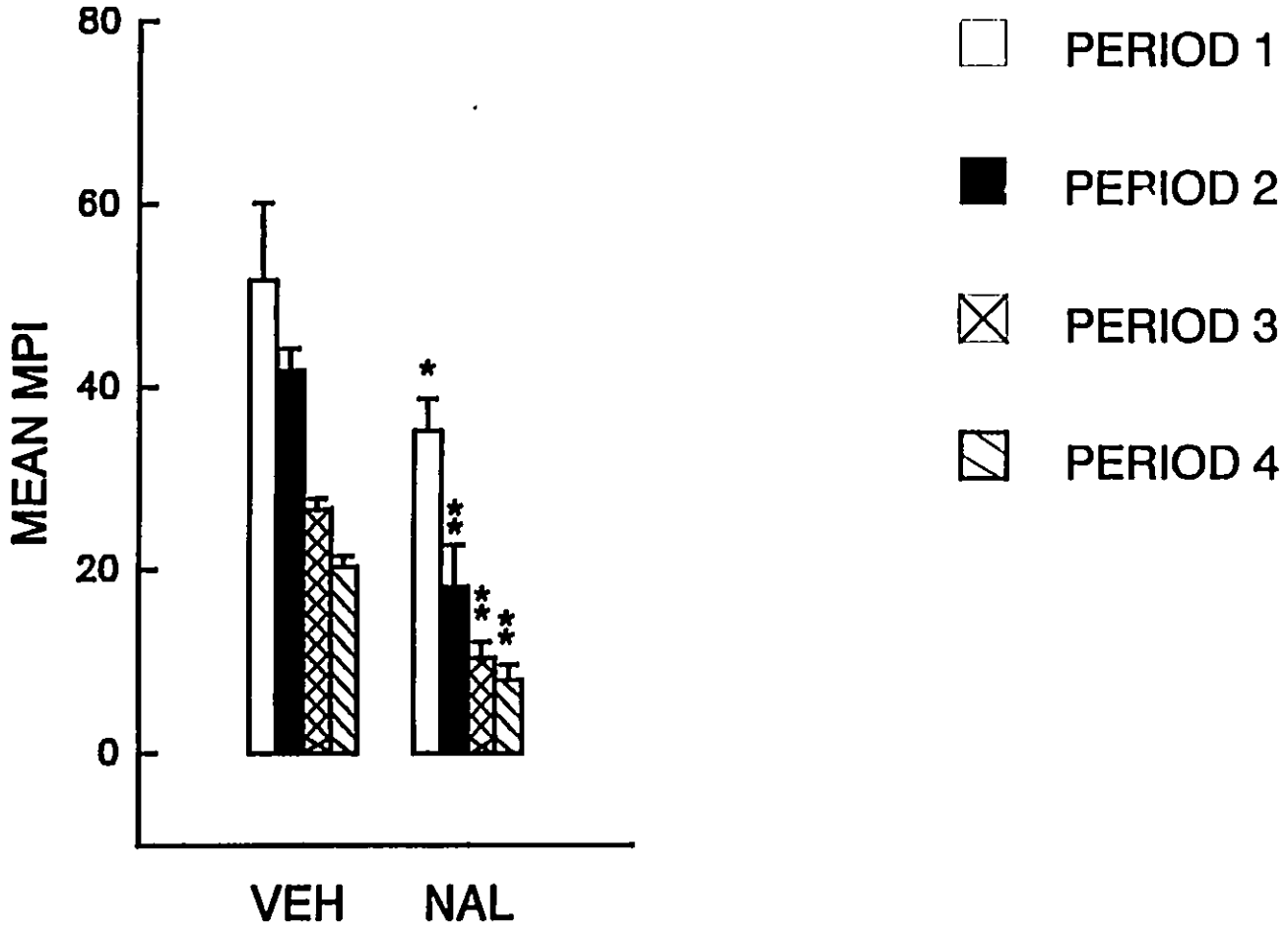
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Figure 2. Effect of naloxone on stimulation-evoked inhibition in spinalized rats three weeks post transection. Transection was at the T6/T7 spinal level. ▵ vehicle (n = 12), ■ naloxone (25 mg/kg; i.p.; n = 11) and ○ needles only (n = 14). Parameters of stimulation are as in Figure 1. ** $p < 0.01$.

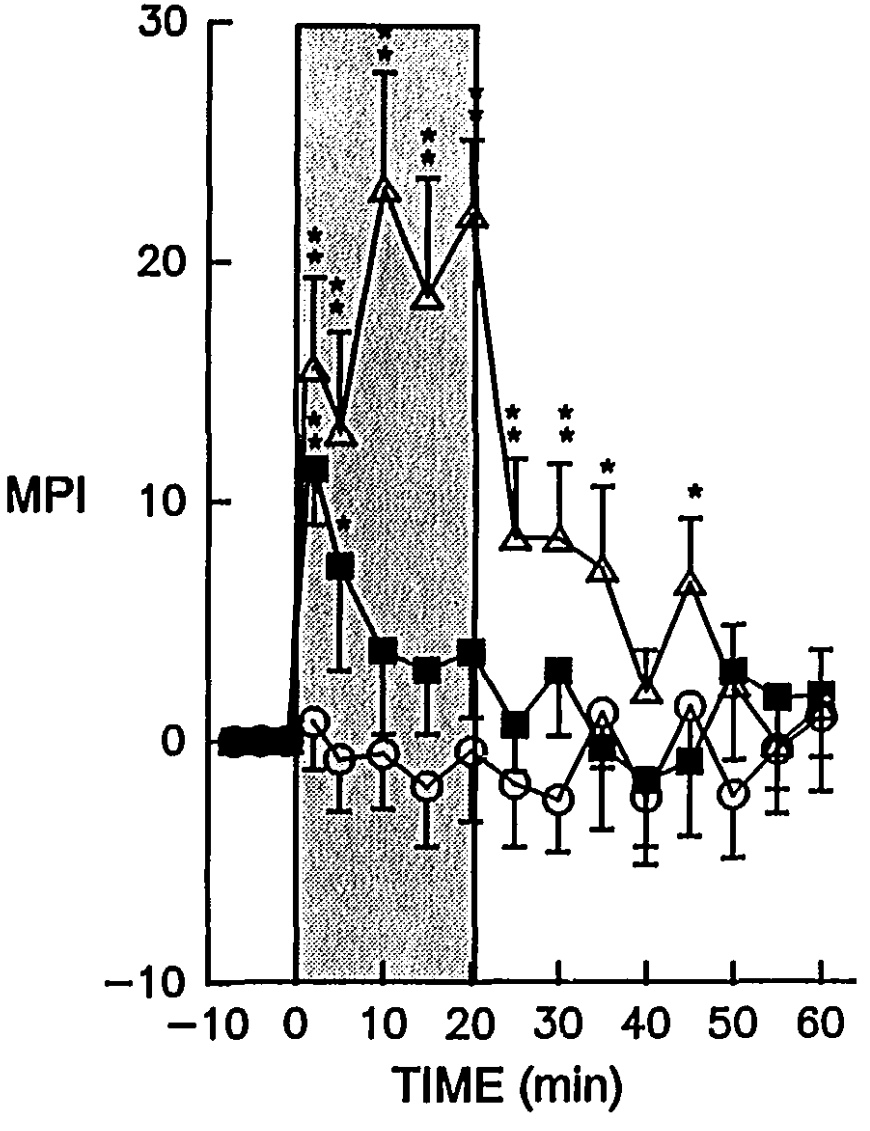
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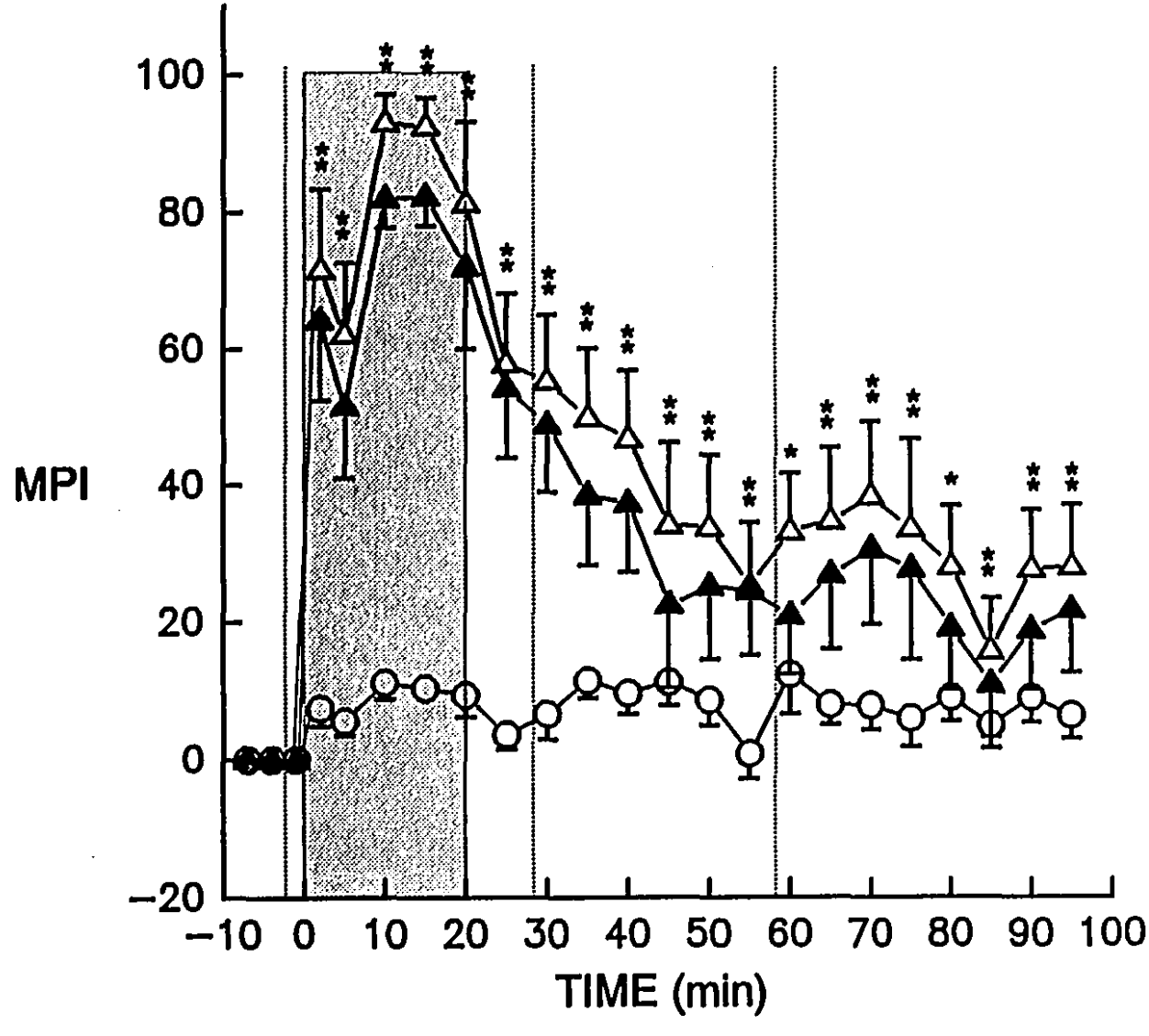
Figure 3. Effect of spinal administration of vehicle on the stimulation-evoked inhibition. Vertical dotted lines indicate times of administration of anaesthetic. Inhibition of the tail withdrawal is expressed as a % of the maximum possible inhibition. ▵ stimulated group (n = 8); ○ needles only (n = 16); ▲ difference between the two groups. ** $p < 0.01$.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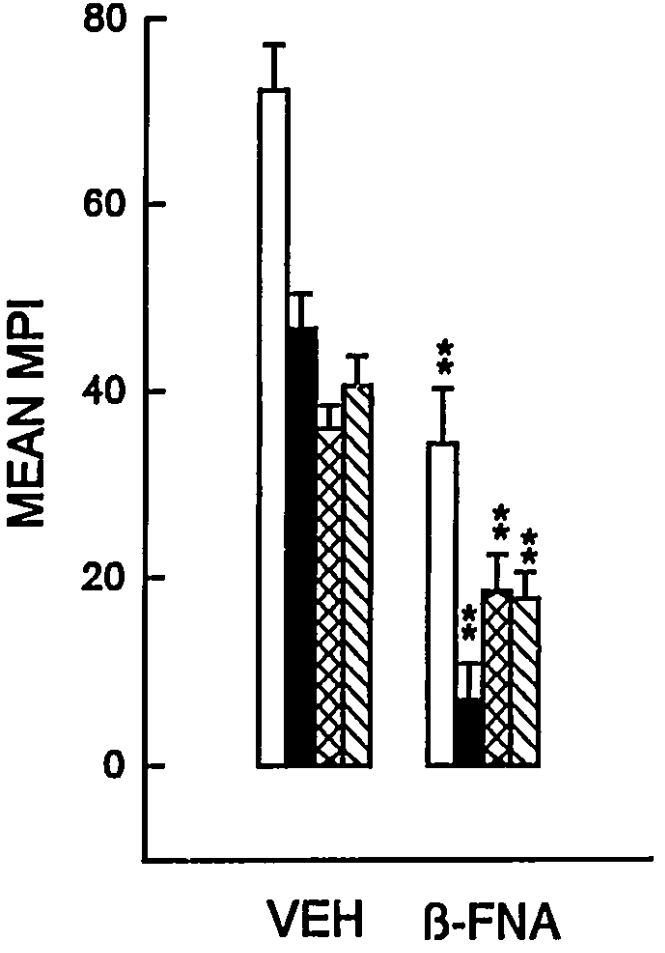
TIME (min)

Figure 4. Comparison of effects of spinal administration of vehicle and of ß-FNA on stimulation-evoked inhibition. ß-FNA (10 nmoles; n = 9) or vehicle (n = 10) was given to the lower lumbar level 1.5 min prior to stimulation. Details are as in Figure 1.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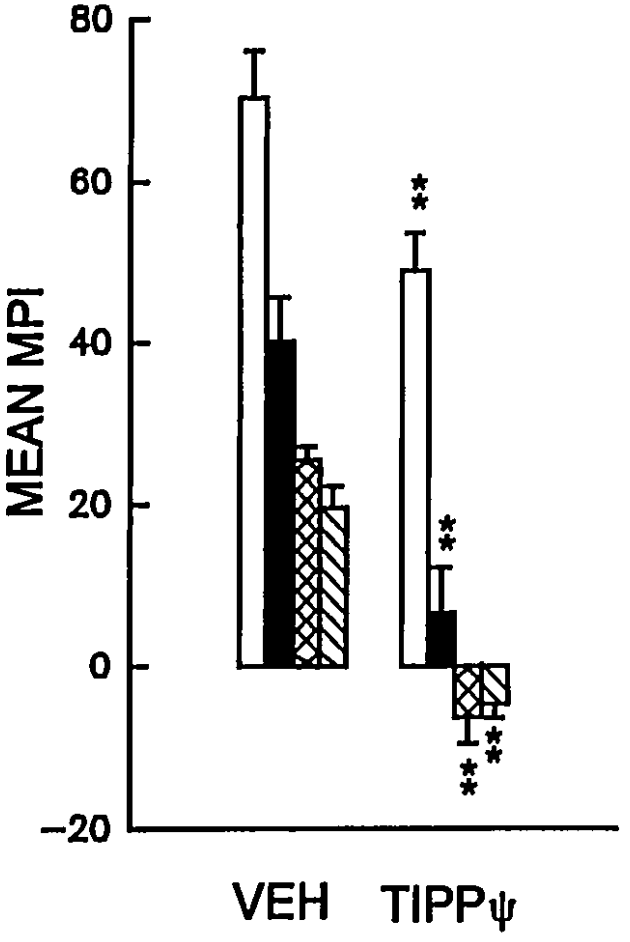
β-FNA

Figure 5. Comparison of effect of spinal administration of vehicle or TIPP[ψ] on stimulation-evoked inhibition. TIPP[ψ] (10 nmoles; n = 7) or vehicle (n = 8) was given to the lower lumbar level 1.5 min prior to stimulation. Details are as in Figure 1.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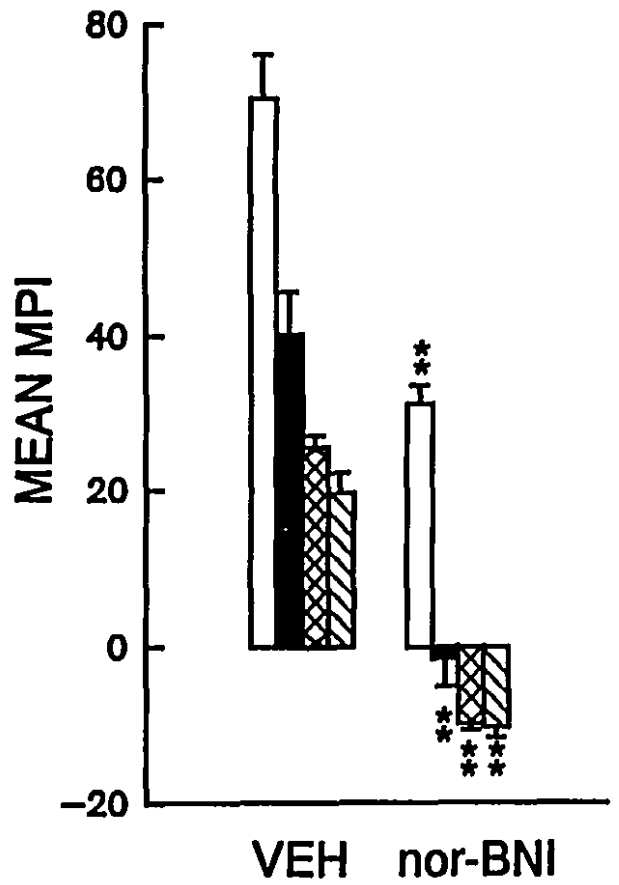
VEH
TIPPψ

Figure 6. Comparison of effect of spinal administration of vehicle and nor-BNI on stimulation-evoked inhibition. Nor-BNI (10 nmoles; n = 13) or vehicle (n = 8) was given to the lower lumbar level 1.5 min prior to stimulation. Details are as in Figure 1.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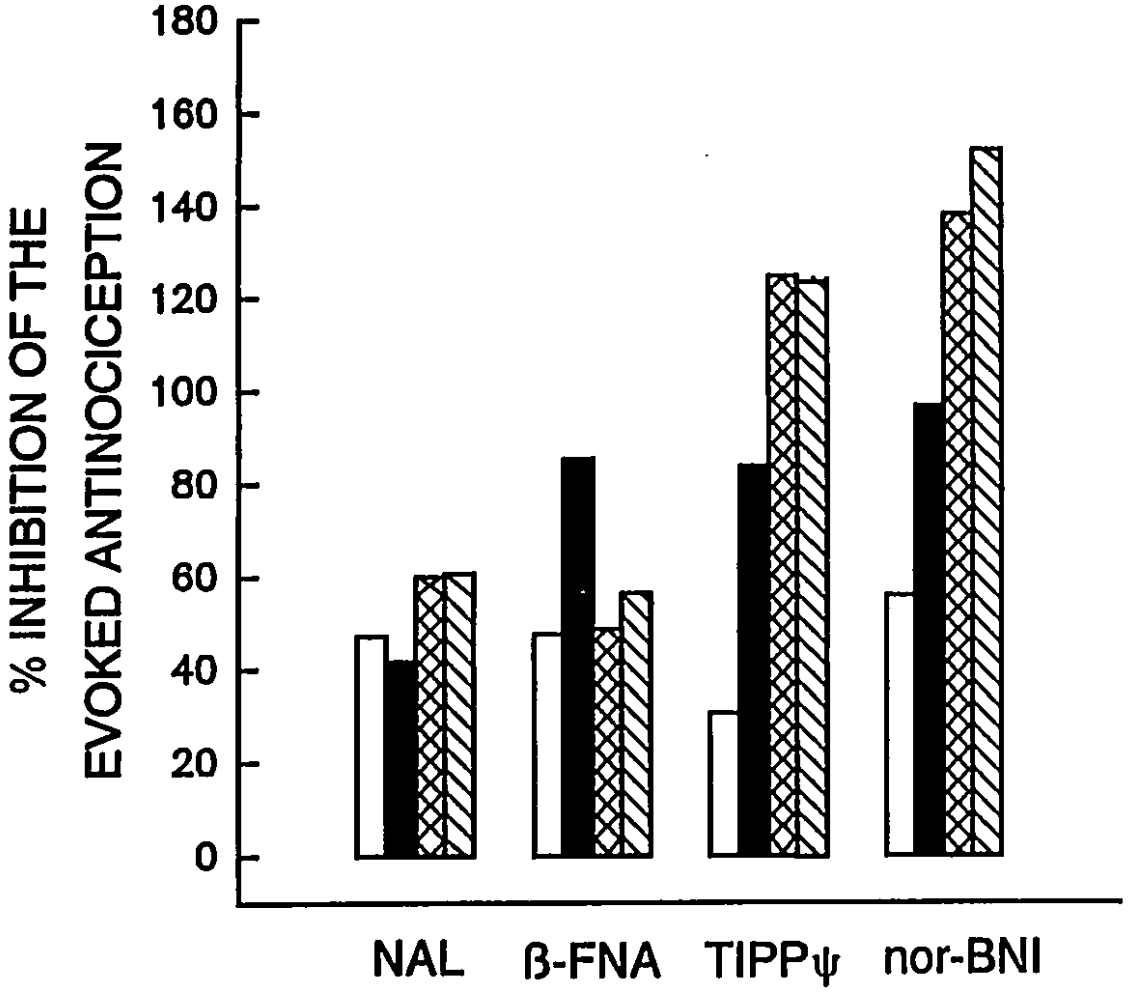
nor-BNI

Figure 7. Percent inhibition of stimulation-evoked antinociception for periods 1 through 4 by naloxone, ß-FNA, TIPP[ψ] and nor-BNI.


% INHIBITION OF THE
EVOKED ANTINOCICEPTION
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20
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NAL
β-FNA
TIPPψ
nor-BNI

PART IV

GENERAL DISCUSSION

Acupuncture Analgesia: Diffuse Noxious Inhibitory Controls, Central Sensitization or a Figment of the Imagination

Introduction

Since the beginning of recorded history man has used counter-irritation techniques to alleviate pain. However, little is known of how these treatments dictate a change in the physiological, motivational, affective and cognitive components of pain. For all practical purposes, how does one approach the problem of determining whether the alleviation of pain is achieved by modulation of somatosensory processes mediated by specific neurophysiological and/or neurochemical mechanisms rather than pain alleviation being an artifact of other psychophysiological phenomena? Is relief from pain produced by counter-irritation or acupuncture-like stimulation manifested by central inhibitory mechanisms provoked by activation of primary sensory afferents or is it a phenomenon evoked by emotional (eg. stress), motivational or suggestive mechanisms (eg. belief or expectation that the treatment will result in a cure)?

It is well documented that the power of suggestion can be effective in control of pain in man. For example, placebo given to treat post-surgical pain is found to produce marked relief in 35 % of the patients tested (Beecher, 1959). Furthermore, it is rather remarkable that in some clinical trials the placebo was 50 % as effective as morphine in producing pain relief (Evans, 1985). This effect has been attributed to both the experimenter's and subject/patient's expectations of the treatment to produce pain relief even in controlled double blind studies. Can this phenomenon be the underlying mechanism by which analgesia is produced by counter-irritation, or by low or high intensity acupuncture-like stimulation? In fact a placebo effect has

been shown to contribute to acupuncture induced analgesia (Mendelson et al., 1983). In animal models, stress becomes an important variable in the outcome of an experimental paradigm. There are several studies demonstrating that long-lasting antinociception can be produced with unaversive but stressful stimuli such as simple restraint (Calcagnetti et al., 1990; Calcagnetti, Holtzman, 1990) or even mild unaversive shock in addition to aversive stimulation (Przewlocka et al., 1990). Therefore, to differentiate between physiological and psychophysiological mechanisms, it is imperative to develop good clinical and experimental paradigms for the study of acupuncture or stimulation-evoked analgesia/antinociception in both human and animals models.

Several studies have demonstrated that low frequency, high intensity stimulation produced a generalized analgesia with a gradual onset and a prolonged aftereffect (Andersson et al., 1973; Andersson, Holmgren, 1975; Chapman et al., 1975,1977; Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Huang et al., 1978; Melzack, 1975; Sjölund, Eriksson, 1979). It is suggested the evoked analgesia is mediated via activation of high threshold fibres (Andersson et al., 1973; Andersson, Holmgren, 1975; Fox, Melzack, 1976; Holmgren, 1975; Melzack, 1975) and that underlying physiological mechanisms may share some similarities with so-called diffuse noxious inhibitory controls, or DNIC (Bouhassira et al., 1990,1992; Le Bars et al., 1979a,b; Villanueva et al., 1986a,b; Willer et al., 1979,1984,1990). However, only one study has shown a direct relationship between the intensity of stimulation and the evoked analgesia within set parameters of stimulation (Holmgren, 1975).

In animal studies in which stress did not appear to be a factor, the antinociception produced by electroacupuncture or direct stimulation of peripheral nerve (Chung et al.,

1983,1984b; Clarke et al., 1989; Han et al., 1980; Kawakita, Funakoshi, 1982; Paik et al., 1981; Taylor et al., 1990) also appeared to be a function of the intensity of the stimulation. Some studies performed in the intact animal suggest that acupuncture stimulation may evoke antinociceptive effects via activation of diffuse noxious inhibitory controls (Bing et al., 1990,1991a,b).

These previous works had a great influence on how I was to approach my objective, which was to study the neurophysiological and neurochemical bases of antinociception elicited by peripheral activation of primary afferents. On the basis of these studies, I have developed a unique animal model to study the effects of activation of peripheral inputs on processing of nociception.

With this model I have attempted to elucidate some of the neurophysiological and neurochemical mechanisms by which activation of primary afferents can modulate the transmission of sensory information at the level of the spinal cord. Based on the results of these experiments, I shall attempt to synthesize the data from the original works presented in this thesis and formulate a working hypothesis. The data will be discussed in an order which will allow for appropriate synthesis. Therefore, the discussion of the results will not necessarily follow the order in which the original manuscripts were presented.

Development of an appropriate animal model

In light of the literature, I decided to use a lightly anaesthetized rat to study stimulation-evoked antinociception. This preparation has several advantages. The anaesthetized rat provided a model that was free of stress and in which a spinal nociceptive reflex could be consistently

provoked and monitored throughout the experiment. In addition, the level of anaesthesia was easily maintained for the duration of the experiment without greatly perturbing the baseline nociceptive reflexes.

The standard parameters of stimulation used in my experiments were based on other studies in which intense, low frequency electrical stimulation produced a consistent inhibition of spinothalamic tract cells (Chung et al., 1984b; Han et al., 1980) and sural gastrocnemius reflexes (Chung et al., 1983; Paik et al., 1981). These parameters resembled those used in humans in that the stimulus was intense in nature and given at low frequency.

Significance of the evoked antinociception

In Chapter one of this thesis I have shown that intense, low frequency stimulation of meridian points in the rat produces inhibition of the nociceptive tail withdrawal reflex both during the stimulation and for more than one hour after the end of stimulation. This effect is consistent with that produced in other studies.

For example, prolonged stimulation of hindlimb acupuncture points (Paik et al., 1981) or peripheral nerve (Chung et al., 1983; Paik et al., 1981) elicited a long-lasting inhibition of the sural-evoked flexion reflex in decerebrated and spinalized cats and depressed the jaw-opening reflex in the rat (Kawakita, Funakoshi, 1982). Furthermore, prolonged intense stimulation applied either to peripheral nerve (Chung et al., 1984a,b) or to cutaneous receptive fields (Han et al., 1980) evoked a long-lasting inhibition of spinothalamic tract cell responses to C fibre inputs in decerebrated and spinalized primates. A similar antinociception evoked by intense electrical peripheral stimulation of C fibres has been reported in the spinalized and decerebrated

rabbit (Taylor et al., 1990; Clarke et al., 1989).

As inhibition of nociceptive pathways mediating the tail withdrawal reflex continued beyond the end of activation of primary afferents, this led to the hypothesis that this stimulation provoked a functional plastic change in inhibitory mechanisms in the CNS.

Significance of remote vs. local stimulation

Analgesia evoked by high intensity electrical stimulation is thought to be a variation of that evoked by counter-irritation (Bing et al., 1990,1991a,b; Fox, Melzack, 1976; Melzack, 1975) and the underlying mechanisms may share some similarities with diffuse noxious inhibitory controls (DNIC) served by a spinal-brain stem-spinal loop (Bouhassira et al., 1990,1992; Le Bars et al., 1979a,b; Villanueva et al., 1986a,b; Willer et al., 1979,1984,1990). In the clinical setting, consistent relief from pain occurred when the intensity of stimulation was applied at levels just tolerable to the patient (Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Levine et al., 1976; Mann, 1974; Melzack, 1975). However, fundamental differences exist in the properties of the analgesia produced by DNIC and brief stimulation vs. that produced by more prolonged stimulation. In common with DNIC, high intensity, low frequency electrical stimulation produced a generalized antinociceptive/analgesic effect, but unlike DNIC this effect required prolonged stimulation (Fox, Melzack, 1976; Mann, 1974; Melzack, 1975) and the effect persisted for hours to days (Eriksson, Sjölund, 1976; Fox, Melzack, 1976; Melzack, 1975; Sjölund, Eriksson, 1979). On the other hand, the antinociception evoked by brief application of aversive stimuli is generally short-lived lasting only minutes (Bouhassira et al., 1990,1992; Bowker et al., 1987; Le Bars et al., 1979b,1992; Ness, Gebhart, 1991b; Willer et al.,

1979,1984,1990).

In Chapter three, experiments were designed to study whether prolonged intense stimulation of remote meridian sites could produce both the brief and persistent effects. The experiments demonstrated that simulation of forelimb meridian sites produced an antinociceptive response similar to that produced with local stimulation of the hindlimb. This observation is consistent with one study where high intensity, low frequency electrical stimulation of Aδ- and C fibres in a peripheral limb nerve or of hindlimb meridian points evoked a relatively long-lasting inhibition of nociception at a remote site as measured by the jaw opening reflex in the rat (Kawakita, Funakoshi, 1982). This is also consistent with data demonstrating that application of brief noxious or intense cutaneous stimuli inhibited nociception in spinal segments remote from the area of stimulation in animals (Cadden et al., 1983; Gerhart et al., 1981; Le Bars et al., 1979a,b,1992; Morton et al., 1988) and in man (Bouhassira et al., 1990; Gammon, Starr, 1941; Kane, Taub, 1975; Willer et al., 1979,1984,1989,1990; Willer, 1985).

Therefore, it is hypothesized further that central mechanisms which manifest both the brief and persistent antinociception are extrasegmental in nature and therefore may share some similarities to mechanisms which underlie brain stem and spinal cord mechanisms of DNIC.

Significance of differential modulation of tail vs. limb reflexes

In Chapter three, it is also shown that intense stimulation of meridian sites only mildly inhibited limb withdrawal reflexes during the stimulation; no post-stimulation effect was evoked. The DNIC mechanisms proposed to mediate the stimulation evoked antinociception in earlier studies (Le Bars et al., 1979a,b,1992) do not fully explain the results obtained here.

The antinociceptive effect elicited by DNIC is proposed to act on convergent dorsal horn cells receiving both nociceptive and non-nociceptive inputs, while purely nociceptive cells receiving only high threshold inputs were unaffected (Le Bars et al., 1979a,b,1992). However, purely nociceptive cells have been shown in several studies to be inhibited by remote noxious stimuli (Gerhart et al., 1981; Morton et al., 1988; Ness, Gebhart, 1991a; Hao et al., 1991; Pubols et al., 1988; Schouenborg, Dickenson, 1985). Furthermore, some convergent dorsal horn cells are not inhibited and may even be excited by remote noxious conditioning stimulation (Gerhart et al., 1981; Morton et al., 1988; Pubols et al., 1988; Schouenborg, Dickenson, 1985; Yezierski, Schwartz, 1986). Therefore, convergent as well as nociceptive specific cells in the dorsal horn may be differentially controlled by central inhibitory mechanisms activated by noxious stimulation. Studies also show that in the hindlimb, cutaneous vs. deep nociceptive inputs are differentially inhibited by descending influences originating from supraspinal pathways (Hoheisel, Mense, 1990; Xian-Min, Mense, 1990); nociceptive inputs arising from deep muscle tissue are under tonic inhibition, while inputs from cutaneous sources are not. A recent study has shown that thermal stimulation applied to the hindpaw increased the tail withdrawal latency (Morgan et al., 1994). However, noxious thermal stimulation of one hindpaw had little effect on the withdrawal latency of the contralateral hindlimb, while noxious thermal stimulation of the tail produced a slight facilitation of the unconditioned hindlimb withdrawal reflex (Morgan et al., 1994). Yet noxious cutaneous stimulation applied extrasegmentally inhibited the activity of nociceptive neurones in lumbar regions (Morgan et al., 1994). In another study, brief noxious pinch applied to the nose of the rat was reported to inhibit or potentiate the nociceptive withdrawal reflexes of different muscles (Kalliomäki et al., 1992), suggesting that there are

separate nociceptive withdrawal reflex pathways to different muscles of the hindlimb and that these are under the influence of different modulatory control mechanisms (Kalliomäki et al., 1992).

In light of the data and arguments presented, the hypothesis is proposed that central inhibitory mechanisms provoked by prolonged activation of high threshold inputs differentially inhibit nociceptive reflexes such as the withdrawal reflexes of tail vs. limb. This differential control may be due to different neuronal circuitry and different control mechanisms within the CNS.

Significance of meridian vs. non-meridian evoked antinociception

In Chapter three, it was demonstrated that the brief inhibition of the nociceptive tail withdrawal reflex was elicited from any site of stimulation (i.e. meridian or non-meridian). However, the persistent effect was only evoked by stimulation of meridian points. The meridian sites chosen in this study were located in proximity to bone and joints and close to major nerve conduits, while non-meridian sites were located deep in muscle. The difference in effect may be due to qualitative and quantitative differences in inputs arising from these two types of site.

There is anatomical and electrophysiological evidence to suggest that there are differences in the number and expression of high threshold fibres arising from deep muscle vs. joint, bone, fascia and cutaneous structures. For example, there are fewer group III and group IV axons in nerve bundles innervating muscle than group III and IV fibres innervating the joint (Boyd, Davey, 1968). Furthermore, it has been demonstrated that dorsal horn neurones receiving exclusive inputs from deep muscle nociceptors are rare in adult cats (Hoheisel, Mense,

1990) and rats (Xian-Min, Mense, 1990). Yet many dorsal horn cells with high threshold mechanoreceptive fields originating from nociceptive inputs from cutaneous or from non-skeletal muscle structures from around the popliteal fossa are found (Hoheisel, Mense, 1990; Xian-Min, Mense, 1990). In addition, nociceptive inputs arising from deep skeletal muscle appear to be under tonic presynaptic inhibition (Hoheisel, Mense, 1990; Xian-Min, Mense, 1990).

It is hypothesized, then, that the attenuated brief inhibition of the tail withdrawal reflex and lack of a post-stimulation effect upon stimulation of non-meridian sites may be a consequence of reduced high threshold input from deep skeletal muscle and/or decreased efficacy in synaptic transmission at the central synapse formed by afferent inputs arising from deep muscle. This is in keeping with the hypothesis that central mechanisms require adequate high threshold afferent input for expression of the persistent antinociception. In support of this hypothesis, it has been demonstrated that 45 Hz stimulation at intensities which recruit Aß fibres produced antinociceptive effects which were not different upon stimulation of meridian or non-meridian sites. However, when stimulation was applied at intensities which recruit Aδ fibres, stimulation of meridian sites produced a greater antinociceptive effect than stimulation of non-meridian sites (Toda, Ichioka, 1978). Therefore, the difference in effect produced by stimulation of these two sites may simply be due to more effective activation and expression of high threshold inputs resulting from stimulation of meridian sites.

Significance of the parameters of stimulation on the evoked antinociception

As inhibition of nociceptive pathways mediating the tail withdrawal reflex continued beyond the end of activation of primary afferents, this led to the hypothesis that this stimulation

provoked a functional plastic change in inhibitory mechanisms in the CNS (*vide supra*). It is evident from the literature that plastic changes in spinal cord function related to transmission of sensory information can occur and have been shown to be dependent on parameters of stimulation, such as intensity and frequency (King et al., 1990; Mendell, 1966; Thompson et al., 1990,1994; Wall, Woolf, 1984). Furthermore, both clinical and human experimental data (Mann, 1974; Holmgren, 1975) as well as some animal studies (Chung et al., 1984b; Clarke et al., 1989; Han et al., 1980; Kawakita, Funakoshi, 1982; Taylor et al., 1990) suggest that parameters of the stimulation may be important for the expression of the evoked analgesia or antinociception. Therefore, experiments were designed to determine if varying the intensity, frequency, pulse duration and the train duration could effect the profile of the evoked response.

Intensity: In Chapter two, it was demonstrated that the nature of the evoked inhibition of the tail withdrawal reflex is a function of the intensity of stimulation. When all other standard parameters of the stimulation were maintained constant and the intensity was varied, the profile of the evoked response also varied. Stimulation at 20 X threshold inhibited the tail withdrawal reflex maximally during the period of stimulation. After the end of the stimulation this inhibition persisted for more than one hour. However, stimulation at 10 X threshold produced inhibition during the stimulation only and the magnitude of this inhibition was smaller. Stimulation at threshold produced no effect. Three conclusions were made on the basis of these results. 1) The data suggest that are two components to the evoked response. 2) The inhibition which occurred only during the period of stimulation, termed the brief inhibition, derives directly from activation of primary afferent inputs. 3) The second component or the persistent

post-stimulation antinociception, is produced only at higher intensities. Therefore, the expression of the brief and the persistent antinociception required activation of high threshold fibres because stimulation at threshold intensities which presumably activated large diameter afferents produced no effect. These findings are consistent with the literature in that low frequency activation of Aß-fibres evoked only weak antinociceptive effects at best and occurred only during the period of stimulation, while stimulation at intensities which recruited Aδ and C fibres produced strong antinociceptive effects with a long-lasting post-stimulation effect (Chung et al., 1984b; Han et al., 1980; Kawakita, Funakoshi, 1982).

This has led to the hypothesis that the central inhibitory mechanisms mediating the evoked inhibition require activation of high threshold inputs. To elicit a long-term plastic change in the CNS which is expressed as a persistent inhibition of nociceptive pathways, further recruitment of high threshold inputs is required. In support of this hypothesis, a recent study by Ness and Gebhart has shown that nocigenic inhibition of dorsal horn neurones evoked by noxious cutaneous stimulation or noxious visceral distension was a function of the intensity of the stimulation. Greater intensities evoked inhibition that was greater in magnitude and duration (Ness, Gebhart, 1991a,b). This phenomenon is also reflected in experimental pain studies demonstrating that the analgesia evoked by electroacupuncture-like stimulation is a function of the intensity of the stimulation and is elicited only when the intensity of the stimulation is at or above the threshold for pain (Holmgren, 1975).

Frequency: In both Chapters one and two, it was shown that evoked responses were dependent upon the frequency of the stimulation. Maintaining all other parameters constant,

stimulation at 2 Hz produced the brief inhibition and a post-stimulation effect lasting only 15 min, while 4 Hz or 6 Hz produced the brief and the full persistent post-stimulation antinociception. On the other hand, 8 Hz elicited only the brief effect. Three major findings should be pointed out. 1) It is important to note that the brief inhibition was evoked independently of the frequency, within the range used. 2) The persistent effect, on the other hand, was elicited only at frequencies less than 8 Hz. 3) The duration of post-stimulation inhibition increased with increasing the frequency from 2 Hz to 4 or 6 Hz.

These data suggest that there are two central mechanisms which mediate the brief and the persistent post-stimulation effect. While the brief effect was elicited independently of frequency, for the persistent effect to be expressed, the induction of long-term plastic changes in the CNS is dependent on the frequencies of stimulation. Furthermore, the absence of the persistent effect at 8 Hz may be due to the inability of the central mechanisms mediating this effect to follow such a frequency of stimulation. In this context, a post-stimulation analgesia evoked by intense stimulation has been reported in an experimental pain study with 2 but not 10 Hz stimulation (Andersson, Holmgren, 1975) and the perceptual response to C fibre stimulation in human peripheral nerves begins to fail at 5 Hz (Liu et al., 1995; Torebjörk, Hallin, 1973). Therefore, it is hypothesized that, in addition to activation and recruitment of high threshold inputs, central summation of effects must occur to elicit an antinociception that is long-lasting in nature.

Central summation may explain how stimulation at 2 Hz produces a post-stimulation effect lasting only 15 min, while at 4 Hz or 6 Hz this effect lasts more than one hour. Other studies have shown plastic changes in spinal cord function have been brought about by prolonged

activation of Aδ and C fibres. For example, wind up is produced optimally with low frequency stimulation (King et al., 1990; Mendell, 1966; Thompson et al., 1990,1994; Wall, Woolf, 1984) and long-lasting summation of ventral root potentials is evoked with 1-5 Hz stimulation of dorsal roots *in vitro* at strengths which recruit C fibres (Thompson et al., 1994). Finally, Ness and Gebhart have demonstrated that the magnitude and duration of the inhibition of neuronal responses to C fibre input evoked by noxious stimulation is a function of the number of preceding conditioning stimuli (Ness, Gebhart, 1991b); they suggest that central sensitization or wind up is the underlying mechanism by which the increase in magnitude and duration of the evoked inhibition is manifested (Ness, Gebhart, 1991b).

Pulse duration: Chapter two, shows that little or no post-stimulation antinociception was produced when the stimulation pulse was 0.2 ms in duration, yet pulse durations of 2 ms or 5 ms produced the persistent antinociception. These data are consistent with the interpretation that recruitment of high threshold, small diameter fibres is required to activate the central mechanisms which produce the persistent post-stimulation antinociception.

Train duration: Chapter two, also demonstrates that the evoked inhibition is dependent on the duration of the train of stimulation. Stimulation with a train duration of 10 min evoked the brief effect only, while 20 min of stimulation elicited long-lasting effects. Similar findings have been reported in the literature. For example, a five min train recruiting Aδ and C fibres elicited a post-stimulation inhibition of spinothalamic tract cells lasting less than 2 min (Chung et al., 1984b). However, when the same stimulus was maintained for 15 min the inhibition

persisted up to 30 min (Chung et al., 1984a). To my surprise, 40 min potentiated the response during the period of stimulation and although a post-stimulation effect was elicited this was shorter in duration than that obtained with 20 min of stimulation. Again three important observations should be highlighted. 1) The brief antinociception could be elicited independently of the duration of the stimulation although the magnitude of this effect increased with the duration of the train. 2) Expression of the persistent effect required a minimum duration of stimulation. 3) Optimal expression of the persistent effect required a maximum duration of stimulation. These data support the hypothesis that there are two independent central mechanisms that bring about the brief and the persistent antinociception. The brief antinociception could be evoked by either long or short trains of stimulation, suggesting that this component of the evoked response is dependent on continuous synaptic activation. On the other hand, expression of the persistent antinociception requires prolonged stimulation, suggesting that adequate summation of input is required to provoke long-lasting plastic changes in inhibitory mechanisms within the CNS. The data also suggest that central mechanisms that mediate the post-stimulation antinociception may be susceptible to a form of tolerance or desensitization when stimulation is applied beyond optimal durations of the train.

Significance of the evoked antinociception in spinal transected rats

In Chapter one, it was demonstrated that the intense, low frequency stimulation of meridian points produced only a short lasting inhibition in acute spinal transected rats. However, in chronic spinalized rats both the brief and a short-lasting post-stimulation effect were produced by the conditioning stimulus. The data suggest that there is a supraspinal component

to both the brief and the persistent effect. As both are elicited in the spinal transected rat, it appears that the spinal cord is capable of sustaining central mechanisms which mediate at least in part the evoked response observed in the intact rat. These data are consistent with other studies demonstrating weak antinociceptive effects evoked by noxious stimulation (Cadden et al., 1983) or electrical stimulation (Woolf et al., 1977) in acutely transected rats. Several studies have shown that high intensity stimulation applied either cutaneously or to peripheral nerves produces segmental inhibition of spinal nociceptive reflexes, lasting 10 min to 1 hour (Chung et al., 1983; Taylor et al., 1990). High intensity stimuli also inhibit convergent dorsal horn neurones (Cadden et al., 1983; Chung et al., 1984a) in acutely spinal transected animals and noxious stimulation produces an extrasegmental antinociception in spinal animals (Ness, Gebhart, 1991a,b; Pitcher et al., 1995). Thus, although supraspinal structures appear to have a dominant role in eliciting antinociception induced by activation of Aδ and C fibres (Cadden et al., 1983), there is sufficient evidence to suggest that intraspinal mechanisms may also provide an important contribution to inhibition of nociceptive mechanisms. These intraspinal mechanisms evoked by high intensity electrical stimulation of hindlimb meridian points may be acting in concert with or independently from supraspinal structures.

In chronically transected rats, appearance of the persistent antinociception was coincident with the return of bladder function, suggesting that after spinal transection these mechanisms may require a period of recovery to be expressed, indicating adaptive changes in spinal analgesic mechanisms.

Central mechanisms

Throughout this discussion I have made the distinction between the brief antinociception and the persistent effect. It has been proposed that these two components are mediated by separate central mechanisms. The brief effects appears to be dependent on the continuous activation of high threshold inputs. On the other hand, the persistent effect is proposed to be the result of long-term plastic changes in central inhibitory mechanisms, as the persistent antinociceptive effect cannot be due to direct synaptic events as it persists 60-90 min after the end of stimulation. These mechanisms may be related to those giving rise to the long-lasting depolarization of cat spinal wide dynamic range neurones evoked by a train of high intensity electrical or mechanical cutaneous stimulation (De Koninck, Henry, 1991; De Koninck et al., 1992). There may also be some similarity to the cellular mechanisms which mediate wind up in spinal neurones, where low frequency repetitive stimulation of Aδ and C fibres, but not of Aβ fibres alone, produced increased excitability which outlasted the stimulation by minutes to more than one hour (Mendell, 1966; Thompson et al., 1990,1992,1994; Wall, Woolf, 1984; Woolf et al., 1989). In fact, others have already alluded to the possibility that nocigenic inhibition brought about by noxious cutaneous or visceral stimulation may be related to mechanisms such as central sensitization or wind up (Ness, Gebhart, 1991a,b).

Long-term changes in spinal function after activation of Aδ and C afferent fibres (Cook et al., 1987; Mendell, 1966; Woolf, 1983) are thought to be mediated via NMDA (Chapman et al., 1994; Dickenson, Sullivan, 1987; King, Lopez-Garcia, 1993; Thompson et al., 1990,1994; Xu et al., 1992) and substance P (De Koninck, Henry, 1991; Thompson et al., 1994) receptors, and by production of nitric oxide (Radhakrishnan, Henry, 1993). These changes, which are variously termed "wind up", "facilitation", "sensitization", "plasticity", etc., are all related to

or expressed as excitatory phenomena. Although these neurochemicals have been implicated in nociception at the spinal level (De Koninck, Henry, 1991; Radhakrishnan, Henry, 1993; Thompson et al., 1990; Xu et al., 1992), it is likely that they may also be the chemical mediators of antinociceptive effects produced by activation of Aδ and C fibre afferents.

Significance of spinal NMDA receptors

In Chapter four, it is shown that activation of the NMDA receptor complex is important in the expression of the brief and the persistent inhibition of the tail withdrawal reflex. This suggests that activation of this receptor complex provokes activity in CNS inhibitory mechanisms which continue long after activation of primary afferents. The cellular mechanisms may be similar to those which mediate long-lasting facilitation of nociceptive pathways via activation of the NMDA receptor complex evoked by prolonged nociceptive input. Activation of the NMDA receptor appears to have critical importance in the development of wind up in the spinal cord (Chapman et al., 1994; Dickenson, Sullivan, 1987; King, Lopez-Garcia, 1993; Thompson et al., 1990,1994; Xu et al., 1992). The wind up phenomenon is thought to be the underlying mechanism by which central sensitization occurs resulting in facilitation of nociception in acute (≤ 48 h) (Chapman et al., 1994; Coderre, 1992; Eisenberg et al., 1993; Malmberg, Yaksh, 1993; Meller et al., 1994; Rice, McMahon, 1994) or chronic (days) neuropathic pain models (Mao et al., 1992a,b,1993; Tal, Bennett, 1993,1994), and in clinical neuropathic and/or neurogenic (Backonja et al., 1994; Kristensen et al., 1992; Vaccarino et al., 1992) or arthritic pain (Wong, 1993). For example, acute administration of NMDA to the spinal cord elicits thermal hyperalgesia (Malmberg, Yaksh, 1993; Meller et al., 1992; Mjellem-Joly et al., 1992;

Raigorodsky, Urca, 1987). Furthermore, spinal administration of NMDA after formalin injection into the rat hindpaw potentiates the second phase of the formalin-evoked behavioral nociceptive responses (Coderre, Melzack, 1992a,b). The selective NMDA antagonist, APV given spinally blocks facilitation of the thermally elicited tail withdrawal reflex produced by noxious thermal cutaneous stimulation (Yashpal et al., 1991) and has been shown to block the second late phase of the formalin induced behavioral response (Coderre, Melzack, 1992b; Vaccarino et al., 1993). Also, thermal (Mao et al., 1992a,b; Meller et al., 1994; Tal, Bennett, 1994) or mechanical (Neugebauer et al., 1993) hyperalgesia evoked by sustained activation of C fibres is thought to be mediated by NMDA receptor activation. On the other hand, NMDA receptor activation does not appear to be involved in acute transmission of sensory information, because NMDA antagonists do not effect the baseline of spinal reflexes such as the visceral bladder reflex (Rice, McMahon, 1994), tail withdrawal reflex (Sher et al., 1992; Yashpal et al., 1991) or limb withdrawal reflex (Tal, Bennett, 1993) initiated by brief (seconds) noxious stimuli.

Although the examples cited pertain to the role of the NMDA receptor in plastic changes leading to facilitation of nociception, similar cellular mechanism may be contributing to the antinociception induced by prolonged activation of nociceptive afferents. In rats made arthritic with injection of Freund's adjuvant, the tail withdrawal latency was greatly increased (Colpaert, 1979). The reported antinociception may be brought about by the sustained activation of nociceptive primary afferents and activation of the NMDA receptor among other receptors. This in turn may induce inhibitory CNS mechanisms leading to long-lasting inhibition of nociceptive processes remote from the site of tonic afferent input. In rats with adjuvant induced arthritis, 5-hydroxytryptamine turnover (Weil-Fugazza et al., 1979) and met-enkephalin levels (Cesselin

et al., 1980) are increased in spinal cord.

Several studies support the involvement the NMDA receptor in antinociceptive processes. For example, low doses of NMDA given by lumbar puncture prior to subcutaneous formalin injection of the hindpaw decrease the nociceptive behaviour of the late phase in mice (Mjellem-Joly et al., 1992) and produced potentiation of morphine-elicited antinociception in intact rats but not in acute ($\leq$ 26 h) spinal rats (Advokat et al., 1994). In addition, intrathecal administration of higher doses of NMDA produces both facilitation of the thermally elicited tail withdrawal reflex in rats and subsequent inhibition of the reflex lasting for several minutes (Raigorodsky, Urca, 1987). This pronociceptive followed by the lasting antinociceptive effect is also produced in chronically spinalized animals ($\geq$ 5 days) (Raigorodsky, Urca, 1987) implicating spinal mechanisms in the NMDA-evoked antinociception.

Evidence in the literature has demonstrated that extrasegmental antinociception produced by Aδ and C fibre activation is mediated by an intact ascending spino-reticular tract (Villanueva et al., 1986a) and descending dorsal lateral funiculi (Villanueva et al., 1986b) but these inhibitory mechanisms, at least in part, can also be sustained in spinal animals (Cadden et al., 1983; Chung et al., 1984a,b; Clarke et al., 1989; Gerhart et al., 1981; Ness, Gebhart, 1991a,b; Pitcher et al., 1995; Pubols et al., 1988). In both intact and spinal animals, this inhibition is found to be naloxone-reversible (Bing et al., 1990; Chung et al., 1983; Clarke et al., 1989; Taylor et al., 1990). Furthermore, the late inhibition of the tail withdrawal reflex elicited by intrathecal administration of NMDA is reversed by methysergide, phentolamine or naloxone (Raigorodsky, Urca, 1987). In support of purely spinal inhibitory mechanisms evoked by NMDA receptor activation, there is anatomical evidence demonstrating substance P containing

primary afferent synaptic boutons projecting onto enkephalinergic neurones in the dorsal horn (Ribeiro-da-Silva et al., 1992). Glutamate is co-localized with substance P (Battaglia, Rustioni, 1988; De Biasi, Rustioni, 1988). Perhaps both glutamate and substance P are released by activation of small diameter fibres or from intraspinal neurones and act post-synaptically to evoke release of opioid peptides which then inhibit nociception either in concert with or independently of descending inhibitory influences. In this context, intrathecal administration of substance P to the lower lumbar level produces facilitation of the nociceptive tail withdrawal reflex (Yashpal et al., 1982; Yashpal, Henry, 1983) followed by a naloxone reversible inhibition of this reflex (Yashpal, Henry, 1983); similar facilitation and inhibition of the tail withdrawal reflex is produced with intrathecal administration of NMDA (Raigorodsky, Urca, 1987). This is parallelled by evidence showing that in addition to NMDA, administration of substance P by lumbar puncture prior to formalin injection inhibits the late phase of the induced nociceptive behaviour (Mjellem-Joly et al., 1992).

It is hypothesized, then, that activation of primary afferents may elicit the release of endogenous excitatory amino acids from primary afferents, from terminals of descending collaterals originating from supraspinal structures, or spinal interneuronal pools. Activation of the NMDA receptor complex within the spinal cord results in prolonged inhibition of nociceptive pathways mediating the tail withdrawal reflex.

Significance of multiple spinal opioid receptors

In Chapter five, activation of different opiate receptors has been shown to differentially modulate the evoked inhibition of the tail withdrawal reflex. The data suggest that activation

of the three opioid receptors contributes to the evoked inhibition. During the stimulation μ-, κ- and to a lesser extent δ-receptors mediate at least part of the inhibition occurring during stimulation, while δ-, κ- and to a lesser extent μ-receptors mediate most of the post-stimulation inhibition. Interactive or synergistic actions between the three receptors may provide a mechanism by which the persistent effect is expressed. For example antinociceptive effects elicited by spinal administration of μ-opiate receptor agonists are prolonged when κ-receptor agonists are administered concomitantly (Furst, 1991).

The model

The model that I have attempted to develop has now come to its fruition. It is proposed that the brief and the persistent effects are expressed by activation of high threshold primary afferent inputs. Two separate mechanisms are thought to mediate the brief and persistent effects. The central inhibitory mechanisms which mediate both the brief and the persistent inhibition are extrasegmental in nature. These mechanisms differentially modulate tail vs. limb nociceptive pathways. The brief antinociception requires continuous activation of high threshold primary afferents. The brief effect is independent of the site of stimulation, the frequency of stimulation, the pulse duration and the duration of the train. The persistent antinociception requires greater recruitment of high threshold fibres to be expressed. Because this late antinociceptive effect persists long after activity in primary afferent terminals has ceased, it is proposed that long-term plastic changes in central inhibitory mechanisms are provoked and result in a long-lasting inhibition of spinal nociception. The central mechanisms which mediate the persistent antinociception is dependent on the site of stimulation, the frequency, pulse duration and train

duration of the stimulation. Dependence of the persistent effect on these parameters suggests that some form of central summation is required.

The induction of long-term plastic changes in central inhibitory mechanisms is dependent on activation of spinal NMDA receptors. The site of action may be post-synaptic at the first synapse or on some spinal interneuronal pool. Finally, the inhibition is mediated by activation of multiple opiate receptors. The three types of opiate receptor differentially contribute to the evoked inhibition. During the stimulation μ-, κ- and to a lesser extent δ-receptors mediate at least part of the inhibition occurring during stimulation, while δ-, κ- and to a lesser extent μ-receptors mediate most of the post-stimulation inhibition.

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PART V

CLAIMS OF ORIGINALITY

The principal results and conclusions presented in this thesis are original and have not appeared elsewhere except as specifically stated in the text and the Preface. To the best of my knowledge all the relevant literature which pertains to these findings has been cited in the text. The principal original contributions from this thesis are briefly outlined below.

Chapter One:

1) This is the first demonstration that intense, low frequency electrical stimulation applied to hindlimb meridian sites evoked a long-lasting inhibition ($\geq$ 1 h) of the tail withdrawal reflex in the intact, lightly anaesthetized rat.

a) This inhibition was maximum during the stimulation.

b) After the end of stimulation the inhibition persisted for more than one hour.

2) This is the first report showing that the inhibition produced during the stimulation, termed the brief effect, and the post-stimulation effect, termed the persistent effect, could be separated on the basis of the frequency of stimulation.

a) Inhibition elicited during the stimulation was unaffected by the range of frequencies used.

b) The post-stimulation effect increased in duration by increasing the frequency of stimulation from 2 Hz to 4 Hz or 6 Hz.

c) The post-stimulation inhibition was absent at 8 Hz.

3) This is the first report demonstrating that the evoked response could be differentiated on the

basis of the site of stimulation.

a) Stimulation of non-meridian sites evoked a brief effect only; the magnitude was smaller than that produced by stimulation of meridian sites.

b) The persistent effect was absent upon stimulation of non-meridian sites.

4) This is the first report demonstrating that recovery of spinal antinociceptive mechanisms occurs over time after spinal transection and is coincident with the return of bladder function.

a) Prolonged stimulation of meridian sites produced only the brief effect in acute spinal transected rats.

b) In chronic spinal transected animals, prolonged stimulation produced both a brief and a post-stimulation inhibition.

Chapter Two:

1) This is the first report demonstrating an intensity dependent effect on both the magnitude and the duration of the inhibition and that the brief and the persistent inhibition could be differentiated on the basis of the intensity of stimulation.

a) Stimulation at threshold produced no effect.

b) Stimulation at 10 X threshold produced only the brief effect.

c) Stimulation at 20 X threshold evoked both the brief and the persistent inhibition.

2) The evoked inhibition is a function of the frequency of stimulation (see Chapter One).

3) This is the first report demonstrating that the evoked response could be differentiated on the basis of the duration of the pulse.

a) The brief effect was elicited with short or long-pulse durations.

b) The persistent effect was elicited only with long pulse durations ($\geq$ 2 ms).

4) This is the first direct demonstration that the evoked response could be differentiated on the basis of duration of the train of stimulation.

a) The brief effect was elicited independently of the duration of the train.

b) The persistent effect was elicited only with long train durations.

c) The persistent effect was less when train durations were beyond an optimal limit.

Chapter Three:

1) This is the first report demonstrating that the inhibition evoked by stimulation at remote sites can be differentiated on the basis of the site of stimulation.

a) Stimulation of meridian sites whether local (i.e. sites extrasegmental but local to the test stimulus) or remote (i.e. sites extrasegmental and remote form the test stimulus) produced both the brief and the persistent inhibition of the tail withdrawal reflex.

b) Stimulation of local or remote non-meridian sites evoked only the brief inhibition.

2) This is the first report demonstrating that bilateral stimulation potentiates the brief inhibition.

3) This is the first report demonstrating that prolonged stimulation of meridian sites only produces little inhibition of limb reflexes.

Chapter Four:

1) This is the first report to implicate activation of spinal NMDA receptors in the mediation of acupuncture-evoked antinociception.

a) Both the brief and the persistent inhibition were blocked by spinal administration of 5-amino-2-phosphonovaleric acid.

Chapter Five:

1) This is the first report to demonstrate the evoked inhibition of the tail withdrawal reflex is differentially blocked by the spinal administration of μ-, δ- and κ-opiate receptor antagonists

a) Systemic naloxone, a wide spectrum opioid antagonist, attenuated the brief and the persistent effects.

b) The μ- and κ- receptor antagonists ß-funaltrexamine and nor-binaltorphimine were more effective in attenuating the brief effect than the δ-opiate receptor antagonist TIPP[ψ].

c) δ- and κ- opiate receptor antagonists were more effective in blocking the post-stimulation effect than the μ-opiate receptor antagonist.

d) In spinal transected rats systemic naloxone blocked the evoked inhibition.