A STUDY OF SPINAL PROSTAGLANDINS IN EXPERIMENTAL ALLODYNIA

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A STUDY OF SPINAL PROSTAGLANDINS IN EXPERIMENTAL ALLODYNIA

by

Zizhen Zhang

A thesis submitted to the School of Graduate Studies in

partial fulfillment of the requirements for the degree of

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Specialization: Neuroscience

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ABSTRACT

Cumulative evidence indicates that the release of spinal prostaglandins (PGs) is increased in hyperalgesia and persistent pain states following peripheral inflammation or injury, events that are associated with repetitive C-fiber stimulation. Non-steroidal anti-inflammatory drugs (NSAIDs), given intrathecally (i.t.), inhibited PG release and attenuated persistent pain and hyperalgesia, approximately 500-1000 more potently when compared with systemic administration suggesting a central site of action. Cyclooxygenase (COX), the enzyme for PG synthesis, is present in the spinal dorsal horn where the nociceptive C-fibers terminate. Furthermore, i.t. PGE_2 induces hyperalgesia and allodynia in conscious mice. All these observations support the hypothesis that PGs are involved in C-fiber mediated spinal sensitization processes underlying hyperalgesia and allodynia. Tactile stimulation (Aβ-fiber input) induces prominent, well-defined allodynia after i.t. bicuculline (BIC) in the rat. However, the mechanism of allodynia is unclear. The objective of the present study was to determine whether the low threshold mechanoreceptive (Aβ) primary afferent fibers activate a similar prostanoid-sensitizing mechanism in the rat spinal cord in allodynia. Male Sprague Dawley rats (325-400g) were anaesthetized with halothane and maintained with urethane for the continuous monitoring of blood pressure (MAP), heart rate (HR) and cortical electroencephalogram (EEG). A laminectomy was performed to expose the dorsal surface of the spinal cord. Unilateral application of bicuculline (0.1 μg in 0.1 μl) to the L5 or L6 spinal segment induced a highly localized allodynia (e.g., one or two digits) on the ipsilateral hind paw. Thus, hair deflection (HD) (brushing the hair with

a cotton-tipped applicator) in the presence, but not absence of bicuculline, evoked an increase in MAP and HR, abrupt motor responses (MR) (e.g., withdrawal of the hind leg, kicking, and/or scratching) on the affected side, and desynchrony of the EEG. Bicuculline-allodynia was dose-dependent, yielding ED_{50} values (95% CI) of 0.055 mg (0.035-0.085) for MAP; 0.075 mg (0.048-0.118) for HR and 0.097 mg (0.078-0.122) for MR. Allodynia was sustained for up to 2 h with repeated bicuculline doses without any detectable change in the location or area of peripheral sensitization. Pretreatment with either the EP-receptor antagonist, SC-51322, the cyclooxygenase (COX)-2 selective inhibitor, NS-398, or the NMDA-receptor antagonist, AP-7, inhibited bicuculline-allodynia in a dose-dependent manner. Innocuous tactile stimulation in the presence of i.t. PGE_2 induced nociceptive-like behavioural responses (allodynia) in conscious rats. These allodynic responses were attenuated by SC-51322, or AP-7. Bicuculline, given i.t. 5 min before PGE_2, enhanced PGE_2-induced allodynia and shifted the PGE_2 dose-response curve to the left. The spontaneous behavioural response after i.t. PGE_2 was also enhanced by bicuculline, but to a much less extent. The present results demonstrate: a) the utility of topical drug delivery for inducing highly localized and sustained allodynia in the lightly-anaesthetized rat; b) that PGs, synthesized by constitutive COX-2 in the spinal cord in response to NMDA-dependent afferent input, contribute to the abnormal processing of tactile input via spinal EP-receptors; c) i.t PGE_2 induces allodynia in conscious rats which is mediated by EP and NMDA receptors, and is potentiated by pretreatment with i.t. bicuculline. These results suggest that low threshold afferent input acquires access to a PG-sensitizing process during

bicuculline-disinhibition in the rat.

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LIST OF ABBREVIATIONS AND SYMBOLS

07:00h	7 o'clock in the morning
±	plus and minus, in addition to, subtracted from (about the mean)
%	percent
>	greater than
<	less than
°C	degrees Celsius
μg	microgram, 10^{-6} grams, unit of mass
μl	microliter, 10^{-6} liters, unit of volume
μm	micrometer, 10^{-6} meters, unit of length
A-fiber	a class of primary afferent neurons
ANOVA	analysis of variance
AP-7	(±)-2-amino-7-phosphonoheptanoic acid
BIC	(-) bicuculline [(-)bicuculline methiodide]
bpm	beats per minute
BP	blood pressure
C-fiber	a class of primary afferent neurons
Ca^{++}	calcium ion
CGRP	calcitonin gene-related peptide
CI	confidence interval
CNS	central nervous system
Co.	company
COX	cyclooxygenase
CSF	cerebrospinal fluid
DMSO	dimethyl sulfoxide, an organic solvent
EAA	excitatory amino acid
ED_{50}	effective dose for 50 percent response
EEG	electroencephalogram(s)/graph(s)

g	gram(s)
GABA	γ-aminobutyric acid, γ-aminobutyrate
Glu	glutamate
Gs	stimulatory G-protein
Gi	inhibitory G-protein
h	hour(s)
HD	hair deflection
Hg	elemental symbol for mercury
HR	heart rate
IASP	International Association for the Study of Pain
ID_{50}	inhibitory dose decreasing a response by 50 percent
i.e.	id est (that is)
i.p.	intraperitoneal(ly)
i.t.	intrathecal
i.v.	intravenous(ly)
L5	lumbar vertebra number 5
L6	lumbar vertebra number 6
LI	-like immunoreactivity
MAP	mean arterial pressure
MK-801	dizocilpine maleate
min	minute(s)
MR	motor response or motor response duration
mRNA	messenger ribonucleic acid
n	number of determinations
NMDA	N-methyl-D-aspartic acid
NS-398	N-(2-cyclohexyloxy-4-nitrophenyl)- methanesulfonamide
PE-10	size 10 polyethylene tubing (diameter~0.61mm)
PG	prostaglandin
S	second(s)
s.c.	subcutaneous(ly)

SC-51322	[8-chlorodibenz(b,f)(1,4)oxazepine-10[11H]-carboxyl acid, 2-(3-(2-[fury-anylmethyl]thio)-1-oxopropyl)hyrazide
SD	standard deviation
SEM	standard error of the mean
SP	substance P, a neurotransmitter
STR	strychnine
T	time
WDR	wide dynamic range

1 INTRODUCTION

1.1 Neuropathic Pain and Allodynia

Neuropathic pain is defined by the International Association for the Study of Pain (IASP) as "pain initiated or caused by a primary lesion or dysfunction in the nervous system" (Merskey, 1994). It usually arises from trauma or disease in the peripheral nerves, the posterior spinal roots, the spinal cord itself, or certain regions of the brain. Examples include phantom limb pain; central post-stroke pain; diabetic, alcoholic, nutritional, traumatic or cancerous neuropathy; anterior spinal artery syndrome; postherpetic neuralgia; reflex sympathetic dystrophy; plexus avulsion; postcordotomy dysesthesia and painful conditions associated with paraplegia and multiple sclerosis (Shibasaki and Kuroiwa, 1974; Boivie, 1989; Tasker, 1990; Portenoy and Hagen, 1990; Tanelian and Brose, 1991; Price et al., 1992; Triggs and Beric, 1992; Baron and Saguer, 1993; Portenoy, 2000).

The rate of occurrence of neuropathic pain varies with the causative event. For example, poststroke pain was found in 16 out of 207 (8%) stroke patients (Andersen et al., 1995). Postherpetic neuralgia (PHN), defined as neuropathic pain persisting for 1 month or longer after herpes zoster infection, affects about 10% of all patients who have contracted the disease (Watson, 1995). This increases to approximately 50% of patients infected with herpes zoster who are older than 50 years (Beydoun et al., 1999).

Spinal cord injury, which affects about one in 40 patients who present to a major trauma centre (Kearney et al., 1991), is another common cause of neuropathic pain. Although the annual incidence in developed countries is relatively

low (from 11.5-53.4 per million population) (Botterell et al., 1975; Kurtzke 1975), victims frequently develop chronic musculo-skeletal and neuropathic pain, in addition to the loss of motor control. Approximately 30% of patients with spinal cord injury experience symptoms of neuropathic pain. This neuropathic pain is a relatively rare and idiosyncratic outcome of nerve injury (Noordenbos and Wall, 1981; Arner and Meyerson, 1988; Tasker, 1990). However, it is extremely debilitating, often intractable and a major burden on the health and social systems (Arner and Meyerson, 1988; Rowbotham et al., 1991; Baron and Saguer, 1993; Schmader, 1998).

Neuropathic pain differs from normal nociceptive pain in a number of important ways. The former is chronic in nature, persisting for years or even decades after the initial injury has healed. The onset of neuropathic pain is normally delayed for weeks to months after the causative event (Tasker, 1990). For example, 82% of patients with spinal cord lesions experienced a delay in the onset of pain, which ranged from less than a month to more than one year after injury (Tasker et al., 1992). Neuropathic pain is frequently described by patients as a burning, ripping, tearing, pressing or twisting pain, terms normally associated with physical injury. Patients are often unable to identify or locate the inciting stimulus and radiation of sensation, abnormal temporal summation, and after-sensations are frequent sequelae of this syndrome (Lindblom and Verrillo, 1979; Noordenbos and Wall , 1981; Price et al., 1992).

One of the major problems of neuropathic pain is its poor response to treatment. Surgical interventions, intended to alleviate neuropathic pain, usually

provide only incomplete and temporary relief, with the pain eventually returning (Tasker et al., 1992; Eide, 1998). Pharmacotherapy with drugs such as opioid analgesics, tricyclic antidepressants, anticonvulsants, barbiturates, local anesthetics and/or use-dependent sodium channel blockers is highly variable from patient to patient, and rarely successful in effecting complete pain control. As a result, the majority of patients with neuropathic pain are inadequately controlled, making neuropathic pain a serious clinical problem and a major therapeutic challenge (Eide, 1998).

Surveys of patients with neuropathic pain report that the most common and troublesome symptom is allodynia (Campbell et al., 1988; Raja et al., 1988). Allodynia is defined by the IASP as "pain arising from a stimulus that does not normally evoke pain" (Merskey, 1986). Thus, a cold draft of air or the light touch of clothing can acquire the ability to evoke excruciating pain after nerve injury. Mechanical (tactile) allodynia is the most common type, occurring in 54% of patients with central neuropathic pain and 48% of patients with peripheral neuropathic pain (Nurmikko and Hietaharju, 1992), and it is the major clinical form of allodynia (Woolf and Doubell, 1994; Ma and Woolf, 1996).

1.2 Putative Mechanisms of Allodynia

A fundamental characteristic of allodynia is the altered afferent input (Aβ- vs C-fibers) that elicits pain. Thus, ischemic nerve block or nerve compression eliminated allodynia and the sensation of light touch on adjacent normal skin in patients with neuropathic pain (Campbell et al., 1988; Price et al., 1989). These

results indicated that both sensations are mediated by the same neural elements, namely Aβ fibers. In contrast, temperature discrimination in the same region was unaffected, indicating that functional Aδ and C-fibers do not mediate the allodynia (Campbell et al., 1988). Studies of the response latencies for the detection of mechanical stimuli showed that the conduction velocity for detection of pain in the nerve-injured limb was similar to that for detection of touch in the normal limb (Lindblom and Verrillo, 1979; Campbell et al., 1988; Gracely et al., 1992). Additional evidence for the involvement of Aβ-fibers in allodynia is derived from the observation that high-frequency, low-intensity electrical nerve stimulation exacerbates allodynia, rather than eliciting the analgesic effect seen with nociceptive pain (Price et al., 1992). Since Aβ-fibers do not normally cause pain, their acquired ability to do so after peripheral or central nerve injury implies a dramatic change in somatosensory processing at the spinal and/or supraspinal level.

In the periphery, nerve injury has been shown to result in a variety of changes. These include: i) spontaneous afferent activity in the injured terminals and/or the dorsal root ganglion cells of the injured axons (Devor 1991); ii) increased mechanosensitivity of the neuroma leading to increased Aβ-fiber discharges (Babbedge et al; 1996); iii) infiltration of inflammatory cells such as macrophages into the myelin lamellae similar to that seen in inflammatory neuropathies (Nukada et al., 2000); iv) development of post-ganglionic sympathetic sprouts around type A dorsal root ganglion cells (McLachlan et al., 1993); and v) ephaptic connections (abnormal electrical connections between demyelinated adjacent axons, (Jänig et

al., 1988). Each of these has the ability to generate an excitatory barrage reaching the spinal cord. Sustained excitatory input is known to induce a facilitated central hyperexcitability in the spinal cord (Coderre, 1993; Millan, 1999).

Nerve injury is also known to elicit pronounced changes in the spinal cord. These include: i) the sprouting of nerve terminals of large primary afferent (Aβ) fibers into lamina I and II of the dorsal horn of spinal cord (LaMotte et al., 1991; Woolf et al., 1992); ii) changes in the postsynaptic function of spinal neurons including the loss of opioid binding sites in the superficial dorsal horn (Besse et al., 1992; Molander et al., 1992); iii) the appearance of new neurotransmitters such as neuropeptide Y, galanin, and vasoactive intestinal peptide in spinal afferent terminals (Wakisaka et al., 1992); iv) alterations in intracellular and cell surface markers (e.g., glial fibrillary acidic protein) (Garrison et al,. 191992); v) the upregulation of several immediate early gene products in the spinal cord such as *c-fos* and *c-jun* which are implicated in the increased responsiveness of second-order neurons (Herdegen et al., 91, Cameron-Curry et al., 1991); and vi) the appearance of dark staining neurons in the dorsal horn thought to be deteriorating interneurons (Sugimoto et al., 1989; 1990; Mayer et al., 1999).

These presynaptic and postsynaptic changes suggest a major reorganization of spinal cord connections and/or nerve function after nerve injury. Many of these are directly or indirectly correlated with the development of hypersensitivity in the spinal cord (Millan, 1999). Although the functional significance of these changes remains to be determined, it is clear that the spinal cord is a major site of injury-induced adaptations to neuropathic pain.

1.3 Spinal Mechanisms of Allodynia

The spinal cord contains the first synaptic connection in somatosensory pathways, and represents the first site of signal processing. Thus, primary afferent fibers form extensive synaptic contacts with second order neurons in the dorsal horn. The latter are either relay cells, with axons projecting to the brain stem or thalamus, or interneurons that transfer information locally to other interneurons or to relay cells in adjacent spinal segments. Functionally, second order neurons are classified into three general types (Besson and Chaouch, 1987; Henry, 1989): 1) non-nociceptive (those receiving input from Ab primary afferent fibers), 2) nociceptive specific (those receiving input from C and Ad fibers), and 3) nociceptive non-specific or wide dynamic range (WDR) neurons. The latter group, which are known to be involved in normal pain processing, receive convergent input from both nociceptive and non-nociceptive primary afferent fibers (Figure 1A). This synaptic arrangement and the ability of these neurons to elicit a graded response to a range of stimulus intensities make WDR neurons a logical site for the investigation of allodynia. A basic question related to WDR neurons and allodynia is why, under normal conditions, Aβ input is not routinely perceived as pain.

The ultimate perception of a stimulus as tactile or nociceptive appears to be governed by the balance of excitatory and inhibitory input that determines the discharge of second order neurons (see reviews by Yaksh et al., 1999b; Milan, 1999; Figure 1A, B). Convergent lines of evidence suggest that low threshold Aβ afferent fibers activate inhibitory interneurons in the spinal dorsal horn which modulate the evoked discharge of WDR neurons (Millan, 1999) (Figure 1A). These

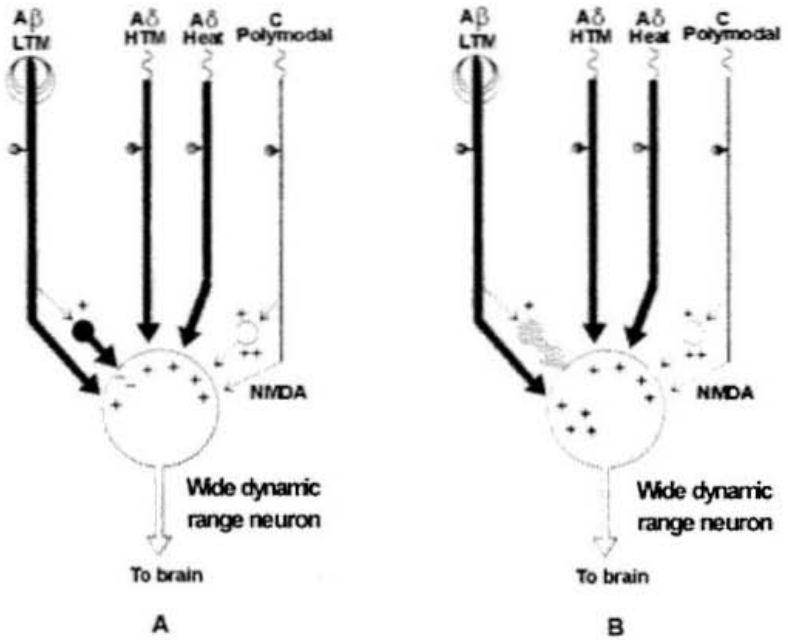


Figure 1. A schematic diagram showing the convergent input of primary afferent fibers onto a wide dynamic range (WDR) neuron in the spinal dorsal horn. A: In normal conditions, WDR neurons receive input from large diameter, myelinated, low-threshold mechanoreceptive (LTM) Aβ fibers; intermediate diameter, myelinated, high threshold mechanoreceptive (HTM) and thermosensitive (heat) Aδ fibers, and small diameter, unmyelinated, polymodal C fibers. Collateral axons of LTM fibers make synaptic contacts with small inhibitory interneurons (small filled circle and arrow) that modulate the response of WDR neurons to LTM input. B: Central or peripheral nerve injury may induce dysfunction and/or loss of these interneurons. The removal of this inhibitory modulation results in an exaggerated response to normal low threshold mechanoreceptive (Aβ) input that could be mistakenly interpreted as pain. The small open circle and arrow represents an excitatory interneuron. The shaded circle and arrow represents damaged inhibitory interneuron. The + and - symbols indicate excitation and inhibition, respectively (adapted from Wall et al., 1989).

inhibitory interneurons contain GABA and glycine (Todd and Sullivan; 1990; Carlton et al., 1996). GABA-like immunoreactivity (LI) is concentrated in the somata, dendrites and axon terminals within laminae I-III of the spinal dorsal horn (Todd, 1990). In the spinal cord, GABAergic dendrites receive synaptic input from the central terminals of primary afferent fibers, especially those of the low-threshold, myelinated, Aβ-type (Todd, 1990; Todd et al., 1996). In turn, GABA-containing neurons synapse directly on the proximal dendrites or cell bodies of spinothalamic tract (STT) cells (Carlton and Hayes; 1990; Carlton et al., 1992) which relay information to sensory nuclei in the thalamus.

Immunohistochemical studies have also confirmed the presence of $GABA_A$ receptors in the synaptic regions formed by these interneurons (Solodkin et al., 1984). Indeed, GABA interneurons are thought to effect both presynaptic inhibition at axoaxonic synapses (Solodkin et al., 1984), and postsynaptic inhibition at axodendritic and axosomatic synapses by means of the $GABA_A$ receptors present at these sites (Todd et al., 1996).

Likewise, glycinergic cells are most abundant in lamina III and IV of the spinal dorsal horn (less in lamina II, and V) (Carlton et al., 1996) where glycine appears to co-exist with GABA in many (but not all) GABAergic cells (Todd, 1990). The dendrites of neurons exhibiting glycine-LI in laminae II and III are postsynaptic to the central axons of type II (myelinated axons), but not type I (unmyelinated axons) glomeruli (Todd, 1990). Thus, glycinergic interneurons appear to receive a major monosynaptic input from myelinated primary afferent fibers (Todd 1990; Carlton et al., 1996). Glycinergic interneurons also make functional synaptic contacts with

STT cells in the spinal dorsal horn (Hori and Endo, 1992; Antal et al., 1996). Consistent with these data are reports of glycine receptor- and $GABA_A$ receptor-LI in the synapses formed by these interneurons. Glycine and $GABA_A$ receptor immunoreactivity was enriched in the postsynaptic dendrites, cell bodies as well as presynaptic axons. These results suggest that glycine effects both pre-synaptic and post-synaptic inhibition of second order neurons in cats and rats (Solodkin et al., 1984; Todd et al., 1996) and provide further anatomical evidence for the model illustrated in Figure 1A.

Glycine and GABA are known to exert powerful inhibitory effects on spinal neurons. For example, in cats and monkeys, iontophoretic delivery of GABA or glycine onto STT cells elicited a profound and dose-related inhibition of their responses to cutaneous mechanical stimuli (Lin, 1996a; 1996b; Sorkin and Puig, 1996; Sorkin et al., 1998). Likewise, iontophoresis of glycine or GABA in the cat dorsal horn diminished the responsiveness of spinal neurons to light tactile stimulation (light touch/light pressure), and decreased the size of the cutaneous receptive fields (Zieglgänsberger and Herz, 1971). Conversely, application of the $GABA_A$ receptor antagonist, bicuculline, or the glycine receptor antagonist, strychnine into the dorsal horn increased both the background activity and the responses to cutaneous mechanical stimuli (Lin et al., 1996; Sorkin et al., 1998; Willcockson et al., 1984). These data suggest a tonic inhibition of STT cells by GABAergic and glycinergic neurons. The intrathecal (i.t.) injection of bicuculline and strychnine has also been shown to induce a long-lasting increase in the responsiveness of dorsal horn neurons to low intensity, Aβ-fiber stimulation

resembling the hyperexcitability of allodynia (Sivilotti and Doubell, 1994). Finally, genetic variants such as the Poll Hereford calf (Gundlach et al., 1988) and the spastic mouse (White and Heller, 1982), which exhibit up to 10-fold decrease in STR binding in the spinal cord, display exaggerated sensitivity to even modest cutaneous stimulation.

Collectively, these studies provide convergent evidence that low threshold Aβ-fibers activate local GABAergic and glycinergic interneurons that regulate the excitability of WDR neurons (Carlton et al., 1996; Millan, 1999), and support for the modulatory effects of GABA and glycine in spinal sensory processing. Thus, the encoding of a low-threshold mechanical stimulus as an innocuous event may depend on the presence of intrinsic GABAergic and/or glycinergic inhibition in the spinal dorsal horn.

1.4 Evidence for Spinal Disinhibition After Neural Injury and Experimental Models of Allodynia

By virtue of its location and anatomy, the spinal cord is vulnerable to injuries that commonly precede the onset of clinical allodynia (Tasker, 1990). For example, infarction of the anterior spinal artery (anterior spinal artery syndrome) leads to painful burning dysesthesia below the level of the spinal lesion which is refractory to opioid, anticonvulsant and antidepressant therapy (Triggs and Beric, 1992). Traumatic spinal cord injury is also a common cause of neuropathic pain. This is generally characterized by diffuse burning dysesthetic sensations (including allodynia) distal to the level of spinal injury (Davidoff et al., 1987; Yezierski, 1996).

That the spinal cord is a major site of dysfunction in neuropathic pain is not surprising given the fact that the dorsal horn contains the first synapse in pathways subserving nociception.

Animals subjected to central or peripheral nerve injury exhibit a reduction in the functional tone of spinal GABAergic and glycinergic interneurons. For example, photochemically-induced spinal cord ischemia in the rat produced severe mechanical allodynia, and a significant decrease in GABA-LI in lamina I-III of the irradiated dorsal horn (Demediuk et al., 1989; Hao et al., 1991; Zhang et al., 1994). Electrophysiological studies revealed enhanced sensitivity of spinal WDR neurons to electrical, and low threshold mechanical stimulation that persisted 10-20 days after focal spinal ischemia corresponding to the behavioural data (Hao et al., 1992a). This sensory dysfunction was limited to Aβ afferent fiber input (Hao et al., 1992a, b); the responses to Aδ/C fiber activity remained normal. Of relevance to the spinal disinhibition model of allodynia is the observation that the recovery from allodynia parallelled the return of GABAergic tone in the spinal dorsal horn (Zhang et al., 1994).

Similar observations have been reported following partial ligation of the sciatic nerve; an experimental model of peripheral neuropathy. Thus, GABA-LI was significantly decreased on the side ipsilateral to the sciatic nerve ligation (Ibuki et al., 1997; Eaton et al., 1999). Abnormal pain-related behaviour, including cold and tactile allodynia and thermal and tactile hyperalgesia, parallelled these immunohistochemical changes in the spinal dorsal horn. Extracellular recordings of WDR neurons revealed a significant increase in the frequency of spontaneous

discharges, and the responsiveness to brush and light pressure (Yakhnitsa et al., 1999). Interestingly, spinal cord stimulation (SCS) depressed the activity of WDR neurons, and produced a marked and long-lasting increase in the threshold of activation by tactile input (Meyerson et al., 1995; Yakhnitsa et al., 1999). These inhibitory effects were associated with a significant increase of GABA concentration in spinal microdialysate samples (Linderoth et al., 1994; Stiller et al., 1996). These results provide further evidence for the role of GABAergic modulation of the low threshold (A-fiber) mediated WDR neuron hyperactivity in peripheral neuropathy.

In turn, pharmacological blockade of spinal GABAergic and glycinergic tone with bicuculline and strychnine enhanced the already established hyperalgesia and allodynia following sciatic nerve ligation (Yamamoto and Yaksh, 1993; Satoh and Omote, 1996; Hwang and Yaksh, 1997). Conversely, tactile and cold allodynia and hyperalgesia were completely blocked by a single dose of i.t. GABA, and significantly reduced by cultured GABAergic cells grafted into the subarachnoid space close to the spinal dorsal horn (Eaton et al., 1999). These results suggest that a local supply of GABA in the spinal dorsal horn was able to reverse the development of allodynia following peripheral nerve injury. Finally, a bilateral reduction in the population of STR-sensitive glycine receptors in the spinal dorsal horn has been reported after unilateral constriction of the rat sciatic nerve (Simpson and Hwang, 1998).

Even in the absence of nerve injury, a reduction in spinal GABAergic and/or glycinergic tone evokes robust allodynia in experimental animals. Cumulative evidence indicates that normally innocuous mechanical stimulation acquires

nociceptive characteristics during the pharmacological blockade of spinal glycine or $GABA_A$ receptors (Yaksh, 1989; Sherman and Loomis, 1994). Thus, the i.t. injection of sub-convulsive doses of strychnine or bicuculline induced an exaggerated excitatory state in spinal cord whereby innocuous tactile stimulation evoked behavioral, autonomic and neurochemical responses characteristic of a noxious event (Beyer et al., 1985; Yaksh, 1989; Sherman and Loomis, 1994; 1995). The resulting allodynia was: 1) evoked by low threshold Aβ-fiber activity; 2) only elicited by light brushing of the hair at circumscribed sites corresponding to the spinal segments affected by i.t. strychnine or bicuculline (segmentally organized; Sherman and Loomis 1994); 3) reversible and reproducible; 4) unaffected by pharmacological treatments that interfere with high-threshold C-fiber input (e.g., morphine, capsaicin, substance P antagonists) (Triggs and Beric, 1992); and 5) attenuated by i.t. glycine, the glycine pro-drug, milacemide or the $GABA_A$ agonist, muscimol (Hwang and Yaksh, 1997; Khandwala and Loomis, 1998).

In summary, there is growing evidence that disruption of GABAergic and/or glycinergic modulation in the spinal dorsal horn, either pharmacologically or by nerve injury, yields an exaggerated sensitivity to otherwise innocuous stimuli (Figure 1A, B). In the absence of this inhibitory modulation, Aβ-fibers appear to activate cellular mechanisms normally limited to nociceptive signalling; and only recruited by repeated, high-threshold C-fiber input.

1.5 Evidence for the Role of Prostaglandins in Normal Pain and Hyperalgesia

One of the notable biochemical mechanisms enhancing pain signalling in the

spinal cord is the production of prostaglandins (PGs). This mechanism is completely independent of the well-known role of peripheral PGs in pain and inflammation. That PGs might be generated within the central nervous system (CNS) in response to repeated C-fiber input was first suggested by Malmberg and Yaksh (1992a). This was based on the observation that non-steroidal anti-inflammatory drugs (NSAIDs), injected directly into spinal subarachnoid space of conscious rats, elicited dose-dependent inhibition of the behavioral responses evoked by formalin injection into the foot pad (Malmberg and Yaksh, 1992a). NSAIDs, given spinally, were 100-1000 times more potent than systemic administration in inhibiting the second phase of the formalin test (Malmberg and Yaksh, 1992a). Intrathecal NSAIDs were also effective in inhibiting glutamate- or substance P-induced hyperalgesia in the rat (Malmberg and Yaksh, 1992b). These results implicate a spinal antinociceptive effect of NSAIDs that is distinct from their usual anti-inflammatory actions, and are consistent with a central as well as peripheral site of action in pain. The spinal antinociceptive effect of NSAIDs was found to be stereospecific (Jett et al., 1999), suggesting that the inhibition of cyclooxygenase in the spinal cord, and thus the suppression of central PG synthesis, is the mechanism responsible.

The central production of PGs in response to high-threshold nociceptive input has been confirmed in *in vivo* and *in vitro* experiments. The injection of carrageenan/kaolin into the knee joint of the rat evoked persistent pain behaviour and a time-dependent increase in the concentration of PGE_2 in spinal microdialysis samples (146 ± 11% and 143 ± 18% of baseline 10 min and 24 h after knee joint

carrageenan/kaolin injection, respectively) (Yang et al., 1996). An increase in PGE_2 concentration of 109 ± 10% and 83 ± 15% of baseline was also reported during the first and second phase of the rat formalin test, respectively (Malmberg and Yaksh, 1995b,c). Whereas both pain behaviour and the increase in PGE_2 concentration were suppressed by i.t. S (+)-ibuprofen, the R(-)-enantiomer had no effect (Malmberg and Yaksh, 1995c; Yang et al., 1996). The i.t. injection of substance P, which induces hyperalgesia in the rat, also elicited a dramatic increase (362 ± 37% of baseline) in PGE_2 concentration in spinal CSF (Hua et al., 1999).

The ability of the spinal cord to generate PGs has been verified using an *in vitro* spinal superfusion model (Dirig and Yaksh, 1999). Spinal cords, harvested from rats that were pretreated with kaolin/carrageenan into knee joint for 5-72 h, exhibited an increased release of PGE_2 into the perfusion medium compared to control. Further elevations in PGE_2 concentrations were evoked by perfusion of these same spinal cords with substance P (0.1-1.0 μM) or capsaicin (0.1-10 μM). Thus, PGE_2 synthesis in the spinal cord is triggered by peripheral inflammation and by direct exposure to receptor ligands known to induce hyperalgesia in response to peripheral input.

The concept that PGs are generated centrally has been strengthened by reports that cyclooxygenase (COX), the prostanoid-forming enzyme, is constitutively present in the brain and spinal cord. Immunocytochemical and autoradiographic studies of the CNS have verified the presence of COX-LI in nociceptive pathways in the spinal cord (Goppelt-Struebe and Beiche, 1997; Willingale et al., 1997; Beiche et al., 1998a,b). This immunoreactivity was especially abundant in the

superficial dorsal horn where the nociceptive primary afferent fibers are known to terminate. COX-2-LI (one of the two isoforms of COX) was found in neurons of laminae II-III, motorneurons of lamina IX and in glial cells located in the spinal cord of untreated rats (Goppelt-Struebe and Beiche, 1997).

Cyclooxygenase in the spinal cord is also subject to induction. A transient increase (2-fold) in COX-2 mRNA, and a smaller increase in COX-2 protein, were detected bilaterally in the lumbar spinal cord of the rat following acute carrageenan-induced, peripheral inflammation (Goppelt-Struebe and Beiche, 1997; Ichitani et al., 1997; Hay et al., 1997). Western blot analysis revealed a 1.6-fold increase in the level of COX-2 protein in the lumbar dorsal horn (lamina II-III) 22 days after the onset of adjuvant-induced arthritis in the rat (Goppelt-Struebe and Beiche, 1997; Beiche et al., 1998a,b). These results indicate that the COX-2 is also inducible in the CNS, and may be responsible for the increase in spinal prostanoid synthesis and release during peripheral inflammation.

The localization of COX in the dendrites of central excitatory neurons in the spinal cord supports a role for PGs in the modulation of synaptic signalling (Yamamoto and Yaksh, 1993). This hypothesis is supported by the abundance of PGE_2 binding sites in the spinal dorsal horn (Onoe et al., 1992; Matsumura et al., 1992; 1995). These appear to be located on the terminals of primary afferent fibers, since these binding sites almost completely disappeared after dorsal rhizotomy. Their density on the operated side was only 4 ± 4% of the control side, 8 days after surgery (Matsumura et al., 1995). This pattern of PGE_2 receptor binding is consistent with a presynaptic facilitation of nociceptive signalling.

In summary, there is considerable evidence that PGs are synthesized and released in the spinal cord in response to nociceptive (C-fiber) input. The temporal correlation of this central biochemical process with pain behaviour and peripheral inflammation indicates that spinal PGs may be critical in the induction of a central pain state at the spinal level.

1.6 Central NMDA Receptor Activation and the Effects of Spinal Prostaglandins

Persistent C-fiber input from peripheral inflammation or repeated high-threshold electrical stimulation effects a rapid change in the sensitivity of spinal dorsal horn neurons. This process, known as wind-up, reflects the slow temporal summation of C-afferent fiber-evoked responses and is thought to be an important central mechanism of hyperalgesia (Mayer, et al., 1999). This process is triggered by the co-release of glutamate and substance P from the terminals of primary afferent C-fibers. Acting at NMDA- and NK1-receptors on the membranes of dorsal horn neurons (Wall and Woolf, 1986; Dickenson et al., 1997; Yaksh et al., 1999b), these neurotransmitters elicit prolonged depolarizations. Pharmacological studies have consistently identified the NMDA receptor as an essential feature in the development of wind-up (see Figure 2) (Dickenson and Sullivan, 1990; Yaksh et al., 1999b). Indeed, all aspects of the hyperalgesic state, regardless of the initiating event, are reversed by agents that block spinal NMDA receptors (Chaplan et al., 1997; Yaksh, 1999a).

The NMDA receptor normally remains inactive, even in the presence of released glutamate, because it is partially blocked by Mg^{++}. Prolonged depolariza-

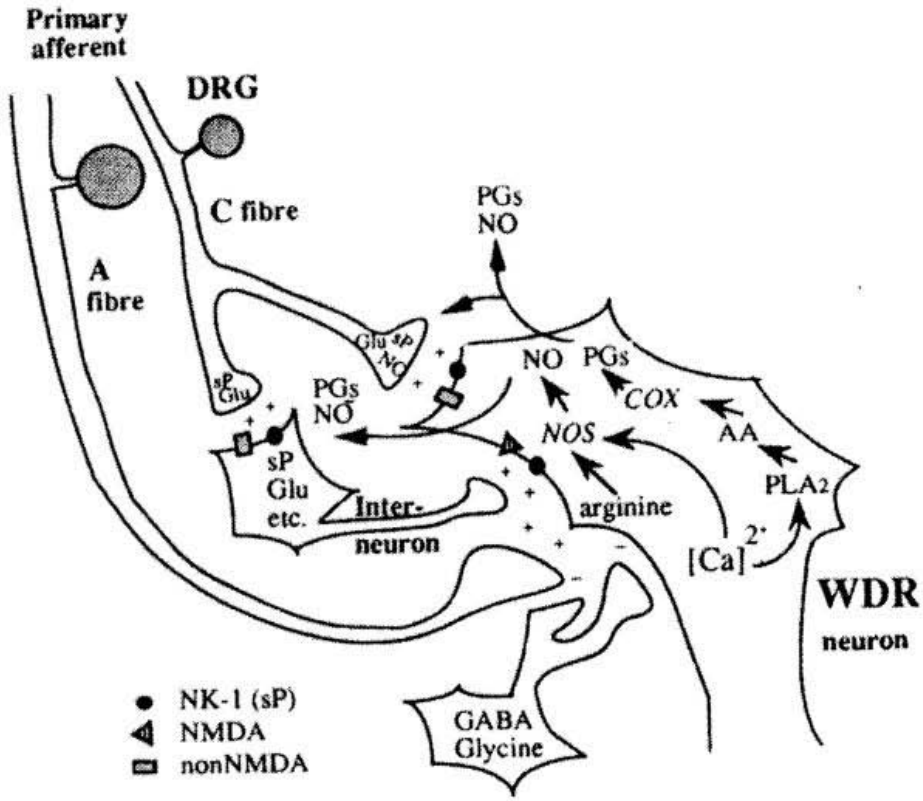


Fig. 2 A schematic summary of the functional organization of elements in dorsal horn showing the NMDA receptor activation and PG release. The primary afferent C-fibers contain and release both peptide and excitatory amino acid products. Primary A-fibers contain and release excitatory amino acids. It is believed that glutamate activates the second order neurons via non-NMDA receptors on sites postsynaptic to A or C afferent input. Excitatory interneurons, excited by an appropriate afferent barrage, activate second order neurons via NMDA receptors. This leads to an increase in intracellular Ca^{++} and the activation of a number of enzymes (e.g., COX, NOS) (see text for details). Inhibitory interneurons, activated by A fibers (not shown in the graph), release GABA or glycine and modulate the excitability of second order neurons. Abbreviations: AA: arachidonic acid, COX: cyclooxygenase, DRG: dorsal root ganglion, Glu: glutamate, non-NMDA: non-N-methyl-D-aspartic acid receptor, NK-1: neurokinin-1 receptor, NO: nitric oxide, NOS: NO synthase, NMDA: NMDA receptor, PG: prostaglandin, PLA_2: phospholipase A_2, sP: substance P, WDR: wide dynamic range (Adapted from Yaksh et al. 1999b).

tion evoked by substance P is thought to remove the voltage-dependent Mg^{++} block of the NMDA receptor. This allows glutamate to exert its agonist effect at the NMDA receptor-channel complex. The opening of this channel leads to a large influx of Ca^{++} into the cytosol and the initiation of a cascade of intracellular events (MacDermott et al., 1986; Chaplan et al., 1994) including: 1) the translocation/activation of protein kinases such as protein kinase C (Sluka and Willis, 1997; Ramakers, et al., 1997; Millan, 1999); 2) the release of arachidonic acid from the cell membrane into the cytosol; 3) the activation of COX and nitric oxide synthase (NOS); and 4) the generation of prostanoids and NO within the cell. The latter are thought to diffuse from the perikarya to adjacent cells where they promote excitatory neurotransmitter release from primary and non-primary afferent terminals (Yaksh, 1999a; Yaksh et al., 1999b; Milan, 1999) (Figure 2).

The exact mechanisms by which central PGs enhance nociceptive signalling have yet to be elucidated. One strong possibility is that PGs exert their effects through excitatory receptors located on primary afferent terminals that form synaptic connections with second order neurons and/or excitatory interneurons in the dorsal horn (Millan, 1999). In this regard, PGE_2 has been shown to evoke Ca^{++}-dependent release of glutamate from synaptosomes of rat spinal cord (maximum effect at 1 nM) (Nishihara et al., 1995), and substance P from cultured rat dorsal root ganglion (DRG) cells (Vasko et al., 1994) or spinal cord slices (Vasko, 1995). The effect of PGE_2 (100nM) on substance P release was inhibited by the selective EP_1-receptor antagonist, SC19220, and by guanosine-5'-[beta-thio] diphosphate, an inhibitor of stimulatory G-protein (Gs) to which the EP_1 receptor is coupled (Cui and Nicol,

1995; White, 1996). Conversely, guanosine-5'-[gamma-thio] triphosphate, an activator of Gs protein, enhanced PGE_2-evoked release of substance P. Perfusion with low concentrations of PGE_2 has also been shown to facilitate capsaicin- or bradykinin-evoked release of substance P or CGRP from rat spinal cord slices (Hingtgen et al., 1995; Vasko, 1995). Collectively, these results indicate that PGE_2, acting through G-protein-coupled receptors, can directly effect neurotransmitter release from the spinal cord, as well as augment the release of neuropeptides from the spinal terminals of C-fibers evoked by known algogenic agents.

PGs may also enhance the excitability of dorsal horn neurons to afferent input by a direct postsynaptic effect. In an early study, microiontophoretic application of PGE_1 to motorneurons and interneurons in the isolated spinal cord of the frog induced an abrupt excitatory effect (Coceani and Viti, 1975). More recently, PGE_2 was shown to induce a long-lasting facilitation of evoked excitatory postsynaptic currents of dorsal horn neurons in mice (Minami et al., 1999). The "wind-up" of a spinal C-fiber nociceptive reflex, induced by repeated electrical stimulation of the sural nerve, was also dose-dependently inhibited by the i.t. or i.v. administration of indomethacin, and by i.v. administration of the selective COX-2 inhibitor, SC58125 (Bustamante et al., 1997; Willingale et al., 1997). While these studies do not preclude a change in neuronal excitability secondary to an increase in glutamate and neuropeptide release, they do provide further support for the positive modulatory effect of PGs on spinal neurotransmission.

In summary, there is substantial evidence that: 1) PGs are synthesized in and released from the spinal cord in response to noxious (high-threshold) C-fiber

input; 2) central PG synthesis is coupled to NMDA receptor activation and the intracellular activation of COX; and 3) PGs, generated locally in the spinal cord, play a role in the cellular events that underlie hyperalgesia.

1.7 Central Prostaglandins and Allodynia

The ability of PGs to sensitize spinal cord neurons to high-threshold (C-fiber) somatosensory input raises an important question about their possible role in abnormal pain states (e.g., neuropathic pain and allodynia). Specifically, do low threshold mechanoreceptive (Aβ) primary afferent fibers activate a similar prostanoid-sensitizing mechanism in the spinal cord in allodynia?

Preliminary evidence suggests that the answer to this question is yes. Intrathecal ketorolac or S (+)-ibuprofen suppressed the hair deflection (HD)-evoked increase in heart rate, blood pressure and catechol oxidation current in the locus coeruleus of i.t. strychnine-treated rats (Hall et al., 1999). The inactive R (-) isomer of ibuprofen was without effect indicating that the attenuation of these allodynic responses was related to the inhibition of COX in the spinal cord. Messenger RNA hybridization analysis revealed a 3-fold increase above control in the level of COX-2 mRNA in the rat lumbar spinal cord 2-4 h after unilateral intraplantar injection of Freund's complete adjuvant (FCA) (Hay et al., 1997). This was followed by a significant increase in the spinal concentration of 6-keto $PGF_{1\alpha}$ and PGE_2 (maximal effect 8 h after FCA injection). These changes were temporally correlated with a decrease in the weight-bearing capacity of the affected paw (a quantitative measure of allodynia). The s.c. administration of indomethacin, or the COX-2 selective

inhibitor, flosulide, attenuated the increase in spinal PG concentration, and inhibited allodynia by 80-100%. These drugs had no effect on the development of mechanical hyperalgesia (Hay et al., 1997). The results of these studies provide indirect evidence for the role of spinal PGs in the development of allodynia, whether induced by peripheral inflammation or by central disinhibition.

If spinal PGs are relevant to the cellular changes that underlie allodynia in the CNS, then their direct injection into the spinal subarachnoid space should yield a measurable allodynic state. The i.t. injection of PGE_2, PGD_2 or $PGF_{2\alpha}$ in conscious mice elicited dose-dependent, touch-evoked agitation. The allodynic effect of PGE_2 was blocked by the receptor antagonist, NON-NT-012 suggesting that this was mediated by EP_1 receptors (Uda et al., 1990; Minami et al., 1995b). Delivery of PGE_2 through a microdialysis probe implanted in the spinal subarachnoid space of rats triggered an immediate increase in the concentration of glutamate, aspartate, taurine, glycine and GABA in dialysate samples (Malmberg et al., 1995a). These neurochemical responses were temporally correlated with the onset and decline of behaviourally-defined allodynia (Malmberg et al., 1995a). The combination of PGE_2 (10 μM) and capsaicin (0.1 or 1.0 μM), concentrations that individually had no effect, evoked a significant increase (60–100%) in glutamate, aspartate, taurine, glycine and GABA concentration and produced tactile allodynia. Thus, the introduction of exogenous PGs into the spinal subarachnoid space induces neurochemical changes and behavioural responses consistent with the development of tactile allodynia in experimental animals. However, the localization and spinal pharmacology of this prostanoid modulation have not been well investigated,

especially in the bicuculline model of allodynia.

1.8 Hypothesis and Specific Objectives

Increased glutamatergic tone activating spinal NMDA receptors leads to a state of facilitated neurotransmission of high-threshold (C-fiber) input and hyperalgesia in the spinal cord. PGs, synthesized and released within the CNS in response to C-fiber input, appear to play a major role in this facilitation. Sustained C-fiber activity induces a rapid increase in the expression of COX-2 and the synthesis of PGs. Because this central sensitization process is dependent on NMDA-receptor activation, it is normally only recruited by high-threshold (nociceptive) input.

Allodynia is an abnormal sensory state in which pain is triggered by innocuous stimuli. Peripheral or central nerve injury appears to reduce the functional tone of spinal glycine and/or $GABA_A$ inhibitory modulation, thereby permitting innocuous tactile stimulation to be interpreted as pain. Indeed, the pharmacological blockade of spinal glycine receptors with i.t. strychnine or $GABA_A$ receptors with i.t. bicuculline induces a selective and reversible allodynic state. In lightly anaesthetitized rats given i.t. strychnine or bicuculline, HD induces cardiovascular, motor and neurochemical responses comparable to those evoked by noxious thermal, mechanical or chemical stimulation in the absence or bicuculline or strychnine (Sherman and Loomis, 1994, 1995; Khandwala et al., 1997; Loomis et al., 2001). The hypothesis of this thesis research is that during allodynia, low threshold mechanoreceptive input activates a PG-mediated

sensitization mechanism in the spinal cord. The overall goal of this research was to characterize the spinal pharmacology of bicuculline-allodynia with an emphasis on the role of spinal PGE_2(the prostanoid most frequently associated with facilitated pain states in the spinal cord). The specific objectives of the research were:

1. To determine the magnitude and area of allodynia following the single topical application of bicuculline to the dorsal surface of the rat spinal cord (spinal topical application).
2. To determine the time course and area of allodynia following repeated spinal topical application of bicuculline.
3. To determine the effect of the COX-2 inhibitor, NS-398, on allodynia following spinal topical application of bicucullline.
4. To determine the effect of AP-7 (an NMDA receptor antagonist) and SC-51322 (EP-receptor antagonist) on allodynia following spinal topical application of bicuculline.
5. To determine the effect of i.t. AP-7 and SC-51322 on i.t. PGE_2-induced allodynia in conscious rats.
6. To determine effect of i.t. pretreatment with bicuculline on PGE_2-induced allodynia in conscious rats.

2 METHODS

2.1 Animals

All experiments were conducted using male, Sprague-Dawley rats (330-400g at the time of experiments), obtained from the Vivarium of Memorial University of

Newfoundland. Animals were housed in the Animal Care Facility, with a room temperature of 22° C, and a 12 h light-dark cycle (lights on 0700 h). Rats had free access to rat chow and tap water. All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Memorial University Animal Care Committee.

2.2 Anaesthetized Animal Experimentation

2.2.1 Laminectomy and Recording

Surgical anaesthesia was induced with halothane (4% in oxygen) until the left jugular vein was cannulated. Thereafter, anaesthesia was maintained with i.v. urethane (10% w/v in saline; Sigma Chemical, Inc.). The initial dose of urethane (1.1g/kg) was infused slowly over 5-10min as the anaesthetic effect of halothane declined. Anaesthesia was supplemented with i.v. urethane (0.1g/kg) as required during the experiment. The trachea was cannulated and the animal was allowed to breathe spontaneously. The left carotid artery was cannulated for monitoring blood pressure and heart rate. The incision was then closed and the rat was placed in a supine position for laminectomy.

A skin incision (3-4cm in length) was made along the midline of the back, corresponding to the thoraco-lumbar region of the spinal cord. The muscles were then separated from the spinal column by blunt dissection. The spinous processes and part of the laminae of the vertebrae (T13-L1) were removed carefully using a rongeur. The rat was then placed in a stereotaxic apparatus (Narishige, Tokyo, Japan) with the head firmly secured using ear bars. The dura mater and arachnoid

membrane were gently removed with the aid of a dissecting microscope to expose the dorsal surface of the spinal cord. Endogenous CSF production was sufficient to keep the exposed surface of the spinal cord (length by width, 1.5 x 0.5cm) from drying throughout the experiment. Excess CSF was absorbed with a soft paper wick.

Blood pressure and heart rate were continuously monitored using a pressure transducer (P23XL) and polygraph (Model 79E, Grass Instruments, Mass, USA). Cortical EEG activity was recorded continuously using two subcutaneous needle electrodes (E2, Grass Instruments) placed 2 mm left of the midline, one extending rostrally, entering the skin near bregma, the other extending caudally, entering the skin about 2 mm caudal to the first. Body temperature was maintained at 36-37°C with a thermostatically regulated blanket (Harvard Apparatus). The animal was allowed to stabilize for at least 1h before experiments. All the experiments were conducted at a light plane of anaesthesia. Light anaesthesia was defined as the presence of an EEG pattern fluctuating between synchrony and desynchrony, with synchrony present for not more than 60% of the time (Sherman and Loomis, 1994). Animals were killed with an overdose of urethane at the end of the experiment.

2.2.2 General Protocol

Following a 1-h stabilization period, rats were given a single application of 0.9% saline (0.1 μl) to the dorsal surface of the spinal cord. Brushing the hair (HD; hair deflection) was performed with a cotton-tipped applicator using no more force than required to move the applicator through the hair such that only the pelage was

disturbed. The HD stimulus (2-min duration repeated every 5 min) was applied bilaterally to the caudal dermatomes including the hind leg, foot and lower back. Allodynia was induced by applying bicuculline (in 0.1 μl sterile saline) to the left or right side of the spinal cord followed 5 min later by HD. The HD stimulus was used to identity the sites of allodynia; subsequent stimulation was restricted to that sites(s). The maximum HD-evoked change in mean arterial pressure (MAP), heart rate (HR) and the duration of motor responses (MR) were recorded as previously described (Sherman and Loomis, 1994). HD was repeated every 5 min until no evoked responses were detected. All animals were allowed to recover for at least 30 min before further drug application.

To determine the dose-response relationship of spinal topical bicuculline, four separate sub-convulsive doses (0.01, 0.03, 0.1, 0.3 μg) were tested (maximum of 2 doses per animal). In separate animals, the effect of repeated spinal topical dosing with bicuculline was also determined (0.1 μg bicuculline given once every 20 min for 2h). All drugs were applied to the same site on the spinal cord as defined by anatomical landmarks observed through the dissecting microscope (Olympus 255040, Tokyo, Japan).

In a further group of rats, 0.1 μg of bicuculline (in 0.1 μl saline) was given topically to the spinal cord as a positive control test. After a complete recovery from allodynia, rats were pretreated with one of the following: NS-398 (0.1, 1.0, 1.5, 5.0 μg), 20-min pre-treatment; SC-51322 (0.1, 1.0, 4.0, 8.0 μg), 15-min pre-treatment; and AP-7 (0.01, 0.1, 1.0 μg), 20-min pre-treatment. Bicuculline (0.1 μg) was repeated every h thereafter until the effects of the pretreatment had completely

disappeared. The maximum HD-evoked change in MAP, HR and MR following each dose of bicuculline was determined. All drugs, including bicuculline, were applied to the same site on the dorsal surface of the spinal cord.

To examine the extent of drug diffusion after topical drug administration to the spinal cord, a fluorescent tracer, bisbenzimide, was added to the bicuculline solution (0.1% w/v each) and applied to the dorsal spinal cord. Twenty minutes later, the animals were perfused with 4% paraformaldehyde transcardially and spinal cords (2 cm, including lumbar segments) were removed. Transverse sections (40 μm) of the lumbar spinal cord were cut using a vibratome (Vibratome Series 1000 Technical Products International, Inc. St. Louis, MO, USA). The sections were analyzed and photographed under UV illumination using a fluorescent microscope (Carl Zeiss MC 63, Germany).

2.2.3 Drugs

Bicuculline [(-)-bicuculline methiodide, MW: 509.3], AP-7 [(+/-)-2-amino-7-phosphono-heptanoic acid, MW: 219.2], and bisbenzimide (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) were obtained from Sigma (St. Louis, USA). NS-398 [N-(2-cyclohexyloxy-4-nitrophenyl)-methanesulfonamide, MW: 314.4] and SC-51322 [8-chlorodibenz(b,f)(1,4)oxazepine-10[11H]-carboxyl acid, 2-(3-(2-[furyanylmethyl]thio)-1-oxopropyl)hyrazide: MW: 457.9) and were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, USA). Bicuculline and AP-7 were dissolved in 0.9% sterile saline (Astra Pharma Inc.). Bisbenzimide was

dissolved with bicuculline in sterile saline. NS-398 and SC-51322 were dissolved in 70% and 90% DMSO (in water), respectively (BDH Inc., Toronto, Canada).

The volume of drug solution applied to the dorsal surface of the spinal cord ranged from 0.05-0.2 μl. All drugs were administered by means of a hand-held microsyringe (1 μl) with the aid of a dissecting microscope (Olympus 255040, Tokyo, Japan). Specific structures on the dorsal surface of the spinal cord such as blood vessels and nerve root bundles were used as landmarks to apply drugs precisely to a specific site each time. Drugs were applied immediately after soaking up the CSF with a paper wick. Only one dose of a pretreatment drug was used in each animal.

2.2.4 *Data Analysis*

The maximum HD-evoked change in MAP (mm Hg), HR (bpm) and MR (min) was used for all data analyses. The change in MAP and HR was calculated relative to the immediate pre-stimulus control (not relative to T=0). The maximum change in MAP or HR observed in the 1-minute interval immediately before stimulus application was subtracted from the maximum value observed during HD. Variability associated with single measurements is indicated by the SEM, while variability associated with blocks of data is indicated by the pooled 95% confidence intervals (95%CI). Repeated measures one-way analysis of variance (ANOVA) followed by the Neuman-Keuls multiple comparison test was used to detect statistical differences within treatment groups over time (pretreatment data). Completely randomized ANOVA followed by the Neuman-Keuls multiple

comparison test was used to identify statistical differences between treatment groups (dose-response data). Dose-response curves were fitted using least squares regression analysis. $P<0.05$ was considered to be statistically significant. The methods of data analysis were based on general statistics texts and the program Sigmastat for Windows Version 2.03 (SPSS Inc.).

2.3 Conscious Animal Experimentation

2.3.1 Implantation of Intrathecal Catheter

The i.t. catheter was constructed from a segment of PE-10 tubing stretched to two times its original length to reduce its diameter. A small loop was made at one end of the tubing and secured with nail polish. The tubing was cut to a length of 8.5 cm from the loop for insertion into the spinal subarachnoid space. The tubing was filled with sterile saline before implantation.

Surgical anaesthesia was induced by placing the rat in a transparent plexiglass box filled with 4% halothane in oxygen. The rat was then transferred to a stereotaxic apparatus (Narishige, Tokyo, Japan) with the head firmly secured using ear bars. Anaesthesia was maintained with 2.5% halothane in oxygen. The hair was shaved at the back of the head and swabbed with 10% povidone iodine topical solution U.S.P. and 75% ethanol. An incision (approximately 1 cm in length) was made along the midline at the base of the skull. The muscles were cut transversely and carefully separated to expose the dural membrane of the cisterna magna. A small puncture was made in the dural atlanto-occipital membrane using a 16G needle. Leakage of a small amount of cerebrospinal fluid (CSF) through the

hole verified penetration into the subarachnoid space. This was immediately absorbed with a cotton-tipped applicator.

The i.t. catheter was inserted through the hole and carefully guided 8.5 cm caudally in the spinal subarachnoid space so that the catheter tip reached the L5-L6 spinal segments. The catheter was anchored in place by suturing the fixed loop at the rostral end to the overlying muscle and skin. The rostral tip was externalized through the skin on the top of the skull and sealed with a stainless steel plug. The incision was closed with sutures and swabbed with 10% povidone iodine solution. Ten ml of normal saline was then injected s.c. on the back of the animal for hydration.

Rats recovering from anaesthesia were immediately observed for signs of neurological damage, including hind limb weakness or paralysis, abnormal gait, locomotor difficulty and atypical behaviour. Rats free of neurological sequelae were returned to the Animal Care Facility where they were housed individually in regular plexiglass cages. They were allowed to recover for at least 3 days prior to experimentation. Animals exhibiting symptoms of neurological damage were immediately killed by an overdose of urethane.

2.3.2 *General Protocol*

On the day before the experiment, rats were transported from the Animal Care Facility to a quiet behavioural room in the research laboratory. They were kept in their home cages at a room temperature of 22° C using a 12-h light-dark cycle (lights on 0700 h). Free access to rat chow and tap water was provided. All

behavioural experiments were conducted at the same time period (14:00-20:00 h) each day. Rats were gently and repeatedly handled by the investigator for one h before the start of the experiment. During this time, rats were also brushed with a cotton-tipped applicator at 5-min intervals to acclimatize them to the HD stimulus. All drugs were injected i.t. by slow infusion (20 μl over 1.5~2 min). Injections were made using a hand-held 25-μl microsyringe to which was attached a segment of PE-10 tubing. The latter was used as a flexible extension to connect the microsyringe to the externalized tip of the i.t. catheter. Drug solutions were loaded into the PE-10 extension from the microsyringe such that all drugs were injected in a volume of 10 μl and flushed with 10 μl of normal saline (i.t. catheter volume: 6~8 μl).

To assess baseline responses, animals were injected with 20 μl of normal saline (equivalent to 10 μl of drug solution + 10 μl saline flush) and continuously observed for spontaneous (without HD) and HD-evoked behaviour. These were scored according to the systems described below. One h later, a single dose of PGE_2 (0.8, 3.0, 8.0, 20 μg) or vehicle (3% ethanol in water) was injected and the behavioural scoring repeated until no further spontaneous or evoked responses were observed. In separate experiments (designed to study the pharmacology of the PGE_2-allodynia), a fixed dose of PGE_2 (8 μg) was injected i.t. in rats pretreated with one of the following: AP-7: (0.4 μg), 20-min pretreatment; SC-51322: (6, 30, 100 μg), 15-min pretreatment; Bicuculline: (0.01 μg), 5-min pretreatment; or Vehicle: water or 90% DMSO in water. The spontaneous and HD-evoked behaviours were

scored as described below. All animals were monitored individually throughout the experiment and were used no more than twice. Rats were killed with an overdose of urethane. In all experiments, the investigator was blinded to the identity of the drug/vehicle treatment.

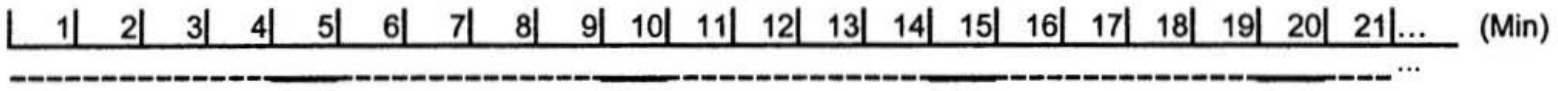
2.3.3 Behavioural Scoring System

The protocol for the evaluation of behaviour, with and without HD, is illustrated in Figure 3. The behaviour of each rat observed without HD was monitored continuously for a 4-min period. The maximum score achieved was recorded in a computer every 30 sec for a total of 8 scores per rat per 4-min observation period. At the conclusion of this observation period, HD was applied continuously for 1 min to the affected dermatome(s). The latter were determined by brushing the hair over the back, flanks, limbs, and hind paws until a response was observed. The maximum score evoked by HD was recorded in a computer at the end of the 1-min stimulus period. This cycle was then repeated until no behavioural responses (spontaneous or HD evoked) were detected.

Behaviour in the absence of HD was rated using the following scoring system (as modified from Malmberg et al., 1995a, and Ishikawa et al., 2000).

Score	**Behaviour**
0	Normal behaviour (bright, alert, and exploring)
1	Huddling, burrowing or hiding
2	One of the following: stationary with one paw elevated, limping on movement, piloerection, occasional vocalization, attention directed to the affected site

Figure 3. Protocol for the evaluation of behaviour in conscious PGE_2 treated rats.



Note: Each number indicates the time in minutes. Intrathecal PGE_2 was given at time t=0. The solid bars under the time scale indicate the time periods during which HD-evoked behaviour was evaluated. During intervals indicated by interrupted line, the behaviour in the absence of HD stimulus was scored. All behaviour was scored according to the scale modified after Malmberg (Malmberg and Yaksh 1995a).

Score	Behaviour
3	Two or more of following together: stationary with one paw elevated, limping on movement, piloerection, occasional vocalization, attention directed to the affected site
4	Frequent vocalization, circling (very agitated), licking, biting and scratching the affected dermatome(s)

HD-evoked behaviour was rated using the following (as modified from Malmberg et al., 1995a, and Ishikawa et al., 2000):

Score	Behaviour
0	Normal (curious, responsive and exploring)
1	Moderate effort to escape: walking away from the stimulus source (avoidance) or protecting the affected dermatome(s)
2	Moderate agitation: paw withdrawal, piloerection (back and flank), or occasional vocalization upon stimulation
3	Strong agitation (two or more of the following together): paw withdrawal; piloerection (back and flank); occasional vocalization upon stimulation
4	Vigorous effort to escape: attacking the applicator, frequent and persistent vocalization, circling the cage, licking, biting and scratching the affected dermatome(s)

2.3.4 Drugs

PGE_2 and SC-51322 were purchased from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, USA). AP-7 and bicuculine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PGE_2 was dissolved in ethanol and diluted with

sterile saline such that the maximum concentration of ethanol never exceeded 3% (v/v). AP-7 and bicuculline were dissolved in 0.9% sterile saline (Astra Pharma Inc.). SC-51322 was dissolved in DMSO (max 90% in water, BDH Inc., Toronto, Canada). All drug doses were administered in a volume of 10 μl.

2.3.5 Verification of Catheter Position

After the completion of the experiment, animals were injected with 10 μl of 5% lidocaine followed by 10 μl saline. Rats were observed for evidence of hind limb weakness or paralysis, indicative of the correct placement of the spinal catheter at the L5 or L6 lumbar levels. A laminectomy was also performed to visually confirm the position of the i.t. catheter in randomly selected rats.

2.3.6 Data Analysis

For behaviour observed without HD stimulation, the average of 8 scores was calculated for each rat per 4-min observation period and expressed as the percent of the maximum possible score (4). The HD-evoked score for each rat is also expressed as the percent of the maximum possible score (4). Time-course data represent the mean ± SEM of the percent maximum possible score of all animals in each treatment group. The cumulative behavioural score was calculated from the entire time-course curve (120 min) for each rat (equivalent to the area-under-the-time-course curve). Dose-response data represent the mean ± SEM of the cumulative behavioural scores for each dose of PGE_2. Completely randomized ANOVA followed by the Neuman-Keuls multiple comparison test was used to

identify the statistical differences between multiple treatment groups (dose-response data). The paired Student t-test was used to detect significant differences between two experimental conditions (before and after pretreatment) within animals. $P<0.05$ was considered to be statistically significant.

3 RESULTS

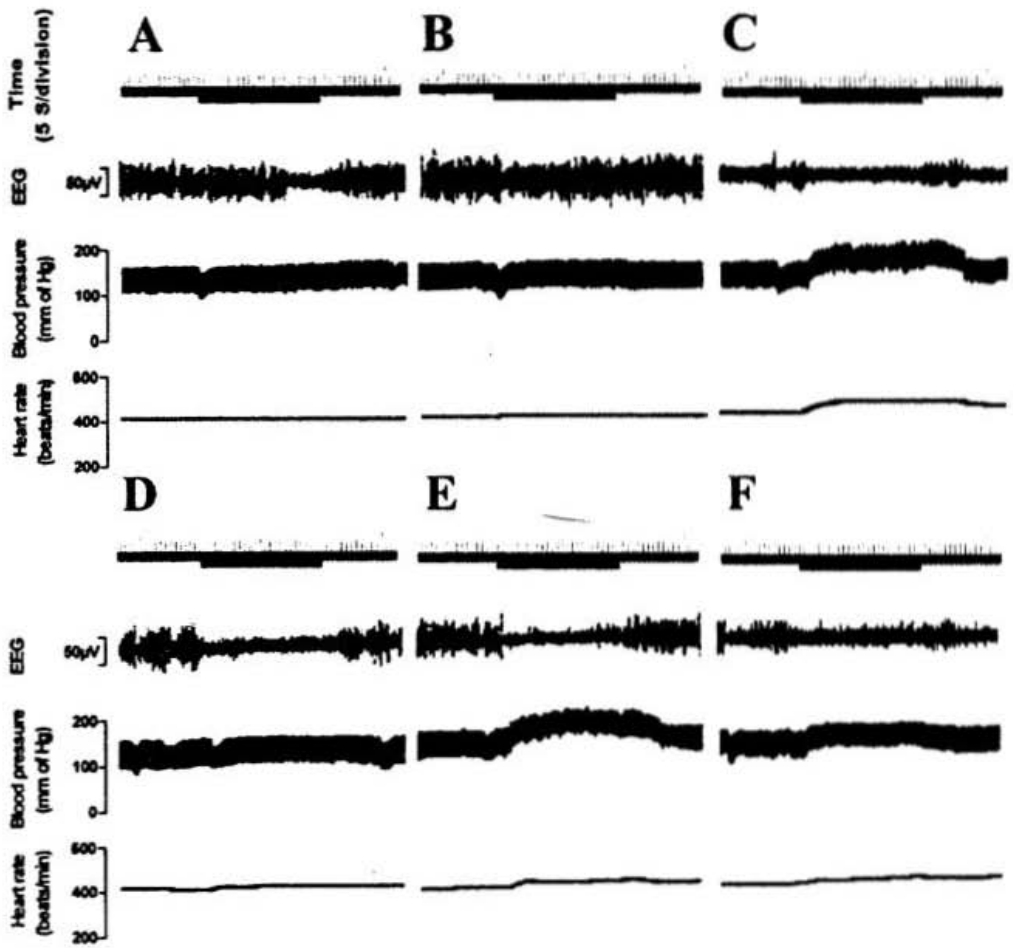
3.1 Spinal Topical Bicuculline-Induced Allodynia in Anaesthetized Rats

3.1.1 Unilateral Application of Bicuculline to the Spinal Cord Induces Localized Allodynia

Mild brushing of the hair (HD) with a cotton-tipped applicator evoked no cardiovascular changes or motor responses after saline application (0.1 μl) to the dorsal surface of the spinal cord (Figure 4B). In contrast, an identical HD stimulus applied to the hind leg or foot ipsilateral to the site of bicuculline application evoked a progressive increase in mean arterial pressure (MAP) and heart rate (HR) (Figure 4C) that persisted beyond the duration of HD. The allodynia arising from this unilateral treatment was normally restricted to a circumscribed area such as a single digit or the area between two digits on the ipsilateral hind paw (Figure 4G, Table 1). Maximum HD-evoked cardiovascular responses were normally observed during the first 2-min stimulus (5 min after bicuculline). These were accompanied by abrupt motor responses (MR) on the affected side (e.g., withdrawal of the hind leg, kicking, and/or scratching), as well as desynchrony of the EEG (Figure 4).

The cardiovascular responses disappeared gradually (0.5-3 min) after the discontinuation of HD. In most cases, termination of HD coincided with a change

Figure 4. Representative tracings of hair-deflection (HD)-evoked changes in blood pressure, heart rate and EEG in a urethane-anesthetized rat. **The top trace in each panel denotes time. HD (2 min duration), applied before and every 5 min after BIC, is indicated by the solid bar below the time trace. All the drugs or vehicle were applied topically to the right or left side of the dorsal spinal cord (see Methods). A fixed dose of BIC (0.1 μg) was used. A: Before any drug administration. B: 5 min after saline (0.1 μl) on the right side; HD on the right side. C: 5 min after BIC (0.1 μg) on the right side; HD on the right side. D: 25 min after NS-398 (1.0μg), 5 min after BIC (BIC was applied 20min later after NS-398. Both agents were applied to the same spot on the right side), HD on the right side. E: 2 h after NS-398 on the right side, 5 min after BIC (0.1 μg) on the left side (contralateral to NS-398 site), HD on the left side. F: 3 h after NS-398, 5 min after BIC (0.1 μg) on the same site as NS-398 (right side), HD on the right side. G: Shading indicates the areas of allodynia on the right hind paw following topical application of BIC (0.1 μg) to the right side of the dorsal spinal cord.**


A
B
C
D
E
F
Time
(5 S/division)
EEG
50µV
Blood pressure
(mm of Hg)
200
100
0
Heart rate
(beats/min)
600
400
200

G

Table 1. Comparison of Topical and Intrathecal Bicuculline-Induced Allodynia in the Rat.

Parameters	Topical	Intrathecal§
Cardiovascular and motor responses at equi-effective dose	MAP: 18.5 ± 3.21mmHg HR: 35 ± 5.75 bpm MR: 30.83 ± 3.96 min (BIC 0.1 μg)	MAP:16.67 ± 1.66 mmHg✳ HR: 30.83 ± 3.96 bpm* MR: 35 ± 5 min* (BIC 0.75 μg)
Injection volume	0.1 μl (on spinal cord)	5 μl (into CSF)
Area of allodynia	Unilateral, highly restricted (e.g., one digit on hind paw)	Bilateral, large (e.g., flanks and hind legs)
Duration of allodynia	30 min after single dose, sustained up to 2 h with repeated dosing.	20-30 min
Drug delivery	Multiple sites available for topical application on spinal surface	Single route into spinal CSF

Abbreviations: MAP: mean arterial pressure

HR: heart rate

MR: motor response

CSF: cerebrospinal fluid

✳ No significant difference compared with the corresponding values from topical BIC administration.

§ Data from Loomis et al., 2001.

in the EEG from a desynchronous to a synchronous pattern. The HD-evoked cardiovascular responses outlasted the motor responses; the former persisted for an additional 5-10 min. The duration of bicuculline-allodynia (from the time of drug application until the disappearance of cardiovascular responses) ranged from 30-50 min for 0.1 μg bicuculline, and increased with bicuculline dose. There was a linear relationship between the log dose of spinal bicuculline (0.01-0.3 μg) and the maximum HD-evoked change in MAP, HR, and MR (Figure 5). The corresponding ED_{50} values and 95% confidence intervals (CI) are shown in Table 2. Allodynia remained localized to the ipsilateral hind paw with all bicuculline doses, and there was no detectable expansion of the allodynic area over time.

3.1.2 Multiple Doses of Bicuculline Induce Sustained Allodynia

When repetitive doses of bicuculline (0.1 μg) were applied to the same site on the left or right side of the spinal cord every 20 min, allodynia was sustained for up to 2 h. As shown in Figure 6, HD-evoked responses in MAP, HR and MR were significantly different from their respective control after each dose of bicuculline (n = 5-9). There were no significant differences across the 6 doses of bicuculline within each evoked response (MAP, HR and MR). In addition, there was no detectable change in the location of allodynia over time, nor was there an expansion of the allodynic area with the multiple bicuculline dosing.

3.1.3 Distribution of Drug Solution after Topical Application to the Spinal Cord

To assess the extent of drug distribution, bisbenzimide was added to the

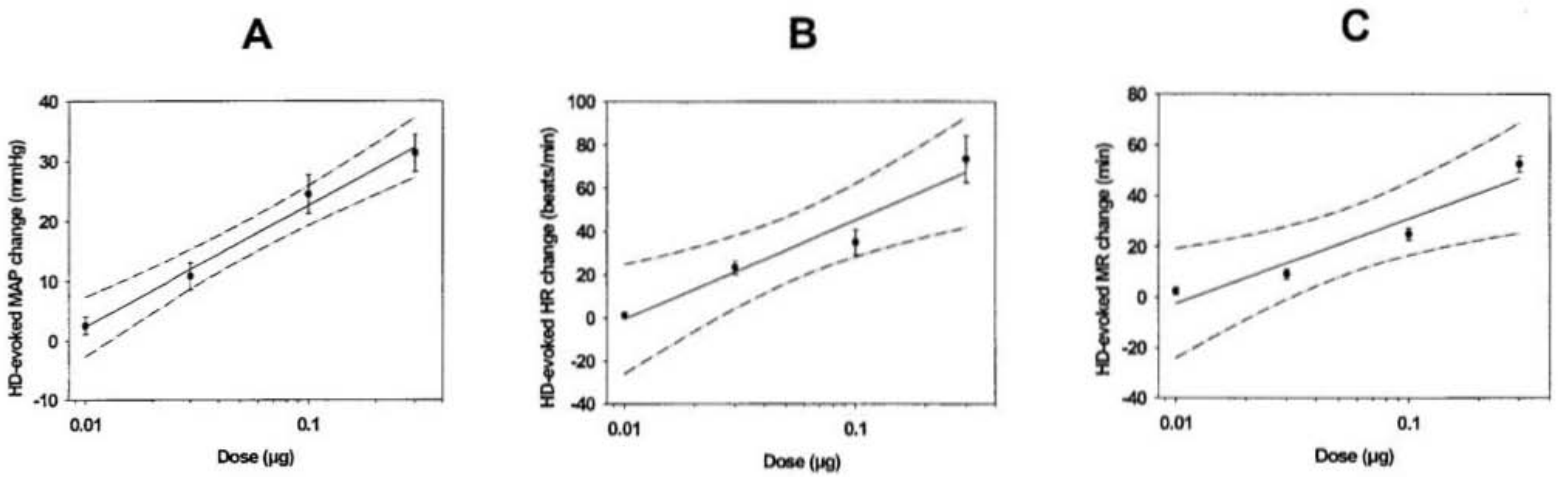


Figure 5. The dose-response relationship of topical bicuculline (BIC) on hair deflection-evoked changes in mean arterial pressure (MAP; Panel A), heart rate (HR; Panel B) and motor responses (MR; Panel C). Bicuculline (0.01, 0.03, 0.1, 0.3 µg) was applied topically on the left or right side of the dorsal spinal cord (see Methods). Doses greater than 1.0 µg produced exaggerated allodynia and convulsions in the lower quadrants and were excluded from the data analysis. Each point represents the mean ± SEM of 5-8 rats. The solid line is the least squares regression line and the dashed lines indicate the 95% confidence intervals (CI). The ED_{50} values and 95% CI are listed in Table 2.

Table 2. ED_{50} Values and 95% Confidence Intervals (CI) of Spinal Topical Application of Bicuculline

Parameters	**ED_{50} (95% CI) (μg)**
Mean Arterial Pressure	0.055 (0.035-0.085)
Heart Rate	0.075 (0.048-0.118)
Motor Response	0.097 (0.078-0.122)

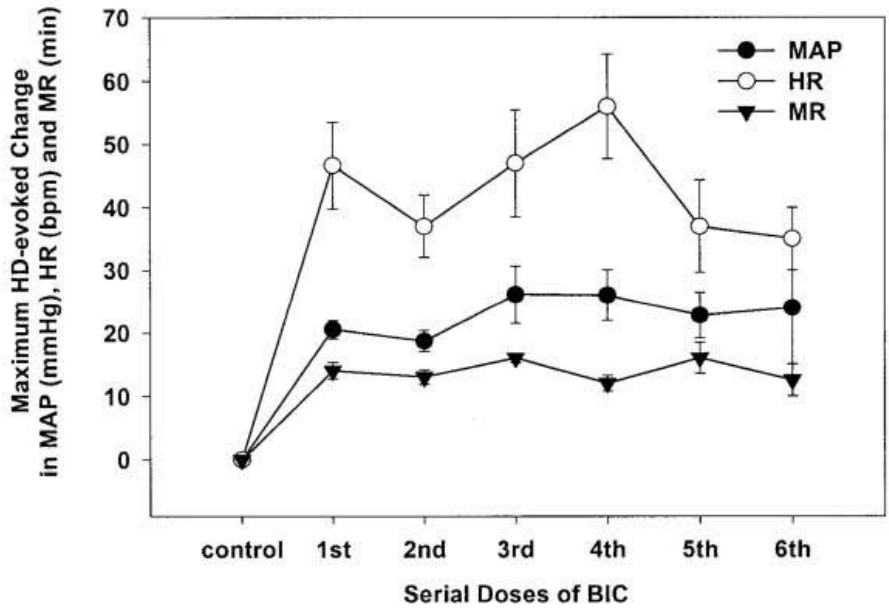


Figure 6. The maximum hair deflection-evoked change in mean arterial pressure (MAP), heart rate (HR) and motor response (MR) after repeated doses of bicuculline. Bicuculline (0.1 μg) was applied unilaterally to the same site on the dorsal spinal cord every 20 min for 2 h. All points are significantly different from their respective (saline) control ($P < 0.05$). Each point represents the mean ± SEM of 5-9 rats.

bicuculline solution (0.1% v/w for bisbenzimide and bicuculline). Fluorescence was restricted to the ipsilateral dorsal spinal cord following either a single application (Figure 7C and D) or 6 consecutive applications of the bisbenzimide/ bicuculline solution (Figure 7A and B). Penetration of the fluorescent tracer was clearly observed in the superficial laminae of the dorsal horn (laminae I-III) (Figure 7A, Figure 7D). In contrast, i.t. bisbenzimide displayed an extensive bilateral distribution over the surface of the spinal cord (data not shown).

3.2 Inhibitory Effect of COX the Inhibitor, NS-398 on Bicuculline-Allodynia

After complete recovery from bicuculline allodynia (control), animals were pretreated with NS-398 (1.0 μg) 20 min before further bicuculline (0.1 μg) application. Both drugs were applied unilaterally to the same site on the spinal cord. Figure 8 illustrates the time-course of inhibition of allodynia by NS-398 (1 μg). All indices of bicuculline-allodynia were significantly reduced. The maximum inhibition achieved with this dose of NS-398 was 66% for MAP, 70% for HR and 62% for MR, and lasted for 1-2 h (Figure 8, Figure 4). The inhibitory effect of NS-398 was confined to the ipsilateral spinal cord. When bicuculline (0.1 μg) was applied to the mirror site (contralateral side) 2 h after NS-398 (time of near maximal inhibition; n=3), no attenuation of allodynia was observed (Figure 4E).

As shown in Figure 9, the inhibitory effect of NS-398 was dose-dependent (n=5~8 for each). The ID_{50} values ranged from 0.49-0.91 μg, although these were not significantly different from one another (Table 3). The maximum inhibitory effect of NS-398 obtained with the highest dose tested (5 μg) was 81% for MAP, 86% for

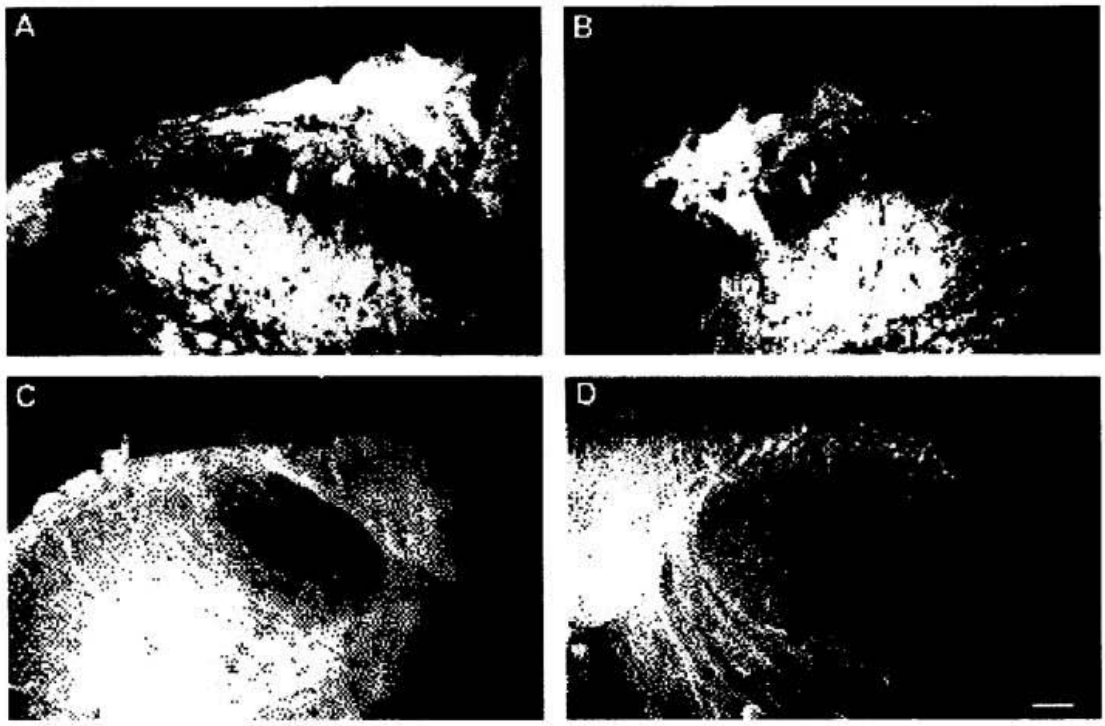


Figure 7. **Fluorescent photomicrograph showing transverse sections of the left and right lumbar dorsal horn. Rats received either multiple doses of a bicuculline/bisbenzimide (0.1% w/v each) solution applied to the dorsal surface on the left side of the spinal cord every 20 min for 2 h (A), or a single dose of the same solution on the right side of the spinal cord (D). All doses were delivered in a volume of 0.1 μl (see Methods). The animals were perfused transcardially 20 min after the last dose. Note that fluorescence is restricted to the drug application side of the drug application (A, B: repeated dose; C, D: single dose). Fluorescence was evident in lamina I-III of dorsal horn after repeated (A) or single drug application (D). Scale bar, 100 μm.**

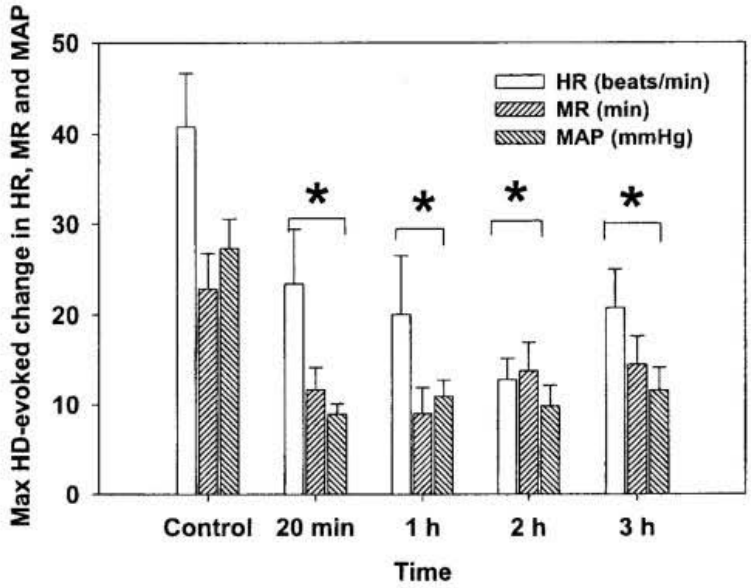


Figure 8. Time course of the inhibitory effect of NS-398 (1.0 μg) on the maximum hair deflection (HD)-evoked change in mean arterial pressure (MAP), heart rate (HR) and motor responses (MR) in bicuculline (BIC)-treated rats. Control indicates the HD-evoked responses to BIC (0.1 μg) before NS-398. Animals were pretreated with NS-398 twenty min before BIC (0.1 μg). Both drugs were applied topically to the same site on the dorsal spinal cord. HD-evoked responses were determined 20min, 1h, 2h and 3h after NS-398; BIC (0.1 μg) was applied immediately before each time point. The maximum HD-evoked change in HR, MR and MAP occurred five min after each BIC application. Each column represents the mean ± SEM of 5-8 rats. The asterisk at each time point indicates a significant difference from the respective control ($P < 0.05$). NS-398 reduced all the indices of BIC-induced allodynia.

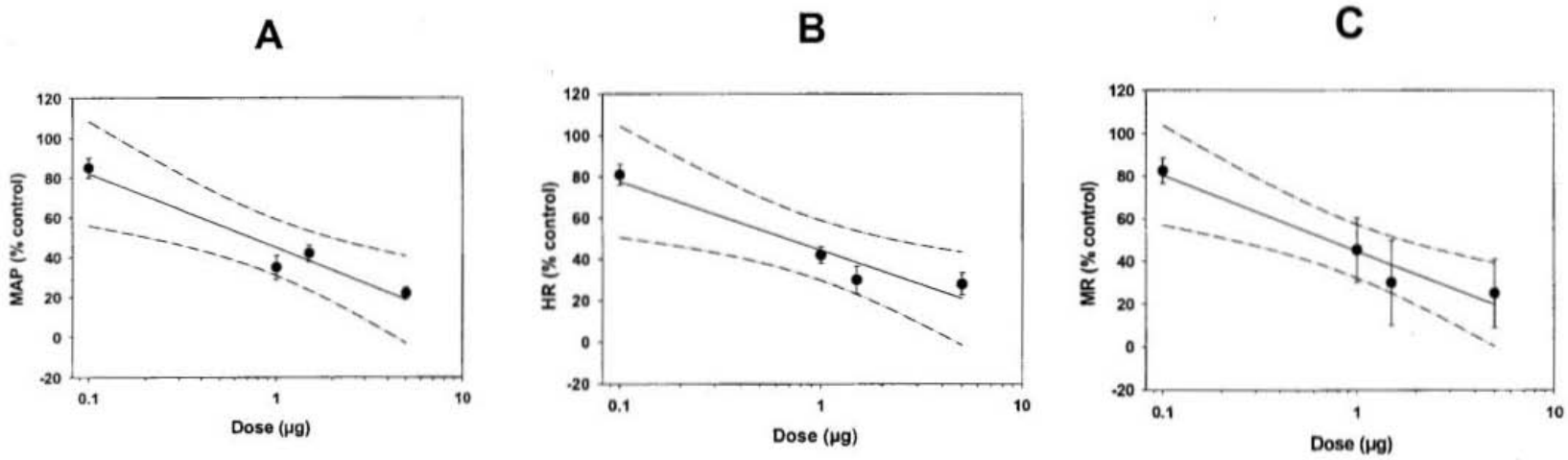


Figure 9. The dose-response relationship of NS-398 on hair deflection (HD)-evoked allodynia in bicuculline (BIC)-treated rats. Rats were pretreated with NS-398 (0.1, 1.0, 1.5 or 5.0 μg) fifteen min before BIC (0.1 μg). Both drugs were applied topically to the same site on the dorsal spinal cord (see Methods). Maximum HD-evoked changes in mean arterial pressure (MAP; Panel A), heart rate (HR; Panel B) and motor responses (MR; Panel C) are shown. Each point represents the mean ± SEM of 5-8 rats. The solid line is the least squares regression line and the dashed lines indicate the 95% confidence intervals (CI). The slopes of the regression lines were significantly different from 0 (slope $\beta \neq 0$, one-way ANOVA, $P < 0.05$). The ID_{50} values and 95% CI are listed in Table 3.

Table 3. Summary of the ID_{50} Values and 95% Confidence Intervals (CI) for NS-398, AP-7 and SC-51322 in Bicuculline-Treated Rats.

DRUGS	MAP	HR	MR
NS-398 (μg)	0.50 (0.32-0.79)	0.49 (0.27-0.94)	0.91 (0.38-1.32)
AP-7 (μg)	0.23 (0.09-0.56)	0.17 (0.07-0.42)	0.09 (0.01-0.31)
SC-51322 (μg)	2.11 (0.65-6.86)	1.36 (0.67-2.75)	1.74 (0.91-3.32)

Note: All drugs were applied unilaterally to the dorsal surface of the spinal cord (see Methods). Allodynia was evoked by repeated hair deflection to the affected dermatome(s). The dose-response curves are shown in Figure 9, 10, and 13. Abbreviations: MAP - mean arterial pressure; HR - heart rate; MR - motor response.

HR and 75% for MR. Vehicle pretreatment (DMSO 70% + 30% water) had no significant effect on bicuculline-allodynia.

3.3 Inhibitory Effect of NMDA Receptor Antagonist, AP-7 on Bicuculline-Allodynia

Following complete recovery from bicuculline allodynia (control), rats were treated with AP-7 (0.01, 0.1, 1.0, or 4.0 μg) for 20 min. Bicuculline, applied topically to the same site of the spinal cord, was given 20 min, 1 h and 2 h after AP-7. The HD-evoked cardiovascular and motor responses were dose-dependently inhibited by AP-7 (n = 4-6 for each dose) (Figure 10). The ID_{50} values and 95%CI shown in Table 3 were obtained at the time of maximum inhibition (i.e. the first dose of bicuculline after AP-7). The time course of the inhibitory effect of AP-7 (0.1 μg) on bicuculline-allodynia is shown in Figure 11. At the maximum dose of 4 μg, AP-7 produced an almost complete blockade of bicuculline allodynia (Figure 10). The cardiovascular responses to phasic noxious pinch remained unaltered after AP-7 (data not shown).

3.4 Inhibitory Effect of EP-Receptor Antagonist SC-51322 on Bicuculline-Allodynia

Pretreatment with SC-51322 (0.1, 1.0, 4.0, 8.0 μg) 15 min before bicuculline (0.1 μg) attenuated the HD-evoked cardiovascular and motor responses of bicuculline-allodynia. Figure 12 shows the time course of the inhibitory effect of 4.0 μg of SC-51322 on bicuculline-allodynia. This inhibitory effect was statistically significant from control for up to 2 h. This effect was also dose-dependent (n = 4-6 for each dose) (Figure 13), yielding ID_{50} values of 2.1, 1.4 and 1.7 μg for MAP, HR

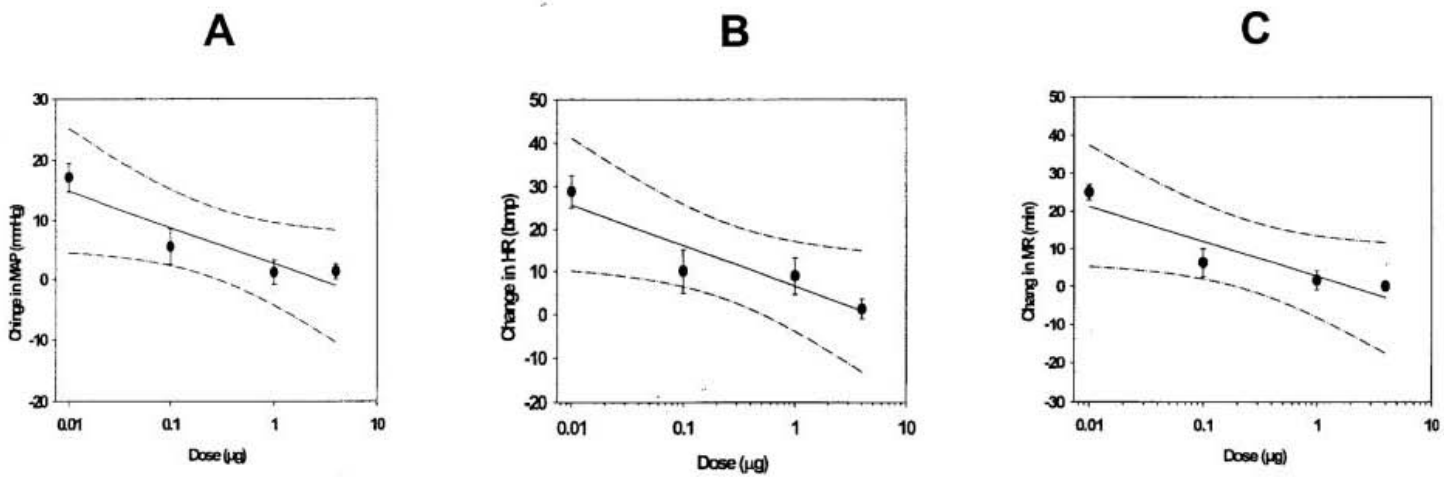


Figure 10. The dose-response relationship of AP-7 on hair deflection (HD)-evoked allodynia in bicuculline (BIC)-treated rats. Rats were pretreated with AP-7 (0.01, 0.1, 1.0 or 4.0 µg) twenty before BIC (0.1 µg). Both drugs were applied topically to the same site on the dorsal spinal cord (see Methods). Maximum HD-evoked changes in mean arterial pressure (MAP; Panel A), heart rate (HR; Panel B) and motor responses (MR; Panel C) are shown. Each point represents the mean ± SEM of 5-8 rats. The solid line is the least squares regression line and the dashed lines indicate the 95% confidence intervals (CI). The slopes of the regression lines were significantly different from 0 (slope $\beta \neq 0$, one-way ANOVA, $P < 0.05$). The corresponding ID_{50} values and 95% CI are summarized in Table 3.

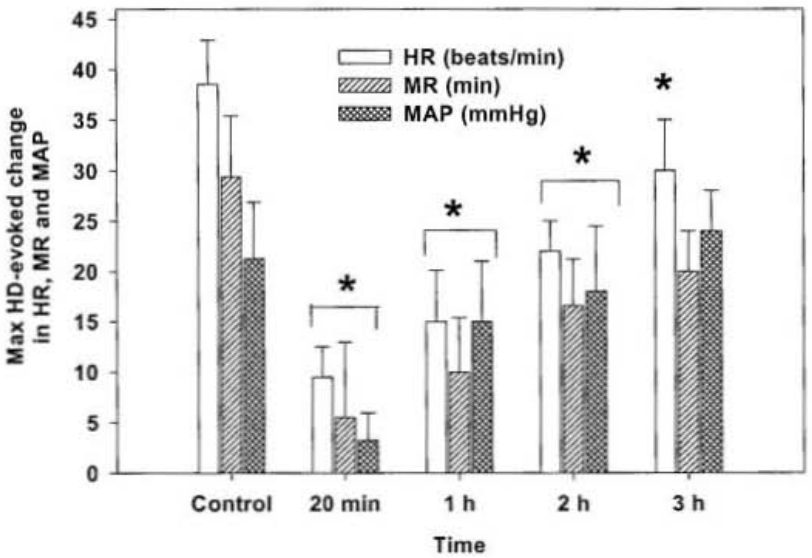


Figure 11. The time course of the inhibitory effect of AP-7 (0.1 μg) on the maximum hair deflection (HD)-evoked change in mean arterial blood pressure (MAP), heart rate (HR) and motor responses (MR) in bicuculline (BIC)-treated rats. Control indicates the HD-evoked responses to BIC (0.1 μg) before AP-7. Animals were pretreated with AP-7 twenty min before BIC (0.1 μg). Both drugs were applied topically to the same site on the dorsal spinal cord. HD-evoked responses were determined 20min, 1h, 2h and 3h after AP-7; BIC (0.1 μg) was applied immediately before each time point. The maximum HD-evoked change in HR, MR and MAP occurred five min after each BIC application. Each column represents the mean ± SEM of 5-8 rats. The asterisk at each time point indicates a significant difference from the respective control ($P < 0.05$). AP-7 reduced all the indices of BIC-induced allodynia.

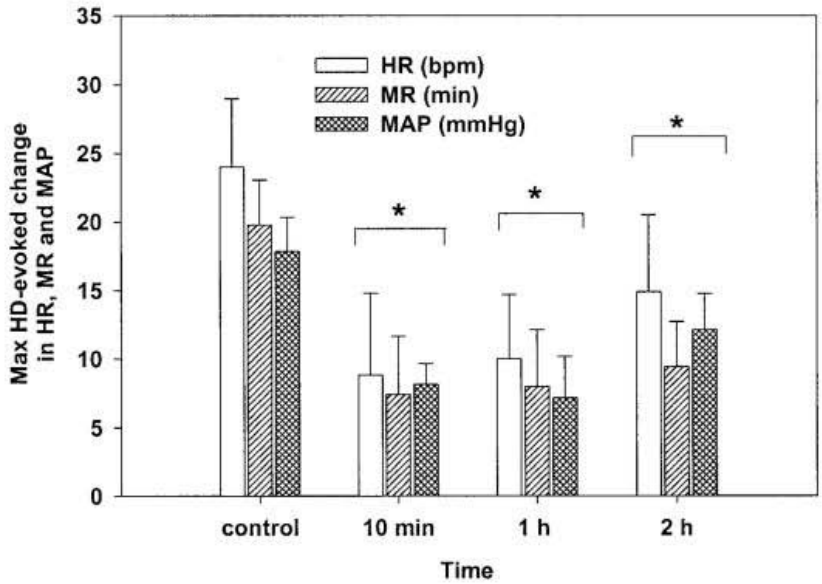


Figure 12. The time course of the inhibitory effect of SC-51322 (4.0 μg) on the maximum hair deflection (HD)-evoked change in mean arterial pressure (MAP), heart rate (HR) and motor responses (MR) in bicuculline (BIC)-treated rats. Control indicates the HD-evoked responses to BIC (0.1 μg) before SC-51322. Animals were pretreated with SC-51322 ten min before BIC (0.1 μg). Both drugs were applied topically to the same site on the dorsal spinal cord. HD-evoked responses were determined 10min, 1h and 2h after SC-51322; BIC (0.1 μg) was applied immediately before each time point. The maximum HD-evoked change in HR, MR and MAP occurred five min after each BIC application. Each point represents the mean ± SEM of 4-9 rats. The asterisk at each time point indicates a significant difference from the respective control ($P < 0.05$). SC-51322 reduced all the indices of BIC-induced allodynia.

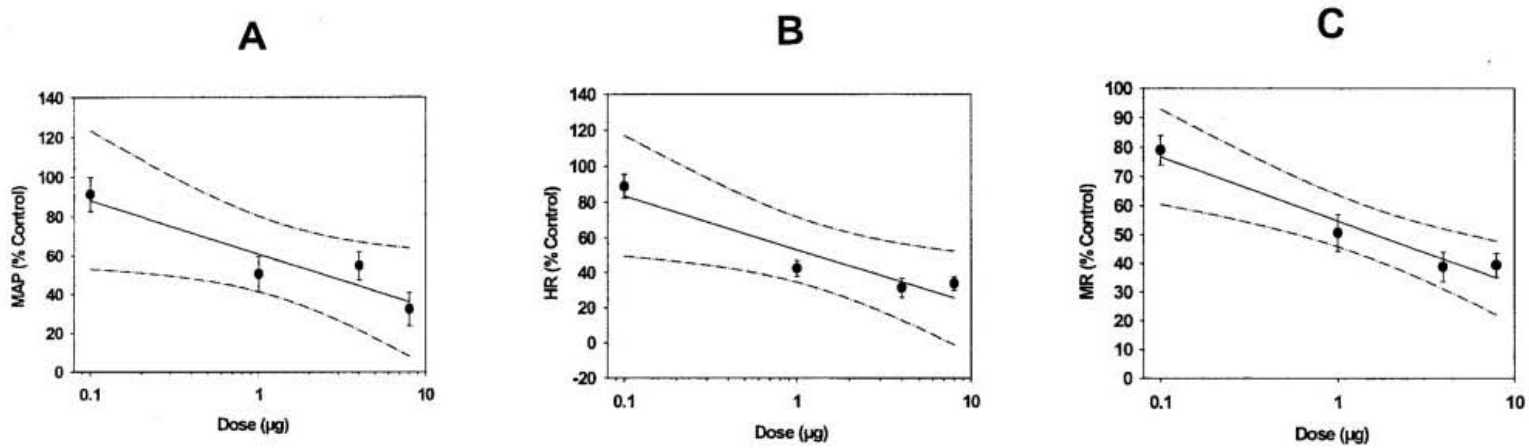


Figure 13. The dose-response relationship of SC-51322 on hair deflection (HD)-evoked allodynia in bicuculline (BIC)-treated rats. Rats were pretreated with SC-51322 (0.1, 1.0, 4.0 or 8.0 µg) before BIC (0.1 µg) treatment. Both drugs were applied topically to the same site on the dorsal spinal cord (see Methods). Maximum HD-evoked changes in mean arterial pressure (MAP; Panel A), heart rate (HR; Panel B) and motor responses (MR; Panel C) are shown. Each point represents the mean ± SEM of 4-9 rats. The solid line is the least squares regression line and the dashed lines indicate the 95% confidence intervals (CI). The slopes of the regression lines were significantly different from 0 (slope $\beta \neq 0$, one-way ANOVA, $P < 0.05$). The ID_{50} values and 95% CI are summarized in Table 3.

and MR, respectively (Table 3).

3.5 Intrathecal PGE_2 Elicits Allodynia-like Behaviour in Conscious Rats

3.5.1 Effects Observed in the Absence of Hair Deflection

PGE_2 elicited behavioural responses ranging from mild burrowing and hiding behaviour to vocalization and biting or scratching the caudal dermatomes. These behaviours were transient, lasting only during the period of i.t. injection, and yielded scores in the 2-3 range. After injection, these responses rapidly decreased in frequency and severity yielding scores in the 1-2 range (i.e. 20-45% of the maximum possible score depending on the dose). These remained relatively stable for the next 19-29 min and declined gradually thereafter (Figure 14). The i.t. injection of vehicle (3% ethanol in water) yielded very mild effects as indicated by the low behavioural scores (5-10% of the maximum possible score) throughout the observation period. Although there appeared to be some relationship between the magnitude of the scores for the behavioural changes observed in the absence of HD stimulation and the dose of PGE_2 (Figure 15), statistical analysis of the regression line indicated that the slope was not significantly different from 0.

3.5.2 Hair Deflection-Evoked Allodynia

Brushing the hair on the lower dermatomes (hind limbs, flanks or lower back) with a cotton-tipped applicator evoked abrupt and robust behavioural responses in rats treated with i.t. PGE_2. These included avoidance behaviour (hiding the affected dermatome); paw withdrawal, vocalization, piloerection; and aggressive nocifensive

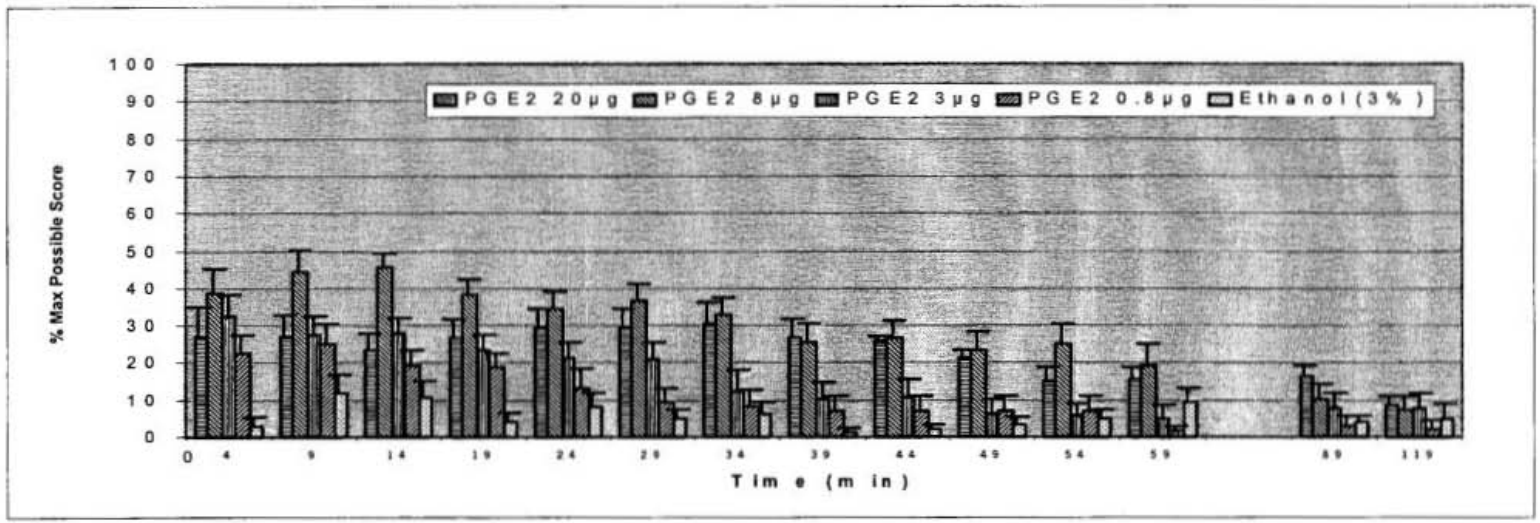


Figure 14. The time course of behavioural responses without hair deflection in PGE_2-treated rats. Animals were injected with intrathecal PGE_2 (0.8, 3.0, 8.0 or 20 µg) or vehicle (3% ethanol in water) at time 0. Each column represents the mean ± SEM of the percent maximum possible score of 5-8 rats for each 4-min observation period. The only time period that exhibited a significant dose-dependent effect was 14 min (one-way ANOVA; $P < 0.05$).

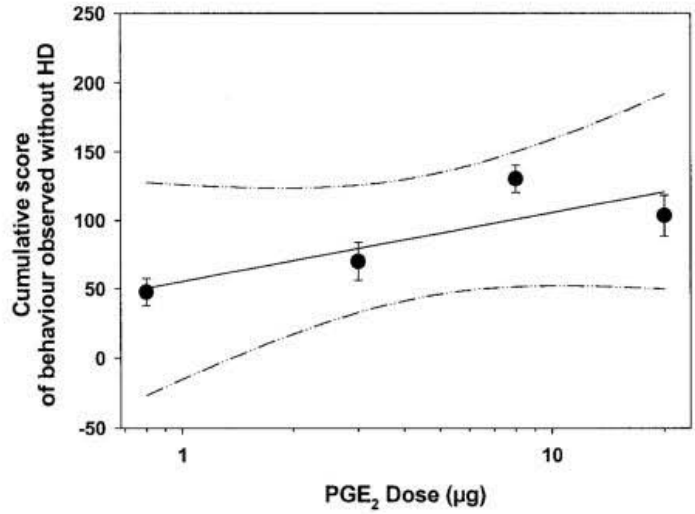


Figure 15. The dose-response relationship of intrathecal (i.t.) PGE_2; behavioural responses observed without hair deflection. Rats were injected with i.t. PGE_2 (0.8µg, 3µg, 8µg and 20µg) and behaviour was rated using a behavioural scoring system. In all cases, the investigator was blinded to the nature of the treatment. Each point represents the mean ± SEM of the sum of the behavioural scores per rat over the 2-h time course (n= 5-8). The solid line is the least squares regression line and the dashed lines indicate the 95% confidence intervals. The slope of the linear regression line was not significantly different from 0 (slope $\beta = 0$, one-way ANOVA, $P = 0.066$).

behaviours such as attacking and biting the stimulus applicator. Identical brushing of the face, head and fore paws of PGE_2-treated rats was without effect. The time-course of HD-evoked allodynia for four separate doses of PGE_2 (0.8, 3.0, 8.0 and 20 μg) is shown in Figure 16. Peak allodynia occurred 5-15 min after injection depending on the dose. Recovery from allodynia was complete 90 min after injection except for the 20 μg dose (Figure 16). Brushing of the caudal dermatomes in vehicle (3% ethanol in water)-treated rats yielded very low behavioural scores (<10% of the maximum possible score; Figure 16). These were not significantly different from those evoked by HD in i.t. saline-treated controls (data not shown). As illustrated in Figure 17, the effect of PGE_2 on HD-evoked allodynia was linearly related to the log of the dose. The slope of the linear regression line was significantly different from 0 and the maximum allodynia appeared to occur between 3 and 20 μg. Dose-response analysis yielded an ED_{50} and 95% CI of i.t. PGE_2 = 2.6 μg (1.6-4.2).

3.6 Effect of SC-51322 on Hair Deflection-Evoked Allodynia in PGE_2-Treated Rats

Pretreatment with i.t. SC-51322 (100 μg), 15 min before i.t. PGE_2 (8 μg), significantly reduced HD-evoked allodynia as compared to control (Figure 18B). Maximum inhibition occurred 15 min after i.t. PGE_2 (30 min after SC-51322) and the inhibitory effect remained significantly different from that of vehicle control for up to 60 min. Pretreatment with i.t. DMSO (the injection vehicle for SC-51322) had no effect on HD-evoked allodynia in PGE_2-treated rats as compared to rats treated only with i.t. PGE_2 (except at 55 and 60 min after injection)(Figure 18B).

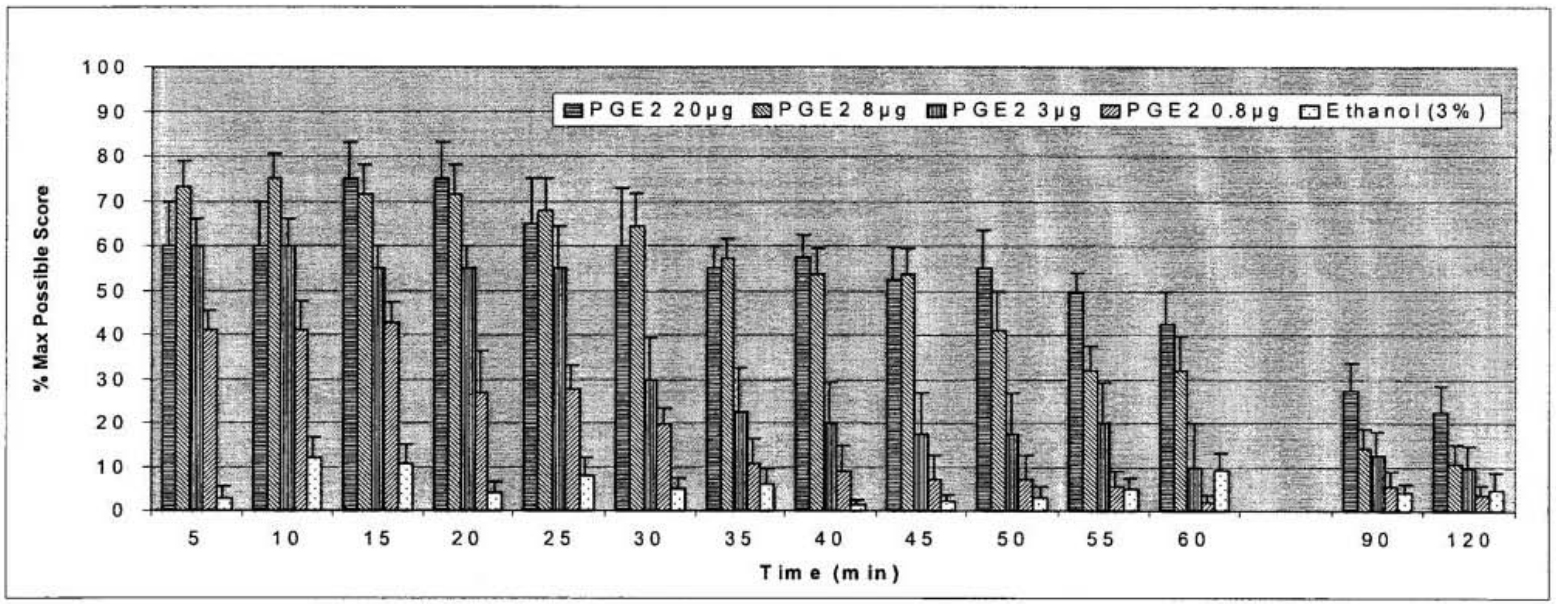


Figure 16. The time course of hair deflection (HD)-evoked behavioural responses in PGE_2-treated rats. Rats were injected with intrathecal PGE_2 (0.8, 3.0, 8.0 or 20 µg) or vehicle (3% ethanol in water) at time 0. Each column represents the mean ± SEM of the percent maximum possible score of 5-8 rats evaluated for each 5-min observation period. All time periods exhibited a significant dose-dependent effect except 120 min (one-way ANOVA; $P < 0.05$).

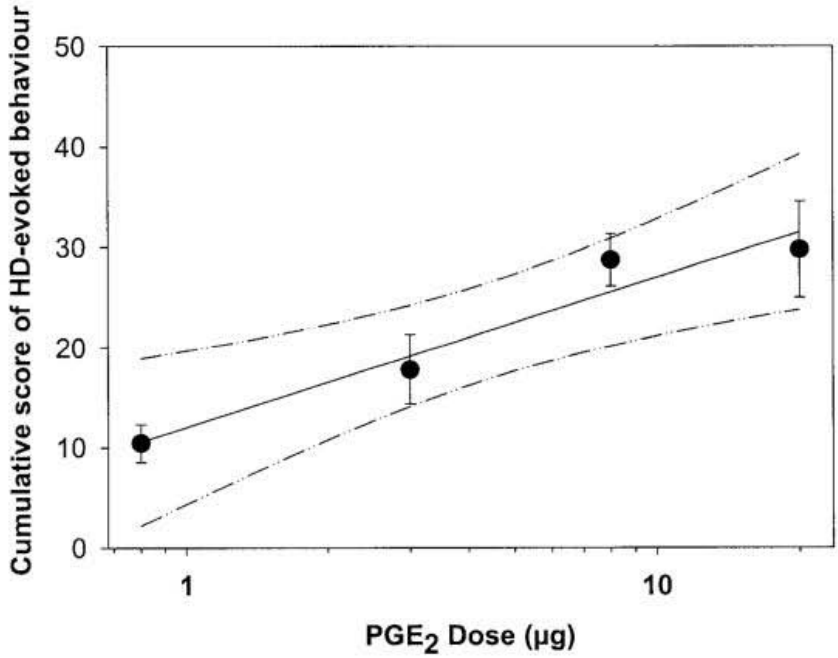
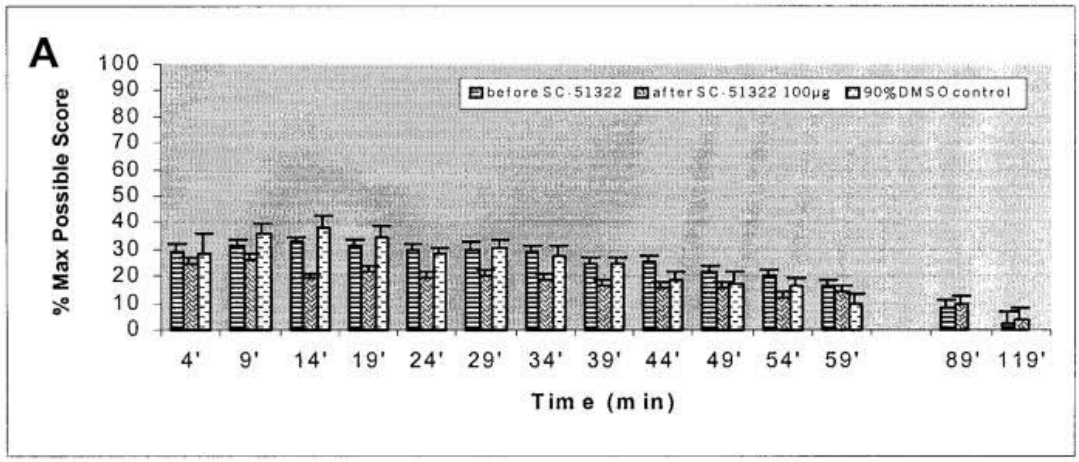


Figure 17. The dose-response relationship of intrathecal (i.t.) PGE_2; hair deflection (HD)-evoked allodynia. Rats were injected with i.t. PGE_2 (0.8μg, 3μg, 8μg and 20μg) and HD-evoked behavioural responses were rated using a behavioural scoring system. In all cases, the investigator was blinded to the nature of the treatment. Each point represents the mean ± SEM of the sum of the behavioural scores per rat over the 2-h observation period (n= 5-8). The solid line is the least squares regression line. The dashed lines indicate the 95% confidence intervals. The slope of the linear regression line was significantly different from 0 (slope $\beta \neq 0$, one-way ANOVA, $P = 0.0019$).



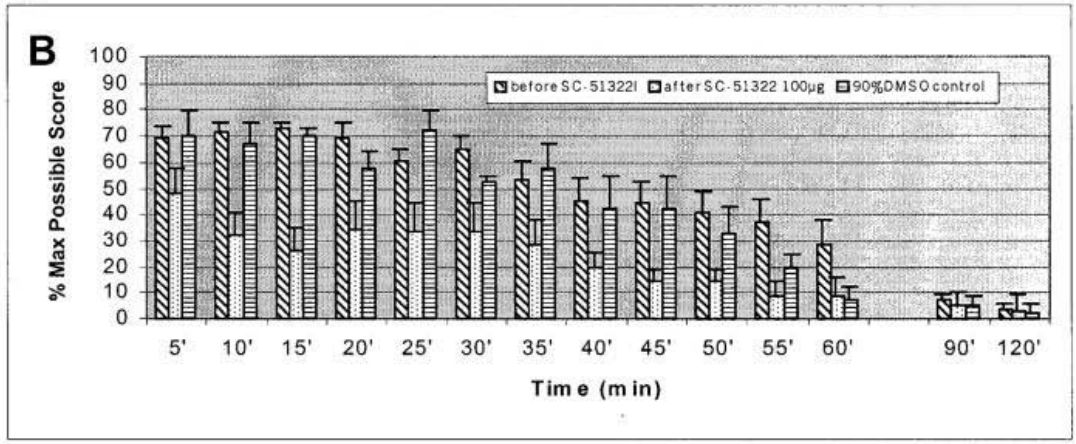


Figure 18. The time course of the inhibitory effect of SC-51322 in PGE_2-treated rats. Intrathecal (i.t.) PGE_2 (8 µg) was given alone or fifteen min after i.t. DMSO (90% in water) or i.t. SC-51322 (100 µg). Spontaneous (A) and hair deflection (HD)-evoked (B) behaviours were rated using behavioural scoring systems with the investigator blinded to the nature of the treatment. Each column represents the mean ± SEM of the percent maximum possible score of 7 rats. SC-51322 significantly inhibited PGE_2-induced behaviour without HD at the 14' and 19' time periods (Panel A), and HD-evoked behaviour at 10', 15', 20', 25' and 30' (Panel B) (one-way ANOVA, $P < 0.05$).

The inhibitory effect of SC-51322 on PGE_2-allodynia was dose-dependent (Figure 19). The reduction in the mean cumulative behavioural score was linearly related to the log dose of i.t. SC-51322 over the range of doses that were tested (6, 30 and 100 μg). At the highest dose (100 μg), SC-51322 decreased the cumulative behavioural score evoked by HD by approximately 60%; behavioural scores evaluated between the HD tests (without HD) were reduced by 48% (Figure 20). SC-51322 attenuated the latter (Figure 18A) in a dose-dependent manner (Figure 20), although the slope of the dose-response was markedly lower than that of the HD-evoked responses (Figure 19).

3.7 Effect of AP-7 on Hair Deflection-Evoked Allodynia in PGE_2-Treated Rats

The i.t. AP-7 experiments were conducted on another separate group of freely moving conscious rats. In order to prevent any nonspecific effect of AP-7 such as immobilization, pilot experiments were performed to choose a maximum dose of AP-7 devoid of any overt behavioural effect. Intrathecal injection of AP-7, at a dose less than 0.4 μg, did not induce any obvious behavioural changes or decreases in mobility. Thus, a maximum dose of 0.4 μg (in 5 μl saline) was chosen for i.t. injection. Pretreatment of the rat with AP-7 (0.4 μg) 20 min before PGE_2 (8 μg) significantly attenuated the HD-evoked allodynia-like responses. The inhibitory effect became significant 15 min after PGE_2 treatment, then gradually decreased and disappeared 90 min thereafter (Paired t test, $P < 0.05$, Figure 21). The mean cumulative behavioural score of HD-evoked responses was significantly decreased by AP-7 (Paired t test, $P < 0.001$, Figure 22). The behaviour observed in the

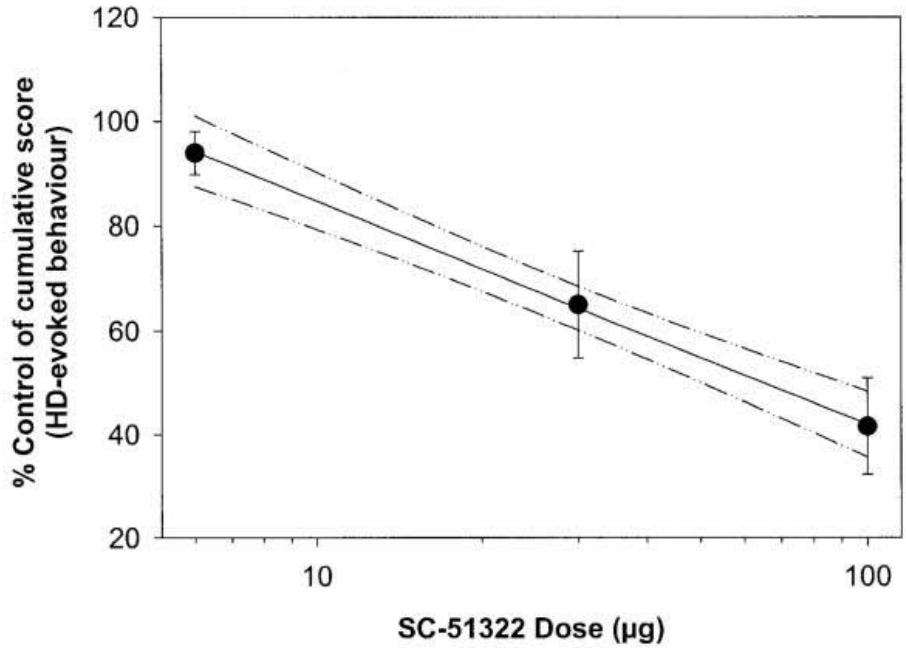


Figure 19. Dose-response relationship of intrathecal (i.t.) SC-51322 on hair deflection (HD)-evoked allodynia in PGE_2-treated rats. Rats were pretreated with i.t. SC-51322 (6 µg,, 30 µg, or 100 µg) 15 min before i.t. PGE_2 (8 µg). Hair deflection-evoked behavioural responses were rated by means of a behavioural scoring system with the observer blinded to the nature of the treatment. Each point represents the mean ± SEM (5-7 rats) of the cumulative score of HD-evoked behaviour per rat over the 2-h experimental time period, expressed as the percent of control (no SC-51322). The solid line is the least squares regression line and the dashed lines indicate the 95% confidence intervals. All points were significantly different from one another except 6 and 30 µg (one-way ANOVA, $P < 0.05$). The slope of the linear regression line was significantly different from 0 (slope $\beta \neq 0$, one-way ANOVA, $P = 0.0036$).

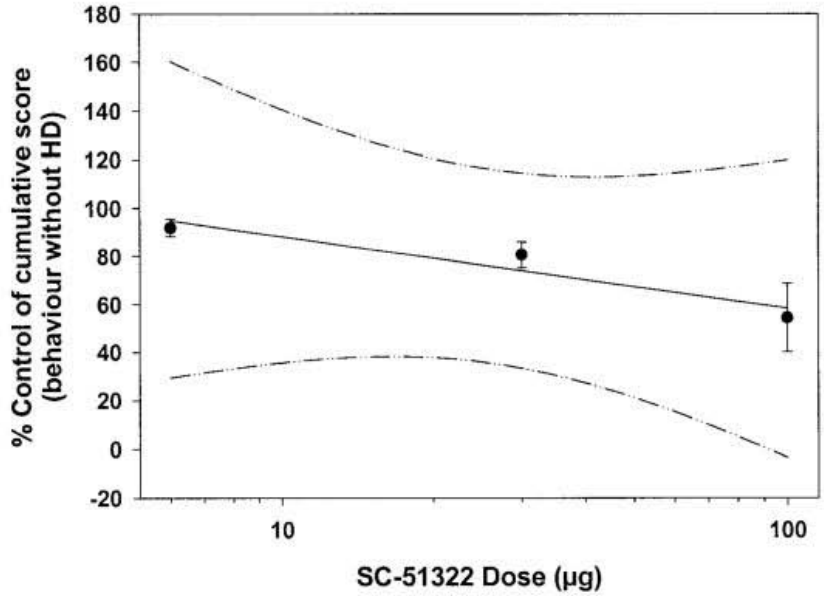


Figure 20. Dose-response relationship of intrathecal (i.t.) SC-51322 on PGE_2-evoked behaviour without hair deflection (HD). Rats were pretreated with i.t. SC-51322 (6 µg, 30 µg or 100 µg) fifteen min before i.t. PGE_2 (8 µg). Behaviour in the absence of HD was rated using a behavioural scoring system with the observer blinded to the nature of the treatment. Each point represents the mean ± SEM (5-7 rats) of the cumulative score of the observed behaviour per rat over the 2-h experimental time period, expressed as the percent of control (no SC-51322). The solid line is the least squares regression line and the dashed lines indicate the 95% confidence intervals. The effects of 6 µg and 100 µg SC-51322 were significantly different from each other (Kruskal-Wallis ANOVA on ranks, $P < 0.05$). The slope of the linear regression line was significantly different from 0 (slope $\beta \neq 0$).

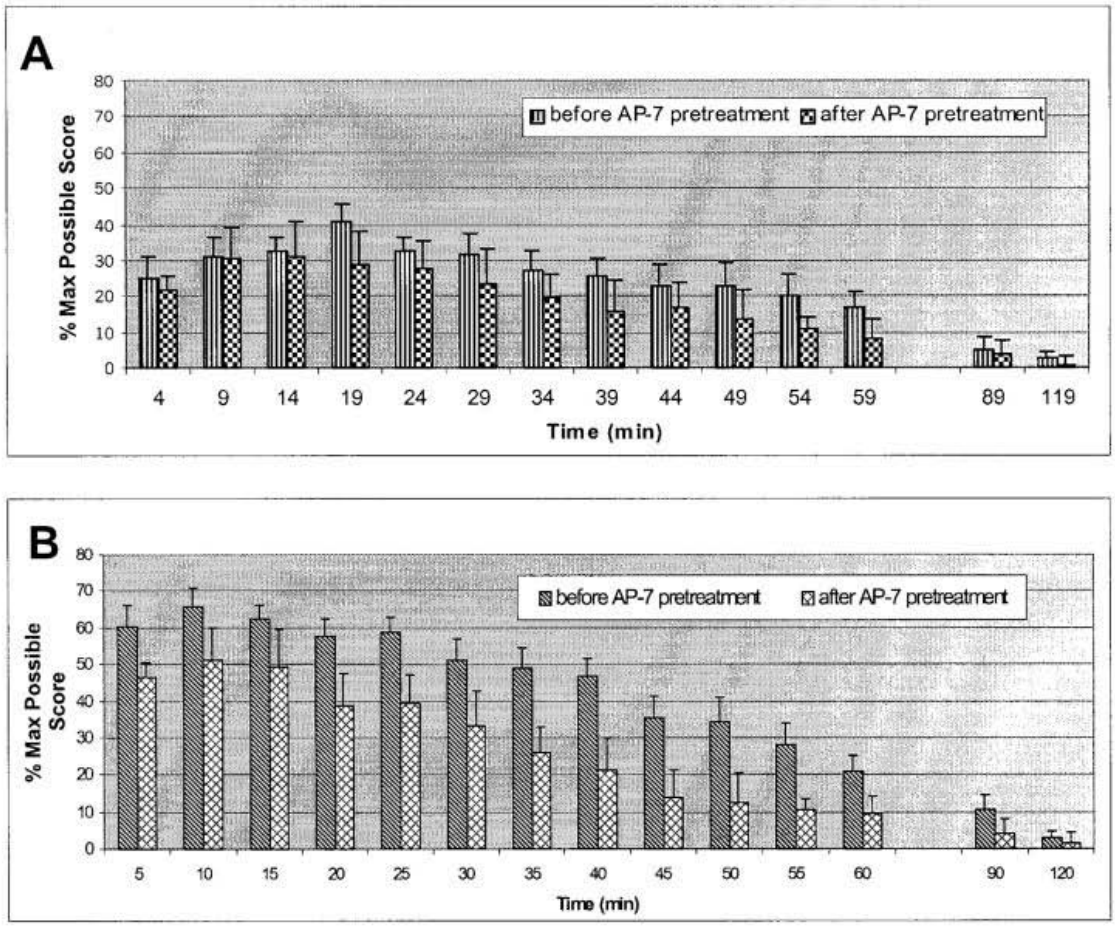


Figure 21. The time course of the inhibitory effect of AP-7 in PGE_2-treated rats. Intrathecal (i.t.) PGE_2 (8 µg) was given before (control) or twenty min after i.t. AP-7 (0.4 µg). Spontaneous (A) and hair deflection (HD)-evoked (B) behaviours were rated using behavioural scoring systems with the observer blinded to the nature of the treatment. Each column represents the mean ± SEM of the percent maximum possible score of 12 rats. AP-7 significantly inhibited PGE_2-induced behaviour without HD at the 19 and 29-59 min time periods (Panel A) and HD-evoked behaviour at all time points except 10, 15, 90 and 120 min (Panel B) (Paired t-test, $P < 0.05$).

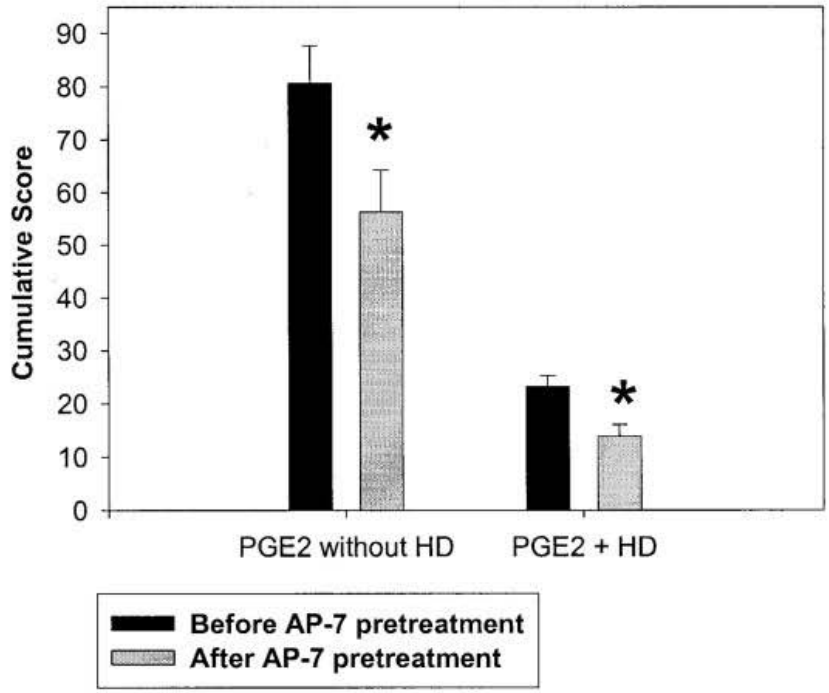


Figure 22. The effect of intrathecal (i.t.) AP-7 on PGE_2-evoked behaviour with and without hair deflection (HD). Rats were pretreated with vehicle or AP-7 (0.4 μg) twenty minutes before i.t. PGE_2 (8 μg). Behavioural responses were rated using behavioural scoring systems with the observer blinded to the nature of the treatment. The cumulative score was calculated from the entire time-course curve (120 min) for each rat (see Data Analysis; 2.3.6). Each bar represents the mean ± SEM of 12 animals. The asterisks indicate a significant difference from the corresponding control (Paired t-test, *P* <0.001).

absence of HD stimulation was also significantly inhibited by AP-7 pretreatment during 16-19, 26-29, 31-34, 36-39, 1-44, 46-49, 51-54 and 56-59 min time intervals during the 2-h experimental period (Paired t test, $P < 0.05$, n=12, Figure 21). The sum of the behavioural scores per rat without HD was significantly reduced after AP-7 (Paired t test, $P < 0.001$, n=12, Figure 22).

3.8 Effect of Bicuculline on Behavioural Responses in PGE_2-Treated Rats

In pilot experiments, i.t. bicuculline dose-dependently induced allodynia-like behavioural responses in conscious rats (data not shown). After a sub-threshold dose of i.t. bicuculline (0.01 μg in 5 μl saline), rats did not show any overt behavioural changes; an HD stimulus to the lower dermatomes of the rat did not evoke obvious behavioural agitation. This dose of bicuculline (0.01 μg in 5 μl saline) was chosen for i.t. injection.

Pretreatment with this sub-threshold dose of bicuculline (0.01 μg in 5 μl saline) 5 min before PGE_2 enhanced PGE_2-induced allodynia. Figure 23B shows the time course of the effect of bicuculline pretreatment on the HD-evoked behavioural responses in 8 μg PGE_2-treated rats; the percent maximum possible score was significantly increased at all time point except 90' and 120' during the 2-h experimental time period (Paired t test, $P < 0.05$). The percent maximum possible scores for behaviour in the absence of HD stimulation was also increased during 1-4, 6-9, 11-14, 16-19, 21-24, 26-29, 36-39, 41-44 and 56-59 min time periods (Figure 23A).

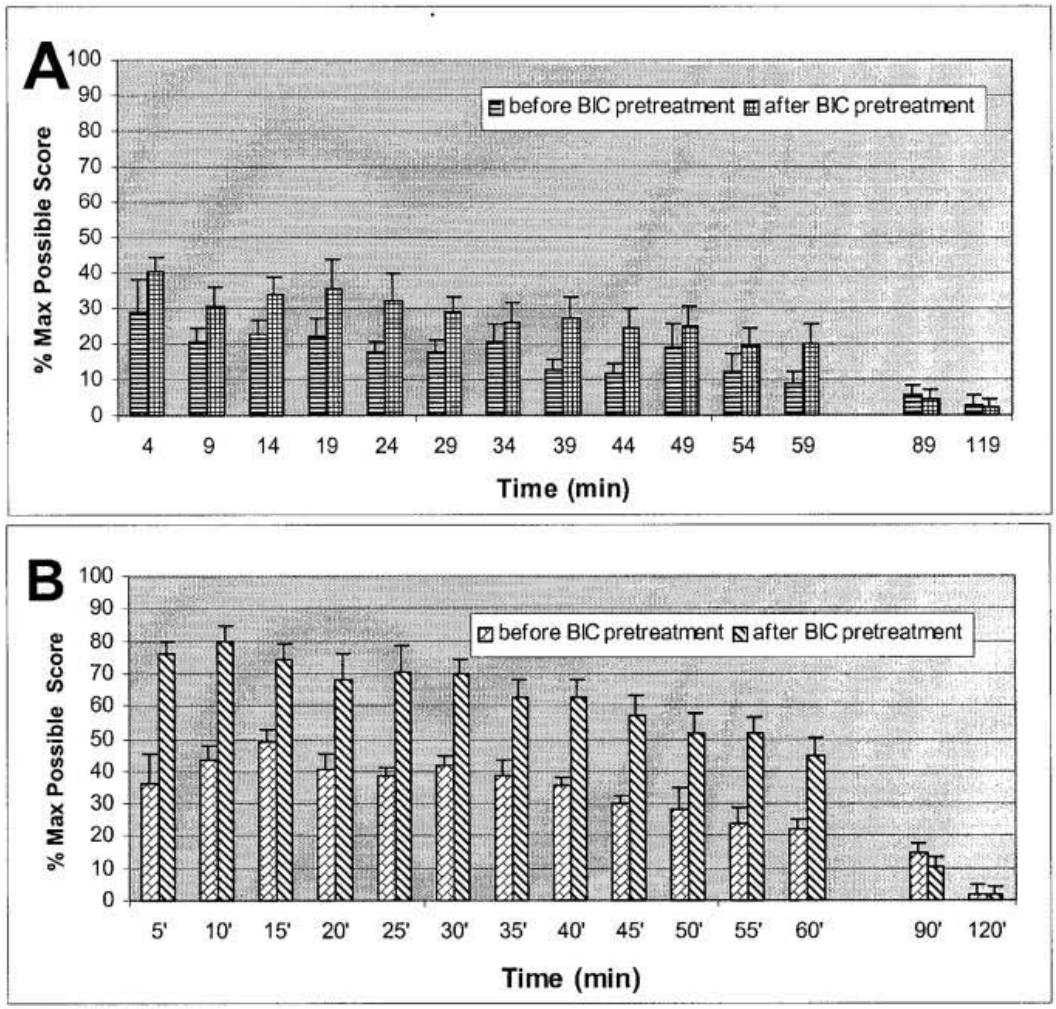


Figure 23. The time course of the effect of bicuculline (BIC) in PGE_2-treated rats. Intrathecal (i.t.) PGE_2 (8 μg) was given before (control) or five min after a sub-threshold dose of i.t. BIC (0.01 μg). Spontaneous (A) and hair deflection (HD)-evoked (B) behaviours were rated using behavioural scoring systems with observer blinded to the nature of the treatment. Each column represents the mean ± SEM of the percent maximum possible score. Bicuculline significantly enhanced PGE_2-induced behaviour without HD at the 4-29, 39, 44 and 58 min time periods (Panel A) and HD-evoked behaviour at all time points except 90 and 120 min (Panel B) (Paired t-test, $P < 0.05$).

Intrathecal PGE_2 (0.1 μg) had no effect on the spontaneous and HD-evoked behaviour in conscious rats. After pretreatment of rats with subthreshold dose of bicuculline (0.01 μg in 5 μl saline), intrathecal 0.1 μg of PGE_2 indeed induced obvious behavioural responses. Figure 24 and Figure 25 show the dose-response curve of PGE_2 (with or without HD, respectively) before and after bicuculline pretreatment. The dose response-curves of PGE_2 were shifted to the left.

4 DISCUSSION

4.1 Topical Application of Bicuculline to the Dorsal Spinal Cord Induces Localized Allodynia

The acute blockade of spinal glycine- or $GABA_A$-receptors with sub-convulsive doses of intrathecal strychnine or bicuculline, respectively, induces a rapid, reversible and highly selective allodynic state in conscious and lightly-anaesthetized animals (Yaksh 1989; Sherman and Loomis, 1994; Onaka et al., 1996). Thus, in the presence, but not absence, of i.t. strychnine or bicuculline, mild brushing of the hair on the affected dermatomes evokes behavioural, autonomic and neurochemical responses that are strongly indicative of a nociceptive event. In the present study, we have shown that bicuculline, applied locally to the surface of the dorsal spinal cord, is equally effective in altering somatosensory processing, but in a much more circumscribed cutaneous field (Table 1). Indeed, the allodynia induced by the unilateral application of bicuculline (0.1 μg in 0.1 μl saline) to the L5 or L6 spinal segment was limited to one to two digits on the ipsilateral hind paw (Figure 4G; Table 1). This is in sharp contrast to the bilateral and more widespread

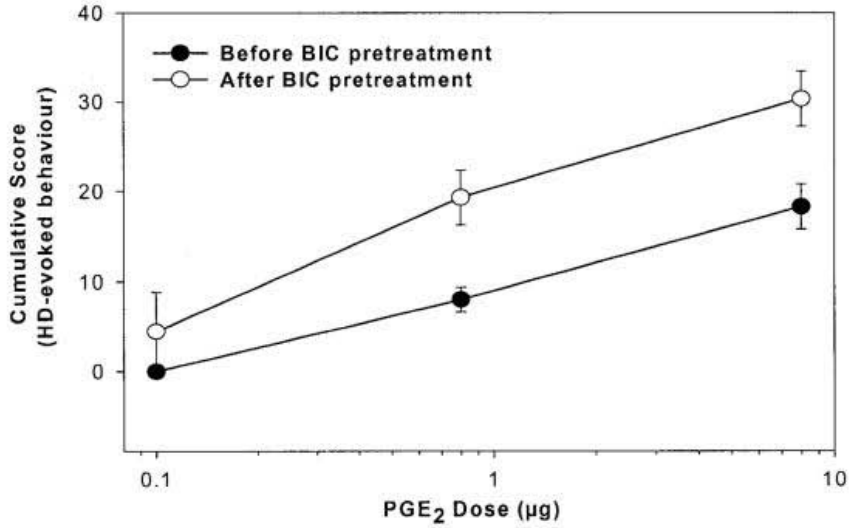


Figure 24. The effect of intrathecal (i.t.) bicuculline (BIC) on the dose-response relationship of i.t. PGE_2. Rats were pretreated with a sub-threshold dose of BIC (0.01 µg) five min before PGE_2 (0.1, 0.8 or 8 µg). Hair deflection-evoked behavioural responses were rated using a behavioural scoring system with the observer blinded to the nature of the treatment. Each point represents the mean ± SEM (5-7 rats) of the cumulative scores of HD-evoked behaviour per rat over the 2-h experiment. Bicuculline significantly enhanced the HD-evoked responses of 0.8 and 8 µg PGE_2 (Paired t-test: $P = 0.006$ and $P = 0.012$, respectively).

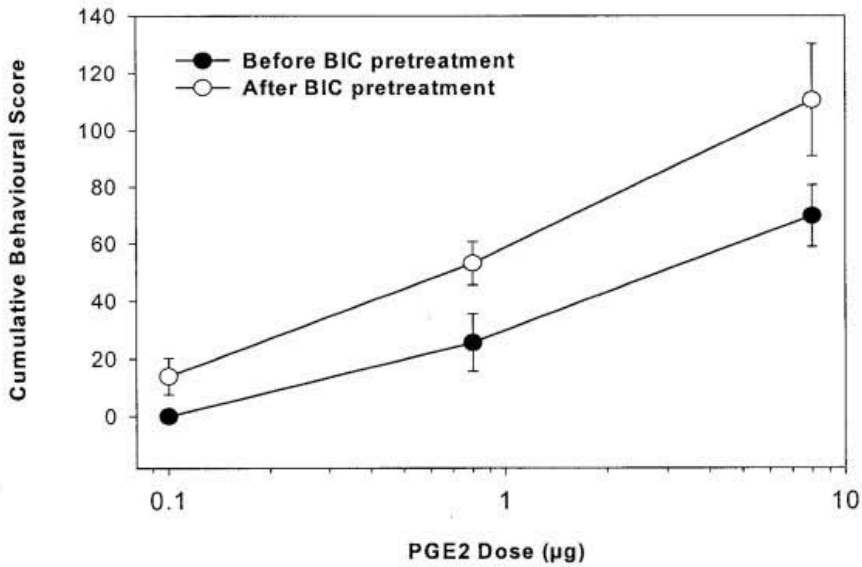


Figure 25. The effect of intrathecal (i.t.) bicuculline (BIC) on the dose-response relationship of i.t. PGE_2 (no hair deflection). Rats were pretreated with a sub-threshold dose of BIC (0.01 µg) five min before PGE_2 (0.1, 0.8 or 8 µg). Behavioural responses were rated using a behavioural scoring system with the observer blinded to the nature of the treatment. Each point represents the mean ± SEM (5-7 rats) of the cumulative scores of the observed behaviour over the 2-h experiment. Bicuculline significantly enhanced the behavioural responses of 0.8 and 8.0 µg PGE_2 (Paired t test: $P = 0.042$ and $P = 0.049$, respectively).

sensitization of cutaneous dermatomes induced by i.t. strychnine or bicuculline (Table 1) (Yaksh 1989; Sherman and Loomis, 1995; Onaka et al., 1996). Allodynic responses were evoked by hair deflection only on the affected digits of the hind paw; they occurred without general or localized convulsive activity, and the sensitization did not expand to the contralateral extremity over time or after repeated bicuculline dosing (up to 6 consecutive doses every 20 min).

The magnitude of allodynia, determined from the maximum HD-evoked responses, was dependent on the topical dose of bicuculline. These results provide further evidence for the role of GABA in modulating low-threshold afferent input at the spinal level, and indicate that topical bicuculline elicits allodynia by blocking $GABA_A$-receptors at discrete sites with the spinal segment(s) on which it is applied. Clearly, allodynia is not the result of a generalized increase in the sensitivity to afferent input along the spinal cord.

The localized effect of bicuculline in this study is also consistent with the unilateral distribution of fluorescence following the topical application of a bicuculline/bisbenzimide solution. There was no evidence of spread to the contralateral cord after single or multiple applications. In terms of penetration into the dorsal horn, fluorescence was highest in laminae I-III. While this fluorescence pattern does not necessarily reflect the penetration of bicuculline into the dorsal horn, it is noteworthy that fluorescence was highest in those laminae known to contain the highest density of $GABA_A$-receptors in rat spinal cord (laminae II-III; Persohn et al. 1991), and where GABAergic terminals contact primary afferent nerve endings (Barber et al, 1978, Carlton and Hayes, 1990, Alvarez et al, 1992)

or the cell bodies and dendrites of second order neurons (Barber et al, 1978; Magoul et al, 1987; Spike and Todd, 1992). Indeed, bicuculine, delivered into the spinal cord of the monkey or cat by means of a microdialysis probe placed transversely through the mid-dorsal horn, enhanced both the background activity and the evoked responses of spinal projection neurons (Lin et al., 1996; Sorkin et al, 1998). Conversely, the delivery of GABA or the $GABA_A$ receptor agonist, muscimol, elicited dose-dependent inhibition. These results indicate that GABAergic neurons modulate the transfer of low threshold mechanical input from primary afferent fibers to second order neurons in the spinal dorsal horn. That spinal GABAergic modulation has relevance to abnormal pain states is indicated by the marked allodynia arising from the blockade of spinal $GABA_A$ receptors with bicuculline (Yaksh, 1989, Onaka et al, 1996, Loomis et al, 2001), and by the loss of GABA-like immunoreactive cells in lamina I-III following focal spinal cord ischemia (Demediuk et al, 1989; Zhang et al, 1994; Hao et al, 1991). Bicuculline applied to the dorsal surface of the spinal cord appears to mimic the acute features of GABA deficiency but in a highly site-specific and circumscribed manner.

In an effect to prolong a bicuculline effect, the drug was reapplied to the same spot on the dorsal spinal cord every 20 min for 2 h. Allodynia was sustained at a reproducible level throughout the experiment with no detectable change in the size or location of cutaneous sensitization. While a similar approach is possible using i.t. drug delivery, this is often complicated by the volume and pressure changes introduced by repeated i.t. injections, and the attendant problem of non-specific effects. The results of present study demonstrate the ability to induce

highly localized and sustained allodynia using repeated topical drug administration to the dorsal spinal cord. Sustained disinhibition will be important in determining the immediate and delayed changes in somatosensory processing and for comparison with changes induced by spinal cord injury.

4.2 Effect of NS-398 on Blcuculline-Allodynia: Evidence of Prostaglandin Synthesis

In the present study, the topical application of NS-398 to the dorsal surface of the spinal cord dose-dependently inhibited bicuculline-allodynia. NS-398 is a potent and selective inhibitor of COX-2 (Copeland et al, 1994; Yamamoto, 1996b), exhibiting a selectivity ratio (IC_{50} COX-1/IC_{50} COX-2) of 163 in human whole blood cells (Panara et al, 1995), 300 in COX-1 and COX-2 transfected Chinese hamster ovary cells (Riendeau et al, 1997a, b) and 1263 in intact human platelets and synovial cells (Kawai et al, 1998) by measuring the inhibition of PGE_2 production. These data, combined with our previous report of stereo-selective inhibition of STRYCHNINE-allodynia with i.t. S(+) ibuprofen (Hall et al., 1999), support the hypothesis that PGs, synthesized and released in the rat spinal cord, facilitate the abnormal processing of low threshold mechanoreceptive input during bicuculline- or strychnine-disinhibition.

The synthesis of PGs from arachidonic acid is catalyzed by the enzyme COX. There are two known isoforms of this enzyme (COX-1 and COX-2) that reside in the membranes of the endoplasmic reticulum and nuclear envelope. Although both have considerable sequence homology, COX-1 and COX-2 are differentially expressed and thought to subserve different biological functions (Otto and Smith,

1995; Herschman, 1996). COX-1 is the constitutive isoform in most tissues including spinal cord, and is thought to be responsible for the synthesis of PGs mediating homeostatic regulation. Conversely, COX-2 is primarily an inducible isoform whose expression is triggered by a variety of chemical (e.g., growth factors, cytokines) and electrical (depolarizing) stimuli. As such, COX-2 is thought to be responsible for the generation of PGs mediating pathogenic events. However, COX-2 mRNA and protein exhibited basal expression in the brain and spinal cord (Breder et al., 1995; Beiche et al., 1996; Willingale et al., 1997; Vane et al., 1998), suggesting that constitutive COX-2 also plays an important regulatory role within the CNS.

This concept is supported by the results of the present study with NS-398. Indeed, the rapid onset (5 min after topical application) and short duration of allodynia (35-40 min) after a single dose of bicuculline (0.1 g), and the time course of inhibition by NS-398, argue against the recruitment of inducible COX-2 in this experiment model. These results are in agreement with earlier reports showing that: 1) the COX-2 selective inhibitor, celecoxib, but not the highly selective COX-1 inhibitor, SC-560, blocked the release of spinal PGs following the injection of carrageenan into the hind paw of the rat (Smith et al., 1998); and 2) PGD_2 levels in the Hippocampus of the gerbil increased ~50 fold within 5 min of a seizure and returned to near pre-seizure levels by 30 min (Forstermann et al., 1984; Hertting and Seregi, 1989). In addition, the restricted area of spinal cord over which the inhibitory effects of NS-398 were observed strongly suggests that the PGs facilitating allodynia in this model are products of COX-2 within the affected spinal

segment(s). Whether HD ultimately triggers the expression of COX-2 during $GABA_A$-receptor blockade remains to be determined. However, this would presumably require a more prolonged allodynic state (>2 h) which could be induced with repeated bicuculline application.

4.3 Effect of AP-7 on Bicuculline-Allodynia: Role of NMDA Receptors

Pretreatment of the rat with the NMDA receptor antagonist, AP-7, markedly suppressed the responses evoked by hair deflection during $GABA_A$ receptor blockade. This dose-dependent effect was not due to a general depression of spinal reflexes. Cardiovascular responses to phasic noxious pinch remained unaltered after AP-7 (data not shown). This finding suggests that low threshold afferent input acquires access to an NMDA receptor-dependent mechanism(s) during $GABA_A$ receptor blockade. It is well established that the activation of NMDA receptors is a very important component for both hyperalgesia and mechanical or thermal allodynia in the acute or the chronic facilitated pain animal models (Davar et al., 1991; Kim et al., 1997; Khandwala et al., 1997; Yamamoto et al., 1996a), while the COX-prostanoid pathway is also implicated in these abnormal pain states (Yaksh and Malmberg, 1993; Malmberg, 1995b,c; Hay et al., 1997). Ample evidence demonstrated a sequential functional link between NMDA receptor activation and the production of prostanoids in nociceptive neurotransmission in the spinal cord (Yaksh, 1999a). For example, in an *in vitro* perfusion study, PGE_2 levels in the perfusion medium were increased after NMDA incubation of the spinal cord of naive rats or rats with thermal hyperalgesia after peripheral inflammation (Dirig

and Yaksh, 1999). Intrathecal NMDA-induced mechanical allodynia and hyperalgesia were attenuated by the NMDA receptor antagonist MK801, the COX-2 selective inhibitor DFU or NS-398 and the non-selective COX inhibitor indomethacin, but not by the metabotropic glutamate receptor antagonist MCPG or the non-NMDA receptor antagonists DNQX in sheep or rats (Dolan and Nelan, 1999; Yamamoto and Sakashita, 1998). Therefore, it appears that there exists an NMDA receptor-COX-prostaglandin pathway in the spinal nociceptive neurotransmision (Yaksh, 1999a) and that spinal bicuculline induces allodynia, at least in part, through prostaglandin release (Fig 2).

$GABA_A$ receptors are distributed throughout rat spinal cord dorsal horn, especially, abundant in laminae II-III (Persohn et al. 1991). GABAergic neurons are presynaptic to primary afferent input terminals or axons of interneurons and appear to modulate glutamate release (Persohn et al. 1991, Barber et al., 1978, Carlton and Hayes, 1990; Alvarez et al., 1992). *In situ* hybridization analysis has confirmed the presence of $GABA_A$ receptor subunit mRNAs and protein within both small and large diameter dorsal root ganglion cells and in spinal cord dorsal horn (Persohn et al., 1991). As much as 30% of $GABA_A$ receptors in the spinal cord dorsal horn are believed to be on primary afferent terminals (estimates from the effects of rhizotomies and neonatal capsaicin). Therefore, the development of allodynia may result from an excessive glutamate release due to a loss of GABA inhibition on the presynaptic input. Indeed, GABA released from inhibitory interneurons of the spinal cord is believed to activate $GABA_A$ receptors in the afferent terminals and to reduce transmitter release (Rudomin, 1999); intrathecal bicuculline induces a transient

glutamate release in the CSF of the rat (Ishikawa and Yaksh, 1996). On the other hand, GABAergic neurons may produce postsynaptic inhibition by contacting the dendrites and cell bodies of the second order neurons. Thus, the blockade of GABA function with bicuculline may produce a partial depolarization in the membrane of the second order neurons and remove the Mg^{++} blockade from the NMDA receptor ion channel. In the presence of glutamate, this partial depolarization produces a facilitated sensitization in the spinal cord, thus, HD induces allodynia.

In a more recent study (Ishikawa et al., 2000) using a loop dialysis catheter, intrathecal strychnine or bicuculline yielded a touch-evoked agitation. Intrathecal bicuculline also evoked a transient spinal release of glutamate in the 0-10 min sample, while strychnine did not affect spinal transmitter release at any time. As intrathecal bicuculline but not strychnine increases glutamate release, while the allodynia of both is blocked by NMDA receptor antagonism, this further supports the hypothesis that GABA(A) sites regulate presynaptic glutamate release, while glycine regulates the excitability of neurons postsynaptic to glutamatergic terminals.

In the present study, the NMDA receptor antagonist AP-7 dose-dependently inhibited bicuculline-allodynia. This provides further evidence that NMDA receptor activation mediates the facilitated sensory signal processing of bicuculline-allodynia.

4.4 Effect of AP-7 on PGE_2-Allodynia in Conscious Rats: Role of NMDA Receptors

Experiments using conscious rats afford several advantages over anaesthetized animal experiments. Conscious experimental animals have similar physiological conditions to those of naive animals. This is especially important in

studies of sensory processing and pain. In this study, PGE_2-allodynia was investigated using conscious rats.

In the present study, intrathecal injection of PGE_2 to conscious rats dose-dependently induced nociceptive-like behavioural responses to innocuous tactile stimuli. These data agree with other studies which have shown that i.t. administration of PGE_2 caused allodynia in conscious mice (Minami et al., 1994a,b; 1995a) and rats (Malmberg et al., 1995a). The spontaneous behavioural response (without HD stimulation) showed a trend to increase with the PGE_2 dose, however, statistical analysis indicated that this response was not dose-dependent (the slope of linear regression line $\beta = 2.95$, $P = 0.066$).

The mechanism(s) of this innocuous tactile stimulus evoked nociceptive-like responses (allodynia) in PGE_2-treated rats remain(s) unclear. As shown in previous studies using HPLC and fluorescence detection method PGE_2 induces an immediate increase in glutamate and aspartate release in intrathecal dialysis perfusate of the rat spinal cord and in the superfusion of rat spinal cord synaptosomes. Thus, one may infer that the release of excitatory amino acid (EAA) glutamate and aspartate represents an important mechanism in PGE_2 -allodynia. Indeed, in the present study, intrathecal pretreatment of sub-immobilization dose of an NMDA receptor antagonist AP-7 significantly inhibited PGE_2-allodynia in conscious rats. This is consistent with other data obtained in conscious mice. Co-administration of MK-801, a non-competitive NMDA receptor channel blocker, or D-AP-5, a selective NMDA receptor antagonist, with PGE_2, dose-dependently blocked PGE_2-induced allodynia. In contrast, none of these NMDA receptor antagonists

inhibited $PGF_{2\alpha}$-allodynia (Minami et al., 1994b). These results are also supported by the preliminary data of our laboratory showing that perfusion of lumbar spinal cord slices with PGE_2 increases glutamate release. All these results suggest a presynaptic action of PGE_2 on the primary afferent terminals.

EAA such as glutamate and aspartate have been proposed as primary afferent neurotransmitters involved in nociception in the spinal cord (Watkins and Evans, 1981; Salt and Hill, 1983; Merighi et al., 1991). The results of electrophysiological and immunohistochemical studies suggest that these EAAs are involved in the neurotransmission between low-threshold afferent and dorsal horn neurons (Kangrga and Randic, 1991; Schneider and Perl 1994). Although the precise mechanism of allodynia induced by PG is not known at present, our results with AP-7 suggest that the PGE_2 receptor activation gives rise to an increased spinal EAA release or prolongs the release of EAA in response to low-threshold tactile stimuli and that spinal NMDA sites activated by the released EAA may initiate a facilitated state of spinal sensory signal processing leading to the PGE_2-induced allodynia.

In conscious mice, post-treatment with MK-801, the NMDA receptor antagonist, 5 min after i.t. PGE_2 failed to block PGE_2-allodynia (Minami et al., 1994b). It is suggested that this facilitated state, once activated, does not require glutamate receptor sites. Our preliminary experiments (data not shown) suggest that post-treatment with AP-7, 5 min after i.t. PGE_2 does not block innocuous tactile stimulus-induced allodynia-like behavioural responses. These results combined with other data from post-treatment experiments suggest that in the presence of

PGE_2, once an afferent barrage from Aβ fibers has generated a threshold effect on neuronal function, the resulting activation of glutamate receptors may cause a series of subsequent events (e.g., the production of PGs, NO) that maintain the established allodynia without further requirement of NMDA receptor activation.

In the present study, a low dose of AP-7 (0.4 μg) was used in order to prevent drug diffusion-induced immobilization effects. AP-7 significantly but only partially inhibited PGE_2-allodynia suggesting that the dose employed may not have been sufficient to produce a stronger or even complete blockade of the PGE_2 effect. Another strong possibility is that PGE_2-induced glutamate release may only represent one mechanism that underlies the allodynic effect of i.t. PGE_2.

An alternative mechanism of PGE_2-allodynia is that PGE_2 may act via neuropeptide release. PGE_2 has been shown to increase the release of substance P and to facilitate depolarization-evoked substance P release from both dorsal root ganglion neurons in culture (Nicol et al., 1992) and spinal cord slices *in vitro* (Vasko et al., 1994; Vasko, 1995). Substance P and glutamate, acting at NK1- and NMDA-receptors of dorsal horn neurons, respectively, (Wall and Woolf, 1986; Dickenson et al., 1997; Yaksh et al., 1999b), elicit prolonged depolarization and initiate the facilitated excitation state in the spinal cord. Furthermore, there is cumulative evidence showing that PGE_2 may directly increase the excitability of sensory neurons. For example, in patch clamp studies of sensory neurons isolated from embryonic rats and grown in culture, PGE_2 enhances the excitability through the suppression of an outward potassium current that may modulate the firing threshold for generation of the action potential (Nicol et al., 1997; Evans et al., 1999), through

the activation of the cyclic adenosine 3',5'-monophosphate pathway. PGE_2 also enhanced the capsaicin-evoked current in rat sensory neurons (by 2- to 3-fold) (Lopshire and Nicol, 1997; 1998). Intrathecal injection of an inactive dose of PGE_2 enhanced capsaicin-evoked amino acid release in the spinal cord perfusate of the conscious rat. The hypothesis that PGE_2 has a direct action on the primary afferent terminals is also supported by the finding that PGE_2 enhanced CGRP release from the spinal cord *in vitro* in response to dorsal root stimulation (Andreeva and Rang, 1993). CGRP is contained exclusively in primary afferent nerve terminals in the dorsal horn (Chung et al., 1988).

Although there is no direct evidence showing the existence of postsynaptic PGE_2 receptors in the dorsal horn, the possibility of a PGE_2 postsynaptic action on the intrinsic spinal dorsal horn neurons still cannot be excluded. For example, nerve ligation central to the ganglion (central ligation) yielded an accumulation of iloprost binding sites (PGI_2 binding sites) in the ganglion side proximal to the ligature. On the contrary no clear accumulation of PGE_2 binding sites could be demonstrated. It is suggested that iloprost binding sites are transported from ganglion cell body to afferent input terminals (Matsumura et al., 1995). Thus, iloprost binding sites may exist at the presynaptic sites while PGE_2 binding sites may exist on postsynaptic sites as suggested by the absence of PGE_2 accumulation after ligature. The decrease of PGE_2 binding sites after rhizotomy may possibly only suggest that the expression of the binding sites is under the regulatory control of primary sensory input (Matsumura et al., 1992, 1995).

Taiwo and Levine (1988) proposed another possible mechanism of PGE_2 action at the spinal cord level. Intrathecal PGE_2 antagonized the analgesia produced by both nucleus reticularis paragigantocellularis (NRPG) electrical stimulation and intracerebroventricular morphine. In contrast, the NSAIDs synergized with brain stimulation and morphine-induced analgesia. The α-adrenergic antagonist phentolamine and the catecholaminergic selective neurotoxin 6-hydroxydopamine (used to block tonic catecholamine neuron activity in endogenous opioid-mediated analgesia systems) prevented the hyperalgesia induced by intrathecal PGE_2. Phentolamine did not, however, block the hyperalgesia caused by intradermal PGE_2. These results suggest that PGE_2 could block both electrical stimulation-produced analgesia and endogenous opioid mediated analgesia by inhibiting the noradrenergic synaptic transmission in the spinal terminals of bulbospinal noradrenergic projection neurons of this analgesia pathway. The observations that PGE_2 induced-allodynia was dose-dependently relieved by a high dose of the α_2-adrenoceptor agonist clonidine (Minami et al., 1994b) and that PGE_2 could inhibit the synaptic release of norepinephrine in the brain (Bergstrom et al., 1973) provide further evidence for the hypothesis that PGE_2 may act at spinal postsynaptic sites in nociceptive neurotransmission.

Collectively, PGE_2 may facilitate spinal sensory neurotransmission by both postsynaptic and presynaptic mechanisms, for example, by increasing glutamate release from primary afferent terminals as supported by the present study, by increasing the release of neuropeptide (the latter may in turn increase glutamate release) or by the inhibition of opioid and adrenergic mediated analgesia mechanisms.

4.5 Effect of SC-51322 on Bicuculline- and PGE_2-Allodynia: Role of EP Receptors

There is considerable evidence that PGE_2, released in the rat spinal cord in response to noxious stimuli, is the major PG that facilitates spinal nociceptive processing (Willingale et al., 1997; Malmberg and Yaksh, 1992a; Yang et al, 1996; Hay et al., 1997; and Goppelt-Struebe and Beiche, 1997). For example, superfusion of the lumbar spinal cord of normal rats with artificial CSF and subsequent radioimmunoassay revealed the presence of $PGE_2 > PGD_2$, but not PGI_2 (determined by measurement of the stable metabolite, 6-keto-$PGF_{1\alpha}$) or $PGF_{2\alpha}$.

If central PGs are relevant to the abnormal processing of sensory input during spinal disinhibition, then their effects should be attenuated by the local application of a PG receptor antagonist. In the present study using SC-51322, we have demonstrated that bicuculline-allodynia is sensitive to the effects of EP-receptor blockade; an effect that was dose-dependent. PGE_2-allodynia was also dose-dependently inhibited by SC-51322 in conscious PGE_2-treated rats. These data are consistent with previous studies demonstrating: a) the involvement of spinal EP receptors in the induction of allodynia by i.t. PGE_2 (Minami et al., 1995b; Sakai et al., 1998); b) PGE_2 as the major arachidonic acid product facilitating spinal nociceptive processing (Malmberg and Yaksh, 1994; Yang et al., 1996; Hay et al., 1997; and Goppelt Struebe and Beiche, 1997); and c) the high affinity of PGE_2 to EP receptors (Lawrence et al., 1992). They are also in agreement with preliminary experiments in our laboratory showing a significant increase in the concentration of PGE_2 in spinal microdialysis samples during but not after strychnine-allodynia (Hall

et al., unpublished observation). These results, combined with the local inhibitory effect of NS-398, provide further evidence that spinal PGs contribute to the cellular events which effect bicuculline-allodynia.

At least four subtypes of the PG E-series receptors have been identified: EP_1, EP_2, EP_3, EP_4. The four PGE_2 receptor subtypes couple to different major signal transduction pathways: namely elevation of intracellular calcium (EP_1), stimulation (EP_2, EP_4) or inhibition of adenylyl cyclase (EP_3). Several lines of evidence indicate that sensory neurons express EP receptors (Bley et al., 1998). Although [^{3}H]PGE_2 binding sites are present at a high density in rat dorsal horn of the spinal cord, the identity of the receptor subtypes is unclear. For example, using *in situ* hybridization techniques, EP_3, and to a lesser extent EP_1 and EP_4 receptor mRNAs have been detected in mouse dorsal root ganglia neurons (Oida et al., 1995; Sugimoto et al., 1994). EP_2 mRNA was detected in the spinal dorsal horn of rats (Kawamura et al., 1997). A recent study showed that the EP_3 receptor is also expressed in the spinal cord of the rat (Beiche et al., 1998a). Immunocytochemical staining with EP_3 antibody revealed a spatially contained expression of EP_3 IR in the superficial dorsal horn laminae I-II, where the nociceptive afferent terminates (Beiche et al., 1998a). It seems that multiple EP receptors are associated with the PGE_2 action in the sensory processing of spinal cord dorsal horn. In the present study, the EP-receptor antagonist SC-51322 partially inhibited bicuculline-allodynia as well as PGE_2-allodynia suggesting that PGE_2, at least partially, mediate this abnormal sensory processing of allodynia. While this distortion of sensory processing appears to be mediated by spinal EP receptors, the contribution of specific EP-receptor subtypes remains to be determined.

4.6 Bicuculline- and PGE_2-Allodynia

Bicuculline selectively induced an augmented excitatory state in the spinal dorsal horn; an innocuous stimulus in the presence of bicuculline evoked allodynia. This allodynic state is sensitive to inhibition of COX, or blockade of EP and NMDA receptors. These results combined with the observations that PGE_2 dose dependently induced allodynia-like behaviour in conscious rats provide further evidence for the possible role of PGE_2 in bicuculline-allodynia. These results suggest that PGs, especially PGE_2, may mediate bicuculline-allodynia via affecting EP receptors, and that the activation of NMDA receptors is important for the abnormal sensory processing of bicuculline-allodynia. Furthermore, in the present study, pretreatment of bicuculline enhanced PGE_2-induced allodynia as evidenced by the leftward shift of the PGE_2 dose-response curve. This result indicates that bicuculline may act, at least partially through a PGE_2 pathway; in the presence of bicuculline, administration of PGE_2 may incrementally facilitate the excitatory signal processing and produce an additive effect.

It is obvious that there is a functional link between bicuculline-induced disinhibition and PGE_2 release. This link may include: 1) bicuculline-induced glutamate release, 2) NMDA receptor activation, 3) activation of a cascade of signal transduction pathways including PGE_2 release. PGE_2 would induce more glutamate release and further NMDA receptor activation. This positive feedback process could represent an important mechanism in bicuculline-allodynia and PGE_2 allodynia. There also seems to be some evidence showing the direct effect of PGE_2 on GABAergic systems. For example, in the rat brain PGE_2 inhibited spontaneous

inhibitory postsynaptic currents (IPSCs) of the rat supraoptic neurons in the presence of tetrodotoxin, a blocker of Na^+ channels, suggesting that PGE_2 acts presynaptically on the GABAergic terminals to decrease GABA release (Ibrahim et al., 1999). In a study (Eguchi et al., 1999) using lipocalin-type PGD synthase deficient mice (L-PGDS-/- mice), i.t. PGE_2 and bicuculline failed to induce tactile allodynia, while simultaneous injection of PGE_2 or BIC with a femtogram amount of PGD_2 induced allodynia in L-PGDS-/- mice to the same extent as in wild-type mice. The PGE_2 or bicuculline induced allodynia in L-PGDS-/- mice was blocked by a PGD_2 receptor antagonist given in a femtogram amount. These results combined with the present observations that bicuculline-allodynia was markedly inhibited by the blocking of PGE_2 pathway and that bicuculline enhanced PGE_2 allodynia suggest that bicuculline and PGE_2 may, at least partially, share the same signal transduction pathways in inducing allodynia.

4.7 Summary

Increased spinal glutamatergic tone has been shown to yield a state of facilitated transmission of both low- and high-intensity stimuli (Dougherty et al., 1992). Of the glutamate receptor family, spinal NMDA-receptors are especially important as those receptors that mediate long lasting depolarization including the phenomenon of "wind up" (Dickenson and Sullivan, 1987). Such excitation appears to be an important spinal mechanism underlying the condition of allodynia and hyperalgesia (Yaksh et al., 1999b, Woolf and Thompson, 1991). The present research demonstrates that the abnormal responses evoked by input from low-

threshold mechanoreceptive afferents in the presence of spinal bicuculline and PGE_2 are indeed sensitive to NMDA-receptor blockade by AP-7 or the EP-receptor antagonist SC-51322. In summary, the evidence obtained in this study supports the following conclusions:

1. Bicuculline is a modulator of non-noxious somatosensory input in the spinal cord of the rat.
2. In the presence of spinal topical bicuculline, low threshold afferent input selectively accesses a spinal sensitization mechanism normally activated by nociceptive fibers.
3. Blockade of the PGE_2 system by the selective COX-2 inhibitor, NS-398 or by the EP-receptor antagonist, SC-51322 attenuates bicuculline-allodynia.
4. Intrathecal application of PGE_2 dose-dependently induces nociceptive-like behavioural responses similar to those induced by i.t. bicuculline.
5. Blockade of spinal NMDA receptors inhibits both bicuculline- and PGE_2-allodynia, and at high dose, completely inhibits bicuculline-allodynia.
6. A sub-allodynic dose of bicuculline potentiates PGE_2-induced allodynic behavioural responses.

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6 PUBLICATIONS ARISING FROM THIS WORK

Papers:

Zizhen Zhang, H. Khandwala, M.P. Heferran and C.W. Loomis, Topical bicuculline to the rat spinal cord induces highly localized allodynia that is mediated by spinal prostaglandins, *Pain*, 92 (2001) 351-361.

Abstracts:

Zizhen Zhang, H. Khandwala and C.W. Loomis, A study of the role of spinal prostaglandins in an acute rat model of allodynia, Proceedings of the IASP 9th world Congress on Pain, Page 380, Vienna Austria, 1999.

Zizhen Zhang, M.P. Heferran and C.W. Loomis, Topical bicuculline to the rat spinal cord induces highly localized allodynia that is mediated by spinal prostaglandins, Abstract and Poster, the Canadian Pain Society Annual meeting, St. John's, Canada, 1999.

Michael P. Hefferan, Zizhen Zhang, Hemal Khandwala and C.W. Loomis, Application of strychnine or bicuculline to the dorsal surface of spinal cord produces highly localized allodynia in the rat, Abstract and poster, the Canadian Pain Society Annual meeting, St. John's, Canada, 1999.

Zizhen Zhang, H. Khandwala, M.P. Hefferan and C.W. Loomis, A study of the spinal prostaglandins in an experimental animal model of allodynia, MRC National Graduate Student Research Poster Competition, University of Manitoba, and the Annual meeting of the Canadian Federation of Biological Sciences, Winnipeg, Canada, 1999.