A STUDY OF INTRATHECAL STRYCHNINE-INDUCED ALLODYNIA IN THE LIGHTLY ANESTHETIZED RAT

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by

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ABSTRACT

Allodynia, an outcome of neural injury, is a condition in which non-painful stimuli (e.g. light brushing or light touch of clothing) evoke pain. Unlike normal (nociceptive) pain, allodynia is triggered by input from low-threshold (Aβ) afferent fibers. Normally, the synaptic transmission of Aβ-input appears to be modulated by glycine released from spinal interneurons. Thus, the blockade of spinal glycine receptors with intrathecal (i.t.) strychnine (STR) produces reversible, segmentally localized allodynia in the rat; a condition blocked by the spinal administration of excitatory amino acid (EAA) receptor antagonists. These results suggest that, in the absence of spinal glycinergic modulation, input from Aβ-fibers acquire access to spinal pain-signalling pathways. If this hypothesis is correct, then i.t. STR-induced allodynia should be blocked by antagonists of spinal NMDA-receptors, and by neuromodulatory systems that modulate EAA-mediated synaptic transmission in the spinal cord. Therefore, the specific objectives of this study were to: 1) determine the effect of i.t. AP-7 (NMDA-antagonist), cyclopentyladenosine (A_1-agonist; CPA), CGS-21680 (A_2-agonist), muscimol ($GABA_A$-agonist) and baclofen ($GABA_B$-agonist) on STR-allodynia; 2) compare the effect of intravenous mexiletine on STR-allodynia and calibrated bilateral paw-pinch (without STR); 3) determine the effect of intravenous milacemide (a glycine prodrug and substrate for MAO-B) on STR-allodynia; and 4) test the sensitivity of this milacemide effect to

pretreatment with clorgyline (MAO-A inhibitor) or l-deprenyl (MAO-B inhibitor). Male, Sprague- Dawley rats, fitted with i.t. catheters, were lightly anesthetized with urethane. Stimulus-evoked changes in blood pressure and heart rate, and cortical electroencephalographic (EEG) activity were continuously recorded. After i.t. STR (40 µg), repetitive brushing of the hair with a cotton tipped applicator (a normally ineffective stimulus) evoked a marked and progressive increase in mean arterial pressure (MAP) and heart rate (HR), an abrupt motor withdrawal (WD) response and desynchronization of the EEG; effects comparable to those elicited by intradermal mustard oil without STR. Pretreatment with i.t. AP-7, CPA, CGS and muscimol, but not baclofen, dose-dependently inhibited STR-allodynia without affecting EEG synchrony. The ED_{50}s for i.t. AP-7 were: 1.12 µg for the evoked change MAP, 1.71 µg for the evoked change in HR, and 0.36 µg for the evoked change in withdrawal duration. The corresponding ED_{50}s ranged from 0.02-0.07 µg for CPA; 2.69-3.11 µg for CGS and 0.44-0.51 µg for muscimol. Intravenous mexiletine inhibited the responses evoked by noxious paw-pinch (without STR) and hair deflection in STR-treated rats with equal potency (ED_{50}s = 9-17 mg/kg). There was no concurrent change in EEG synchrony and no blockade of motor efferent pathways. Intravenous milacemide inhibited STR-allodynia (ED_{50}s = 398-404 mg/kg) at doses having no effect on normal nociception. This selective anti-allodynic effect was blocked by pretreatment with l-deprenyl, but not with clorgyline.

The results of this study support the following conclusions. 1) Glycine is a selective modulator of non-noxious somatosensory input in the spinal cord of the rat. 2) In the presence of i.t. STR, low threshold afferent input accesses an NMDA-mediated spinal sensitisation mechanism normally activated by high threshold (nociceptive) fibers. 3) Systemic mexiletine inhibits both abnormal (STR-allodynia) and normal (noxious pinch) nociception at a common central site, most likely the dorsal horn cells receiving convergent Aβ- and C-fiber input. 4) The recruitment of neuromodulatory systems utilizing spinal A_1- and $GABA_A$ -receptors blocks the abnormal responses to hair deflection during STR blockade of spinal glycine receptors. 5) Increasing central glycine concentration by means of a systemic glycine prodrug (milacemide) normalizes the spinal processing of low threshold afferent input in STR-allodynia. 6) The spinal pharmacology of STR-allodynia is similar to that of clinical allodynia, and clearly distinct from that of normal nociception. 7) STR-allodynia is a rapid, selective and valid non-injury model for investigating this abnormal pain state.

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%	percent
°C	degree(s) Celsius
α	alpha, a Greek letter
β	beta, a Greek letter
γ	gamma, a Greek letter
δ	delta, a Greek letter
μ	mu, a Greek letter, used to indicate micro in metric units
μg	microgram(s), 10^{-6} grams, unit of mass
μl	microliter(s), 10^{-6} litres, units of volume
5-HT	5-hydroxytryptamine
Aβ	A-beta, a class of primary afferent neurons
Aδ	A-delta, a class of primary afferent neurons
A_1	adenosine receptor subtype
A_2	adenosine receptor subtype
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
AP-5	D-aminophosphonovalerate
AP-7	(±)-2-amino-7-phosphonoheptanoic acid
Asp	aspartate
ATP	adenosine triphosphate
BBB	blood brain barrier
BP	blood pressure
bpm	beats per minute
C	a class of primary afferent neurons
CGS-21680	2-p-(2-carboxyethyl)phenylamino-5'-N-ethylcarboxamidoadenosine hydrochloride
CI	confidence interval
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CP-96345	(2S,3S)-cis-2-(di-phenylmethyl)-N-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine
CPA	N^6-cyclopentyl adenosine
CPP	3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonoic acid
CSF	cerebrospinal fluid
DGG	γ-D-glutamylglycine
DMSO	dimethyl sulfoxide
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DRG	Dorsal root ganglion

EAA	excitatory amino acid(s)
ED_{50}	effective dose yielding a 50 percent response
EEG	electroencephalogram(s) / graph(s)
e.g.	*exempli gratia* (for example)
EPSP	excitatory postsynaptic potential(s)
GABA	γ-aminobutyric acid
Glu	glutamate
Gs	stimulatory G-protein
Gi	inhibitory G-protein
HCl	hydrochloric acid
HD	hair deflection
Hg	elemental symbol for mercury
HR	heart rate
Hz	hertz, S^{-1}, reciprocal seconds, unit of frequency
IASP	International Association for the study of Pain
i.e.	*Id est,* that is
in vitro	in glassware
in vivo	in the living body
i.p.	intraperitoneal(ly)
IPSP	inhibitory postsynaptic potential(s)
i.t.	intrathecal(ly)
i.v.	intravenous(ly)
I	Roman numeral one, used to denote spinal lamina
II	Roman numeral two, used to denote spinal lamina
III	Roman numeral three, used to denote spinal lamina
IV	Roman numeral four, used to denote spinal lamina
Ka	affinity constant
KA	kainate
Kpa	kilopascal(s), unit of pressure
LI	-like immunoreactivity
L1	lumbar vertebra number 1
L2	lumbar vertebra number 2
M	molar (moles/liter)
MAO-A	monoamine oxidase A type enzyme
MAO-B	monoamine oxidase B type enzyme
MAP	mean arterial pressure
MK-801	(+)-5-methyl-10,11-dihydro-*5H*-dibenzo (*a,d*) cyclo heptene-5,10-imine maleate
N	number of determinations
NaOH	sodium hydroxide

NBQX	2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo (f) quinoxaline
NECA	5'-N-ethylcarboxamidoadenosine
NK	neurokinin
NMDA	N-methyl-D-aspartate
nmol	nanomole(s), 10^{-9} moles, number of molecules
p	probability of error
PE-10	polyethylene tubing (diameter ~0.61 mm)
PP	paw-pinch
RPIA	R(-)-N^6-(2-phenylisopropyl)adenosine
s.c.	subcutaneous(ly)
SEM	standard error of the mean
SP	substance P
STR	strychnine
SYN	synchrony
T	time
WDR	wide dynamic range

1 Introduction

1.1 Statement of the Research Problem

Injury to the peripheral or central nervous system can effect an abnormal sensory state in which innocuous stimuli acquire the ability to evoke intense pain; a condition known as allodynia. Clinically, allodynic pain is triggered by input from low threshold mechanoreceptive primary afferent neurons (Meyer et al., 1985; Campbell et al., 1988; Price et al., 1989; Treede et al., 1991); that is, by peripheral (Aβ) substrates which normally have no role in pain transduction and transmission. Although the pathophysiological mechanisms of allodynia are unknown, the striking change in modality subserved by Aβ-fibers after nerve injury, and the apparently limited role of abnormally sensitized C-nociceptors in this condition (Cline et al., 1989) are highly suggestive of a central dysfunction.

Under normal conditions, the synaptic transmission of low threshold afferent input appears to be modulated by glycine and GABA (γ-aminobutyric acid) released from interneurons in the dorsal horn of the spinal cord (Curtis et al., 1968, 1971; Game and Lodge, 1975; Wilcockson et al., 1984; Todd, 1990; Todd and Sullivan, 1990). The loss of such inhibitory modulation, as might occur after nerve injury, could enhance the synaptic efficacy between Aβ-fibers and pain-signalling pathways, thereby permitting the miscoding of low-threshold input as pain.

Consistent with this central hypothesis of allodynia are reports that the intrathecal (i.t.) injection of strychnine (STR; a competitive glycine receptor antagonist) into the spinal subarachnoid space of rodents induces a pure allodynic state. Thus innocuous hair deflection (HD) in the presence (but not absence) of i.t. STR evokes behavioral, cardiovascular, neurochemical and electrophysiological responses comparable to those elicited by noxious thermal, mechanical or chemical stimulation without STR (Beyer et al., 1985, 1988; Sosnowski and Yaksh, 1989; Yaksh, 1989; Sherman and Loomis, 1994, 1995, 1996; Milne et al., 1996; Sherman et al., 1996; Onaka et al., 1996). Importantly, these tactile-evoked responses are segmentally localized (Sherman and Loomis, 1995) and exhibit a spinal pharmacology that is distinct from that of conventional nociception (Yaksh, 1989; Sosnowski and Yaksh, 1989; Sherman and Loomis, 1994, 1995, 1996). Indeed, glycine appears to be a selective modulator of *non-nociceptive* input in the rat spinal cord as i.t. STR neither enhances nor inhibits responses to high threshold cutaneous stimulation (Sherman and Loomis, 1996). Collectively these data indicate that the acute blockade of spinal glycine receptors with i.t. STR yields an abnormal sensory condition having many of the features of clinical allodynia.

Previous pharmacological studies have shown that STR-allodynia is dose-dependently inhibited by i.t. excitatory amino acid antagonists including NBQX (2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(f) quinoxaline) and γ-DGG (γ-D-glutamylglycine) and by i.t. glycine or betaine (N,N,N-trimethyl glycine) (Sherman

and Loomis, 1995, 1996) (see Table II, page 22). In contrast, STR-allodynia is insensitive to i.t. morphine, CP-96345 [neurokinin(NK_1)-receptor antagonist] or neonatal treatment with capsaicin, at doses which are effective against normal nociception (Sherman and Loomis, 1994, 1996). These results indicate that the spinal pharmacology of STR-allodynia is distinct from the nociception normally conveyed by small diameter (Aδ and C) fibers and evoked by high-threshold thermal, chemical or mechanical stimuli (for review see Yaksh and Malmberg, 1994).

The spinal dorsal horn contains the first synaptic contacts between peripheral afferent fibers and the central neurons conveying somatosensory information to the critical sites in the brain. Insight into the spinal processing of somatosensory input could reveal useful information about the mechanism(s) of allodynia as well as identify modulatory systems that could be exploited pharmacologically to offset the abnormal sensations triggered by light tactile input in allodynia. The neural systems implicated in the spinal processing of Aβ-input are illustrated in Figure 1. To further investigate the spinal pharmacology of STR-allodynia, and to explore the neuromodulatory systems affecting low threshold somatosensory input in the spinal cord, the present study sought to systematically determine the effect of GABA, adenosine and glycine in the STR-model. Also, the effect of mexiletine, AP-7 [D(-)-2-amino-7-phosphonoheptanoic acid; an NMDA-receptor antagonist], and milacemide (a glycine prodrug) on STR-allodynia and

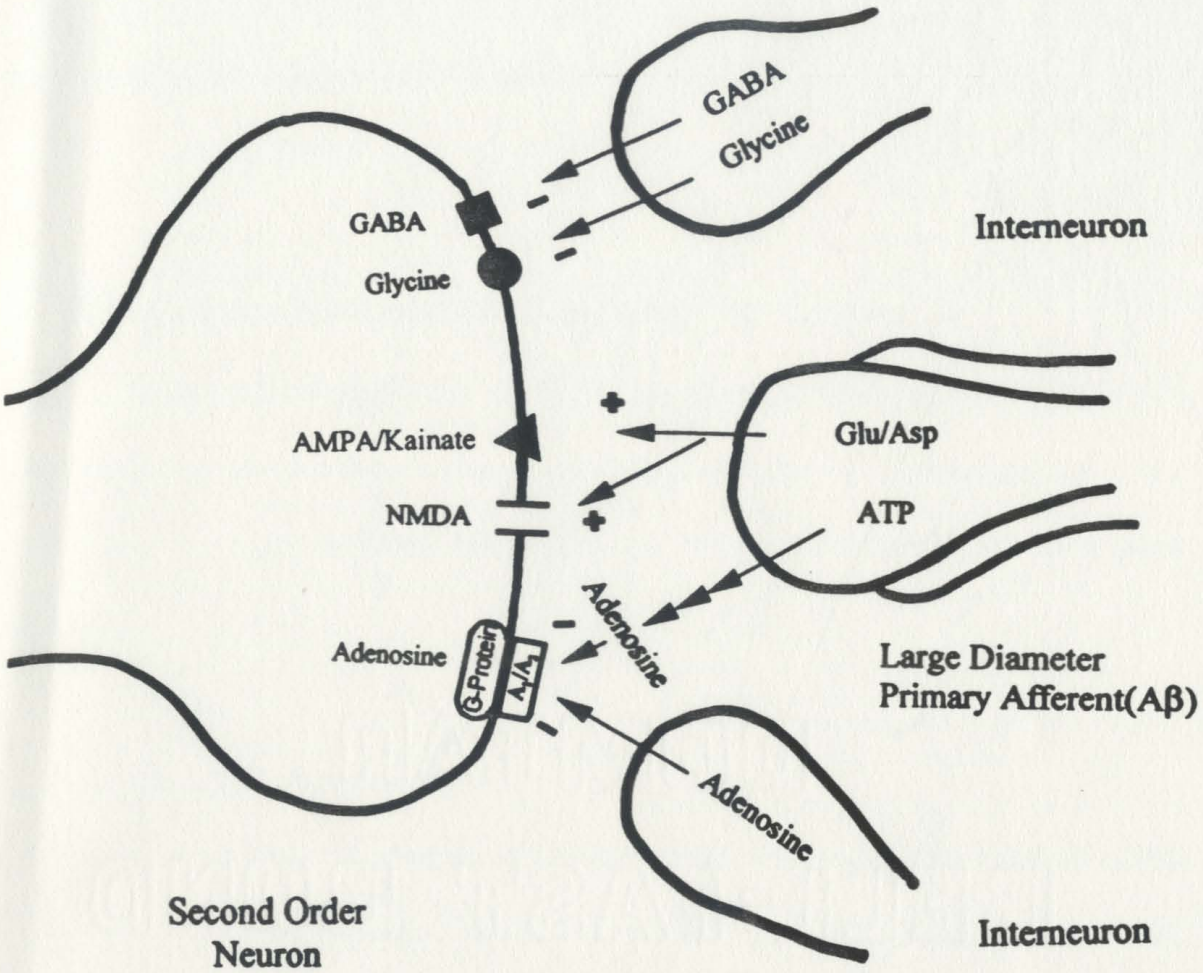


Figure 1. Possible neurotransmitters/neuromodulators and their receptor systems implicated in the spinal processing of low-threshold (Aβ) afferent input. Glutamate (Glu), aspartate (Asp) and ATP are thought to be released from the central terminals of Aβ-fibers. Normally, extracellular glutamate and aspartate act on AMPA- and kainate-receptors. NMDA-receptors, although present on second order neurons, are not normally recruited by low-threshold input (but may be important in abnormal low-threshold input). Local inhibitory systems modulating the response to Aβ-input utilize GABA, glycine and adenosine. The latter, including that derived from the extracellular hydrolysis of ATP, can act on A_1- and/or A_2-receptors. The postsynaptic effects of these neurotransmitters are well established. The presynaptic effects of glycine and adenosine are less clear and have been omitted for clarity. Although, in the present diagram, adenosine is shown to be released from interneurons, its neuronal origin is unclear.

normal nociception (without STR) was compared.

1.2 General Organisation of the Somatosensory System

Sensory systems consist of serial neural pathways linking the periphery with the spinal cord, brain stem, thalamus and cerebral cortex. These pathways are comprised of primary afferent fibers, second order neurons and the third order neurons.

1.2.1 Primary Afferent Neurons

Primary afferent neurons carry somatosensory information from the periphery to the spinal cord where they synapse with second order neurons located in the dorsal grey matter. Thus, the first synapse in this neural circuitry is located centrally in the outer laminae of the dorsal horn. Primary afferent fibers found in mixed peripheral nerves are subdivided into three main types; A, B and C. A-fibers are further divided into four sub-groups (α, β, γ and δ) based on their diameter, degree of myelination and corresponding conduction velocity (Table I). These fibers selectively respond to different modalities in accordance with the types of sensory receptors located on their peripheral terminals. Thus, Aδ-fibers are activated primarily by intense mechanical pressure but can also be activated by noxious thermal or chemical stimuli (Kandel and Schwartz, 1991). In contrast, C-

Table I. Classification of fibers in peripheral nerves

Fiber Type	Innervation	Mean Diameter μm (range)	Mean Conduction Velocity m/sec (range)
Aα	Primary Muscle Spindle To Skeletal Muscles	12 (12-20)	100 (70-120)
β	Cutaneous Touch and Pressure Afferents	8 (5-15)	50 (30-70)
γ	Motor to Muscle Spindle	6 (6-8)	20 (15-30)
δ	Mechanoreceptors, Nociceptors	3 (1-4)	15 (12-30)
B	Sympathetic Preganglionic	3 (1-3)	7 (3-15)
C	Mechanoreceptors, Nociceptors Sympathetic Postganglionic	1 (0.5-1.5)	1 (0.5-2)

fibers are primarily activated by noxious chemical, mechanical and thermal stimuli. Psychophysical studies in humans demonstrate that electrical stimulation of Aδ-fibers evoke a brief pricking or stinging sensation while that evoked by C-fiber stimulation is described as a prolonged burning and unpleasant (Torebjörk and Ochoa, 1980; Konietzny et al., 1981). Stimulation of Aβ-fibers normally evokes light tactile sensations including vibration and mild pressure. These primary afferent fibers enter the spinal cord through the dorsal root entry zone. Large diameter fibers bifurcate centrally with one branch entering the dorsal columns and the second branch penetrating deep into the neck of the dorsal horn where they reverse course and terminate in the nucleus proprius (Figure 2). Small diameter

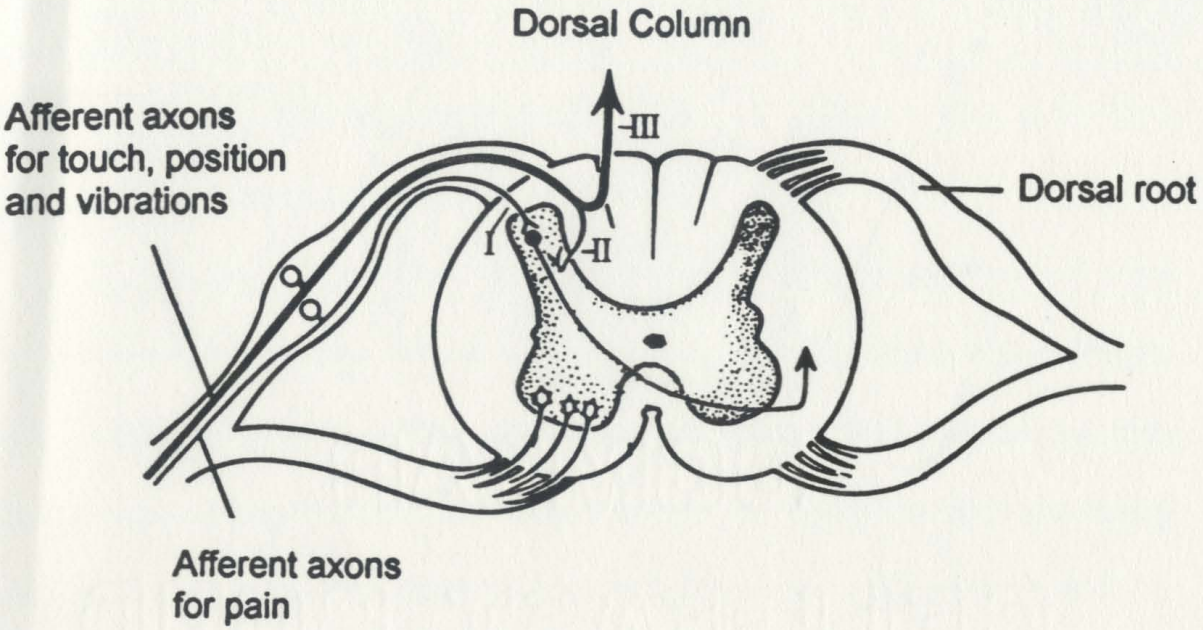


Figure 2. Cross section of spinal cord showing the termination of primary afferent fibers in dorsal grey matter. Small diameter fibers terminate directly in the outer laminae of the dorsal horn where they make synaptic contact with the dendrites of second order neurons (I). Large diameter fibers bifurcate centrally with one branch penetrating deep into the neck of the dorsal horn (II) where they reverse course and terminate in the nucleus proprius, and the second branch entering the dorsal columns (III).

fibers terminate directly in the outer laminae of the dorsal horn where they make synaptic contact with the dendrites of second order spinothalamic tract neurons (Figure 2) (Kandel and Schwartz, 1991).

1.2.2 Second Order Neurons

The primary afferent fibers form the synaptic contacts with second order neurons in the dorsal horn. These are either relay cells, with axons projecting to the brain stem or thalamus, or interneurons that transfer information locally to other interneurons or relay cells in the adjacent segments. Second order neurons are classified into three general types (Besson and Chaouch, 1987; Henry, 1989): 1) non-nociceptive (receiving input from large diameter myelinated fibers), 2) nociceptive specific (those receiving input from C and Aδ fibers), and 3) nociceptive non-specific (those receiving convergent input from both nociceptive and non-nociceptive primary afferent fibers). The latter are also known as wide dynamic range (WDR) neurons. WDR neurons show a brisk transient response to low-threshold mechanical stimuli and a delayed after-discharge in response to noxious stimuli, the latter representing a slow depolarization of the neuron (Henry, 1989). Virtually all sensory information arising from the somatic segment of the body enters the spinal cord through the dorsal root.

1.2.3 Excitatory Neurotransmitters and Neuromodulators in Somatosensory Processing in the Spinal Cord

A variety of substances present in primary sensory neurons have been proposed as possible neurotransmitters or neuromodulators. Putative afferent neurotransmitters include: substance P (SP), neurotensin, cholecystokinin, vasoactive intestinal peptide, calcitonin gene related peptide (CGRP), somatostatin, glutamate, aspartate and adenosine triphosphate (ATP) (Besson and Chaouch, 1987; Henry, 1989; Yaksh and Malmberg, 1994). The precise role of many of these substances has not been fully elucidated. Currently, glutamate and aspartate are believed to be the main excitatory neurotransmitters released from all primary afferent fibers (Kangrga and Randic, 1991; Dougherty et al., 1992). In nociceptive fibers, these excitatory amino acids (EAAs) co-exist with neuropeptides including CGRP and substance P (Battaglia and Rustioni, 1988). ATP has been implicated in the transmission of information from large diameter primary afferent fibers. Its involvement in nociception is also suggested by the depressant effects of adenosine (the product of its hydrolysis) on nociceptive neurons (Henry, 1989).

Synapses of primary afferent and second order neurons are subject to extensive modulation by a number of endogenous systems (Yaksh and Malmberg, 1994). Local inhibition is mediated by glycine, adenosine and GABA (Nagy and Daddona, 1985; Sawynok and Sweeney, 1989; Yaksh and Malmberg, 1994) (see sections 1.4.2, 1.7.1 and 1.7.2). Endogenous opioids, such as enkephalin, are

present in spinal interneurons and mediate the local inhibition of nociceptive input (Yaksh and Malmberg, 1994). Nociceptive transmission is also subject to descending inhibitory modulation originating from sites in the pons (e.g. locus coeruleus) and medulla (i.e. nucleus raphe magnus).

1.2.4 Ascending Neural Tracts Conveying Sensory Information

There are two main pathways conveying sensory information from the dorsal horn to the brain: 1) the dorsal column-leminiscal system; and 2) the anterolateral system. The dorsal column-leminiscal system conveys touch and vibration sense and is generally characterized by a well defined localization of the stimulus source. The anterolateral system is responsible for conveying pain, thermal sensations including cold and warmth, crude touch and pressure sensations, tickle and itch (Kandel and Schwartz, 1991).

Dorsal column medial leminiscal system: The dorsal column pathway relays somatosensory information directly to the medulla. It is comprised of ascending axons of the large diameter primary afferent fibers and axons of second order neurons originating in the dorsal horn. These axons ascend ipsilateraly to the caudal medulla where they synapse onto the cells in the dorsal column nuclei (cuneate and gracile nuclei). Fibers leaving the dorsal column nuclei arch across the midline where they collect into a discrete bundle (the medial lemniscus) and ascend to the thalamus. From the thalamus, the somatosensory information is

conveyed to the anterior parietal lobe by thalamo-cortical neurons.

Anterolateral system: This system is actually composed of two major pathways distinguished by their sites of termination: the spinothalamic tract and the spinoreticular tract. The spinothalamic tract originates principally from cells in lamina I. This tract mediates fast pain, relayed from the periphery to the spinal cord by Aδ-fibers. In contrast, the spinoreticular tract principally originates from cells in lamina V. It transmits both nociceptive and non-nociceptive (Aβ-, Aδ- and C-fibers) information and hence cells of this tract are referred to as WDR nociceptors. This tract is important in slow pain. Axons of spinoreticular tract cells end on neurons in the reticular formation of the brain stem, which then relay information rostrally to the thalamus and other structures in the diencephalon. The spinoreticular tract also relays information to the mesencephalic periaqueductal gray, the region surrounding the cerebral aqueduct.

It is evident from this brief description that the processing of somatosensory information is complex, involving several parallel but discrete systems which extend throughout the neuraxis. These systems utilize multiple neurotransmitters and several levels of regulation (spinal, bulbar, thalamic and cortical). Thus, under normal conditions, nociceptive and non-nociceptive sensory information is transmitted through distinct neural circuits, thereby ensuring appropriate interpretation of the sensory stimulus.

1.3 Neuropathic Pain

1.3.1 General Features of Neuropathic Pain

Acute pain arising from tissue injury has an immediate onset, a short duration, temporally coupled to stimulus exposure and is intimately related to changes induced by peripheral inflammation. Physiologically, it is thought to serve an important protective function. In contrast, chronic pain usually has delayed onset and persists long after the time normally required for tissue repair. Intuitively, chronic pain involves more complex events than those of normal nociception and has no apparent biological usefulness. Along with tissue damage and inflammation, chronic pain is often associated with neuropathology. This includes changes in the CNS leading to an increased sensitivity of central neurons; a condition known as central sensitization (Dray et al., 1994).

Neuropathic pain is a chronic condition resulting from traumatic injury to or pathology of the central or peripheral nervous system. Phantom limb pain, multiple sclerosis, central post stroke pain, neuropathies such as those of diabetic, alcoholic, nutritional or traumatic origin, post herpetic neuralgia, trigeminal neuralgia, and reflex sympathetic dystrophy are some examples of neuropathic pain (Bovie et al., 1989; Tasker, 1990; Portenoy et al., 1990; Triggs and Beric, 1992; Woolf, 1993). It is characterized by allodynia, hyperalgesia and spontaneous pain (Price et al., 1989, 1992; Bonica, 1990; Coderre et al., 1993; Galer, 1995). Spontaneous pain is pain felt in the absence of any apparent physiological

stimulus. Hyperalgesia is an exaggerated response to a normally painful stimulus and allodynia is a condition in which pain is evoked by a normally non-painful stimuli (e.g. touch, vibration). Additional symptoms may include a partial loss of sensitivity, and the spread of pain to uninjured tissue (referred pain) (Price et al., 1989, 1992; Coderre et al., 1993; Woolf, 1993; Rowbotham, 1995).

Unlike regular pain, neuropathic pain often takes weeks or months to develop following the causative event (Tasker, 1990). For instance, in a group of 127 patients with spinal cord lesions, the onset of pain ranged from a month to more than a year in 79% of the patients (Tasker et al., 1992). Neuropathic pain is commonly described by sufferers as a burning, twisting, ripping, tearing or sharp lancinating type of pain (Price et al., 1992; Woolf, 1993; Gonzales, 1995) and is often unbearably intense. It is difficult to treat and symptoms may last indefinitely (Merskey, 1986). Conventional analgesics like opioids and adjuvant drug therapy such as tricyclic antidepressants, barbiturates or anticonvulsants are only partially or temporarily effective (Bowsher, 1991; Triggs and Beric, 1992; Baron and Saguer, 1993).

The use of opioid analgesics in neuropathic pain is highly controversial (Arner and Mayerson, 1988; Portenoy et al., 1990; Rowbotham et al., 1991; Kupers et al., 1991; Jadad et al., 1992). For example, neuropathic pain following amputation or nerve injury has been shown to be unresponsive to opioids (Arner and Mayerson, 1988). In contrast, patients with malignant neuropathic pain have

shown a shift to the right in the opioid dose-response curve such that an increase in the dose will provide effective pain relief (Portenoy et al., 1990). For patients with a demonstrable sympathetic component (i.e. sympathetically maintained pain), interruption of the sympathetic nervous system with local anaesthetics (Price et al., 1989), sympatholytic drugs or by surgery is the major treatment approach (Woolf, 1993).

Surgical intervention in the periphery is also controversial. Pain relief is usually temporary, often ineffective and may, in some cases worsen the situation (White and Sweet, 1969; Wirth and Rutherford, 1970; Noordenbos and Wall, 1981). Neurolytic procedures or electrical stimulation of the dorsal column or the brain, produce partial relief for intractable pain of spinal cord origin (Tasker et al., 1992). However, these procedures are appropriate only for patients with a severe disabling pain that is refractory to all other treatments. Pain of this type is often associated with a terminal disease and limited life expectancy (Woolf, 1993).

1.3.2 Pathophysiology of Neuropathic Pain

Peripheral nerve injury often leads to pathological changes including neural degeneration, neuroma formation, and spontaneous activity in damaged nerve fibers (Coderre et al., 1993). Structural changes within spinal cord, such as the loss of dorsal horn interneurons (Kapadia and La Motte, 1987; Sugimoto et al., 1989), some of which contain GABA and glycine (Todd and Sullivan, 1990), and the

abnormal rearrangement of afferent nerve processes have also been reported. The latter include central sprouting of large afferent fibers from lamina III to lamina II of the dorsal horn, and sprouting of sympathetic nerves into the dorsal root ganglia (Woolf et al., 1992; McLachlan et al., 1993). Functionally, these changes could result in either a reduction in the threshold of activation of nociceptors and/or an increase in the excitability of the central neurons comprising the pain-signalling pathways (e.g. "*central sensitization*"). Central sensitization is characterized by increased spontaneous activity, decreased threshold of activation or increased responsiveness to afferent input, prolonged after-discharge to repeated stimulation, and an expansion of the receptive field of dorsal horn neurons. These changes would explain, at least in part, the phenomena of hyperalgesia, allodynia and spontaneous pain (Dubner, 1991; Coderre et al., 1993; Woolf, 1993).

The mechanisms underlying the development and maintenance of neuropathic pain are unclear. However, the peripheral substrates triggering allodynia and hyperalgesia are known to be different. Hyperalgesia arises from a decrease in the threshold of activation of nociceptive C-fibers (Shir and Seltzer, 1990; Cline et al., 1989; Lee et al., 1994). In contrast, allodynia is triggered by input from low threshold Aβ-fibers (Meyer et al., 1985; Campbell et al., 1988; Price et al., 1989; Treede et al., 1991); that is, by peripheral substrates that normally have no role in pain transduction and transmission. The striking changes in the modality subserved by Aβ-fibers after nerve injury are highly suggestive of a central

dysfunction.

1.4 Allodynia

1.4.1 Role of Aβ-fibers in Allodynia

Allodynia, a characteristic feature of neuropathic pain, is an abnormal condition in which light tactile stimuli (i.e. touch or the brush of clothing against the skin) acquire the ability to evoke intense pain. As noted above, allodynic pain is triggered by input from Aβ-fibers, rather than abnormally sensitized C-fibers (Campbell et al., 1988). Thus selective nerve block using ischemia or nerve compression eliminated both allodynia in the area of nerve injury along with light touch sensation in adjacent normal skin without affecting the temperature discrimination in patients with neuropathic pain suggesting that both sensations were mediated by Aβ-fibers (Campbell et al., 1988; Koltzenburg et al., 1992). Conversely, nerve block with local anesthetics, that affected Aδ- and C-fibers (as demonstrated by absence of temperature sensibility in those patients), but not Aß-fibers did not block allodynia (Campbell et al., 1988). In addition, response latencies for touch evoked-pain on the affected side were nearly identical to those for the same stimulus applied to the homologous area on the normal side. Both latencies were much faster than the latencies for the detection of pain in the normal limb (Lindblom and Verrillo, 1979; Campbell et al., 1988). These results indicate

that allodynic pain is evoked by afferent input conveyed by low threshold Aβ-fibers. In support of this conclusion, high-frequency, low intensity electrical nerve stimulation worsens allodynia, unlike the analgesic effect observed with regular nociceptive pain (Price et al., 1992). Finally the selective destruction of C-fibers in rats using neonatal treatment with capsaicin did not prevent the induction of allodynia following chronic constriction of the sciatic nerve (Shir and Seltzer, 1990). Rather, this treatment blocked the development of thermal hyperalgesia. Overall, these data indicate that Aβ-fibers mediate alldoynia whereas, C- and Aδ-fibers mediate thermal hyperalgesia.

Since under normal conditions, the activity in Aß-fibers is perceived exclusively as touch, light pressure and vibration, the fundamental question arising from the phenomenon of allodynia is how this same neural activity acquires ability to evoke severe pain. One possibility is that inhibitory interneurons normally modulating the response of spinal neurons to low-threshold afferent input may become dysfunctional after injury. Indeed, interneurons containing GABA and glycine are known to be vulnerable to ischemic and excitotoxic damage (Tureen, 1936; Davidoff et al., 1967; Sugimoto et al., 1989, 1990). The functional loss of such inhibition could result in an excessive activation of second order neurons by low-threshold afferent input thereby strengthening the synaptic contact between non-nociceptive fibers and pain-signalling pathways. Thus, Aβ-input could be miscoded as pain.

1.4.2 Role of Spinal Glycinergic Interneurons in Allodynia

GABA and glycine are important in the spinal processing of innocuous tactile information (Yaksh, 1989, 1993). Glycine-like immunoreactivity (-LI), as demonstrated using glycine antisera, is found throughout the gray matter in the spinal cord of the rat (Van den Pol and Grocs, 1988; Todd and Sullivan, 1990). In the dorsal horn, glycine-LI is present in laminae I-IV, but is most densely localized in lamina III and IV (Todd and Sullivan, 1990). These results are consistent with the presence of glycine receptor-LI at axodendritic and axosomatic synapses in laminae I-III and at dendrodendritic synapses in lamina II (Mitchell et al., 1993). Thus, binding and immunocytochemical data indicate that glycine and its corresponding receptors are localized in regions of the dorsal horn containing the central terminations of low-threshold afferent fibers (laminae III-IV). Furthermore, glycinergic neurons in laminae II and III of the rat spinal cord have been shown to receive a major monosynaptic input from myelinated low threshold cutaneous primary afferent fibers (Todd, 1990). Collectively, current anatomical evidence supports the hypothesis that Aβ-fibers activate local glycinergic interneurons in the spinal dorsal horn.

Pharmacological data also substantiate the relevance of glycine to the regulation of the behaviour generated by low-threshold afferent transmission. Light tactile stimuli, typically eliciting no more than an orientation response in rats, evoke overt pain-like behaviours following the blockade of spinal glycine receptors with

i.t. STR (Yaksh, 1989). STR exhibits very high affinity (Ka = 3-10 nM) for the glycine receptor (Young and Synder, 1974; Zarbin et al., 1981). This is distinct from the glycine modulatory site located on the NMDA-receptor complex. The latter is insensitive to STR blockade. The data from STR-treated rats are in agreement with studies of single unit activity in the trigeminal nucleus of the monkey. Intravenous (i.v.) STR induced a marked facilitation of the response of WDR neurons to electrical stimulation of low-threshold mechanoreceptive afferent fibers (Yokota et al., 1979). Iontophoretic delivery of STR in the lumbar spinal cord of cats diminished the inhibition phase otherwise observed following natural and electrical stimulation of low-threshold afferent fibers (Game and Lodge, 1975). Conversely, the iontophoretic delivery of glycine in the spinal dorsal horn diminished the responsiveness of dorsal horn neurons to light tactile stimulation (light touch/light pressure), decreased the size of their cutaneous receptive fields in cats (Zieglgänsberger and Hertz, 1971), and reduced the responses of spinothalamic tract neurons, including low-threshold and WDR neurons, in monkeys (Wilcockson et al., 1984).

These observations suggest that the encoding of low-threshold mechanical stimuli as an innocuous event depends upon the presence of intrinsic glycinergic inhibition in the spinal dorsal horn. Indeed, genetic variants, such as the Poll Hereford calf (Gundlach et al., 1988) and the spastic mouse (White and Heller, 1982) which exhibit up to a 10-fold decrease in STR binding in the spinal cord,

display exaggerated sensitivity to even modest cutaneous stimulation. Consistent with these animal data are clinical reports of pronounced hypersensitivity to light touch in humans during STR intoxication (Arena, 1979). Thus, glycinergic dysfunction in the spinal dorsal horn could be an important central abnormality in the development of allodynia.

1.4.3 Strychnine-induced Allodynia

Further support for the role of spinal glycine in modulating low-threshold input is provided by the observation that pharmacological blockade of spinal glycine receptors with i.t. STR induces a pure allodynia like state in the rat (Beyer et al., 1985, 1988; Yaksh, 1989). In the presence of STR, innocuous mechanical stimulation such as HD evoked high pitched vocalisation, biting and scratching of the stimulated dermatomes and vigorous organised escape behaviour (Yaksh, 1989). These evoked responses resemble the hyperaesthesia reported by humans during the preconvulsive stages of STR intoxication (Arena, 1979). A striking feature of these responses is that they could be reliably driven by HD applied to circumscribed areas of the body surface. These corresponded to the dermatomes innervated by spinal segments near the site of i.t. STR injection. Similar results were obtained using lightly anaesthetized rats pretreated with i.t. STR. Exaggerated autonomic and motor withdrawal responses to repeated HD, comparable to those evoked by noxious stimuli in the absence of STR, were

reported (Yaksh, 1989; Sherman and Loomis, 1994). All HD-evoked responses were observed without convulsions or seizure-like effects and thus did not reflect a general sensitization of spinal neurons by i.t. STR. Subsequent studies in our laboratory have shown that i.t. STR neither enhances nor inhibits responses to noxious bilateral hind paw-pinch or tail immersion in water at 55°C. These results indicate that: a) glycine is a selective modulator of non-nociceptive input in the rat spinal cord; b) the blockade of spinal glycinergic receptors with i.t. STR yields an abnormal sensory condition resembling allodynia; and c) i.t. STR has neither a hyperalgesic nor an antinociceptive effect on normal nociception.

1.4.4 Spinal Pharmacology of Intrathecal Strychnine-induced Allodynia

Clinical studies have shown that allodynic pain is triggered by input from low threshold (non-nociceptive) fibers. Therefore, any valid experimental model of allodynia should exhibit a pharmacology that is distinct from that of normal (C-fiber mediated) nociception. Previous studies of the STR model support this distinction (Table II). For example, chronic treatment of neonatal rats with sub-cutaneous (s.c.) capsaicin at doses that selectively destroyed C-fibers by adulthood and yielded significant antinociception in the paw-pinch (mechanical), tail immersion (thermal) and topical xylene (chemical) tests, had no effect on STR-allodynia (Sherman and Loomis, 1996). Similarly, pain-like responses evoked by HD in STR-treated rats were insensitive to antinociceptive doses of i.t. morphine and CP-96345

Table II. Comparison of the spinal pharmacology of i.t. strychnine-allodynia and normal nociception (without strychnine)

Treatment✳	Strychnine-allodynia	Noxious stimulation	References
Agents affecting C-fibers			
Morphine	-	+	Sherman & Loomis, 1994
Capsaicin	-	+	Sherman & Loomis, 1996
CP-96345 (NK_1-antagonist)	-	+	unpublished observations
Glycine or analogue			
Betaine	+	-	Sherman & Loomis, 1995
Glycine	+	-	Sherman & Loomis, 1995
Excitatory amino acids			
γ-DGG	+	+	Sherman & Loomis, 1996
NBQX	+	+[a]	Näsström et al., 1992 Sherman & Loomis, 1996 Näsström et al., 1992

'+' → blocks; '–' → no effect

✳ All drugs were administered as a single i.t. injection except capsaicin which was given as repeated s.c. injections during the neonatal period.

a The non-NMDA receptor antagonist CNQX was used instead of NBQX by Näsström et al., 1992.

(a NK_1-receptor antagonist) (Sherman and Loomis, 1994; unpublished observation). In contrast, i.t. glycine and betaine selectively inhibited STR-allodynia, although their effect on normal nociception was not systematically determined (Beyer et al., 1985, Sherman and Loomis, 1995). These results demonstrate that agents which

selectively interfere with the function of C-fibers do not attenuate STR-allodynia in the rat.

Glutamate and aspartate are the principal excitatory neurotransmitters in the CNS (Mayer and Westbrook, 1987; Evans, 1989; Perschak and Cuenod, 1990), and are released from central terminals of both low- and high-threshold afferent fibers of rats and monkeys (Skilling et al., 1988; Liu et al., 1989; Kangrga and Randic, 1991; Sorkin et al., 1992). Indeed, glutamate-LI and aspartate-LI are localized in the dorsal root ganglion (DRG) cells of both large- and small-diameter primary afferent fibers (Wanaka et al., 1987; Battalgia and Rustioni, 1988; Westlund et al., 1989, 1990), as well as in interneurons of the dorsal horn of cats, rats and monkeys (Rizzoli, 1968; Fagg and Foster, 1983; Cottman et al., 1987; Storm-Mathisen and Ottersen, 1987). The intraspinal boutons of cat afferent hair follicle fibers, labelled intra-axonally with horseradish peroxidase, are also enriched with L-glutamate-LI (Maxwell et al., 1993). Consistent with the localization and release of EAAs from both low- and high-threshold afferent fibers, and their putative role in somatosensory transmission, EAA receptor antagonists have been shown to block the excitation of spinothalamic tract neurons by noxious thermal, mechanical, chemical and electrical stimuli in monkeys (Dougherty et al., 1992), and to dose-dependently inhibit STR-allodynia in lightly anesthetized and conscious rats (Table II; Yaksh, 1989, Sherman and Loomis, 1994, 1996). Considering the selective effect of i.t. STR on low-threshold afferent input in the spinal cord

(Sherman and Loomis, 1996), and the marked sensitivity of this input to EAA antagonists (Table II), it is possible that the blockade of spinal glycinergic modulation by i.t. STR permits the exaggerated depolarization of second order neurons in pain-signalling pathways by low threshold (Aβ) input, and the miscoding of this input as pain.

1.5 Role of Excitatory Amino Acid Receptors in Sensory Processing

The biological actions of EAAs are mediated by at least three subtypes of ionotropic receptors. These receptor subtypes were identified on the basis of their selective responsiveness to the following agonists; α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate (KA) and N-methyl-D-aspartate (NMDA) (Watkings et al., 1990). Collectively, the AMPA and kainate receptor subtypes are referred to as non-NMDA receptors. While glutamate activates both NMDA and non-NMDA receptors, aspartate is thought to act preferentially at NMDA-receptors (Mayer and Westbrook, 1987; Dickenson, 1990). Non-NMDA receptor-coupled ion channels mediate the fast component of the excitatory post synaptic potential (EPSP). Under physiological conditions, NMDA receptor-coupled ion channels are normally blocked by Mg^{2+} in a voltage-dependent manner. The opening of these channels requires a prior depolarising potential, generated primarily by non-NMDA receptors (for review see Ascher and Nowak, 1987). As a result of this prerequisite

event for activation, NMDA receptors mediate the slow-component of the EPSP (Headly and Griller, 1990; Watkins et al., 1990).

Intracellular studies using a hemisected rat spinal cord-hind limb preparation demonstrated that the amplitude and duration of the EPSPs elicited by low-intensity (touch; Aβ) or high-intensity (pinch; Aδ and C) mechanical stimuli are blocked by non-NMDA receptor antagonist, CNQX and the cell firing of the dorsal horn cells was completely abolished (King and Lopez-Garcia, 1993). In contrast, the NMDA receptor-antagonist, AP-5 (D-aminophosphonovalerate), blocked only the longer latency spikes elicited by high-intensity cutaneous mechanical stimuli. It never abolished the cell firing produced by such stimuli (King and Lopez-Garcia, 1993) and was ineffective against responses evoked by light touch (King and Lopez-Garcia, 1993). Thus, EAAs released from Aβ-fibers selectively activate non-NMDA receptors under physiological conditions. In this regard i.t. γ-DGG (non-selective EAA-antagonist), and NBQX (a selective AMPA-antagonist) have been shown to block STR-allodynia in lightly anaesthetized rats (Sherman and Loomis, 1994, 1996); observations consistent with the model of synaptic transmission illustrated in Figure 1.

The role of spinal NMDA receptors in normal nociceptive transmission has been extensively investigated. If a brief noxious stimulus, sufficient to activate C-fibers, is repeated at frequency $\geq$0.3 Hz, the long latency discharge recorded in second order neurons progressively increases in magnitude; a phenomenon called

"wind up" (Mendell, 1966; Price, 1978). 'Wind up' is a characteristic feature of C-fiber evoked nociception and is mediated by EAAs acting at post-synaptic NMDA-receptors (Dickenson and Sullivan, 1987). In rats, NMDA receptor antagonists such as ketamine, AP-5, dextromethorphan and levorphanol have been shown to block the slow temporal summation of EPSPs evoked by electrical stimulation of C-afferent fibers ('wind up') without reducing the response of these same neurons to A-fiber stimulation (Davies and Lodge, 1987; Dickenson and Sullivan, 1987; Dickenson et al., 1991). Psychophysical studies in humans have also shown that second pain, a physiological correlate of long-latency C afferent-evoked impulses, is sensitive to NMDA-receptor blockade by dextromethorphan (Price et al., 1994). This phenomenon is believed to underlie the development of central sensitization during sustained nociceptive input (i.e. inflammation). Thus, 'wind up' is a physiological process mediated by NMDA receptors and is normally restricted to noxious (C-fiber) afferent input.

It has recently been suggested that under pathophysiological conditions, Aß afferent impulses may acquire access to the same 'wind up' mechanism, thereby allowing low-threshold fibers to trigger pain (Price et al., 1989; 1994a). Unlike normal subjects, patients with nerve injury exhibit temporal summation to repeated activation of Aß afferents at frequencies $\geq$0.3 Hz, using either natural stimuli such as stroking the skin with a gauze pad or low-threshold electrical stimulation (Price et al., 1989). Subjects reported that the pain evoked by these normally innocuous

stimuli had the same burning quality and tendency to radiate as noxious heat evoked pain. Moreover, subanesthetic doses of ketamine (an NMDA-receptor antagonist) attenuated the pain evoked by low-threshold mechanical stimulation in patients with traumatic spinal cord injury, post-herpetic neuralgia, post-stroke pain and peripheral nerve injury (Backonja et al., 1994; Eide et al., 1994, 1995). Collectively, these observations suggest that a process similar to 'wind up' is activated in allodynia and that this process is sensitive to NMDA-receptor blockade. If, in the presence of i.t. STR, low threshold (Aβ) input acquires access to the same sensitization ('wind up') mechanism normally activated by C-fiber input, then STR-induced allodynia should be blocked by NMDA-receptor antagonists. While NMDA receptor antagonists are known to attenuate tactile-evoked agitation in conscious STR-treated rats (Yaksh, 1989), their dose response effects in the anesthetized STR model have not been determined.

1.6 Effects of Local Anaesthetics on Normal Nociception and Neuropathic Pain

Local anaesthetics such as mexiletine, lidocaine and tocainide are known to be effective in the treatment of clinical and experimental neuropathic pain, including allodynia. This includes the treatment of pain arising from peripheral nerve injury, chronic painful diabetic neuropathy, trigeminal neuralgia, causalgia and other

chronic pain syndromes which are resistant to conventional drug therapy (Lindstrom and Lindblom, 1987; Dejgard et al., 1988; Chabal et al., 1992; Colclough et al., 1993). Indeed, a sustained analgesic response has been reported weeks after the termination of infusion in humans (Edward et al., 1985, Steinhaus and Howland, 1958; Kastrup et al., 1986). Systemic mexiletine has also been shown to relieve chronic allodynia-like symptoms in rats with ischemic spinal cord injury (Xu et al., 1992) or spinal nerve ligation (Chaplan et al., 1995).

The pharmacological effect of local anaesthetics is normally associated with frequency- and voltage-dependent blockade of Na^+ channels, resulting in the nerve conduction block. However, the analgesic effect in patients with neuropathic pain is unlikely to result from simple peripheral conduction block. Local anaesthetics do not block nerve conduction when given systemically in clinically effective analgesic doses (Dejgard et al., 1988; Chabal et al., 1989; Rowbotham et al., 1991). Electrophysiological studies have shown that sub-anesthetic doses of lidocaine and tocainide significantly suppress C-fiber evoked polysynaptic responses of spinal neurons, incompletely reduce reflex activity in hamstring flexor α-motoneurons, and completely blocked reflexes to noxious chemical and thermal stimuli in normal rats (Woolf and Wiesenfeld-Hallin, 1985; DeJong et al., 1969; Dohi et al., 1979). C-fiber evoked 'wind up' is also blocked by i.t. lidocaine in the rat (Fraser et al., 1992). *In vitro,* subanesthetic concentrations of lidocaine inhibited direct and synaptically driven NMDA- and neurokinin-receptor mediated post-synaptic depolarizations in

rat spinal neurons (Nagy and Woolf, 1996). These results indicate that under physiological conditions sub-anesthetic doses of local anesthetics selective block C fiber-evoked activity in the spinal cord.

Mexiletine, an orally active congener of lidocaine, is the most commonly used local anesthetic for clinical neuropathic pain (Hegarty and Portenoy, 1994) and should be effective in any valid experimental model of allodynia. Moreover, if Aß-input acquires access to pain signalling-pathways in the spinal cord, then mexiletine should block both the Aß-evoked responses (in presence of STR) and C-fiber evoked responses (without STR) with comparable potency.

1.7 Inhibitory Modulation in Sensory Processing

1.7.1 Role of Spinal Adenosine

There is a growing body of evidence implicating the role of purines in the spinal modulation of afferent neurotransmission (Sawynok et al., 1986, Delander and Hopkins, 1986). Adenosine , a probable purinergic agonist, is thought to exert its effect at specific purine receptor sites. These receptors are G-protein coupled and are classified on the basis of their ability to inhibit (A_1-receptor) or stimulate (A_2-receptor) adenylate cyclase through Gi and Gs proteins, respectively (Van Calker et al., 1979; Marala and Mustafa 1993; Jockers et al., 1994). A_1-agonists such as LPIA [N^6- (L-2-phenylisopropyl)-adenosine] or cyclohexyladenosine in the dose

range of 0.5 - 10 µg, and non-selective (A_1/A_2) agonists like NECA [5'-(N-ethyl carboxamido)-adenosine] or 2-chloroadenosine in the dose range of 0.5 - 3.6 µg, produced potent analgesia in the tail flick and hot plate tests following i.t. administration (Sawynok et al., 1986; Delander and Hopkins, 1986). Conversely, i.t. adenosine receptor antagonists such as theophylline (11.8 µg) and 8-phenyltheophylline (0.12 µg) attenuated adenosine-induced antinociception (Sawynok et al., 1986; Delander and Hopkins, 1986). Such pharmacological studies have consistently implicated A_1-, rather than A_2 -receptors in the spinal modulation of nociceptive input (Fastbom et al., 1990; Karlsten et al., 1991; Poon and Sawynok, 1995; Lee and Yaksh, 1996).

That adenosine affects Aβ-evoked responses was first demonstrated by Salter and Henry (1987). They observed that the depressant effect of vibration, applied to the ipsilateral hind limb, on nociceptive (wide dynamic range) neurons in the cat dorsal horn was potentiated by an adenosine uptake inhibitor (dipyridamole, 1.2 mg/kg, i.v.). This inhibitory effect was reversed by caffeine (20 -60 mg/kg, i.v.), and unaffected by naloxone (0.1 - 0.4 mg/kg, i.v.). They proposed that the analgesic effect of vibration in the spinal cord is mediated primarily by purines and may underlie the mechanism of action of transcutaneous electrical nerve stimulation (TENS) in clinical pain management, as predicted by the gate control theory (Melzack and Wall, 1965). TENS activates large diameter primary afferent neurons and produces analgesia in the area affected by such stimulation.

Conversely, in the inherited nervous system disorder known as the Lesh-Nyhan syndrome, affected children show involuntary movements and self-injurious behaviour (e.g. biting of lips and fingers). Patients with this syndrome are known to lack the enzyme, hypoxanthine-guanine phosphoribosyl transferase, thereby reducing the plasma concentration of adenosine. Whether there is a corresponding decrease in the CNS concentration of adenosine is unknown but caffeine does worsen this syndrome (Stone, 1981).

Based on these experimental and clinical observations, adenosine has been used in the management of neuropathic pain. The effect of systemic adenosine on pain symptoms in seven patients with peripheral neuropathy was evaluated in double-blind, placebo-controlled cross-over study (Balfrage et al., 1995). Adenosine (50 µg/kg/min, i.v.) reduced spontaneous pain, allodynia and hyperalgesia whereas placebo had no effect. In other case reports, adenosine (70 µg/kg/min, i.v.) or R-PIA (19 µg, i.t.) alleviated spontaneous pain and touch-evoked allodynia in patients with peripheral neuropathy (Karlsten and Gordh, 1995; Sollevi et al., 1995). Low dose adenosine (70 µg/kg/min, i.v.) has also been shown to effect analgesia in awake healthy volunteers subjected to experimentally-induced ischemic pain (Segerdhal et al., 1994).

Similar results have been obtained from studies using animal models of neuropathic pain. Yamamoto and Yaksh (1991) reported antinociception in thermal hyperalgesia with i.t. NECA (0.01 - 0.3 nmol) in rats subjected to chronic nerve

constriction injury (Bennet and Xie, 1988). Intrathecal R-PIA (10 ng) and NECA (5 ng) reduced the pain-like behaviours induced by i.t. prostaglandin $F_{2\alpha}$ in mice (Minami et al., 1992). LPIA or NECA, in the range of 0.3-1 nmol i.t., dose-dependently inhibited touch-evoked agitation in conscious STR-treated rats (Sosnowski and Yaksh, 1989). Both drugs were equi-potent in STR model and were significantly blocked by i.p. caffeine. The potent inhibitory effect of these adenosine analogues occurred at doses that were only mildly antinociceptive in the hot plate test (Sosnowski and Yaksh, 1989). These results demonstrate the remarkable sensitivity of abnormal pain states to adenosine or related agonists, in both injury and non-injury model of neuropathic pain. However, the receptor subtypes (A_1 versus A_2) mediating the anti-allodynic effect of adenosine have not been systematically investigated.

1.7.2 Role of Spinal GABA

GABA is densely localized in the spinal cord (Sivilotti and Nistri, 1991; Bohlhalter et al., 1994) where it appears to modulate both nociceptive and non-nociceptive neurotransmission. In case of nociception, $GABA_A$-agonists such as muscimol and $GABA_B$-agonist like baclofen given i.t. in dose range of 0.1-1 µg, significantly increased the response latency in the tail flick and hot plate tests, and vocalization threshold to tail shock in the rat (Roberts et al., 1986; Hammond and Drower, 1984; Wilson and Yaksh, 1978). Intrathecal baclofen and muscimol (0.3 -

flinch response to formaline injection (s.c) in a dose-dependent manner (Diring and Yaksh, 1995). Although these studies did not report any motor dysfunction at antinociceptive doses($\leq$ 1µg, i.t.), comparable doses of muscimol and baclofen ($\geq$1µg, i.t.) produce muscle flaccidity in rats (Hammond and Drower, 1984, Diring and Yaksh, 1995). Conversely, $GABA_A$-receptor antagonists such as picrotoxin (0.25 µg) and bicuculline (1 µg) decreased nociceptive threshold in the tail-shock and tail-flick tests (Roberts et al., 1986). The i.t. administration of CGP 35348 (30 µg) or phaclophen (100 µg) ($GABA_B$-antagonists) also blocked the antinociceptive effects of glutamate micro-injected into the ventromedial medulla of monkeys (McGowan and Hammond, 1993). These pharmacological data are supported by electrophysiological studies demonstrating the inhibition of glutamate- and pinch-evoked activity by GABA, ionophoretically applied onto spinothalamic tract cells in monkeys (Willcockson et al., 1984). *In vitro* studies using rat spinal cord preparation have shown that Aδ-afferent fibers activate GABAergic interneurons which inhibited nearby dorsal horn neurons (Yoshimura and Nishi, 1995). Bath application of musicmol and baclofen also depressed excitatory synaptic transmission (Kangrga et al., 1991; Araki, 1994). Thus, both $GABA_A$ and $GABA_B$ receptors in the spinal cord appear to mediate the antinociceptive effects of exogenously administered GABA agents.

GABA has also been implicated in the spinal modulation of low-threshold afferent input. Thus, i.t. bicuculline (30 µg) ($GABA_A$-antagonist) induced an

allodynia-like state in the rat similar to that produced by i.t. STR (Yaksh, 1989). Systemic administration of baclofen (0.1 mg/kg, i.p.), but not muscimol (1mg/kg, i.p.), inhibited allodynia-like behaviours arising 1-4 days after focal spinal cord ischemia in the rat (Hao et al., 1992). Thus, spinal GABA, specifically $GABA_A$-receptors appears to modulate Aβ transmission in a manner similar to spinal glycine in normal conditions (absence of neural injury); however, in the presence of neural injury, spinal $GABA_B$-receptors seem to play a major role in the modulation.

Consistent with this hypothesis is the observation that GABA and glycine are co-localized in interneurons in lamina I-III of the dorsal horn (Mitchell et al., 1993). In fact glycine immunoreactivity in lamina I-III is virtually restricted to neurons that also exhibit GABA immunoreactivity (Todd and Sullivan, 1990) suggesting that the effects of GABA and glycine on synaptic transmission may be complementary. The receptors mediating the distinct effects of glycine and GABA are co-localized on the same post-synaptic membrane (Mitchell et al., 1993; Todd et al., 1996). Finally, these $GABA_A$- and glycine-receptors are separately coupled to chloride ion channels leading to increased chloride conductance and neuronal hyperpolarisation.

Recent electrophysiological studies in the rat spinal cord slices have demonstrated that evoked inhibitory post synaptic potentials (IPSP's) consist of two distinct components; a short-duration and long-duration IPSP. Bath application of STR and bicuculline blocked the short- and long-IPSP, respectively. These results

suggest that the short IPSP's are mediated by glycine acting at STR-sensitive sites, while the long-IPSP's are mediated by GABA acting at $GABA_A$-receptors (Yoshimura and Nishi, 1995). The time course of GABA- and glycine-mediated IPSP's in this study are similar to those previously recorded in motoneurons of cats (Rudomin et al., 1990). If glycine and GABA are released together into the synapse upon stimulation, and if GABA and glycine effect distinct but complementary inhibition of Aβ-input in the spinal cord, then the allodynia arising from the blockade of spinal glycine receptors should be attenuated by the concurrent activation of $GABA_A$-, but not $GABA_B$-receptors.

1.7.3 Role of Spinal Glycine

The importance of glycine in the processing of Aß input has been extensively discussed (see section 1.4.2). Thus, the disinhibition of spinal glycine by i.t. STR (a competitive glycine antagonist), produces an allodynia-like state in rats, similar to that seen in patients with neuropathic pain. Consistent with the role of STR-sensitive glycine receptors in the dysesthetic state is the observation that glycine and betaine (a glycine analogue) inhibited STR-induced allodynia in lightly anesthtized rats (Sherman and Loomis, 1994). However, the charged nature of these molecules at physiological pH required that they be injected directly into the subarachnoid space.

Milacemide (2-n-pentylaminoacetamide) is an orally active glycine pro-drug.

Serum milacemide rapidly enters and equilibrates with the CNS compartment where it is metabolised primarily by MAO-B to glycinamide (Christophe et al., 1983, De Varebeke et al., 1989) and finally to glycine (see Figure 3). Milacemide (100, 200, 400 mg/kg i.p.) dose-dependently increased the concentrations of glycine and glycinamide (intermediary metabolite) in the cerebrospinal fluid (CSF) of the rat (Semba et al., 1993) and remained significantly elevated 7h after administration (400 mg/kg, i.p.) (Semba and Patsalos, 1993; Semba et al., 1993). Serum glycine concentration were unaffected. Clinical studies have confirmed the anticonvulsant, mood elevating, cognitive and memory enhancing properties of milacemide (Van Dorsser et al., 1983; Houtkooper et al., 1986; Saletu et al., 1986; Handelman et al., 1989; Schwartz et al., 1992). In view of the ability of systemic milacemide to increase central glycine concentration (Semba et al., 1993), and given the locus and mechanism of action of STR-allodynia, milacemide is an obvious candidate for testing in the STR model.

1.8 Rationale and Specific Research Objectives

Allodynia, a symptom of clinical neuropathic pain, is a condition in which normally non-nociceptive tactile stimuli acquire the ability to evoke excruciating pain. Although, the neuropathology is unclear, changes in the spinal processing of low-threshold afferent input are strongly suggested. Under normal conditions,

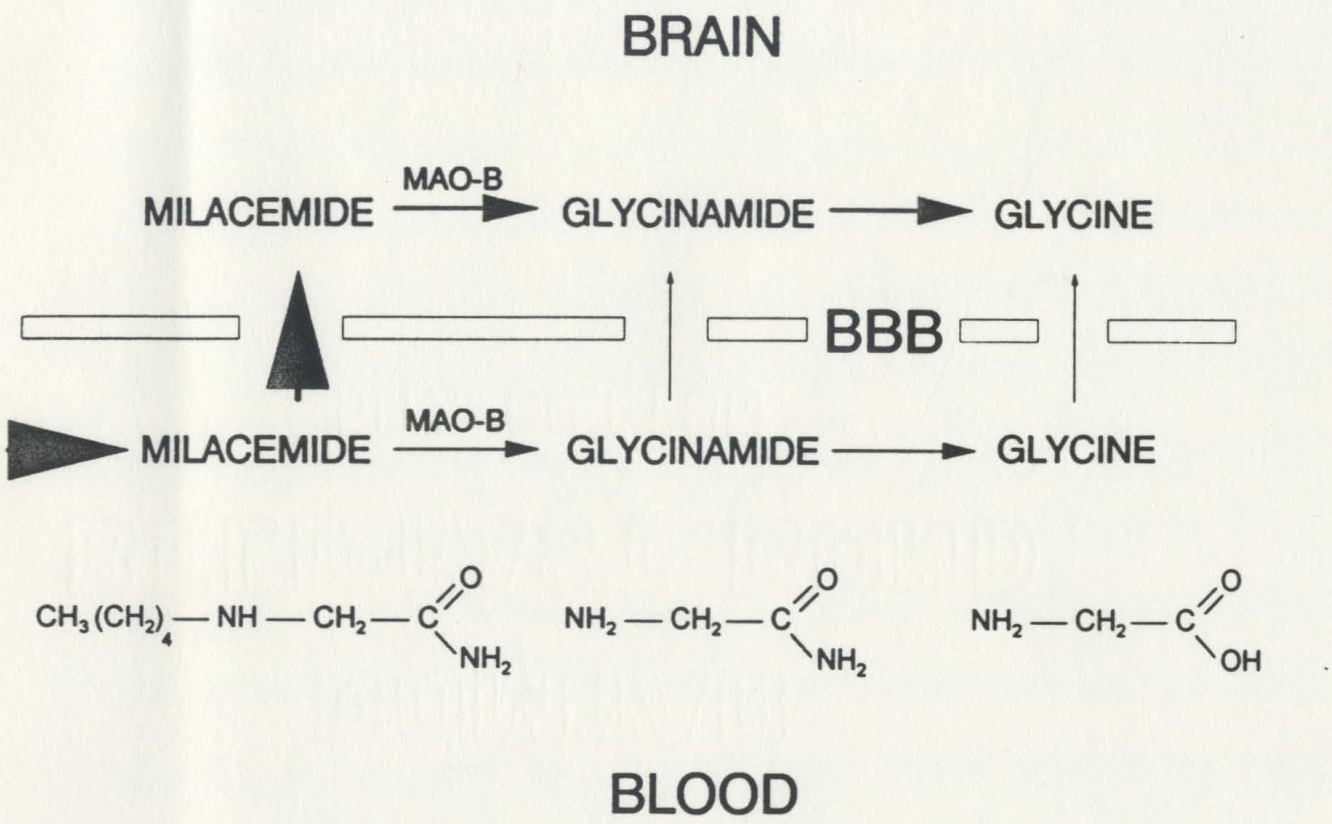


Figure 3. Systemic milacemide readily crosses blood-brain-barrier (BBB) where it is converted to glycine by the action of monoamine oxidase-B (MAO-B). Milacemide rapidly equilibrates between blood and brain. As a substrate for MAO-B, milacemide is first metabolised to glycinamide and subsequently converted to glycine. Milacemide is metabolised primarily in the central rather than the peripheral compartment. While some glycinamide produced in the periphery can cross the blood brain barrier, its contribution to the central concentration is small. (Adapted from Semba et al., 1993)

the synaptic transmission of mechanoreceptive Aβ-fibers is modulated by glycine-containing interneurons in the substantia gelatinosa. Consistent with the concept of a central dysfunction in allodynia is the observation that i.t. STR, (competitive glycine antagonist) produces reversible allodynia in both conscious and lightly anesthetized rats.

Pharmacological studies have shown that STR-allodynia is insensitive to conventional analgesics (morphine, capsaicin and NK_1-antagonist), but is dose-dependently reversed by i.t. glycine and betaine. The latter have no effect on normal nociception at anti-allodynic doses. Thus, STR-allodynia exhibits a spinal pharmacology that is distinct from normal nociception, is qualitatively similar to that of clinical allodynia, and may represent a useful non-injury model for the investigation of allodynia. To further investigate the spinal pharmacology of STR-allodynia and to explore the neuromodulatory systems affecting the spinal processing of Aβ transmission (Figure 1), the aim of the present study was to investigate the role of spinal NMDA-, adenosine-, GABA-, and glycine-receptors in lightly anesthetized STR-treated rats.

The specific objectives of this study were:

1. To determine the effect of i.t. AP-7 (an NMDA-receptor antagonist) on STR-induced allodynia.

2. To compare the effect of i.v. mexiletine on STR-induced allodynia and normal nociception (without STR).

3. To compare the effect of i.t. cyclopentyladenosine (A_1-agonist) and CGS-21680 (A_2-agonist) on STR-induced allodynia and to determine the sensitivity of their respective effects to pretreatment with i.t. DPCPX (A_1-antagonist).

4. To compare the effect of i.t. muscimol ($GABA_A$-agonist) and baclofen ($GABA_B$-agonist) on STR-induced allodynia.

5. To compare the effect of i.v. milacemide (a glycine pro-drug) on STR-induced allodynia and normal nociception (without STR) and to determine the sensitivity of this effect to pretreatment with clorgyline or l-deprenyl.

2 Methods

2.1 Animals

Male, Sprague-Dawley rats (260 - 490 g at the time of the experiments) were obtained from the Vivarium, Animal Care Services, Memorial University of Newfoundland (St. John's, Canada). Animals were housed in the Animal Care Facility, with a 12-h dark/light cycle (lights on 07:00 h), a room temperature of 22°C, and free access to rat chow and tap water. All experiments were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Memorial University Animal Care Committee.

2.2 Implantation of Intrathecal Catheters

Anesthesia was induced with 4% halothane (Halocarbon Laboratories, River Edge, USA) in oxygen and maintained at a concentration of 2.0 - 2.5 %. Rats were fitted with i.t. catheters prepared from stretched polyethylene tubing (PE-10) pulled to ≈1.5 x the original length (Sherman and Loomis, 1994). The catheters were filled with sterile saline (Astra Pharma Inc., Mississauga, Canada), inserted through the cisterna magna into the spinal subarachnoid space, and guided 8.5 cm caudally terminating near the L1-L2 segment. A fixed loop near the rostral end of the catheter was sutured to the overlying muscle to secure the catheter and the incision closed. The rostral tip was externalised on the top of the head and sealed with a

stainless steel plug. Animals with i.t. catheters were then housed individually and permitted to recover for at least 4 days. Only those animals exhibiting normal feeding, drinking and grooming behavior, and having normal gait were used for experimentation.

2.3 Acute Anaesthetized Animal Preparation

On the day of the experiment, animals were anesthetized with halothane and the left jugular vein was cannulated. Thereafter, halothane was gradually discontinued and anesthesia was maintained using i.v. urethane (10% w/w in saline, Sigma Chemical Inc., St. Louis, USA). The initial dose of 1.1 g/kg was administered slowly over 10-15 min as the anesthetic effect of halothane declined. Body temperature was maintained at 37°C with a thermostatically-regulated blanket (Harvard Instruments, St. Laurent, Canada). The left carotid artery was cannulated for continuous monitoring of blood pressure and heart rate using a pressure transducer (P23XL) and polygraph (Model 79E, Grass Instruments, Quincy USA). The trachea was also cannulated and the animal was allowed to breathe spontaneously. The incision was then closed and the animal was positioned in a stereotaxic apparatus (Narishige, Tokyo, Japan) with the head firmly secured using ear bars. The incision and the contact points with the ear bars were coated with 2% lidocaine gel (Astra Pharma, Inc.) to reduce the basal sensory input. Cortical EEG

was monitored using 2 subcutaneous needle electrodes (E2, Grass instruments) placed 2 mm left of midline, one extending rostrally entering the skin near bregma, the other extending caudally and entering the skin about 2 mm caudal to the first. The animal was then permitted to stabilize for one hour. During this time, anaesthesia was adjusted with i.v. urethane as required.

To ensure reproducible touch-evoked responses in STR-treated rats, a light level of anaesthesia is required (Sherman and Loomis, 1994). A reliable correlation has been observed between the proportion of time that the EEG is "synchronized" and the depth of urethane anaesthesia (Angel et al., 1976; Lincoln et al., 1980). For our purposes, light anaesthesia was defined as the presence of an EEG pattern which fluctuates between high amplitude ("synchronized") and low amplitude ("desynchronized") activity, with high amplitude activity present for not more than 60% of the time. The basis for this cut off in the STR model has been reported previously (Sherman and Loomis, 1994). The level of anaesthesia was also assessed by observing the animal's reflex responses to stimulation.

2.4 Application of Stimuli

The innocuous tactile stimulus was applied in the form of hair deflection (HD). The hair on the legs, flanks and lower back was sequentially brushed with a cotton-tipped applicator using an oscillating motion (rate of 1-2 per sec; 2-min

stimulus train applied at 5-min intervals). Oscillating stimuli elicit cardiovascular, motor and neurochemical responses in this model more effectively than stationary ones (Yaksh, 1989; Sherman and Loomis, 1994; Milne et al., 1996). Brushing was done with no more force than required to move the applicator through the hair (hair deflection) such that only the pelage was disturbed. In the absence of spinal STR, this stimulus produced no change in heart rate (HR) or blood pressure (BP) and was not associated with any type of motor withdrawal response. After i.t. STR (40 μg), the same stimulus applied to the affected dermatomes elicited a 15-20 mm Hg increase in mean arterial pressure (MAP), a 15-20 beats/min (bpm) increase in HR, and an abrupt motor withdrawal response (see results).

Noxious mechanical stimulation was performed using bilateral hind paw pinch. Both paws were gripped with hemostats at the post axial border (on the medial surface, distal to the tarsal joint) such that the region of skin covered by each hemostat was ≈20 mm^2. The force of the pinch was produced by 500 g weights attached to the handles of each hemostat. This resulted in a final pressure on each paw of approximately 0.7 kPa. Withdrawal of the hind paws was prevented until the end of the 10-second stimulus period. In some experiments, noxious chemical stimulation was applied in the form of a s.c. injection (0.05 ml) of 50% mustard oil in each of the hind paws.

2.5 Drug Administration

AP-7 [(+/-)-2-amino-7-phosphonoheptanoic acid], N^6-cyclopentyladenosine (CPA), CGS-21680 hydrochloride [2-p-(2carboxyethyl)phenylethylamino-5'-N-ethylcarboxamidoadenosine HCl; CGS], DPCPX (1,3-dipropyl-8-cyclopentylxanthine), muscimol hydrobromide, R(+)-baclofen hydrochloride, and clorgyline hydrochloride were obtained from Research Biochemicals International (Natick, USA). Mexiletine hydrochloride [1-methyl-2-(2,6-xylyloxy)-ethylamine hydrochloride; Boehringer Ingelheim Inc., Ingelheim am Rhein, Germany] and milacemide (2-N-pentylamino acetamide; Searl Inc., Skoki, USA) were generously provided by their manufacturers. Tyramine hydrochloride and l-deprenyl hydrochloride were obtained from Aldrich Chemical Company Inc. (Milwaukee, USA). Mustard oil was obtained from Madison, Colman and Bell (East Rutherford, USA).

All drugs were dissolved in 0.9% saline (Astra Pharma, Inc.) except CGS-21680, which was dissolved in a 0.02 M solution of sodium hydroxide (Fisher Scientific Company, Ottawa, Canada), and DPCPX which was dissolved in dimethyl sulfoxide (DMSO; British Drug House, Toronto, Canada). Mustard oil was diluted with an equal volume of 90% alcohol (Fisher Scientific Inc.). Strychnine hemisulphate (Sigma Chemical Company) was administered i.t. in a volume of 4 µl to reduce the rostro-caudal spread in the CSF. All other i.t. injections were made in a volume of 5 µl. Intravenous injections were made in a volume not exceeding

0.5 ml, and i.p. injections were made in volume of 1 ml. Drug solutions were flushed through i.t and i.v catheter using 10 µl and 1 ml sterile saline, respectively.

2.6 Experimental Protocols

2.6.1 Control Experiment (i.t. vehicle /i.t. STR)

To establish the control responses to STR + HD (in absence of any drug treatment), each rat received i.t. vehicle followed 10 min later by i.t. STR (40 µg) (Figure 4A). This dose of STR elicits optimal allodynia without convulsive activity in lightly anesthetized rats (Sherman and Loomis, 1994). The HD stimulus (2-min duration) was applied at 5-min intervals after vehicle and strychnine injection (Figure 4A). These stimulus trains were repeated until no HD-evoked cardiovascular or motor withdrawal responses were observed (~30 min after STR).

2.6.2 Quantification of Strychnine-induced Allodynia

The ***HD-EVOKED MAXIMUM*** increase in blood pressure and heart rate within 30 min of i.t. STR was used for graphical and statistical analysis (see section 2.8). The total period of time following STR injection, during which a motor withdrawal responses could be evoked by HD was also determined. In order to verify that each animal was maintained at an appropriate level of anaesthesia (less than 60% synchrony in the EEG) throughout the experiment, the % synchrony was

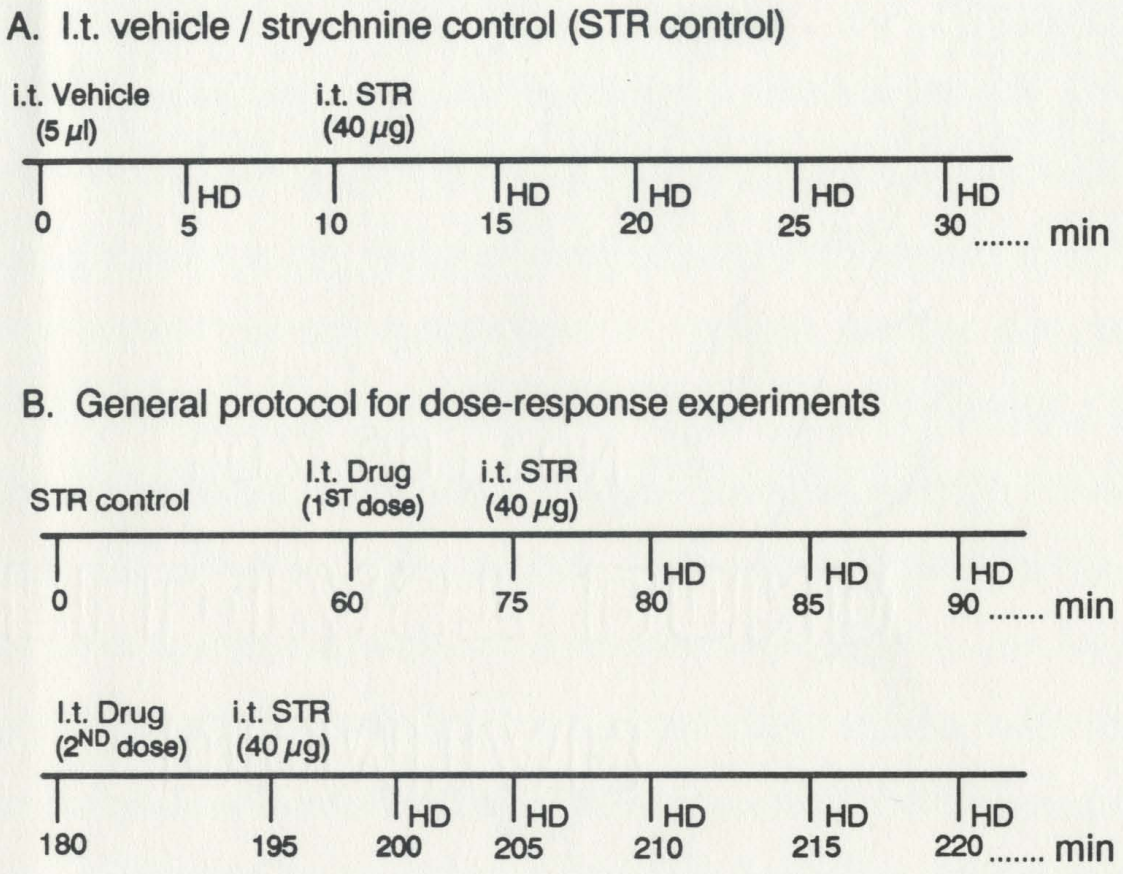


Figure 4. Summary of the Strychnine (STR) control protocol (A) and dose-response protocols (B). All time scales are in minutes. Each hair deflection stimulus (HD) was applied for 2-min using a cotton tip applicator. Following i.t. STR (40 µg), the HD stimulus was repeated every 5 min until the STR effects were no longer detected (~ 30 min) (A, B). One dose of i.t. AP-7, CPA, CGS, muscimol or baclofen was administered 15 min before i.t. STR (B; upper panel). The second dose was administered approximately two hours after the first dose (B; lower panel). Preliminary time-course experiments indicated that a 15-min pretreatment period yielded optimal pharmacological activity for i.t. administration (A).

calculated by determining the number of 1-min intervals with a synchronous EEG (determined visually) and expressing this as percentage of the experimental time.

2.6.3 Dose-response analysis for intrathecally administered drugs

For dose-response studies of i.t. AP-7, CPA, CGS, muscimol and baclofen, control responses to STR + HD were first determined in the absence of any drug treatment (see Figure 4A). Approximately one hour later, one dose of the above mentioned drugs was administered i.t. This was followed 15 min later by i.t. STR (40 µg). Hair deflection was then applied at 5-min intervals as described previously (Figure 4B). All animals received multiple drug doses. The order of dosing was from low to high to reduce the possibility of a carry over effect and thus, the recovery time. No more than three doses were administered to the same animal, and no animal received any further drug treatment once the highest dose had been given. Preliminary time-course experiments indicated that a 15-min pretreatment time was optimal for i.t. drug administration and that 1.5 hr between doses was sufficient for complete recovery from even the highest doses. Therefore, subsequent doses were generally given approximately two hours after the previous dose (lower panel of Figure 4B). For AP-7, mexiletine and CPA dose-response studies, the i.t. vehicle/STR control was repeated after each dose to verify complete recovery from the previous dose, and to ensure the reproducibility of STR-allodynia throughout the experiment. For the remaining dose-response experiments, i.t.

vehicle/STR control responses were determined once at the onset of the experiment. All animals were killed with an overdose of urethane at the end of the experiment.

2.6.4 DPCPX Experiments

The selectivity of i.t. adenosine agonists for A_1- and A_2 -receptors were determined in a separate group of animals. Control responses to STR + HD were initially established (see Figure 4A). Approximately one hour later, rats were pretreated with i.t. DPCPX (10 µg) followed 10 min later by i.t. CPA (0.2 µg) or CGS (5 µg). STR was administered 15 min after CPA or CGS, and STR-allodynia was quantified as described in section 2.6.2. The effect of CPA (0.2 µg) or CGS (5 µg) alone (no DPCPX) on STR allodynia was also established. Thus, each rat was in all of the following protocols:

I.t. DMSO (15 µl) / i.t STR (40 µg) (STR control)

I.t. DPCPX (10 µg) / i.t. CPA (0.2 µg) / i.t. STR

I.t. DPCPX (10 µg) /i.t. CGS (5 µg) / i.t. STR

i.t. CPA (0.2 µg) / i.t. STR

i.t. CGS (5 µg) / i.t. STR

A 1.5-hour recovery period was allowed between protocols (sufficient for the STR-responses to return to the control values). The dose of DPCPX (10 µg) used in this study was selected on the basis of preliminary experiments.

2.6.5 Mexiletine Experiments

Control responses to HD were determined 5 min after the i.t. injection of saline (15 µl, Figure 4A). Ten minutes later, STR (40 µg) was injected i.t. and the HD stimulus was applied at 5-min intervals (i.t. vehicle / STR control, Figure 4A) until all sensory-evoked responses were no longer detectable (~30 min after the STR injection). Approximately 1 h after the first STR injection, control responses to calibrated paw pinch were determined. Animals were then injected with mexiletine (5 or 15 mg/kg i.v., infused slowly over 10 min) and calibrated paw pinch was repeated 5 min after termination of the infusion. When the responses to noxious pinch had normalized (about 1-2 min), i.t. STR (40 µg) was injected and the HD was applied at 5-min intervals for 30 min. Most animals received 2 doses of i.v. mexiletine. Animals were allowed to recover for 2 h after the first mexiletine-STR treatment. Before a second dose of mexiletine (15 or 30 mg/kg i.v.) was given, the STR control was repeated to verify recovery from the initial mexiletine effect, as evidenced by a complete return of the STR control responses.

2.7 Protocols for Milacemide

To establish the time course of activity for each dose of milacemide in STR-model, milacemide (100, 400 or 600 mg/kg) was injected i.v. and the effect on STR-induced allodynia was followed for four hours. These doses and the 4-h time period

were chosen on the basis of preliminary experiments.

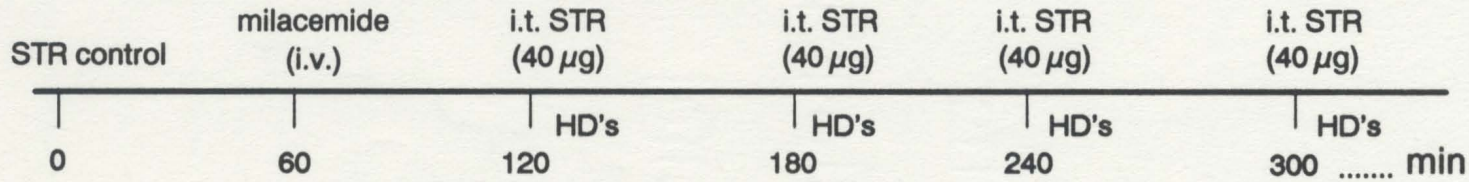
2.7.1 Dose-response Study

The general protocol for the dose-response experiments is shown in Figure 5(A). At the onset of the experiment, i.t. vehicle/STR control responses were obtained as described previously (sections 2.6.1-2; Figure 4A). One hour later, one dose of milacemide (100, 400, 600 mg/kg) was slowly infused i.v. over periods of 10 (for 100 mg/kg) -30 min (for 400 and 600 mg/kg). Every hour thereafter, i.t. STR (40 µg) was injected and the responses to HD were determined as described above (sections 2.6.1-2). Due to the short duration of allodynia (<30 min), STR was administered every hour for four hours. Animals were killed with an overdose of urethane at the end of the experiment.

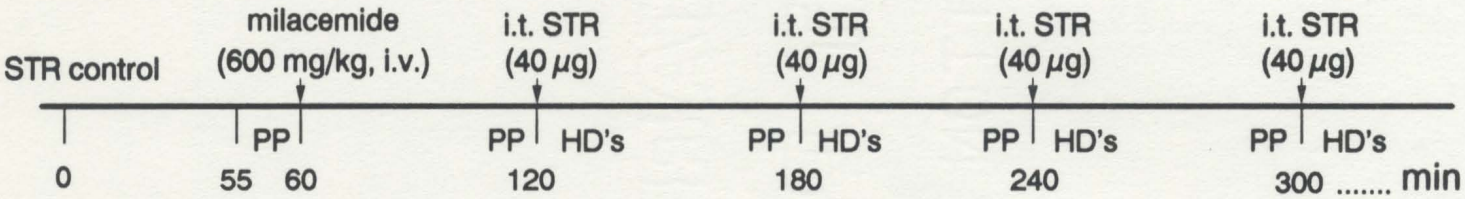
2.7.2 Comparison of the Effect of Milacemide on Strychnine-allodynia and Mechanical Nociception

To compare the effect of milacemide (600 mg/kg) on STR-allodynia and mechanical nociception (without STR), HD-evoked responses in presence of i.t. STR (40 µg), and calibrated bilateral hind paw-pinch in absence of STR were determined every hour for four hours following milacemide administration (Figure 5B). Control responses to i.t. vehicle/STR were initially determined (Figure 4A). Approximately one hour later (after the first STR effect had completely abated)

A. Milacemide dose-response protocol



B. Effect of milacemide on paw-pinch (PP) and STR-allodynia



C. Effect of MAO-A or -B inhibitor

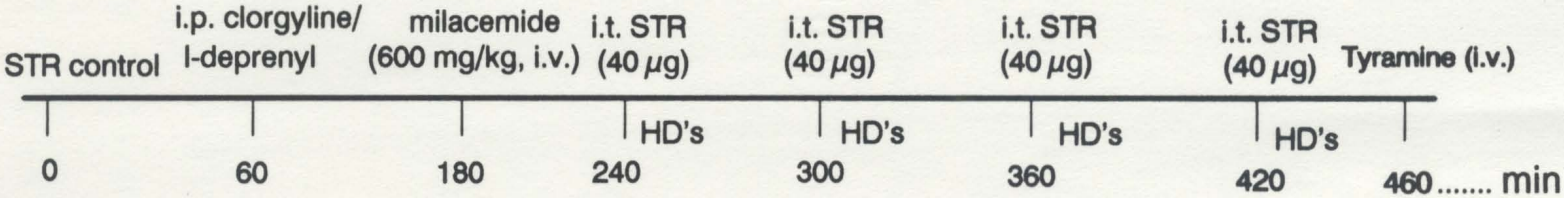


Figure 5. Summary of protocols for the milacemide experiments. All time scales are in minutes. Each hair deflection (HD) stimulus was applied for 2-min using cotton tip applicator (see section 2.4). Following i.t. strychnine (STR; 40 µg), the HD stimulus was repeated every 5 min until the STR effects were no longer detected (~ 30 min) (A, B, C). Hind paw-pinch (PP) was applied bilaterally, for 10 sec (B) using calibrated hemostats. STR was injected every hour for four hours following milacemide infusion. Tyramine was given approximately 40 min after the last STR-injection (C).

control responses to calibrated paw-pinch were determined. Animals were then injected with milacemide (600 mg/kg i.v., infused slowly over 30 min). STR (40 µg, i.t.) was injected every hour for four hours following the termination of infusion. Hair deflection was applied for 2-min every 5-min interval up to 30 min after each STR injection. Paw-pinch was applied once every hour for four hours following milacemide; this was done 5 min before each i.t. STR injection (Figure 5B).

2.7.3 Clorgyline and L-Deprenyl Experiments

The general protocol for assessing the effects of clorgyline or l-deprenyl on the action of i.v. milacemide is shown in Figure 5C. Intrathecal vehicle/STR control responses were initially determined (Figure 4A). Approximately one hour later, rats were pretreated with clorgyline (10 mg/kg, i.p.; Semba et al., 1993; de Varebeke et al., 1988) or l-deprenyl (3 mg/kg, i.p.; de Varebeke et al., 1988; Semba et al., 1993) (Figure 5C). Two hours later, milacemide (600 mg/kg, i.v.) was infused over a period of 30 min. Thereafter, HD-evoked responses in the presence of i.t. STR (40 µg) were determined every hour for four hours. In a separate group of animals, the effect of clorgyline or l-deprenyl on STR-allodynia (no milacemide) was determined every hour for four hours. To assess the selectivity of inhibition of clorgyline and l-deprenyl for MAO-A and MAO-B, respectively, i.v. tyramine was injected at the end of the experiment (Figure 5C). Doses ranged from 6.7 to 20 mg/kg and cardiovascular responses were recorded.

2.8 Data Analysis

All cardiovascular data are presented as the maximum change in MAP (= systolic blood pressure + 1/3 pulse pressure) and HR evoked by the different sensory stimuli. The change in MAP or HR was determined relative to the immediate (1 min) pre-stimulus control period (not relative to T=0). The maximum of each of these evoked responses, observed within 30 min of i.t. STR, was used for graphical and statistical analysis. For the paw-pinch stimulus, the baseline MAP and HR averaged over 1-min interval preceding stimulus application, was subtracted from the maximum response evoked by the stimulus, and this difference was reported.

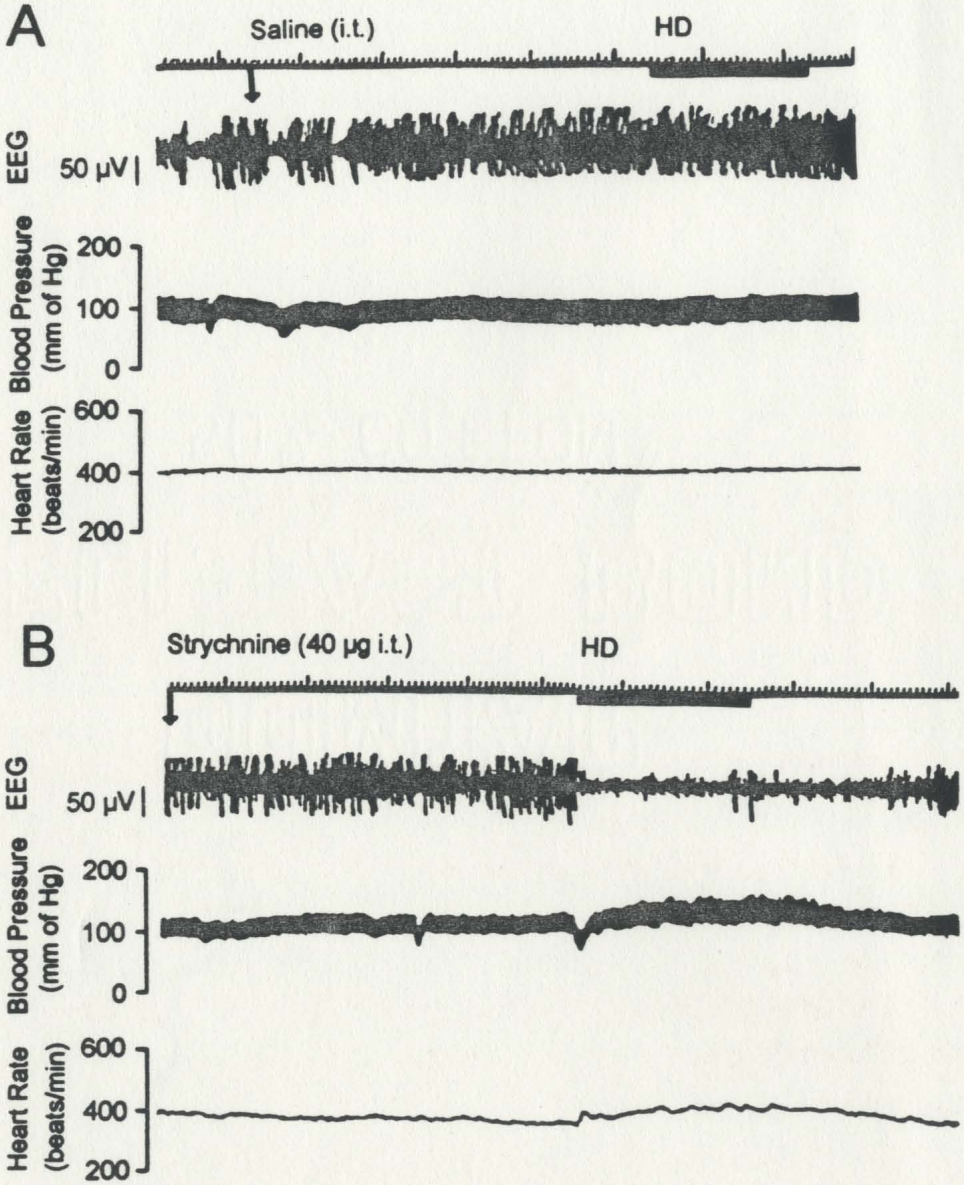
With the exception of milacemide, all dose-response data were analysed using regression ANOVA. A modified t-test was used to determine if the regression lines had significant slopes (=1). Variability associated with single measurements is indicated by the standard error of the mean (S.E.M.), while variability associated with blocks of data is indicated by the pooled 95% confidence interval (CI). ED_{50} and 95% CI values were determined for all dose-response curves (Figure 7, 10-13, 15, 17). The milacemide time-course data and dose-response data were analysed using one-way ANOVA; significant differences were identified using the Neuman-Keuls test (Figure16, 17, 19). Statistically significant differences among multiple independent treatment groups (i.e. DPCPX experiments) were also determined using completely randomised one-way ANOVA followed by the Neuman-Keuls test

(Figure 14). The unpaired, two-tailed students t-test was used to the compare the differences between two groups (Figure 18). Methods of data analysis were based on a general text (Tallarida and Murray, 1987).

3 Results

3.1 General Observations Following Intrathecal Strychnine

Stroking the hair on the legs, flanks, and lower back of the lightly anesthetized animal with a cotton tip applicator was without effect in i.t. saline-treated animals (Figure 6A). An identical stimulus applied to rats pretreated with i.t. STR (40 μg) evoked a progressive rise in HR and BP that persisted beyond the duration of the HD stimulus (Figure 6B). These responses were accompanied by an abrupt scratching response near the site of stimulation and/or motor withdrawal, as well as desynchrony of the EEG (Figure 6B). Normalization of the EEG (return to a synchronous pattern), which occurred 2-3 min after the discontinuation of the HD stimulus, coincided with recovery of baseline BP and HR. These cardiovascular and motor responses were only evoked by HD at discrete sites corresponding to dermatomes innervated by spinal segments near the site of STR injection. The time course of these tactile-evoked cardiovascular responses were comparable to those elicited by the intradermal injection of mustard oil into the hind paw (without STR) and were greater in magnitude (Figure 6C). Average control responses to HD in STR-treated rats were 10-15 mm Hg for MAP, 20-25 beats/min for HR and a duration of 15-20 min during which motor withdrawal responses which could be evoked by HD. These appear as the solid horizontal line in each of the dose-response curves (e.g. Figure 7A-C). The STR effects lasted for approximately 30 min with the peak evoked cardiovascular responses appearing 5 min after i.t. STR


A
Saline (i.t.)
HD
EEG
50 µV
Blood Pressure (mm of Hg)
200
100
0
Heart Rate (beats/min)
600
400
200
B
Strychnine (40 µg i.t.)
HD
EEG
50 µV
Blood Pressure (mm of Hg)
200
100
0
Heart Rate (beats/min)
600
400
200

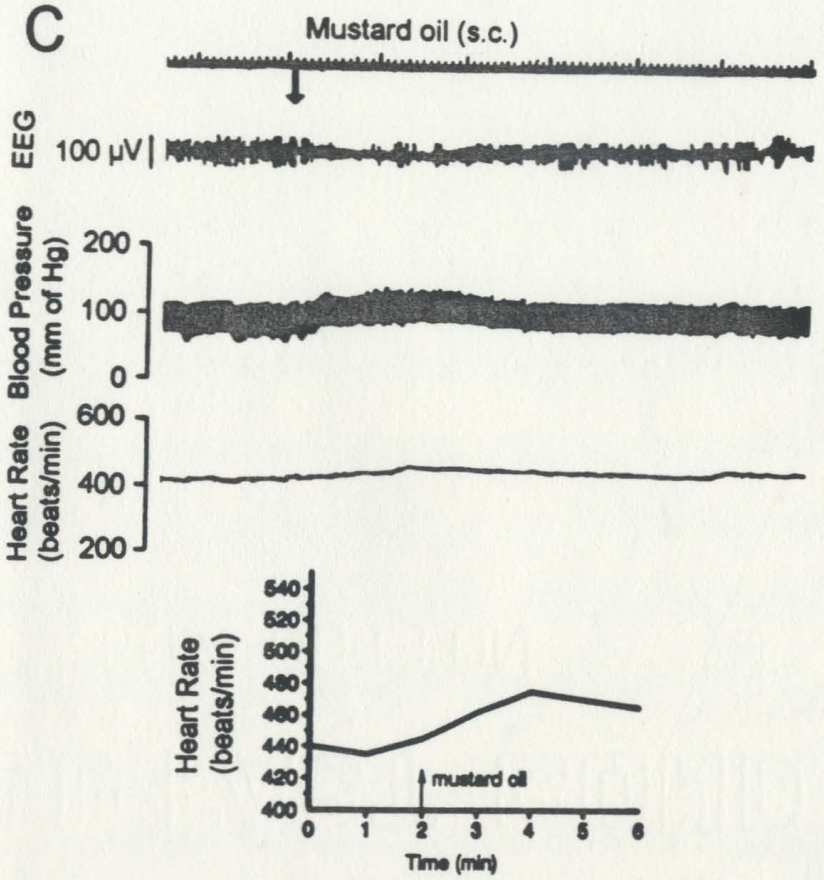


Figure 6. Sample polygraph tracings of arterial blood pressure, heart rate, and cortical EEG illustrating the effect of non-noxious hair deflection in intrathecal (i.t.) saline- and strychnine-treated rats. Hair deflection (HD) to the legs, flank and lower back 5 min after the i.t. injection of saline had little effect on blood pressure, heart rate or EEG (A). The same stimulus applied 5 min after i.t. strychnine (40 µg) evoked a marked and sustained increase in blood pressure and heart rate, and desynchrony of the EEG (B). These evoked responses were localized to dermatomes affected by the i.t. injection of strychnine, and observed without convulsions or spontaneous motor movements. Each large deflection on the time scale (upper trace) indicates 1 min and the horizontal bar below the time trace indicates the application of the HD stimulus. For comparative purposes, the responses evoked by the intradermal injection of mustard oil are shown (C). The heart rate tracing is enlarged for clarity at the bottom of the figure. Arrows indicate the i.t. injection of saline (A), the i.t. injection of strychnine (B) or the intradermal injection of mustard oil into the hind paws (C).

(data not shown).

3.2 Effect of AP-7 on Strychnine-allodynia

Pretreatment with i.t. AP-7 (0.2, 1, 5 µg) produced a dose-dependent reduction of all HD-evoked cardiovascular and motor withdrawal responses in STR-treated rats (Figure 7A-C). The corresponding ED_{50}s and 95% CIs of AP-7 are summarised in Table III. The ED_{50}s ranged from 0.36 - 1.71 µg. The % synchrony of the EEG remained unchanged after AP-7 injection, regardless of the dose (Figure 7D). Baseline HR and arterial blood pressure were also unaffected by AP-7 (data not shown). To compare the spinal effect of AP-7 on STR-allodynia and normal nociception, rats pretreated with i.t. AP-7 received calibrated bilateral hind paw pinch (Without STR). A dose of i.t. AP-7 (5 µg) effecting almost complete blockade of STR-allodynia had no effect on the responses evoked by noxious paw-pinch (Figure 8).

Table III. ***ED_{50}s and 95% C.I. of AP-7***

Treatment	Response	ED_{50} (µg)	95% CI
AP-7 i.t.*	MAP	1.12	0.69 - 1.82
	HR	1.71	0.50 - 5.98
	WD	0.36	0.07 - 2.00

* AP-7 was injected 15 min before STR.

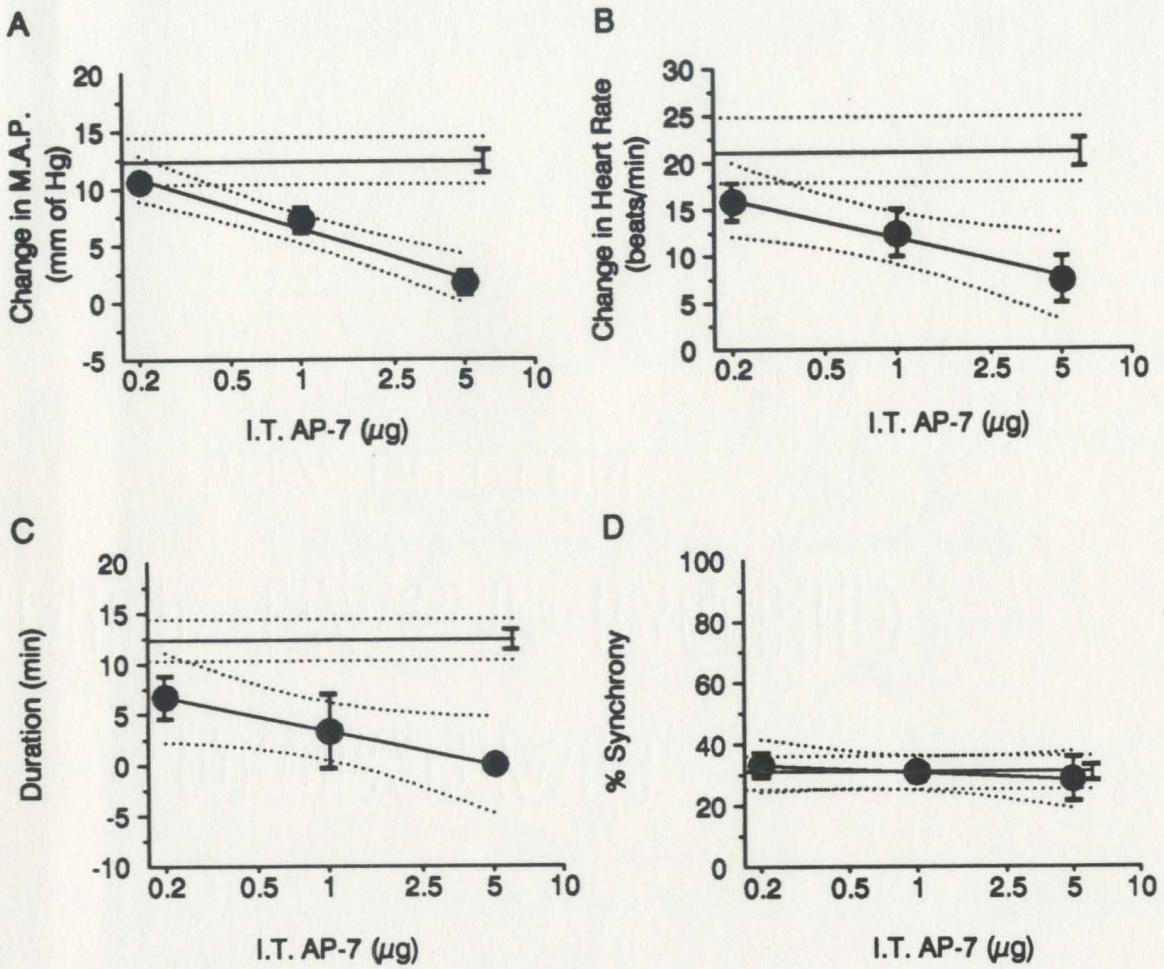


Figure 7. Intrathecal AP-7 dose-dependently suppresses intrathecal strychnine(STR)-allodynia. Following a 15-min pretreatment with i.t. AP-7, responses to hair deflection (HD) were determined in the presence of i.t STR (40 μg). The HD-evoked increase in mean arterial pressure (M.A.P.; A), heart rate (B), the duration for which withdrawal response could be evoked (C) and the % synchrony in the EEG (D) are shown. Each point represents mean ± S.E.M. of 4-6 animals. Least squares regression lines and the corresponding 95% CI (dotted lines) are shown. The horizontal solid lines and adjacent dotted lines indicate the mean ± 95% CI of all STR treatments (N = 14-16) before AP-7.

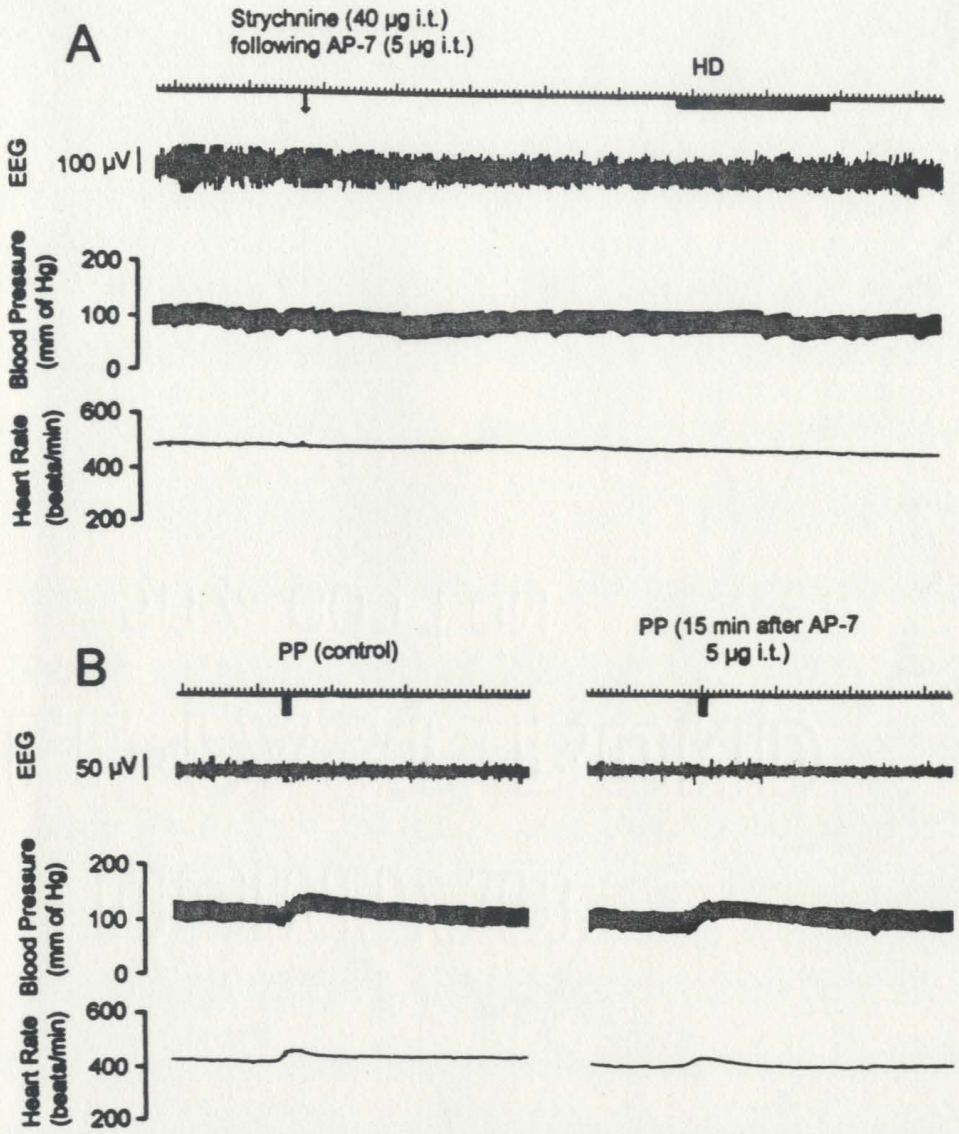


Figure 8. Sample polygraph tracings of arterial blood pressure, heart rate and cortical EEG illustrating the effect of intrathecal AP-7 on strychnine-allodynia (A) and noxious mechanical hind paw-pinch (B) in the rat. AP-7 (5 µg, i.t.) was injected 15 min before the i.t. injection of strychnine (40 µg). The arrow indicates the injection of strychnine (A). Hair deflection (HD) was applied to the legs, flanks and lower back of the rat 5 min after strychnine. Note the complete blockade of all HD-evoked responses by AP-7 in the strychnine-treated rat. However, identical dose of AP-7 failed to block the noxious paw-pinch evoked responses (B). Each large deflection on the time scale (upper trace) indicates 1 min and the horizontal bar below the time trace indicates the application of stimulus.

3.3 Effect of Mexiletine on Strychnine-allodynia and Normal Nociception

Intravenous mexiletine (5, 15 and 30 mg/kg), administered 5 min before i.t. STR, dose-dependently inhibited all the indices of STR-allodynia with ED_{50}s ranging from 13.3 - 16.6 mg/kg (Table IV, upper panel of Figure 9, Figure 10A-C). At 30 mg/kg, there was a small but non-significant increase in % synchrony compared to STR-control (Figure 10D). Nevertheless, this increase was well below the previously established cut-off of 60% synchrony (Sherman and Loomis, 1994). The i.v. injection of mexiletine produced an immediate dose-dependent decrease in baseline BP and HR (i.e. before STR-allodynia). Baseline blood pressure decreased by 10 mm Hg (5 mg/kg) to 20 mm Hg (30 mg/kg); an effect which lasted for approximately 1-2 min. In all cases, blood pressure returned to normal before i.t. STR was injected (data not shown). Baseline HR, which decreased by 30 bpm (5 mg/kg) to 60 bpm (30 mg/kg), was fully recovered 5 min after the injection of mexiletine (5 mg/kg). At higher doses, baseline HR remained partially depressed (~4% of pre-injection values for 15 mg/kg and ~8% for 30 mg/kg) throughout the STR treatment.

The effect of i.v. mexiletine was also determined against noxious paw-pinch using the same experimental animals. Calibrated bilateral hind paw pinch was applied 3 min before the injection of i.t. STR. Mexiletine dose-dependently inhibited pinch-evoked cardiovascular responses (Figure 11A-B), and prevented desynchrony of the EEG (Figure 9, lower panel). All rats receiving 15 mg/kg dose

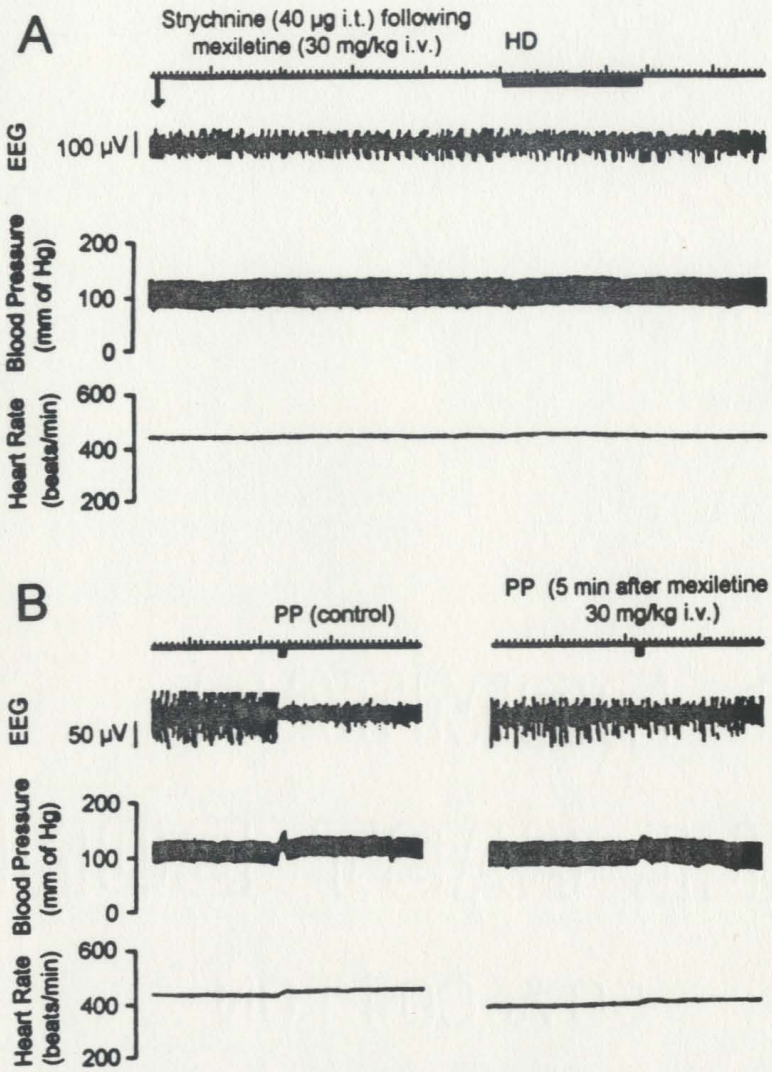


Figure 9. Sample polygraph tracings of arterial blood pressure, heart rate, and cortical EEG illustrating the effect of intravenous mexiletine on strychnine-allodynia (A) and noxious mechanical hind paw pinch (B) in the rat. Mexiletine (30 mg/kg, i.v.) was injected 5 min before the i.t. injection of strychnine (40 µg). The arrow indicates the injection of strychnine (A). Hair deflection (HD) was applied to the legs, flanks and lower back of the rat 5 min after strychnine. Note the complete blockade of all hair deflection (HD)-evoked responses by mexiletine in the strychnine-treated rat (A) and its inhibitory effect against calibrated bilateral hind paw-pinch without strychnine in the same animal (B). Each large deflection on the time scale (upper trace) indicates 1 min and the horizontal bar below the time trace indicates the application of stimulus.

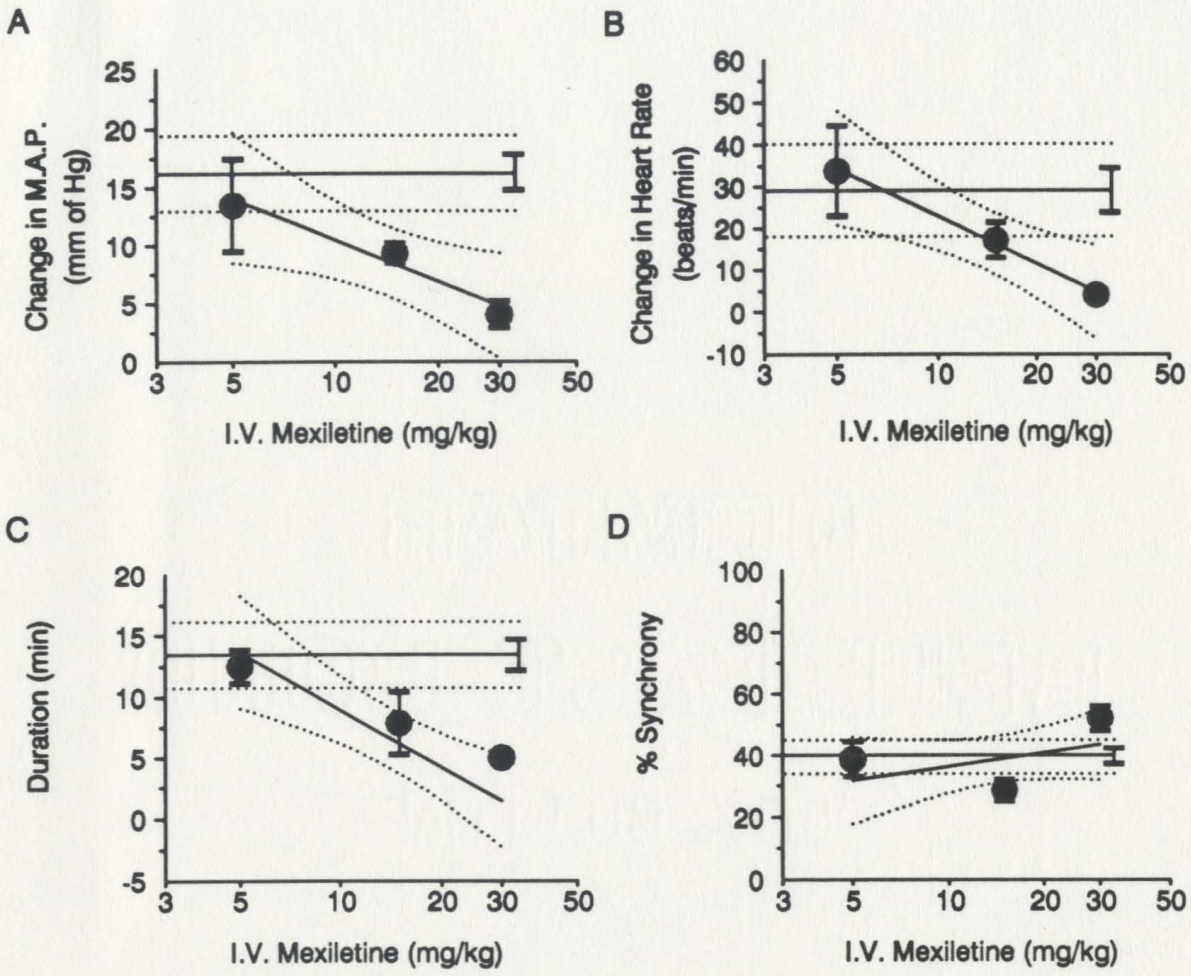


Figure 10. Intravenous mexiletine dose-dependently suppresses intrathecal strychnine(STR)-allodynia. Following a 5-min pretreatment with i.v. mexiletine, responses to hair deflection (HD) were determined in the presence of i.t. STR (40 µg). The HD-evoked increase in mean arterial pressure (M.A.P.; A), heart rate (B), the duration for which withdrawal response could be evoked (C) and the % synchrony in the EEG (D) are shown. Each point represents mean ± S.E.M. of 4-7 animals. Least squares regression lines and the corresponding 95% CI (dotted lines) are shown. The horizontal solid lines and adjacent dotted lines indicate the mean ± 95% CI of all STR treatments (N=16) before mexiletine.

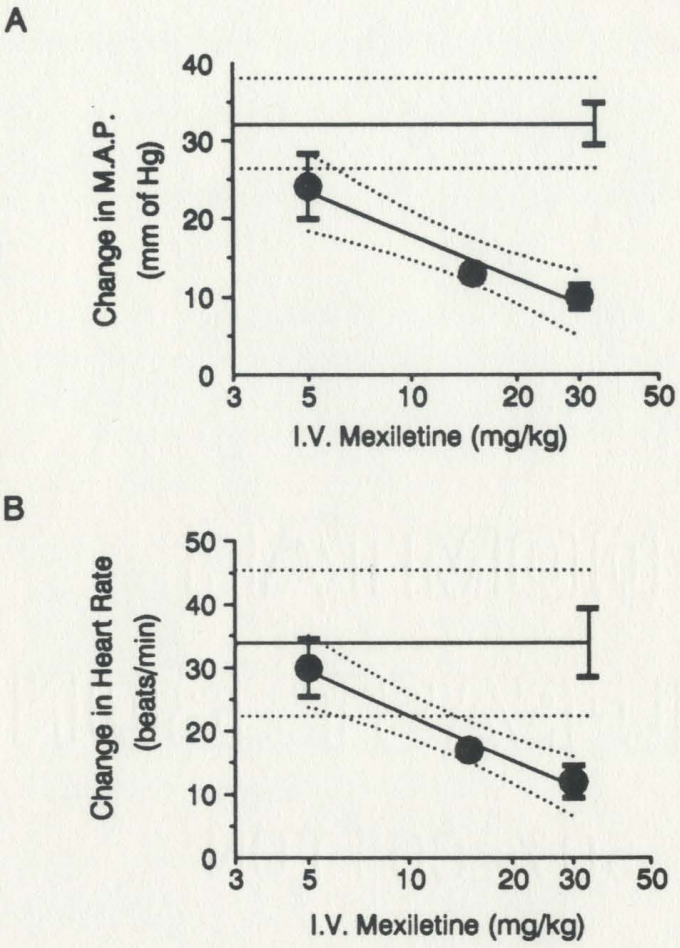


Figure 11. Intravenous mexiletine dose-dependently suppresses paw-pinch induced responses in absence of strychnine (STR). Following a 5-min pretreatment with i.v. mexiletine, responses to calibrated bilateral hind paw-pinch were determined (without STR). The stimulus evoked increase in M.A.P.(A) and HR (B) are shown. Each point represents the mean ± S.E.M. of 4-5 animals. Least square regression lines and the corresponding 95% CI (dotted lines) are shown. The horizontal solid lines indicate the mean ± 95% CI of all paw-pinch evoked responses (N=16) before mexiletine.

attempted to withdraw their hind paws during noxious stimulation but were prevented from doing so to ensure a consistent noxious mechanical stimulation. No withdrawal attempts were recorded in any of the rats receiving the 30 mg/kg dose. The ED_{50}'s of i.v. mexiletine against noxious pinch (without STR) and STR-allodynia were very comparable and exhibited narrow 95% CI's (Table IV).

Table IV. ED_{50}s and 95% C.I. of i.v. Mexiletine

Response	ED_{50}(mg/kg)	95% CI
**STR-allodynia:*		
MAP	15.70	8.67 - 28.42
Heart Rate	16.60	10.46 - 26.42
Duration of Withdrawal	13.83	9.58 - 19.97
Paw-pinch evoked responses:		
MAP	9.10	6.00 - 13.82
Heart Rate	13.31	9.66 - 18.35

* STR was injected 5 min after termination of the mexiletine infusion.

3.4 Effects of Adenosine Agonists on Strychnine-allodynia

Pretreatment with i.t. CPA (0.001, 0.05 and 0.1 µg), 15 min before STR, dose-dependently inhibited the HD-evoked rise in BP (Figure 12A), HR (Figure 12B) and motor withdrawal (Figure 12C). Overall, i.t. CPA had no significant effect

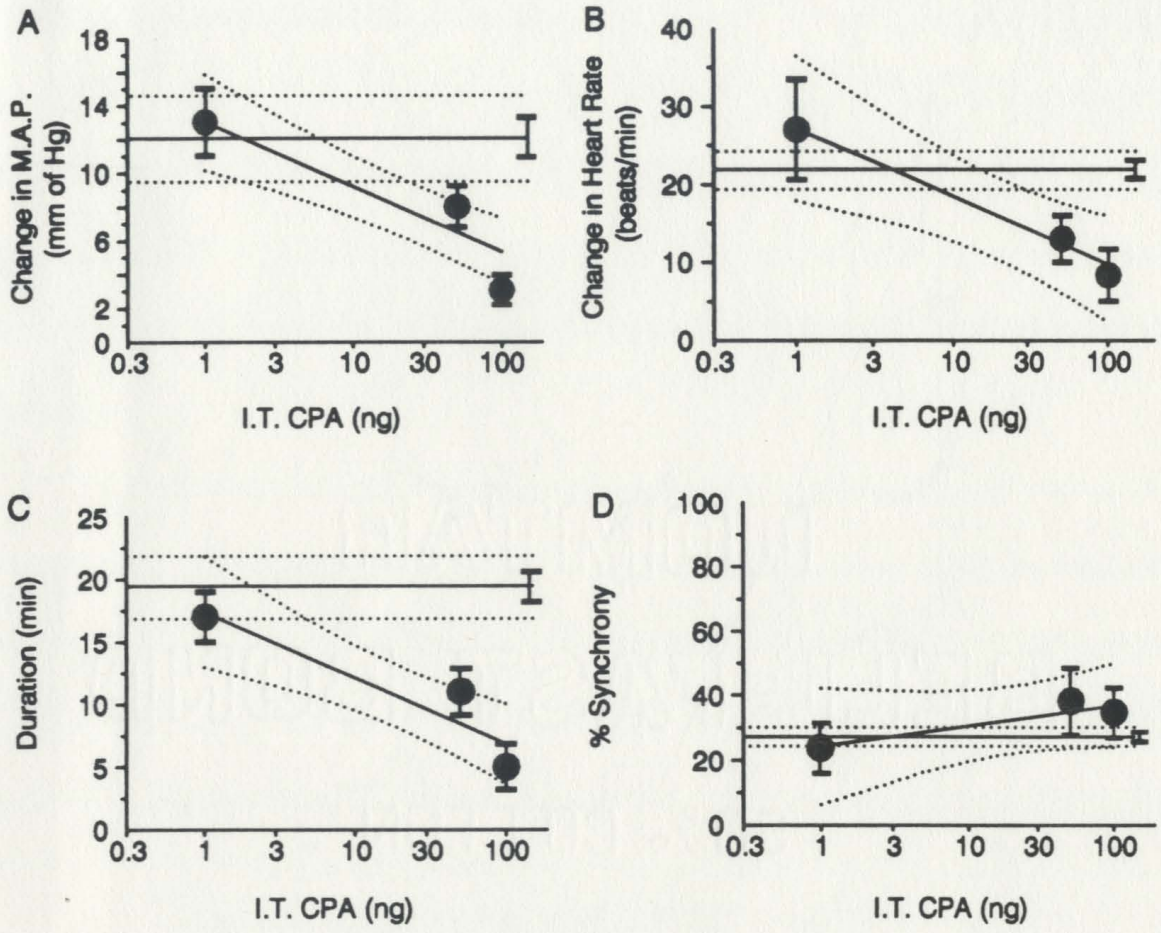


Figure 12. Intrathecal CPA dose-dependently suppresses intrathecal strychnine(STR)-allodynia. Following a 15-min pretreatment with i.t. CPA, responses to hair deflection (HD) were determined in the presence of i.t. STR (40 µg). The HD-evoked increase in mean arterial pressure (M.A.P.; A), heart rate (B), the duration for which withdrawal response could be evoked (C) and the % synchrony in the EEG (D) are shown. Each point represents mean ± S.E.M. of 5-6 animals. Least squares regression lines and the corresponding 95% CI (dotted lines) are shown. The horizontal solid lines and adjacent dotted lines indicate the mean ± 95% CI of all STR treatments (N=16) before CPA injection.

on % synchrony of the EEG (Figure 12D). However, the data from two animals receiving 0.05 µg and one animal receiving 0.1 µg were excluded from the final results as the % synchrony exceeded the 60% cut off. The ED_{50} of i.t. CPA ranged from 0.02 - 0.07 µg (Table V). The inhibitory effect of i.t. CPA (0.2 µg) was blocked by pretreatment with the A_1-antagonist, DPCPX (10 µg i.t.; Figure 13). Interestingly, i.t. DPCPX alone triggered mild spontaneous scratching and HD-evoked withdrawal responses for approximately 5 - 10 minutes after injection in two animals (data not shown).

I.t. CGS also inhibited STR-induced allodynia in a dose-dependent manner (Figure 14). However, doses of 2 - 5 µg were required to achieve inhibition comparable to that of i.t. CPA at nearly 1/60 the dose. The ED_{50}s of i.t. CGS ranged from 2.7 - 3.1 µg (Table V). This anti-allodynic effect was significantly blocked by i.t. DPCPX (Figure 13B). CGS had no detectable effect on level of anesthesia with

Table V. ED_{50}s and 95% C.I. of CPA or CGS

Treatment	Response	ED_{50} (µg)	95%CI
CPA i.t *	MAP	0.07	0.02 - 0.20
	HR	0.06	0.01 - 0.30
	WD	0.02	0.01 - 0.09
CGS-i.t.*	MAP	3.11	2.50 - 3.86
	HR	2.75	2.09 - 3.61
	WD	2.69	1.81 - 3.98

* CPA and CGS were injected 15 min before STR.

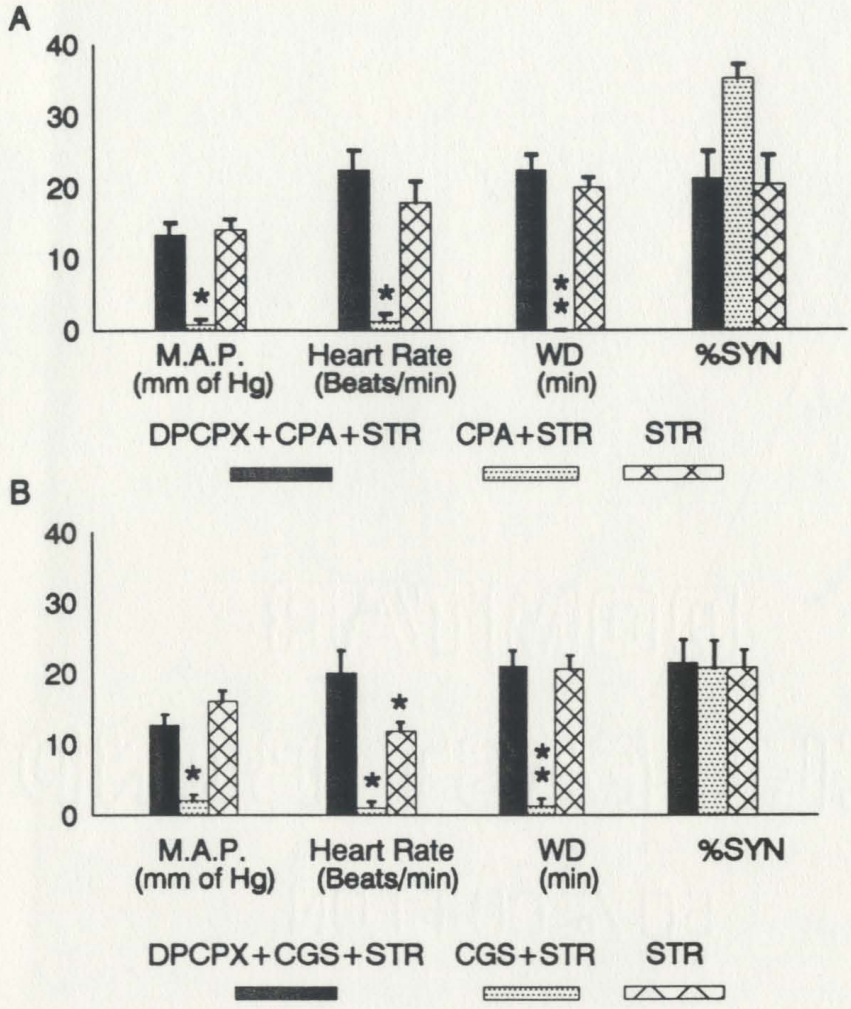


Figure 13. Inhibition of the anti-allodynic effect of CPA or CGS by pretreatment with intrathecal DPCPX. DPCPX (10 µg, i.t.) was administered 10 min before i.t. CPA (0.2 µg) or CGS (5 µg). STR (40 µg, i.t.) was given 15 min later and responses to hair deflection (HD) were determined at 5-min intervals thereafter. The effects of CPA (A) or CGS (B) (in absence and presence of DPCPX) on the HD-evoked increase in mean arterial pressure (M.A.P.), heart rate, duration for which motor withdrawal responses could be evoked (WD) and % synchrony (% SYN) are shown. The solid bar represent the STR-control responses without any drug treatment. Each bar represents mean ± SEM of 6 - 8 animals. (*$p<0.05$, **$p<0.01$)

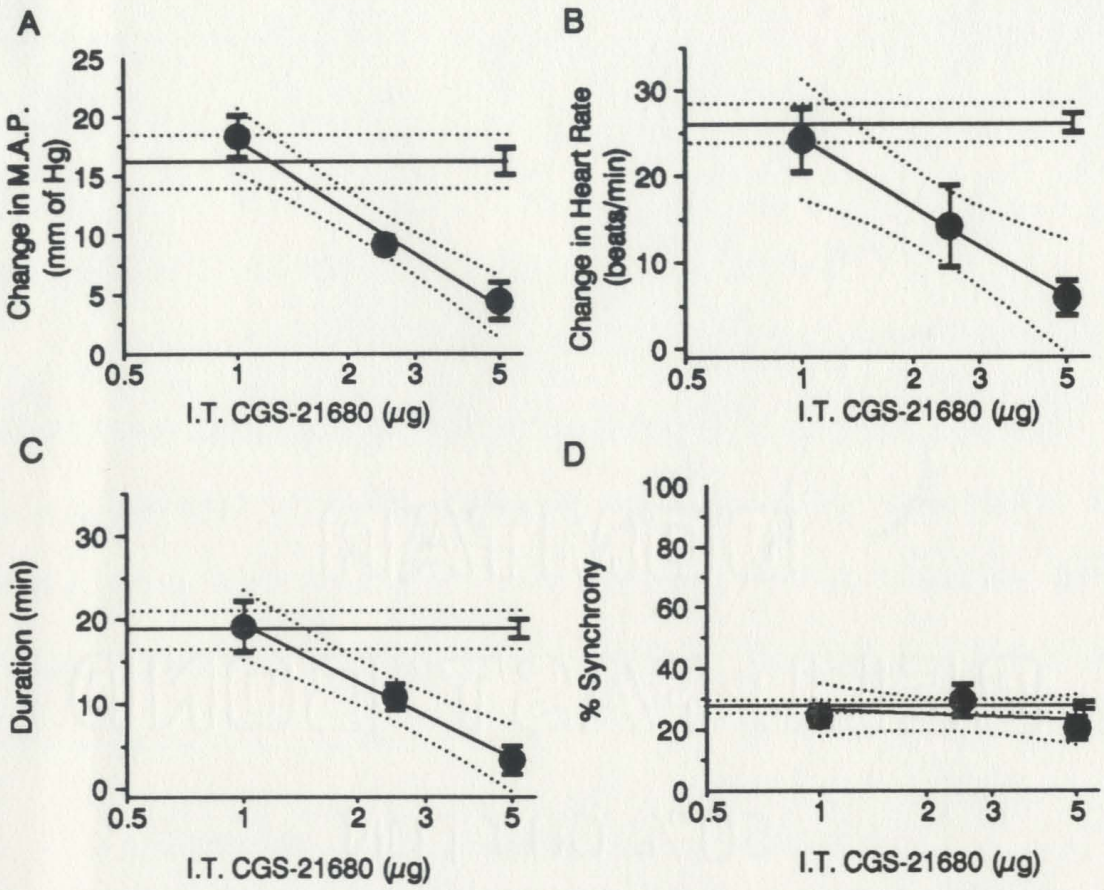


Figure 14. Intrathecal CGS dose-dependently suppresses intrathecal strychnine(STR)-allodynia. Following a 15-min pretreatment with i.t. CGS, responses to hair deflection (HD) were determined in the presence of i.t. STR (40 μg). The HD-evoked increase in mean arterial pressure (M.A.P.; A), heart rate (B), the duration for which withdrawal response could be evoked (C) and the % synchrony in the EEG (D) are shown. Each point represents mean ± S.E.M. of 5-6 animals. Least squares regression lines and the corresponding 95% CI (dotted lines) are shown. The horizontal solid lines and adjacent dotted lines indicate the mean ± 95% CI of all STR treatments (N=18) before CGS injection.

the % synchrony remaining unchanged at all doses. At a dose of 5 µg, i.t. CGS had no antinociceptive effect against noxious bilateral hind paw pinch (n=2, data not shown).

3.5 Effects of GABA Agonists on Strychnine-allodynia

I.t. muscimol (0.1, 0.5, 1 and 2.5 µg), given 15 min before STR (40 µg) injection, dose dependently inhibited all indices of STR-allodynia (Figure 15). This was accomplished without a change in the % synchrony of the EEG, which remained below 60% cut off. The baseline arterial blood pressure and HR were also unaffected by i.t. muscimol (data not shown). The ED_{50} and 95% CI for i.t. muscimol are summarised in Table VI.

Table VI. ED_{50}s and 95% C.I. of Muscimol*

Treatment	Response	ED_{50} (µg)	95% CI
Muscimol i.t.	MAP	0.51	0.37 - 0.72
	HR	0.50	0.34 - 0.74
	WD	0.44	0.32 - 0.61

* Muscimol was injected 15 min before STR

In contrast, baclofen (up to 10 µg, i.t.) failed to inhibit STR-induced allodynia (Table VII). Rather there was a trend towards increased cardiovascular responses

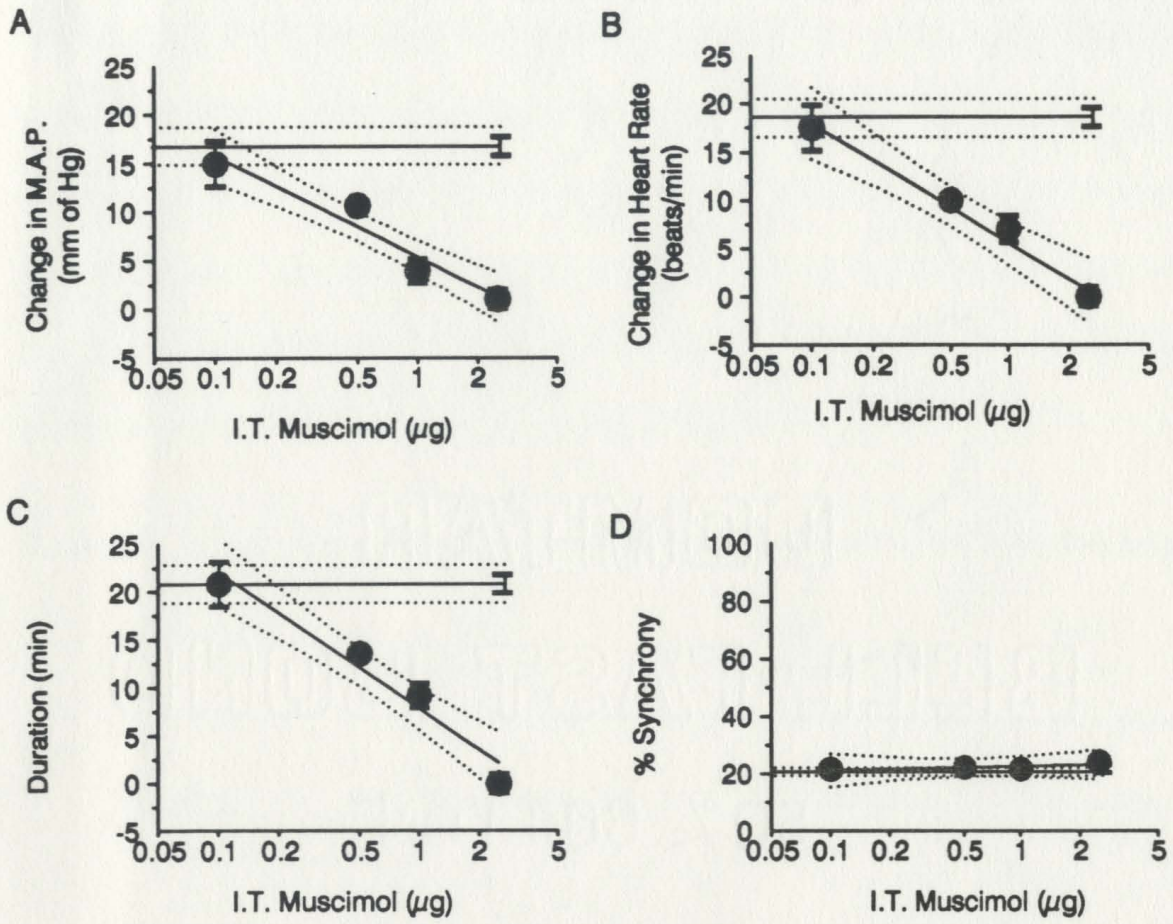


Figure 15. Intrathecal muscimol dose-dependently suppresses intrathecal strychnine(STR)-allodynia. Following a 15-min pretreatment with i.t. muscimol, responses to hair deflection (HD) were determined in the presence of i.t. STR (40 μg). The HD-evoked increase in mean arterial pressure (M.A.P.; A), heart rate (B), the duration for which withdrawal response could be evoked (C) and the % synchrony in the EEG (D) are shown. Each point represents mean ± S.E.M. of 5-7 animals. Least squares regression lines and the corresponding 95% CI (dotted lines) are shown. The horizontal solid lines and adjacent dotted lines indicate the mean ± 95% CI of all STR treatments (N=23-24) before muscimol injection.

after i.t. baclofen. However, HD-evoked increase in MAP and HR following baclofen (Table VII) was not significantly different from vehicle-control in STR-treated rats. The withdrawal responses to HD were significantly inhibited by i.t. baclofen (*$p<0.01$). Indeed, one rat receiving the 1 µg dose, and all rats receiving ≥5 µg exhibited reversible hind limb paralysis. Thus, HD-evoked motor withdrawal responses were completely abated by high doses of baclofen at a time when cardiovascular responses remained unaffected. The baseline arterial blood pressure and HR were also unaffected by i.t. baclofen.

Table VII. Effect of baclofen on HD-evoked responses in STR-treated rats

Treatment	MAP (mm Hg)	HR (beats/min)	WD (min)
Vehicle-control (14)	13.4 ± 1.6	20.7 ± 1.9	25 ± 2.0
Baclofen*			
1 µg (3)	22.8 ± 4.9	33.3 ± 13.0	6.7 ± 3.3*
5 µg (4)	25.4 ± 5.0	40 ± 8.4	Absent
10 µg (5)	22.3 ± 4.8	29 ± 9.4	Absent

Baclofen was injected 15-min before STR. Data are expressed as the mean ± SEM of 3-14 animals (N values shown in the brackets) ($p<0.01$). Mean arterial pressure (MAP); heart rate (HR); withdrawal duration (WD).

3.6 Effect of Milacemide on Strychnine-allodynia and Normal Nociception

Pretreatment with i.v milacemide (400 and 600 mg/kg) significantly inhibited STR-allodynia (Figure 16 and 17). Maximal inhibition was observed 2-3 h after drug administration (Figure 16). The duration of action ranged from 2 h for 400 mg/kg dose to 4 h for 600 mg/kg, and the % synchrony of the EEG was unchanged at all doses throughout the experiment (Figure 16D and 17D). The ED_{50}s at the time of maximum inhibition (i.e. 2 h after milacemide infusion) were approximately 400 mg/kg i.v. (Table VIII). Baseline BP and HR were dose-dependently depressed during milacemide infusion (i.e. before STR-allodynia). Baseline BP decreased by 15 (400 mg/kg) to 25 mm Hg (600 mg/kg) and HR decreased by 25 (400 mg/kg) to 50 bpm (600 mg/kg). For the 400 mg/kg dose, BP and HR return to normal before i.t. STR was injected (i.e. 1 h after milacemide infusion). At 600 mg/kg, BP and HR returned to normal 3 h after infusion, except in two animals where baseline HR and BP remained depressed by approximately 10% and 8% of pre-injection values throughout the experiment.

[Note: Dose-dependent depression of respiration was observed (visual examination) during the first 15-20 min following the termination of milacemide infusion].

In a separate group of animals, responses to noxious hind paw pinch (without STR) and STR-allodynia were recorded every hour for 4 h after milacemide infusion. At the time of maximum inhibition of STR-allodynia (2 h post-infusion),

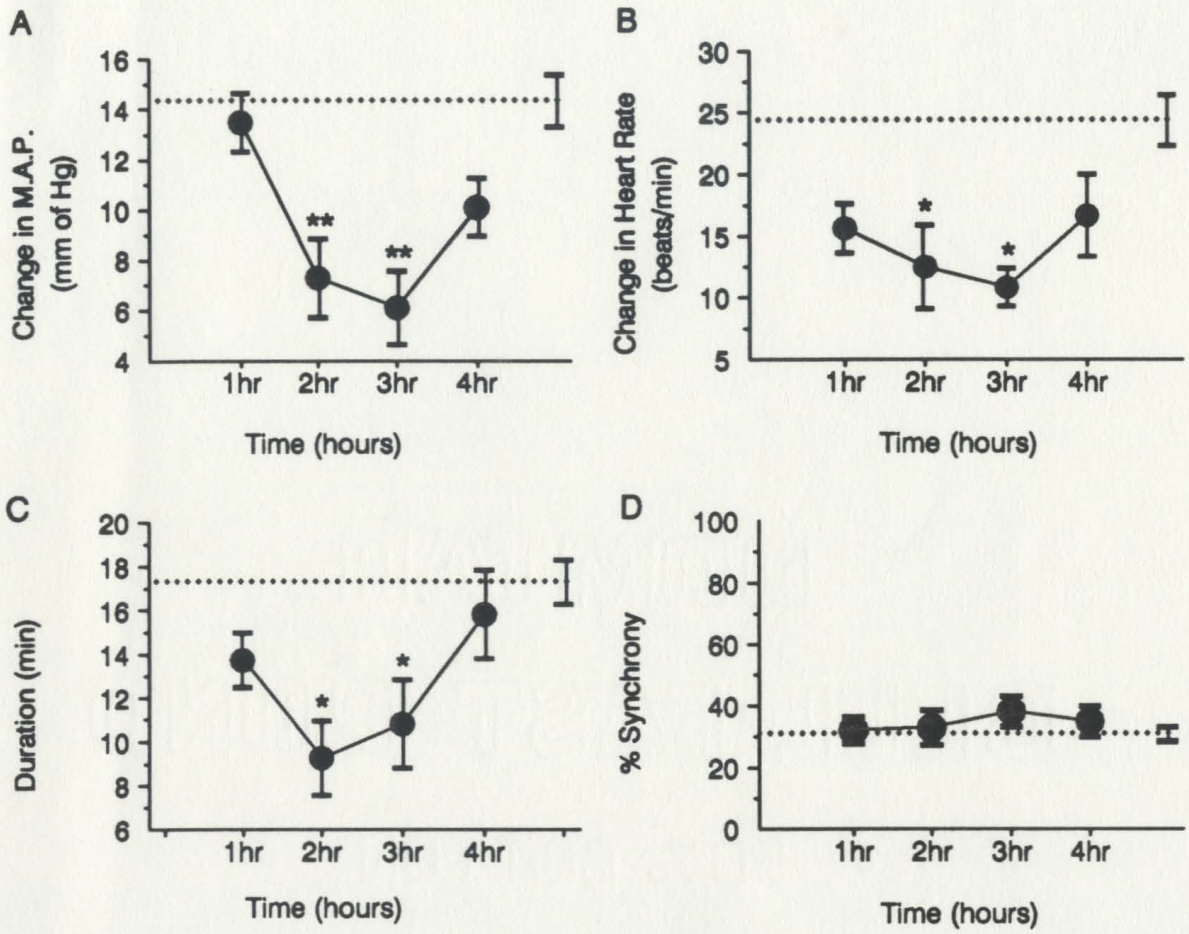


Figure 16. Intravenous milacemide exerts its maximum effect on intrathecal strychnine(STR)-allodynia two hours after injection without affecting EEG synchrony. Hair deflection (HD)-evoked responses in the presence of i.t. STR (40 µg) were determined every hour for four hours after i.v. milacemide (400 mg/kg; approximate ED_{50} value). Because of the short duration of allodynia (<30min), i.t. STR was repeated every hour. The maximum evoked increase in mean arterial pressure (M.A.P.; A), heart rate (B), the duration for which a motor withdrawal response could be evoked (C) and the % synchrony in the EEG (D) are shown. Each point represents mean ± SEM of 6-8 animals. The horizontal dotted line is the maximum evoked response to STR (mean ± SEM) in absence of any further treatment (N=24). (*$p<0.05$, **$p<0.01$)

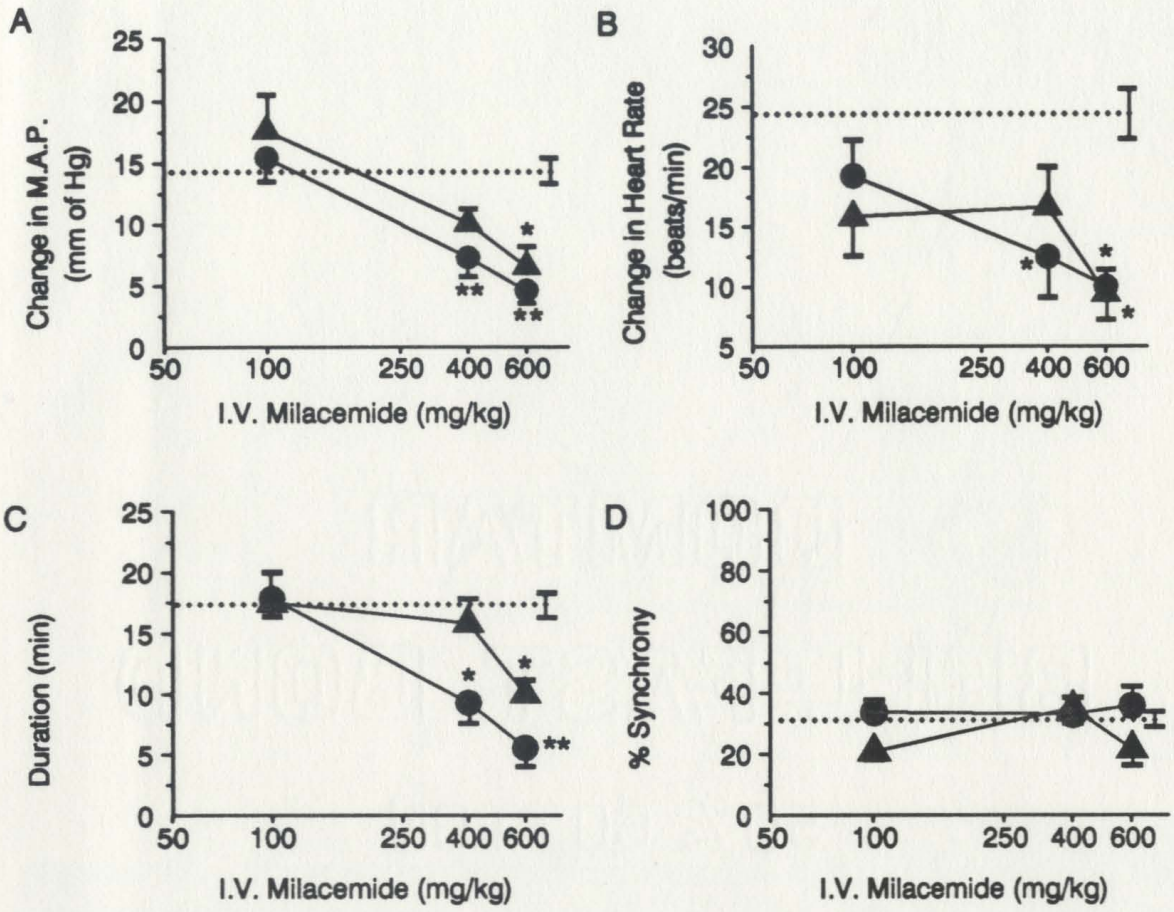


Figure 17. Intravenous milacemide suppresses intrathecal strychnine(STR)-allodynia in a dose- and time-dependent manner, without affecting EEG synchrony. Hair deflection (HD)-evoked responses in the presence of i.t. STR (40 µg) were measured every hour for four hours after i.v. milacemide (100, 400 and 600 mg/kg). Dose-response curves for the change in mean arterial pressure (MAP; A), heart rate (B), the duration for which withdrawal responses could be evoked (C) and the % synchrony in the EEG (D) were determined 2h (● time of maximum effect) and 4h (▲) after injection. Each point represents the mean ± SEM of 6-8 animals. The horizontal dotted line is the maximum evoked response in STR-treated rats (mean ± SEM) before milacemide (N=24). (*$p<0.05$, **$p<0.01$)

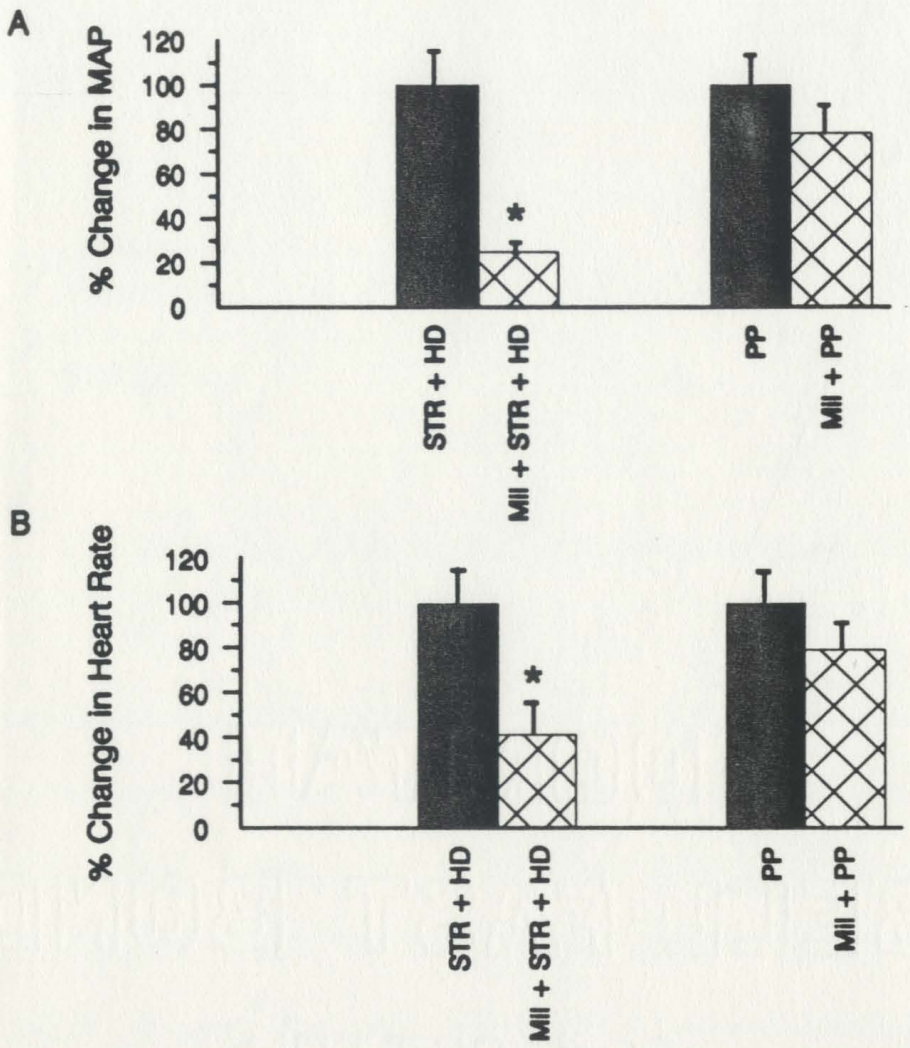


Figure 18. Milacemide (600mg/kg, i.v.) significantly inhibits intrathecal strychnine(STR)-allodynia but has no effect on normal mechanical nociception. Hair deflection (HD)-evoked cardiovascular responses in STR-treated rats, and responses to noxious paw-pinch (without STR) were determined in the same animals two hours after intravenous milacemide (600mg/kg) (time of maximum inhibition of STR-allodynia). Noxious hind paw-pinch was applied bilaterally using calibrated hemostats. The maximum evoked increase in mean arterial pressure (MAP; A) and heart rate (B) are presented. The solid bars represent the control responses to HD in STR-treated rats (STR + HD) or noxious paw-pinch (without STR). The hatched bars represent the corresponding response following i.v. milacemide (Mil). Each bar represents mean ± SEM of 4 animals. (*$p<0.05$)

responses evoked by noxious pinch were unaffected by milacemide (Figure 18).

Table VIII. ED_{50} and 95% C.I. of milacemide

Treatment	Response	ED_{50}(µg)*	95% CI
Milacemide	MAP	398.3	292.5 - 542.3
	HR	413.2	195.6 - 872.6
	WD	404.5	301.1 - 543.4

calculated from dose-response curve determined 2h after milacemide administration

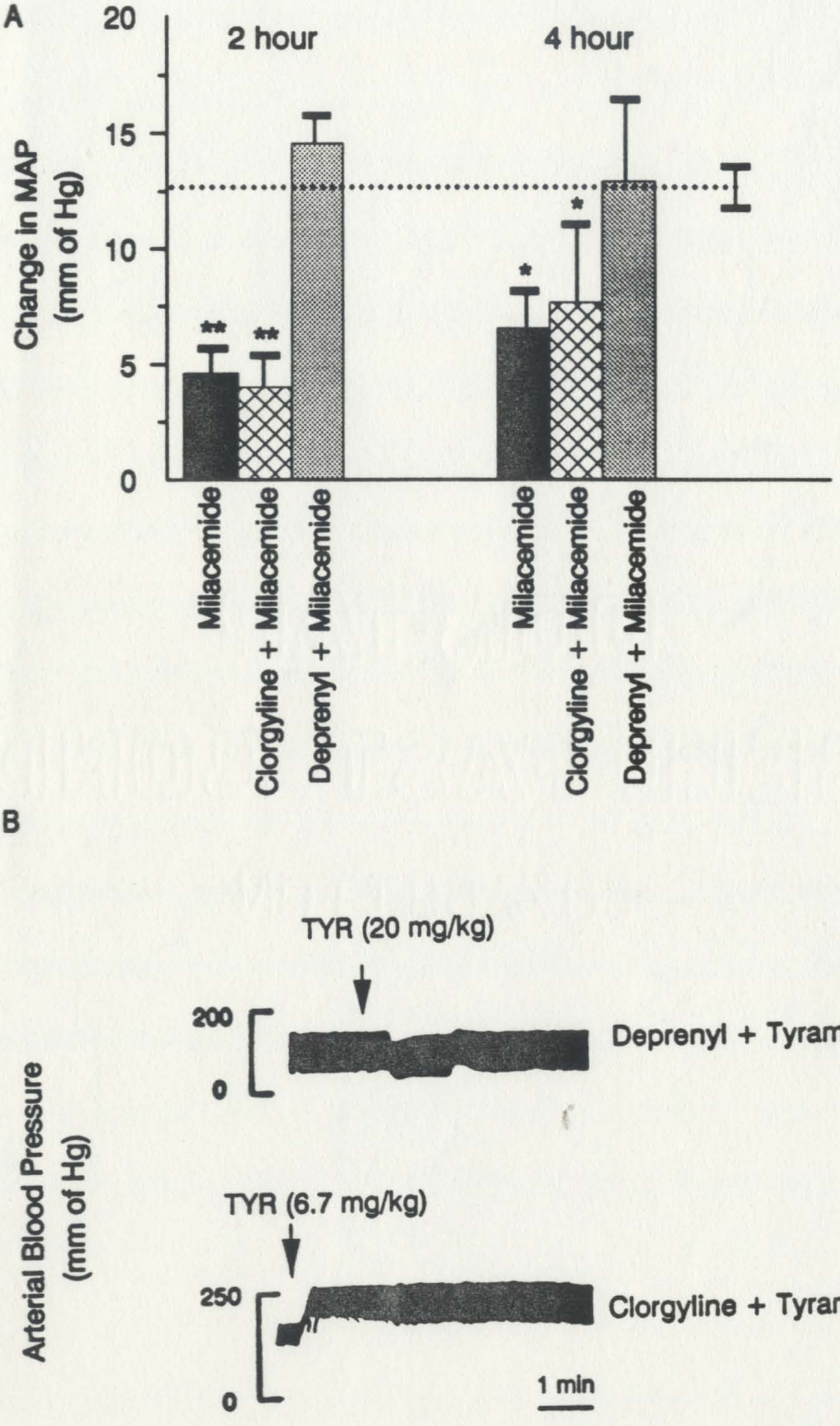
3.6.1 Effect of Clorgyline and L-Deprenyl on the Action of Milacemide

The effect of i.v. milacemide (600 mg/kg) on STR-allodynia was determined in a separate group of rats pretreated with either l-deprenyl (specific MAO-B inhibitor) or clorgyline (MAO-A inhibitor). Clorgyline had no effect on milacemide inhibition of STR-allodynia at either 2h or 4h based on HD-evoked changes in MAP (Figure 18A), HR and withdrawal duration (data not shown). In contrast, l-deprenyl significantly blocked the anti-allodynic effect of milacemide for up to 4 hours (Figure 18A). EEG synchrony was unaffected by any treatment, and clorgyline or deprenyl by themselves had no effect on STR-allodynia (data not shown). Tyramine (6.7 mg/kg, i.v.) injected at the end of the experiment, induced an immediate and sustained elevation in arterial blood pressure in clorgyline-treated rats (Figure 18B). Tyramine, up to 20 mg/kg i.v., had no effect on blood pressure in l-deprenyl-treated rats.

Figure 19. The suppression of strychnine(STR)-allodynia by intravenous milacemide is blocked by l-deprenyl, but not clorgyline.

A. Rats were pre-treated with clorgyline (10 mg/kg, i.p.) or l-deprenyl (3 mg/kg, i.p.) two hours before milacemide (600 mg/kg, i.v.). Hair deflection (HD)-evoked responses in the presence of i.t. STR (40 µg) were determined every hour for four hours after milacemide. Because of the short duration of allodynia (<30 min), STR was repeated every hour. The maximum evoked increase in mean arterial pressure (MAP), determined 2h and 4h after milacemide, is presented. The horizontal dotted line represents the maximum response (mean ± SEM) in STR-treated rats in absence of any other treatment (N=14). Each bar represents the mean ± SEM of 3-5 rats.(*p< 0.05, **p<0.01)

B. Tyramine (6.7 mg/kg, i.v.) triggered immediate and sustained increase in arterial blood pressure in clorgyline-treated rats. Even at three times the dose (20 mg/kg, i.v.) tyramine had no effect in rats pre-treated with l-deprenyl.


A
20
15
10
5
0
2 hour
4 hour
Change in MAP
(mm of Hg)
**
**
*
*
Milacemide
Clorgyline + Milacemide
Deprenyl + Milacemide
Milacemide
Clorgyline + Milacemide
Deprenyl + Milacemide
B
TYR (20 mg/kg)
200
0
Deprenyl + Tyramine
TYR (6.7 mg/kg)
250
0
Clorgyline + Tyramine
1 min
Arterial Blood Pressure
(mm of Hg)

4 Discussion

4.1 Strychnine-allodynia

Hair deflection, applied to the dermatomes of rats pretreated with i.t. STR, evoked physiological responses greater than those elicited by the chemical nociceptive agent, mustard oil (see section 4.2). All STR-dependent responses were: a) evoked (not spontaneous); b) observed without convulsions (no generalized disinhibition of spinal neurons); c) only elicited by light brushing of the hair at circumscribed sites corresponding to the spinal segments affected by i.t. STR and d) completely reversible. These data indicate that robust allodynia can be selectively induced with i.t. STR in animals whose somatosensory systems are otherwise normal. They are also consistent with previous studies of STR-allodynia (Beyer et al., 1985, 1988; Yaksh, 1989; Sherman and Loomis, 1994, 1995), many of which used phasic noxious stimuli for comparative purposes, demonstrating that normally innocuous mechanical stimulation acquires nociceptive characteristics during the pharmacological blockade of spinal glycine receptors. Indeed, there is growing evidence that low threshold afferent input activates central nociceptive pathways in the presence of i.t. STR (Milne et al., 1996; Sherman et al., 1996).

4.2 Effect of AP-7 on Strychnine-allodynia: role of NMDA receptors

STR-allodynia was dose-dependently inhibited by pretreatment with the

selective NMDA receptor antagonist, AP-7. This effect was not due to a general depression of cardiovascular reflexes as i.t. AP-7 did not alter baseline cardiovascular activity, nor did it inhibit the responses evoked by a phasic noxious pinch. The lack of an effect on cortical EEG synchrony, the i.t. route of administration and the correspondingly low ED_{50} values of AP-7 (0.4 - 1.7 µg) are all consistent with a spinal site of action in this study. Normally, spinal NMDA receptors play little or no role in the synaptic transmission of low-threshold (A-fiber) input (Urban et al., 1994). Rather, they are known to mediate the phenomenon of 'wind up' (e.g. the temporal summation of long latency discharges from afferent C-fibers and a characteristic feature of pain) in spinal neurons during repeated high-threshold (C-fiber) stimulation (Dickenson and Sullivan, 1987). Indeed, NMDA receptor antagonists have been shown to block 'wind up' *in vivo* without affecting the neuronal responses to peripheral A-fiber stimulation (Price et al., 1994). The observation that STR-allodynia is highly sensitive to spinal NMDA receptor blockade suggests that, in the presence of STR, low-threshold afferent input acquires access to a central sensitization mechanism normally restricted to repeated noxious stimulation.

That this sensitization process might be 'wind up' is supported by the temporal pattern of BP and HR responses evoked by tonic brushing of the hair in STR-treated rats. This consisted of a progressive increase in cardiovascular responses during the stimulus train which persisted after the stimulus was

discontinued. A similar pattern of HD-evoked neurochemical responses has been observed in the locus coeruleus of STR-treated rats using differential normal pulse voltammetry (Milne et al., 1996). This pattern of evoked responses was matched by the s.c. injection of mustard oil without STR. Mustard oil is a selective activator of C-nociceptors (Woolf and Wall, 1986) that triggers 'wind up' in the rat; an effect blocked by pretreatment with the NMDA antagonist, MK-801 (Woolf and Thompson, 1991). These results, and the recent report that rat dorsal horn neurons exhibit 'wind up' to repeated A-fiber activation following the iontophoretic application of STR (Wilcox et al., 1996), indicate that: a) normally innocuous mechanical stimuli undergo nociceptive-like processing in the spinal cord of the rat during glycine receptor blockade; and b) i.t. STR induces an abnormal pain state in experimental animals consistent with the IASP definition of allodynia.

The present dose-response data for i.t. AP-7 are comparable to those of other NMDA antagonists (MK-801 and AP-5) that inhibited tactile-evoked agitation in conscious STR-treated rats (~ ED_{60}s= 3 μg) (Yaksh, 1989), and in conscious mice pretreated with i.t. prostaglandin E_2 or $F_{2\alpha}$ (ED_{50} = 1.6 μg for AP-5, and 0.52 μg for MK-801; Minami et al., 1994). The similarity of these data, determined in lightly anesthetized and conscious rats, indicate that: a) the spinal pharmacology of STR-allodynia is unaffected by controlled urethane anesthesia; and b) experimental conditions mitigating many of the ethical issues arising from studies of neuropathic pain in animals can be used reliably in the STR-model.

Previous studies conducted in our laboratory showed that STR-allodynia is inhibited by i.t. γ-DGG and NBQX at doses that do not affect cardiovascular or motor reflexes, or the plane of anesthesia (Sherman and Loomis, 1994, 1996). The sensitivity of STR-allodynia to spinal NMDA and non-NMDA antagonists is in agreement with our current understanding of sensory transmission at the level of the spinal cord (Figure 20), and the hypothesis that allodynia arises from the exaggerated depolarization of spinal neurons in pain pathways by low-threshold afferent input. Hence, agents that negatively modulate such excessive excitatory drive should block STR-allodynia.

4.3 Effect of Mexiletine on Strychnine-allodynia and Normal Nociception

Mexiletine, an orally active congener of lidocaine, is the most commonly used local anesthetic for clinical allodynia (Hegarty and Portenoy, 1994) and should be effective in any valid model of allodynia. In the present study, mexiletine dose-dependently inhibited STR-allodynia and paw-pinch induced normal nociception (without STR) as determined in the same experimental animals. Importantly, mexiletine was equipotent in inhibiting the responses to normal and abnormal nociceptive input (ED_{50}'s = 9.1-17 mg/kg, i.v.). Below 30 mg/kg, this effect was achieved without a change in EEG synchrony (cortical activity reflecting the level of anesthesia) and without affecting motor efferent pathways; the motor withdrawal

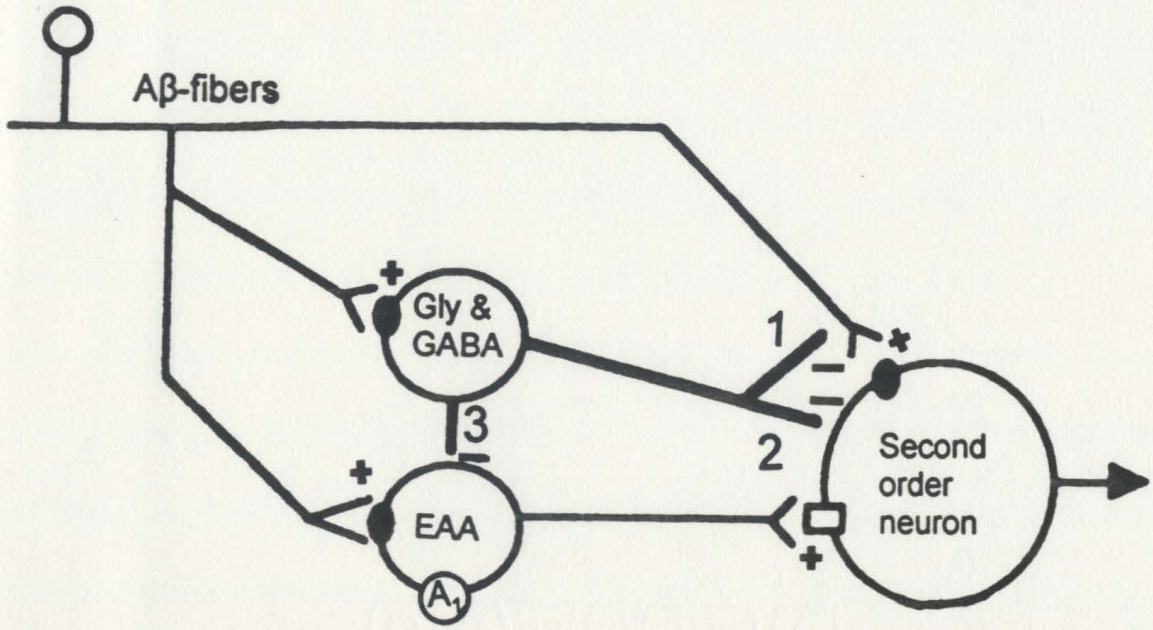


Figure 20. Schematic diagram illustrating the postulated role of excitatory amino acid (EAA)-receptor systems and neuromodulatory systems in the spinal processing of low-threshold (Aβ) afferent input. Glutamate, released from the central terminals of Aβ-fibers, combine with AMPA/kainate-receptors (closed ovals) on both second order neurons and interneurons in the dorsal horn. The latter include excitatory (EAA) and inhibitory [GABA and glycine (Gly)] substrates. Current evidence suggests that post-synaptic NMDA receptors (open square) do not lie adjacent to primary afferent terminals but rather to those of excitatory interneurons (Davies and Watkins, 1983; Evans and Long, 1989; Schouenborg and Sjolund, 1986; Morris, 1989). Thus, glutamate from EAA interneurons is thought to interact with NMDA receptors on the second order neuron (Davies and Watkins, 1983; Evans and Long, 1989; Headley and Grillner, 1990; Yoshimura and Nishi, 1995). The negative modulation of adenosine, GABA and Gly on EAA-mediated transmission is also indicated. For clarity, only the adenosine (A_1) receptor (open circle) is shown. Probable sources of adenosine include low-threshold primary afferents and interneurons (Nagy and Daddona, 1985; Sawynok and Sweeney, 1989) (not shown).

responses to calibrated paw-pinch were preserved in mexiletine-treated rats. At 30 mg/kg or above, motor withdrawal responses were almost absent and EEG synchrony was slightly but non-significantly increased, consistent with the report of sedation, reduced locomotor activity and a slowed righting reflex in conscious rats given a similar dose of mexiletine (Xu et al., 1992).

The mexiletine doses used in the present study are similar to those reported to inhibit allodynia-like symptoms after ischemic spinal cord injury in the rat (Xu et al., 1992). Spinal ischemia was induced photochemically by laser irradiation at mid-thoracic region immediately after the i.v. administration of Erythrocin B. Allodynia-like behaviours developed 6-8 months after irradiation and persisted for at least 4 months. Mexiletine (15 mg/kg and 30 mg/kg, i.p.) significantly inhibited allodynia in these rats for 45-120 min; a duration of action similar to that observed in the STR model. While a direct comparison of mexiletiine in injury vs non-injury (e.g. i.t. STR) models of neuropathic pain will require more extensive and detailed experiments, the present results suggest that chronic spinal cord injury yielding localized allodynia does not display enhanced sensitivity to the anti-allodynic effect of mexiletine when compared to the present pharmacological model. Strychnine-allodynia may be useful for estimating the dose-response relationships of local anesthetics in pathological central pain states, and for investigating their central mechanism(s) of action.

Systemic local anesthetics, in doses ranging from 1-25 mg/kg, do not block

conduction in injured or non-injured peripheral nerve fibers (Woolf and Wiesenfeld-Hallin, 1985; Chabal et al., 1989; Tanelian and MacIver, 1991). In the case of mexiletine, i.v. doses of 10-15 mg/kg yield peak serum/plasma concentrations of 1-5 µg/µl in the rat (Igwemezie et al., 1991, 1992). These are within the range reported for clinical analgesia and well below the concentration required to block electrical conduction of peripheral Aδ and C-fibers *in vitro* (>250 µg/ml for lidocaine) (Tanelian and MacIver, 1991). Pain in peripheral neuropathic conditions has been attributed to an abnormal afferent impulse barrage into the CNS from the trapped end of the injured peripheral nerves (neuromas) and from the axotomised sensory cell bodies in dorsal root ganglia (DRG). In turn, peripheral neuromas and their associated DRG's are known to be sensitive targets for local anesthetics in cases of experimental or clinical nerve injury (Wall and Devor, 1983; Chabal et al., 1989; Devor et al., 1992). For example, systemic local anesthetics suppress ectopic neuroma and DRG discharge without blocking initiation or propagation of impulses by electrical stimulation of sciatic nerve in rats with chronic sciatic nerve ligation (Wall and Devor, 1983; Chabal et al., 1989; Devor et al., 1992). However, these targets are not relevant to the STR model as no peripheral injury is induced. Thus, there is no obvious peripheral site(s) of action of mexiletine in STR-treated rats.

In contrast, sub-anesthetic doses of local anesthetics are known to: a) block conventional nociceptive transmission in the spinal cord of cats (DeJong et al., 1969; Dohi et al., 1979), b) depress a C-fiber mediated polysynaptic reflex in the

spinal cord of normal rats without blocking conduction in primary afferent fibers (Woolf and Wiesenfeld-hallin, 1985), c) block C-fiber mediated 'wind up' in rats (Fraser et al., 1992) and d) inhibit both direct and synaptically driven NMDA- and neurokinin-receptor mediated postsynaptic depolarization in the rat spinal neurons (Nagy and Woolf, 1996). In the present study, i.v. mexiletine blocked the responses of both noxious pinch and STR-allodynia with equal potency. These results suggest that mexiletine inhibits both normal and abnormal nociception at a common central site in the pain-signalling pathway. At the very least, a spinal site of action (e.g., spinal nociceptive neurons receiving convergent low- and high-threshold afferent fiber input) is likely given the locus of action of i.t. STR in this model. Such a site would explain the identical inhibitory potency and parallel dose-response curves of i.v. mexiletine against noxious paw-pinch and STR-allodynia. However, supra-spinal sites of action cannot be excluded. Electrophysiological experiments will be required to test this hypothesis.

In summary, i.v. mexiletine inhibits STR-allodynia in the anesthetized rat at doses that also block normal nociception. Sub-anesthetic doses of mexiletine can suppress centrally induced allodynia in experimental animals that are devoid of peripheral or central nerve injury. Mexiletine appears to have an important spinal site of action in abnormal pain states.

4.4 Effect of Adenosine Agonists on Strychnine-allodynia: role of A_1 receptors

The i.v. infusion of adenosine or the i.t. injection of adenosine analogue, LPIA (A_1-agonist) has been shown to alleviate spontaneous pain, allodynia and pin-prick hyperalgesia in cases of peripheral nerve injury (Sollevi et al., 1995; Karlsten and Gordh, 1995; Belfrage et al., 1995). Consistent with this effect, CPA (an A_1-selective agonist) and CGS (an A_2-selective agonist) dose-dependently inhibited STR-allodynia. However, the ED_{50} of i.t. CGS (2.7-3.1 µg) was approximately 50 times greater than that of CPA (0.02 - 0.07 µg) raising concerns about the selectivity of CGS at spinal A_1-receptors in the present study. This was confirmed by the ability of a fixed dose of DPCPX to block the anti-allodynic effect of both CPA and CGS. These data are in agreement with previous reports that the spinal analgesic action of adenosine is selectively mediated by A_1-receptors whereas the motor dysfunction induced by spinal adenosine is mediated by A_2-receptors (Fastbom et al., 1990; Karlesten et al., 1991; Poon and Sawynok, 1995; Lee and Yaksh, 1996). In this regard, it is important to note that motor withdrawal responses evoked by noxious paw-pinch (without STR) persisted after the i.t. injection of CGS ($\leq$ 5µg) (data not shown). These results indicate that CGS did not adversely affect the motor function in the STR-model.

The ED_{50}s of CPA in the present study are comparable in order of magnitude to those reported for RPIA and NECA in conscious STR-treated rats (ED_{50}s = 0.1

μg RPIA and NECA; Sosnowski and Yaksh, 1989) and for RPIA (ED_{50} = 0.2 μg) in rats with peripheral nerve injury (chronic ligation of L5 and L6 spinal nerves distal to DRGs; Kim and Chung model) (Lee and Yaksh, 1996). A similar ED_{50} of CGS (8 μg) was also reported in the spinal nerve ligation model (Lee and Yaksh, 1996). Unlike the present study however, the anti-allodynic effect of CGS in the Kim and Chung model, was not blocked by pretreatment with i.t. CPT (a selective A_1-receptor antagonist). There are several possible explanations for this difference. First, the dose of CGS used in the injury model was rather high (21 μg). Second, 10 μg of DPCPX was used in the present study compared to 3 μg of i.t. CPT. Alternatively, nerve injury may trigger the expression of A_2-receptors through which adenosine agonists exert their effects. This apparent difference in the pharmacology of the STR- versus the spinal nerve ligation model could have important implications for the investigation of adenosine agonist and needs to be clarified in future experiments.

The absence of a significant effect on cortical EEG synchrony and the low ED_{50} values of CPA and CGS, support a spinal site of action in the present study. Indeed the analgesic action of i.v. adenosine in patients with peripheral nerve injury appears to be mediated by central adenosine receptors as the activation of peripheral adenosine-receptors is known to result in painful symptoms (Belfrage et al., 1995). LPIA and NECA, given i.t. in doses ranging from 0.3 - 1 nmol (doses inhibiting STR-allodynia in conscious rats), also produced dose-dependent

antinociception in rats using the hot-plate and tail-flick tests (Sosnowski et al., 1989). Similar doses of i.t. adenosine analogs also inhibited the scratching and biting behaviour evoked by i.t. SP and NMDA; an effect antagonized by theophylline (Delander and Wahl, 1988). These results indicate that the inhibitory effect of adenosine is not restricted to abnormal pain states.

The mechanism(s) by which adenosine modulates sensory processing in the spinal cord is unclear. However, the sensitivity of experimental models of allodynia (both injury and non-injury) to i.t. EAA antagonists and adenosine A_1-agonists suggests a functional interaction between dorsal horn glutamate- and adenosine-systems. One possibility is that A_1-receptors, present on glutamate-containing interneurons, inhibit glutamate release and attenuate EAA-mediated synaptic transmission (see Figure 20). Indeed, recent electrophysiological recordings in spinal cord slices of the hamster have shown that a principal effect of adenosine is to inhibit interneuronal communication in the substantia gelatinosa (Li and Perl, 1994). Superfusion of adenosine (20-100 µg) resulted in the inhibition of all polysynaptic excitatory post-synaptic currents (EPSPs) evoked by dorsal root stimulation. Moreover, binding studies have shown that A_1- and A_2-receptors are located primarily on intrinsic neurons in the rat spinal cord (Choca et al., 1988). Although the identity of these neurons was not determined, they could include glutamate-containing interneurons, second-order neurons, or both. A second possibility is that A_1-receptors are present on second order neurons which suppress

glutamate-evoked depolarization following glutamate release from the central terminals of primary afferent fibers and/or excitatory interneurons. In either case, these postsynaptic mechanisms would counteract the exaggerated excitatory input from low threshold afferent fibers during spinal glycinergic disinhibition.

The pre-synaptic effects of adenosine are suggested by the reports demonstrating the inhibition of glutamate release *in vitro* using brain slice preparations from the hippocampus and dentate gyrus of the rat (Fastboom and Freedlom, 1985; Dolphine and Prestwich, 1985). This effect was abolished by 8-phenyltheophylline (10 μm) suggesting that A_1-receptors were involved. While a presynaptic effect on primary afferent terminals has been proposed (Li and Perl 1994), immunocytochemical studies failed to detect either A_1 or A_2 receptor-like immunoreactivity on these spinal substrates (Choca et al., 1988). Thus, current evidence supports a postsynaptic inhibitory effect of adenosine (Delander and Wahl 1988; Li and Perl, 1994), presumably on spinal substrates receiving low- and/or high-threshold afferent input. Regardless of the exact mechanism(s), A_1-receptors appear to be strategically located in the spinal cord to modulate somatosensory input.

4.5 Effect of GABA Agonists on Strychnine-allodynia: role of $GABA_A$ receptors

The results of the present study indicate STR-allodynia is selectively modulated by spinal $GABA_A$ receptors. Thus, the i.t. muscimol dose-dependently inhibited STR-allodynia in lightly anesthetized rats. Such an effect on STR-allodynia has not been shown previously but is consistent with reports of allodynia in rats and mice following i.t. bicuculline, a selective $GABA_A$ antagonist (Yaksh, 1989; Onaka et al., 1996; unpublished results). That this represented a selective spinal site of action is indicated by the lack of the effect on cortical EEG synchrony and the low ED_{50} values (0.4 - 0.5 μg). In contrast, i.t. baclofen failed to suppress the abnormal responses to HD in presence of STR, even at doses which produced pronounced motor dysfunction. These data are in agreement with a previous report demonstrating the lack of effect of baclofen against STR-allodynia in conscious rats (Yaksh, 1989) and electrophysiological data demonstrating the lack of effect of intra-arterial CGP 35348 (60 mg/kg) on the spontaneous activity of hamstring flexor α-motoneurons, touch evoked-responses or touch threshold in the spinal cord of the rat (Sivilotti and Woolf, 1994).

The present results are not surprising given the fact that a) $GABA_A$ and glycine receptors independently open chloride channels to hyperpolarize neurons (Bormann et al., 1987; Cohen et al., 1989); b) $GABA_A$ and glycine receptors are co-contained on the same postsynaptic membrane in the spinal dorsal horn (Todd et

al., 1996) consistent with the co-localization of glycine and GABA in the same presynaptic axons (Todd et al., 1996); and c) glycine immunoreactivity is virtually restricted to neurons that also exhibit GABA immunoreactivity in the laminae I-III of the dorsal horn (Todd, 1990; Mitchell et al., 1993). Glycine interneurons receive a major monosynaptic input from large diameter myelinated primary afferents in laminae II and III. These anatomical studies provide evidence for the neuronal circuitry by which glycine and GABA might normally modulate non-nociceptive transmission. They also suggest that GABA and glycine, co-released from the same interneurons might effect discrete but complementary inhibition of relevant spinal neurons. Indeed, electrophysiological studies using spinal cord slices from the rat and cat have shown that $GABA_A$- and glycine-mediated inhibition have a distinct time-course (Game and Lodge, 1975; Baba et al., 1994; Yoshimura and Nishi, 1995). The long and short IPSP, generated in substantia gelatinosa by stimulation of Aδ-fibers was blocked by bicuculline and STR, respectively (Yoshimura and Nishi, 1995). The bicuculline-sensitive IPSP was up to 3 times slower than that of STR-sensitive inhibition reflecting the different kinetics of $GABA_A$- and glycine-receptors, or a different time course of the neurotransmitter uptake. It has been suggested that the STR sensitive IPSP may be important in limiting the firing of the neurons and curtailing the amplitude and the ability of the EPSP to generate an action potential. In contrast, the bicuculline sensitive IPSP which develops more slowly and has a longer duration may be effective in preventing longer lasting

repetitive activation (Yoshimura and Nishi, 1995). While the exact role and significance of glycine- and GABA-inhibition remains to be determined, the apparently complementary effects of glycine and GABA on somatosensory input are consistent with the inhibition of STR-allodynia by i.t. $GABA_A$ agonists. Indeed, one would expect glycine or GABA (acting at $GABA_A$-receptors), to attenuate the exaggerated firing evoked by Aβ-input in presence of STR.

The role of $GABA_B$-receptors in the modulation of low-threshold afferent transmission is controversial. The failure of i.t. baclofen to inhibit STR-allodynia in the present experiment suggests that, in absence of nerve injury, spinal $GABA_B$-receptors do not contribute significantly to such modulation. However, i.p. baclofen (but not muscimol) inhibited touch-evoked allodynia following focal spinal cord ischemia in the rat (Hao et al., 1992); an effect attributed to the activation of $GABA_B$-receptors present on the spinal terminals of Aβ-fibers. Baclofen also reduced the hypersensitivity of spinal WDR neurons to low-intensity mechanical and electrical stimulation in rats with spinal cord ischemia (Hao et al., 1992). The ability of baclofen to effect such changes in this ischemic model may represent an up-regulation of $GABA_B$-receptors in response to central injury and/or altered synaptic connections. Although $GABA_B$ binding sites have been reported on the terminals of large diameter primary afferent fibers in lamina IV of normal rats (Price et al., 1987), $GABA_B$-receptors may not become functionally active until after central nerve injury. These data also suggest that the STR model is relevant for

investigating the somatosensory processing but may not reflect upon the altered pharmacology induced by nerve injury.

4.6 Effect of Milacemide on Strychnine-allodynia: role of glycine receptors

Systemic administration of the glycine prodrug, milacemide dose-dependently inhibited STR-allodynia for up to 4 hours. The time course of this effect corresponds to the increase in glycine concentration in the CSF of rats treated with milacemide (400 mg/kg, i.p.) (Semba et al., 1993). The peak inhibition of STR-allodynia and the maximum increase in $[glycine]_{CSF}$ were achieved 2-3 h after mialcemide administration. That the blockade of STR-allodynia was due to glycine and not milacemide itself was confirmed by the dependency of this effect on MAO-B. Thus, pre-treatment with l-deprenyl, but not clorgyline, significantly blocked the anti-allodynic effect of milacemide.

There is strong pharmacokinetic and biochemical evidence that serum milacemide rapidly enters and equilibrates in the CNS compartment where it is metabolized by MAO-B (Semba and Patsalos, 1993; Semba et al., 1993). In fact, over 90% of the milacemide dose is metabolized by MAO-B to glycinamide and finally glycine (Figure 3). The remaining 10% is metabolized by different routes depending on the species. In support of this metabolic route, and consistent with the results of the present study, pretreatment with l-deprenyl (2 mg/kg, i.p.)

inhibited: a) the conversion of milacemide to glycine *in vivo*; and b) the ability of milacemide to increase the seizure threshold induced by hyperbaric oxygen in rats (Yodium et al., 1988). Indeed, pretreatment with l-deprenyl (2 mg/kg, i.p.) almost completely blocked the formation of glycinamide, and increased milacemide accumulation in rat CSF (Semba et al., 1993). Pretreatment with clorgyline (5 mg/kg, i.p.) yielded only a moderate decrease in glycinamide concentration (Semba et al., 1993).

The ability of glycine, derived from milacemide through the action of MAO-B, to inhibit STR-allodynia is supported by previous work in our laboratory. Glycine or betaine (N,N,N-trimethyl glycine) injected directly into the spinal subarachnoid space of the rat, dose-dependently inhibited STR-allodynia (Sherman and Loomis, 1995). Moreover, i.v. milacemide had no significant effect on normal nociception (phasic noxious paw-pinch without STR) in the present study. The persistence of these noxious-evoked responses indicates that cardiovascular efferent fibers were unaffected by i.v. milacemide, and that the suppresion of cardiovascular responses to HD in the STR model represented a true anti-allodynic effect. These results also support the hypothesis that glycine is a selective modulator of non-noxious somatosensory input in the spinal cord of the rat.

In addition to the STR-sensitive glycine receptor, glycine is also known to bind to a STR-insensitive co-agonist site on the NMDA-receptor complex. In the case of the latter, glycine augments the excitatory effects of NMDA (Rosse et al.,

1991). Considering the sensitivity of STR-allodynia to NMDA antagonists (see section 4.2), the potentiation of NMDA-receptor mediated depolarization by glycine would be expected to worsen the allodynia. We observed no evidence of this in the present study, but subtle changes in NMDA neurotransmission are unlikely to be detected using cardiovascular and motor end-points. Such an outcome will require more sensitive electrophysiological techniques. Nevertheless, the primary effect of milacemide in STR-allodynia was inhibitory. In this regard, it has been suggested that the accumulation of unmetabolized milacemide following high-dose administration may antagonize the possible enhancement of NMDA-mediated transmission by glycine (Rosse et al., 1991).

Besides glycine, systemic milacemide (400 mg/kg, i.p.) is known to increase the concentration of serine and taurine in rat CSF by 20-25% and to decrease alanine concentration by an equal percentage (Semba and Patsalos, 1993). GABA and glutamine concentrations in the CSF were unchanged. The relevance of the decrease in alanine concentration, if any, is not yet known, but a significant increase in taurine and serine concentration could be of importance to the effect of milacemide in the STR model. Taurine, alanine, and serine are agonists at the STR-sensitive glycine receptor having the following order of potency: glycine >>β-alanine > taurine > > L-alanine > L-serine (Pan and Slaughter, 1995). Taurine is also shown to act on $GABA_A$ receptors in the cerebral cortex (Pan and Slaughter, 1995) but such effect has not yet been shown in the spinal cord. The comparatively

small increase in serine and taurine concentration (20-25% versus 75-80% for glycine), their lower affinity for the glycine receptor compared to glycine, and the lack of evidence for distinct taurine and serine receptors suggest that the reported changes in serine and taurine concentrations are unlikely to play a major role in the anti-allodynic effect of milacemide. Nevertheless, their contribution cannot be excluded at the present time.

Like l-deprenyl, milacemide is also a selective inhibitor of MAO-B, both *in vivo* and *in vitro* (de Varebeke et al., 1989; O'Brien et al., 1994). Ten hours after a single injection of milacemide (500 mg/kg, i.p.) to the rat, the MAO-B activity in the brain was reduced to approximately 40% of control. This activity was significantly but incompletely recovered (approximately 75% of control) 40 hours after milacemide administration. As a result of this effect on MAO-B, milacemide is self-limiting during long term therapy. In the present study, only one dose of milacemide was injected per animal to avoid the complications of residual enzyme inhibition and thus a decrease in glycine production with subsequent doses. The eventual inhibition of MAO-B by milacemide, and thus the possible progressive decrease in the yield of glycine, may explain the use of such high doses of the prodrug *in vivo* to achieve a pharmacological effect. Clearly, glycine prodrugs that lack such enzyme inhibitory activity would be preferable for chronic use. While the present study investigated the acute effects of milacemide in the STR model, these data indicate the effectiveness of a systemically administered glycine prodrug in

attenuating abnormal sensitivity to low-threshold afferent input arising in the spinal cord. To the extent that the loss of spinal glycinergic modulation might underlie clinical allodynia, this pharmacological approach may be worth investigating, particularly in patients who respond poorly to current therapy.

4.7 Future Directions

From an experimental point of view, the STR model affords some unique advantages. First, STR-allodynia is reversible, making it possible to test drugs under normal and abnormal (i.t. strychnine) nociceptive conditions in the same animal. Second, it is highly reproducible (with repeated STR injection) allowing for quantitative dose-response analysis (as indicated by the narrow 95% confidence intervals in Tables III-VI, VIII). Finally, it is centrally induced, of known cause, and achieved without injury to the peripheral or central nervous system, thereby allowing certain conclusions to be made about the site, and to a lesser extent, the mechanisms of drug action. However, the experiments that can be undertaken are limited by the short duration of allodynia (peak effect is approximately 5 min after STR injection; duration of 20-30 min). It is not possible to test the chronic effects of glycinergic dysfunction using this model or the long term effectiveness of pharmacological interventions known to inhibit STR-allodynia. Although the pharmacology of STR-allodynia mimics, qualitatively, that observed in humans, it

is not yet known whether the loss of glycinergic modulation underlies clinical allodynia. Therefore, further investigation is required to test this hypothesis. One possible way to induce a more prolonged yet reversible glycinergic dysfunction is to "knock out" spinal glycine receptors using antisense oligonucleotides. Such a model might prove useful to supplement and extend the information determined in the STR model.

The interaction between glycine and GABA in modulating low-threshold afferent input, and the consequences of simultaneous dysfunction of both inhibitory inputs in the development of allodynia have not yet been investigated. Given their known co-localization in spinal interneurons, a more realistic model of spinal disinhibition and allodynia must take into account, the complementary role played by these inhibitory amino acids. This could be achieved initially by co-administering i.t. GABA and glycine-antagonists. However, longer term spinal glycinergic and GABAergic dysfunction would require a different approach, possibly using appropriate antisense oligoneuclotides.

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 these receptors mediate long lasting depolarisation including the phenomenon of 'wind up'. Such augmentation appears to be an important spinal mechanism underlying the condition of allodynia and hyperalgesia (Yaksh and Malmberg, 1994). Moreover, interventions that counteract such exaggerated neurotransmission in the spinal cord should alleviate these sensory events and provide pain relief. The present research demonstrates that the abnormal responses evoked by input from low-threshold mechanoreceptive afferents in presence of spinal STR are indeed sensitive to NMDA-receptor blockade, to i.t. glycine-, GABA- and adenosine-agonists and to sub-anesthetic doses of mexiletine. The evidence obtained in this study supports the following conclusions:

1 Glycine is a selective modulator of non-noxious somatosensory input in the spinal cord of the rat.

2 In the presence of i.t. STR, low threshold afferent input accesses a spinal sensitisation mechanism normally activated by nociceptive fibers.

3 Mexiletine inhibits both abnormal (STR-allodynia) and normal nociception at a common site, most likely dorsal horn cells receiving convergent Aβ- and C-fiber input.

4 The recruitment of neuromodulatory systems involving spinal adenosine A_1 and $GABA_A$ receptors blocks the abnormal sensory responses of allodynia following glycinoceptor blockade in the spinal cord.

5 Increasing central glycine concentration by means of a systemic glycine

prodrug (milacemide) is effective in blocking STR-allodynia.

6 The spinal pharmacology of STR-allodynia is distinct from that of normal nociception, and is similar to that currently understood in clinical allodynia.

7 STR-allodynia is a useful, acute, non-injury model for the investigation of this abnormal pain state.

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