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PERIPHERAL REGULATION OF INFLAMMATORY PAIN AND NEUROPATHIC PAIN BY ADENOSINE

by

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Submitted in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

at

Dalhousie University

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Xue Jun Liu (author)

This thesis is dedicated to my parents and my grandparents.

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ABSTRACT

There is increasing evidence indicating that adenosine systems have the potential to be developed as a novel approach to the treatment of neuropathic and inflammatory pain. The present study investigated the involvement of the peripheral adenosine system in a neuropathic pain model and an inflammatory pain model using behavioral and microdialysis approaches. In *behavioral studies*, tight ligation of the rat spinal nerves L5/L6 induced thermal hyperalgesia and mechanical allodynia. Intraplantar injection of adenosine A_1 receptor agonists, an adenosine kinase inhibitor, 5'-amino-5'-deoxyadenosine (NH_2dAD) and an adenosine deaminase inhibitor, 2'-deoxycoformycin (DCF), into the *nerve injured paw*, but not the *contralateral* paw, reversed the thermal hyperalgesia, indicating a local, peripheral effect. The non-selective adenosine receptor antagonist, caffeine, inhibited antihyperalgesic effects, indicating an adenosine receptor mediated mechanism. A_1 receptor agonists, but not inhibitors of adenosine metabolism, produced overt paw edema involving mast cells and sensory afferents. *Microdialysis studies* were conducted in anaesthetized rats to determine subcutaneous adenosine levels in the rat hind paw and the mechanisms mediating the peripheral adenosine release in an inflammatory pain and a neuropathic pain model. Intraplantar injection of formalin, glutamate and capsaicin induced an increase in extracellular adenosine levels. Lower concentrations (0.5-2.5%) of formalin evoked a rapid-onset, short lasting release of adenosine, while at a high concentration (5%), the adenosine release lasted 60min with a much higher magnitude. Intraplantar injection of glutamate (0.03-100μmol) evoked a rapid adenosine release, which can be blocked by N-methyl-D-aspartate (NMDA) and non-NMDA receptor antagonists. In the neuropathic pain model, saline injection evoked a significant rapid-onset short lasting adenosine release in the ipsilateral paw in the nerve injured rats, but did not induce a release of adenosine in naïve rats or sham operated rats. Destroying nociceptive primary sensory afferents with systemic capsaicin pretreatment significantly inhibited adenosine release evoked by 1.5% formalin, the early 20min release evoked by 5% formalin, the glutamate-evoked adenosine release and the saline evoked adenosine release following nerve injury. Destroying sympathetic postganglionic nerves with 6-hydroxydopamine significantly inhibited the later phase of adenosine release evoked by 5% formalin, but did not have an effect in other circumstances. Thus, noxious chemical stimuli such as formalin and glutamate, as well as nerve injury, evoked adenosine release through a nociceptive sensory afferent dependent mechanism. The sympathetic nervous system only contributed to peripheral adenosine release in the circumstances where more pronounced inflammation and tissue damage occurred. Modulation of peripheral extracellular adenosine levels by inhibitors of adenosine metabolism was also studied. In the formalin model, NH_2dAD increased adenosine release by 0.5-1.5% formalin and DCF increased the release by 5% formalin. In the neuropathic pain model, both agents did not augment saline-evoked adenosine release. Amitriptyline, an antidepressant producing a peripheral antinociceptive effect through adenosine mechanisms, increased 1.5% formalin and nerve injury-induced adenosine release. In conclusion, noxious chemical stimuli and nerve injury evoke peripheral adenosine release, which can be modulated by inhibitors of adenosine metabolism and other agents to produce a peripheral antinociceptive effect.

LIST OF ABBREVIATIONS

ADO	Adenosine
ADP	Adenosine diphosphate
ADR	Adrenoreceptor
AMI	Amitriptyline
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-5-isoxazolepropionate
ANOVA	Analysis of variance
ASIC	Acid sensing ion channels
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
CAF	Caffeine
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction model
CCK	Cholecystokinin
CGRP	Calcitonin gene related peptide
CGS 21680	2-[*p*-(2-carboxyethyl)phenylethylamino]-5'-*N*-ethylcarboxamidoadenosine
CNQX	6-cyano-7-nitroquinoxaline
CNS	Central nervous system
contr	Contralateral paw
CPA	N^6-cyclopentyladenosine
CPT	8-cyclopentyl-1,3-dimethylxanthine
CPX	1,3-dipropyl-8-cyclopentylxanthine

CSC	8-(3-chlorostyryl)caffeine
DCF	2'-Deoxycoformycin
DMSO	Dimethyl sulphoxide
DRG	Dorsal root ganglion
EAA	Excitatory amino acid
FOR	Formalin
G	G protein
GABA	Gamma amino butyric acid
GDNF	Glial cell-line derived nerve growth factor
GFR	GDNF family receptor
GLU	Glutamate
H	Histamine
5-HT	5-Hydroxytryptamine
HPLC	High performance liquid chromatography
IB-MECA	N^6-(3-iodobenzyl)-5'-(N-methylcarbamoyl)adenosine
iGluR	Ionotropic glutamate receptor
KA	Kainatic Acid
IL	Interleukin
5'-IMP	5'-Inosine monophosphate
IN	Interneuron
IP_3	1,4,5-Inositol triphosphate
L-	Spinal cord dorsal horn lamina
i.p.	Intraperitoneal

L-PIA	L-N^6-phenylisopropyladenosine
ipsi	Ipsilateral
mGluR	Metabotropic glutamate receptor
min	minute
MK-801	Dizocipine maleate
MRS 1191	3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(+/-)-dihydropyridine-3,5-dicarboxylate
NA	Noradrenaline
NBI	Nitrobenzylthioinosin
NECA	5'-N-ethylcarboxamidoadenosine
NGF	Nerve growth factor
NH_2dAD	5'-amino-5'-deoxyadenosine
NKA	Neurokinin A
NMDA	*N*-methyl-*D*-aspartate
NO	Nitric oxide
NPY	Neuropeptide Y
NSAIDS	Nonsteroidal anti-inflammatory drugs
NT	Nucleoside transporter
6-OHDA	6-hydroxydopamine
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C

PNS	Peripheral nervous system
PSL	Partial sciatic nerve ligation model
PWL	Paw withdrawal latency
PWT	Paw withdrawal threshold
SAL	Saline
SAH	S-adenosyl homocysteine
sec	Second
SEM	Standard error of the mean
s.c.	Subcutaneous
SNK	Student-Newman-Keuls test
SNL	Spinal nerve ligation
SNS	Sensory neuron specific sodium channel
SP	Substance P
SPGN	Sympathetic postganglionic neuron
TNF	Tumor necrosis factor
TrK	Tyrosine kinase receptor
TTX	Tetrodotoxin
TTXr/s	Tetrodotoxin resistant /(or sensitive) sodium channel
VGCC	Voltage-gated calcium channels
VGSC	Voltage-gated sodium channels
VIP	Vasoactive intestinal peptide
VR1	Vanilloid receptor 1
WDR	Wide-dynamic range neurons

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CHAPTER 1

GENERAL INTRODUCTION

The overall objective of the present thesis is to examine the **peripheral regulation** of **inflammatory pain** and **neuropathic pain** by **adenosine mechanisms** using behavioral and microdialysis techniques. A general background of peripheral pain transmission, peripheral sensitization, the formation and metabolism of adenosine, and the regulation of adenosine in pain and inflammation will be summarized in this chapter. Subsequent specific chapters focus on the particular block of experiments undertaken to address specific issues.

1.1 GENERAL INTRODUCTION TO PAIN

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage. Under normal circumstances, an acute noxious stimulus, such as heat, activates the nociceptor to conduct the sensory input into the spinal cord. Postsynaptic projection neurons in the dorsal horn are then activated to transmit signals to the cortex and subcortex areas, via a relay in the thalamus, to elicit pain. Reflex withdrawal responses, as well as emotional, autonomic and neurohumoral responses can be elicited by the noxious stimuli to terminate the contact with the noxious stimuli as well as to help the body fight with the unfavorable condition (Devor, 1994; Millan, 1999; Woolf and Salter, 2000). Pain can be divided into transient, acute, persistent and chronic, depending on the nature of pathological changes and duration (Loeser and Melzack, 1999). Transient or physiological pain is elicited in the absence of any tissue damage, lasts for seconds, and is terminated after disconnection with the stimuli. Acute or persistent pain is elicited by substantial soft tissue injury and inflammation, lasts hours to days, and remits with tissue healing. Chronic pain is initiated by acute pain, but

persists long after healing of the original tissue damage or associated with prolonged, recurring tissue injury (Loeser and Melzack, 1999; Millan, 1999; Woolf and Salter, 2000).

Inflammatory pain and neurogenic pain are two commonly observed pain states. Peripheral tissue injury or the presence of foreign substance initiates inflammation, a complex of responses aimed at removing the damaged tissue and the foreign substance, as well as promoting healing. Inflammatory pain is usually manageable by nonsteroidal anti-inflammatory drugs (NSAIDs) and opioids, and generally resolves after healing (Levine and Taiwo, 1994; Woolf and Doubell, 1994; Millan, 1999). Neuropathic pain, on the other hand, is initiated by injury of the peripheral or central nervous system. It is maladaptive, offering no physiological function. It has poor response to ordinary analgesics, such as NSAIDs or opioids, but is manageable by antidepressants and anticonvulsants (adjuvants), which can have limited efficacy and considerable side effects. Neuropathic pain is usually chronic, lasting years after the original injury has healed (Woolf and Doubell, 1994; Millan, 1999; Woolf and Mannion, 1999).

1.2 PRIMARY SENSORY AFFERENTS AS THE INITIATORS AND TRANSDUCERS OF PAIN SIGNALING

To understand the mechanisms underlying the sensitization occurring in both inflammatory pain and neuropathic pain, it is necessary to first understand the sensory transduction under physiological conditions. Activation of nociceptors occurs via a number of specialized transducers, channels and receptors located on the peripheral terminals of primary sensory afferents (Woolf and Costigan, 1999), with the initial step of generating action potentials in sensory afferents. Voltage-gated Na^+ channels and K^+

channels play a key role in determining the excitability of sensory neurons, and mediate conduction while transferring the signals from nociceptors to their central terminals in the spinal cord.

1.2.1 Characteristics of primary sensory afferents

Nociceptors are specialized receptors located on the peripheral terminal of sensory afferents. According to differences in myelination, there are three major types of sensory afferents (Table 1.1). The large diameter, fast conducting, myelinated Aβ fibers are activated by low threshold mechanical stimuli to convey sensation such as touch and stretch. Under normal conditions, activation of Aβ fibers does not induce pain, but under some pathological conditions, such as after nerve injury, an innocuous stimulus such as a gentle touch can evoke pain (allodynia) through Aβ fibers. The thin myelinated, small diameter Aδ fibers and unmyelinated, small diameter C fibers are nociceptive sensory afferents. Some distinct characteristics of nociceptive sensory afferents distinguish them from low threshold non-nociceptive Aβ fibers, such as the characteristic expression of some neuropeptides, ion channels and receptors (Table 1.1). C fibers are further divided into two subgroups. One group is nerve growth factor (NGF)-dependent, and expressing SP and CGRP, as well as the NGF receptor tyrosine kinase A (Trk A), with central terminals projecting to lamina I the outer part of lamina II (LIIo). The second group is glial cell-line-derived nerve growth factor (GDNF)-dependent, does not express SP and CGRP, but expresses surface c-galactosyl epitopes and binding sites for lectin IB4 as well as the ATP receptor $P2X_3$, with central terminals projecting to the inner part of lamina II (LIIi). SP-positive and IB4-positive C fibers may be differentially involved in chronic inflammatory pain and neuropathic pain, respectively (Basbaum, 1999).

Table 1.1: Physiological properties of primary sensory afferents

Fiber		Receptor	Transmitter	DH	Post-synaptic neurons	Velocity (V) */Diameter (D)*	Sensation
C	**NGF**	**TrKA** $NK_{1,2}$ CGRP	**SP** **CGRP** EAAs ATP BDNF NKA	**LII_o** LI	EXIN ININ WDR NS	V:0.5-2.0 m/sec *D: 0.4-1.2 μm*	**Noxious** mechanical heat chemical cold
	GDNF	**P2X 3** **GFR** **IB4**	EAAs ATP	**LII_i**			
Aδ		$NK_{1,2}$ CGRP	*CGRP* EAAs SP	LI LII_o LV	EXIN ININ WDR NS	V: 12-30 m/sec *D: 2-6 μm*	**Noxious** mechanical heat
Aβ		TTXs AMPA VGCC iGlu mGlu	EAAs	LIII-VI	ININ WDR no-NS	V: 30-100 m/sec *D: 12-30 μm*	**Innocuous** Mechanical

Abbreviations are explained in List of Abbreviations.

Summarized from: Lawson, 1996; Besson, 1999; Basbaum, 1999; Millan, 1999. Woolf and Costigan, 1999.

1.2.2 Ion channels located on peripheral terminals of sensory afferents

Certain cation channels and receptors are important in pain transmission. The capsaicin-gated *vanilloid receptor (VR1)* is selectively expressed by both SP-positive and IB4-positive C fibers, as well as some Aδ fibers (Tominaga, et al., 1998; Caterina and Julius, 1999;). *VR1* is a non-selective cation channel that can be activated by capsaicin and its analogs, heat (>43°C) and acidification, to produce nociceptive sensation (Tominaga et al., 1998; Caterina and Julius, 1999). *Voltage gated sodium channels (VGSC)* produce the inward membrane current necessary for generating action potentials. There are at least six Na^+ channels expressed in primary sensory dorsal root ganglia (DRG) neurons. According to whether or not they are blocked by tetrodotoxin (TTX), they are termed TTX sensitive (TTX-s) or TTX resistant (TTX-r) Na^+ channels. There are two subtypes of TTX-r Na^+ channels, sensory neuron specific (SNS)/PN3 and SNS-2/NaN, selectively expressed in small diameter DRG sensory neurons and peripheral terminals of sensory afferents. Modulation of TTX-r Na^+ channels is important in generating peripheral sensitization, and abnormal expression and distribution of *VGSC* in injured afferents are important in generating ectopic discharge (Gold, 1999; Waxman et al., 1999). *Acid-sensitive ion channels (ASICs)* are gated by protons produced by tissue acidification in inflammation and ischemia (Waldmann et al., 1997). Five subunits of ASICs are expressed, both in large and small diameter sensory afferents, and different combinations of subunits may confer diverse proton-response properties on nociceptor subtypes (Caterina and Julius, 1999). *ATP-gated ion channels* are also involved in peripheral nociceptive transmission, and will be discussed in Section 1.7.3.

1.2.3 Spinal transduction of pain and central sensitization

The present study is focused on peripheral mechanisms of pain transmission. General mechanisms of spinal pain transmission and sensitization will be summarized here and will not be discussed further.

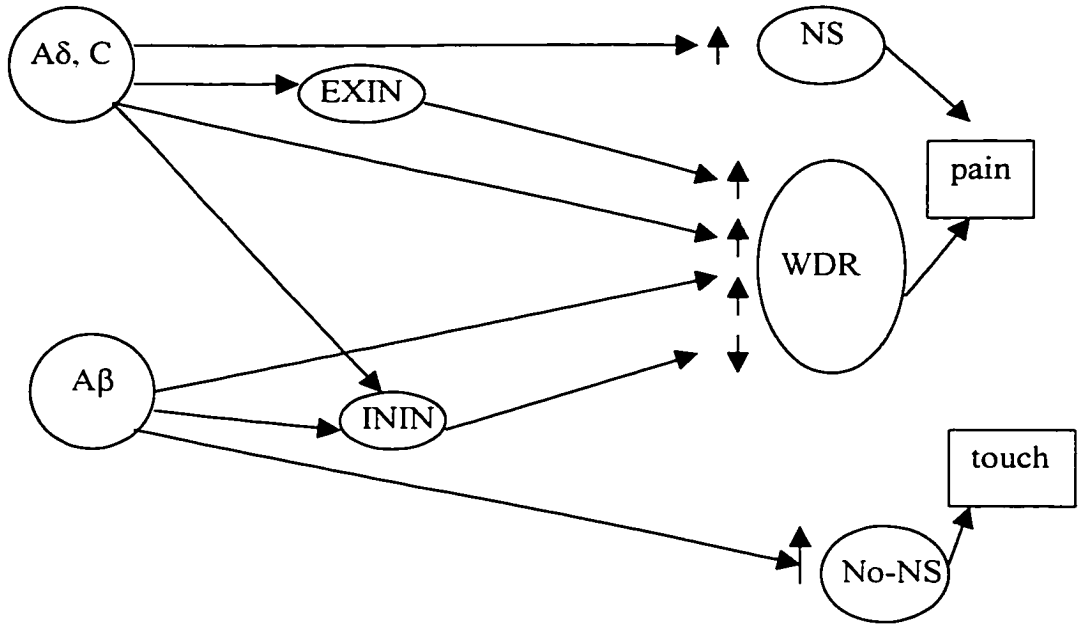
Both Aδ and C fibers primarily project into the superficial laminae (LI and LII, substantia gelatinosa) of the spinal cord dorsal horn while Aβ fibers project into deeper laminae (LIII-VI) (Table 1.1, Fig. 1.1A). Wide-dynamic range (WDR) neurons, predominantly located in LV, are the major secondary neurons and can be activated by a wide range of stimuli, such as thermal, mechanical and chemical (Millan, 1999). Both nociceptive Aδ and C fibers and non-nociceptive Aβ fibers can *directly* activate WDR neurons. Aδ and C fibers also activate *nociceptive specific* projective neurons to convey nociceptive input, while Aβ fibers activate *non-nociceptive* projective neurons to convey touch sensation. Nociceptive and non-nociceptive sensory afferents also activates different *interneurons*. Aδ and C fibers activate *excitatory interneurons*, which subsequently ***activate*** WDR, while Aβ fibers, via activating *inhibitory interneurons*, indirectly ***inhibit*** WDR. Thus, activation of Aβ fibers can ***inhibit*** nociceptive input from Aδ and C fibers by inhibiting WDR projective neurons (Gate control theory, Melzack and Wall, 1965). Following nerve injury, there is a disinhibition in spinal pain transmission. Thus, there is a selective reduction of inhibitory receptors, γ-amino butyric acid (GABA) and glycine, on the central terminal of primary sensory afferents; therefore, the inhibitory effect produced by Aβ activation is decreased (Fig. 1.1. B). Nerve injury also induces sprouting of Aβ fibers into the superficial lamina of dorsal horn and enables the synaptic connection of Aβ fibers with the *excitatory interneuron* and *nociceptive specific*

projective neurons; therefore, the innocuous stimuli are transmitted as noxious stimuli. (Woolf and Doubell, 1994; Baranauskas and Nistri, 1998; Millan, 1999).

Besides disinhibition, an increased *excitation* also occurs at spinal levels in neuropathic pain and inflammatory pain (Woolf and Costigan, 1999; Woolf and Mannion, 1999; Woolf and Salter, 2000). Under physiological conditions, the release of glutamate (Glu) from the central terminal of sensory afferents, and the activation of α-amino-3-hydroxy-5-methyl-5-isoxazolepropionate (AMPA) receptors located on secondary dorsal horn neurons is a key step in spinal pain transmission (Woolf and Costigan, 1999; Woolf and Salter, 2000). Intensive activation of C fibers by nerve injury or inflammation leads to massive release of glutamate, SP, brain-derived neurotrophic factor (BDNF), which will lead to cumulative depolarization in secondary dorsal horn neurons and the subsequent activation of *N*-methyl-*D*-aspartate (NMDA) receptors, which induces sensitization of dorsal horn neurons. The sensitized secondary neurons exhibit an enlarged receptive field, an increased response to suprathreshold input and a decreased threshold, which mediate referred pain, hyperalgesia and allodynia, respectively (Woolf and Costigan, 1999; Woolf and Salter, 2000).

Figure 1.1: Transmission of primary sensory afferents in spinal cord dorsal horn and its modification following nerve injury. A: Transmission under physiological conditions. B: Transmission after peripheral nerve injury. ↑: activating; ↓:inhibiting; EXIN: excitatory interneuron; ININ: inhibitory interneuron; NS: nociceptive specific projective neuron; WDR: wide-dynamic range projective neuron; No-NS, non-nociceptive projective neuron. Modified from Millan (1999).

A:


Aδ, C
EXIN
NS
pain
WDR
Aβ
ININ
touch
No-NS

B:

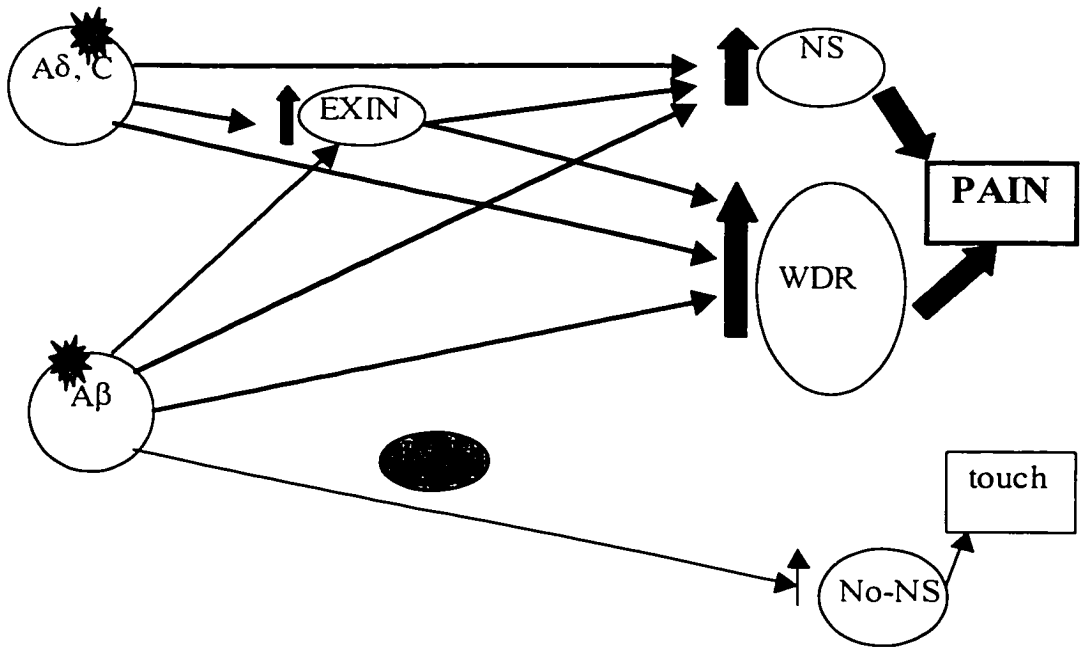

Aδ, C
EXIN
NS
PAIN
WDR
Aβ
touch
No-NS

Fig. 1.1

1.3 NEUROGENIC AND NON-NEUROGENIC INFLAMMATION

Tissue injury and associated inflammation evoke the release of a broad range of inflammatory mediators, neurotransmitters and neuromodulators, including bradykinin, cations (K^+ and H^+), amines (histamine and 5-hydroxytryptamine, 5-HT), prostaglandins (PGs), cytokines (interleukins, IL-1, IL-6; tumor necrosis factor, TNFα), excitatory amino acids (EAAs), nitric oxide (NO), NGF, SP, CGRP, noradrenaline and purines (adenosine triphosphate, ATP and adenosine). These substances are derived from various sources such as damaged tissues, neutrophils, macrophages, endothelial cells, mast cells and nerve terminals (Levine and Taiwo, 1994; Millan, 1999; Woolf and Costigan, 1999). Numerous inflammatory agents produce nociceptive and proinflammatory effects with highly synergistic reciprocal interactions (Millan, 1999).

In addition to pain, erythema and edema are two further salient features of the inflammatory response. The former reflects vasodilation and increased blood flow, while the latter results from increased vascular permeability and consequent plasma extravasation. Primary sensory afferents, sympathetic postganglionic nerves (SPGNs) and mast cells contribute to the inflammatory response with distinct characteristics (Arvier et al., 1977; Coderre et al., 1989).

1.3.1 Primary sensory afferents and neurogenic inflammation

Noxious stimuli activate nociceptors and generate *orthodromic* input to the dorsal horn. However, primary sensory afferents also transmit *antidromic* impulses, propagated via the invasion of collateral fibers (axon reflex), which lead to peripheral release of neuropeptides and neurotransmitters such as SP, CGRP, neurokinin A (NKA), EAAs,

NO, ATP and adenosine. The liberation of these substances from sensory afferents is called the *efferent* function of sensory afferents. These neurogenic agents, on one hand, produce further positive feedback sensitization on primary sensory afferents, and, on the other hand, activate receptors on blood vessels, mast cells and neutrophils to modulate local inflammatory responses (neurogenic inflammation) (Szolcsanyi, 1988). SP and CGRP are the predominant substances modulating neurogenic inflammation. CGRP causes prolonged vasodilation and enhances the vasodilation effect of histamine, PGs and cytokines. SP increases plasma extravasation, and its effect is enhanced by CGRP (Maggi, 1995).

1.3.2 Mast cell degranulation and inflammation

Mast cells have been suggested to provide communication between sensory afferents and immune cells (Suzuki et al., 1999). SP, released from sensory terminals activates neuronkinin receptors (NK_1) and promotes mast cell degranulation (Arvier et al., 1977; Coderre et al; 1989; Tausk and Undem, 1995). Upon activation, mast cells release preformed substances such as histamine, 5-HT, purines, tryptase and NGF, and begin to synthesize and secrete platelet-activating factor, PGs and cytokines (e.g. TNFα, IL-6, IL-8). These agents may act on blood vessels to produce vasodilation and plasma extravasation, and act on sensory afferents to produce hyperalgesia. A very high concentration of SP (>10 μM) is required to elicit mast cell degranulation (Tausk and Undem, 1995), and the amount of SP released by neurogenic inflammation is not necessarily high enough to cause this response (Schmelz et al, 1997; 1999; Tausk and Undem, 1995). Multiple substances, besides SP, can also trigger mast cell

degranulation. For example, adenosine A_{2B} and A_3 receptors are expressed on mast cells and activation of these receptors can trigger mast cell degranulation (Sullivan and Linden, 1998).

1.3.3 Sympathetic postganglionic neurons and inflammation

Several proinflammatory substances induce plasma extravasation via SPGNs (Jänig et al., 1996). Thus, bradykinin produces pronounced plasma extravasation in the synovial joint, and this can be largely blocked by sympathectomy (Coderre et al., 1989; Miao et al., 1996). The acute infusion of 6-hydroxydopamine (6-OHDA), which acts selectively on SPGN terminals to deplete noradrenaline and other neuromodulators, produces plasma extravasation of a similar magnitude to bradykinin (Coderre et al., 1989; Green et al., 1993a). The plasma extravasation produced by both 6-OHDA and bradykinin is largely blocked by indomethacin (Coderre et al., 1989), suggesting the involvement of PGs in plasma extravasation by these agents. Not all contents of SPGNs produce or augment plasma extravasation. Thus, noradrenaline and neuropeptide Y (NPY) decrease the plasma extravasation effect (Green et al., 1993b), as does electrical stimulation of SPGNs (Miao et al., 1996). Purines (ATP and adenosine) are released as co-transmitters along with noradrenaline and NPY following depolarization of SPGNs (Racchi et al., 1999), and contribute to vasodilation via P2Y and A_{2A} receptors, respectively (Green et al., 1993 a; 1993b). Furthermore, ATP can indirectly contribute to plasma extravasation by stimulating synthesis of PGs (Needleman et al., 1974). However, PGs are synthesized *de novo* in SPGNs and the release of PGs is independent of SPGN depolarization and vesicular release of neurotransmitters (Sherbourne et al.,

1992, Miao et al., 1996). Although SPGN-mediated inflammation is quite different from the neurogenic inflammation induced by SP and CGRP, following small diameter sensory afferent depolarization (Coderre et al., 1989), they are linked together by prostaglandins. Indeed, nanomolar concentrations of either PGE_2 or PGI_2 significantly enhances SP and CGRP release from sensory neurons via the cyclic adenosine monophosphate (cAMP) transduction cascade (Hingtgen et al., 1995).

1.4 PERIPHERAL SENSITIZATION FOLLOWING INFLAMMATION

Inflammatory pain and neuropathic pain induce sensitization, observed as (1) being pain independent from external stimuli (spontaneous pain) or long lasting pain after a brief stimulus (on-going pain), (2) being an exacerbated response to a mild noxious stimulus (hyperalgesia) or innocuous stimulus (allodynia). Generally speaking, sensitization means a reduction in the threshold for activation, and an increase in the response to a given stimulus or the appearance of abnormal activity. The molecular mechanisms involved in sensitization include modulation and modification. *Modulation* represents fast, reversible, posttranslational changes induced in receptors/channels to increase the excitability of primary sensory neurons. *Modification* represents late onset, long-lasting changes in expression of transmitters/channels/receptors, the structure/connection changes and changes in specific neuron populations in the system (Coderre and Katz, 1997; Loeser and Melzack, 1999; Millan, 1999; Woolf and Costigan, 1999; Woolf and Salter, 2000).

1.4.1 Peripheral sensitization by modulation of ion channels

Some inflammatory agents, such as protons, ATP and glutamate as well as noxious heat, can directly act on their ionotropic receptors or transducers (ASIC, P2X, iGlu, VR1) to increase the excitability of sensory neurons or generate action potentials. For metabotropic receptors, most of them produce sensitization by phosphorylation of membrane bound receptors and ion channels. Many inflammatory agents, via activation of G protein-coupled or tyrosine kinase membrane-bound receptors, activate protein kinase A (PKA) or protein kinase C (PKC). These kinases phosphorylate ion channels and modulate their function (Millan 1999; Woolf and Costigan, 1999; Woolf and Salter, 2000). Thus, activation of PGE_2, CGRP and adenosine A_{2A} receptors stimulates adenyl cyclase to increase cAMP, and triggers PKA-mediated phosphorylation of K^+ channels, thereby decreasing K^+ conductance. On the other hand, activation of bradykinin B_2, NK_1 and histamine H_1 receptors increases phospholipase C (PLC) and diacylglyceral which, together with intracellular calcium Ca^{2+}, activate PKC, leading to phosphorylation and increased activity of TTX-r Na+ channels and voltage gated calcium channels (VGCC) (Millan, 1999; Reichling and Levine, 1999; Woolf and Costigan, 1999). Modulation of ion channels increases the excitability of primary sensory neurons.

Besides the phosphorylation of ion channels, an activation-dependent sensitization of transducers also contributes to peripheral sensitization. For example, repeated heat stimuli can sensitize VR1 receptors (Millan, 1999; Reichling and Levine, 1999; Woolf and Costigan, 1999). Recruitment of silent nociceptors also contributes to

peripheral hyperalgesia. About 10% of nociceptors are normally unresponsive to acute noxious stimuli but can be sensitized and activated in inflammation (Millan, 1999).

1.4.2 Inflammation-induced phenotype changes

The increase in intracellular Ca^{2+} (via both PKA and PKC pathways), together with the activation of TrkA by NGF, plays a key role in the *modification* of sensory neuron excitability by increasing the expression of certain receptors/effectors (Table 1.2). Thus, inflammation induces the increased expression of VR1 transducers (Tominaga et al., 1998), TTX-r Na^+ channels (eg. SNS/PN3, SNS-2/NaN) (Gold, 1999; Waxman et al., 1999), SP and CGRP (Woolf et al., 1994; Galeazza et al., 1995) and BDNF (Mannion et al., 1999) in small diameter DRG neurons. These entities are transported to both ends of the sensory afferent to participate in peripheral and central sensitization.

Inflammatory agents also induce phenotype changes in myelinated Aβ fibers. Thus, some ligands usually selectively expressed in SP-positive C fibers in normal circumstances are now expressed in large diameter Aβ fibers. Inflammation-induced phenotype changes include the novel expression of SP (Neumann et al., 1996) and BDNF (Mannion et al., 1999) in Aβ fibers. This neurochemical transformation renders Aβ fibers with an electrophysiology resembling that of C fibers, in that an innocuous stimulus will now produce pain (allodynia) in inflamed tissues (Neumann et al., 1996; Mannion et al., 1999).

1.4.3 SPGNs and inflammatory pain

Axons of SPGNs reach the spinal nerves via grey rami. Most of these fibers project distally to peripheral tissues and a few others project proximally to the DRG neurons and spinal roots. Within the DRGs, SPGN terminals are normally found along blood vessels but rarely amongst the cell bodies of afferent neurons (Kummer et al., 1990). Normally, there is no functional communication between SPGN and primary sensory afferents, and stimulation of SPGNs do not produce excitation or sensitization of DRG sensory neurons and axons. However, under tissue inflammation or nerve injury (Section 1.5.3), there is an abnormal coupling between SPGNs and sensory afferents. Thus, stimulation of SPGNs can increase pain, and sympathetic block can decrease pain, in numerous inflammatory or neuropathic pain models (Jänig et al., 1996; Perl, 1999; Michaelis, 2000).

In inflammation, stimulation of SPGNs significantly potentiates the hyperalgesic effect produced by proinflammatory agents (Jänig et al., 1996). It has been demonstrated that SPGNs are involved in the pro-inflammatory and nociceptive effects produced by 5-HT (Pierce et al., 1995), bradykinin (Gonzales et al., 1989), capsaicin (Kinnman and Levine, 1995), complete Freund's adjuvant (Woolf et al, 1996), formalin (Coderre et al., 1984a,b; Fuchs et al., 1999; Hong and Abbott, 1996) and NGF (Andreev et al., 1995; Woolf et al., 1996). Different adrenoreceptor subtypes are involved in SPGN mediated hyperalgesic effects. For example, α_2-adrenoreceptor antagonists inhibit the nociceptive effect produced by noradrenaline (Levine et al., 1986, Gonzales et al., 1991), while an α_{1A}- adrenoreceptor antagonist inhibits formalin and capsaicin induced pain behaviors (Kinnman and Levine, 1995; Hong and Abbott, 1996). Because sympathectomy, and

inhibition of PGs synthesis blocks the sensitizing effect of noradrenaline (Levine et al., 1984), an *indirect* sympathetic-sensory coupling mediated by PGs accounts for the sensitizing effect of catecholamines following inflammation (Jänig et al., 1996). Besides PGs, other neurotransmitters (modulators), such as noradrenaline, ATP, adenosine and NPY may also be involved in SPGN-dependent sensitization. Thus, ATP and adenosine can activate $P2X_3$ and adenosine A_{2A} receptors located on sensory nerve terminals to produce nociceptive effects (Section 1.7).

1.5 PERIPHERAL SENSITIZATION FOLLOWING NERVE INJURY

1.5.1 Experimental models for neuropathic pain

Different animal models have been developed to represent pain induced by complete or partial injury of the sciatic nerves. The sciatic nerve is supplied by the L4, L5 and L6 spinal nerves, while L3 contributes to the innervation of the hind paw by the saphenous nerve (Baron et al., 1988). Sciatic nerve transection (axotomy) is the most commonly used model involving *complete* injury of the sciatic nerve (Kauppila, 1998). The most commonly used *partial* nerve injury models include the chronic constriction injury model (CCI model), which consists of putting four ligatures loosely around the sciatic nerve (Bennett and Xie, 1988), the partial sciatic nerve ligation model (PSL), which consists of tight ligation of part of the sciatic nerve (Seltzer et al., 1990), and the spinal nerve ligation model (SNL) or modified SNL, which consists of tight ligation of the L5 and L6 spinal nerves or transection of the L5 nerve before it joins into the sciatic nerve (Kim and Chung, 1992; Ringkamp et al., 1999a; 1999b; Fig. 5.1). In all partial injury models, a certain degree of ongoing pain, hyperalgesia and allodynia develops as a

result of the injury, but with different characteristics. Thus, the SNL model exhibits the strongest mechanical allodynia and sympathetic dependence on this symptom, while the CCI model exhibits the strongest ongoing pain, but mild mechanical allodynia or sympathetic dependency (Kim et al., 1997).

1.5.2 Nerve-injury induced peripheral sensitization

Peripheral mechanisms mediating nerve injury-induced sensitization include (1) the development of ectopic activities in both damaged and neighboring afferents, (2) changes in the distribution and electrical characteristics of Na^+ channels, (3) local inflammatory responses due to Wallerian degeneration, (4) phenotype changes and structure reorganizations (Coderre and Katz, 1997; Baron 2000; Millan, 1999).

Ectopic discharge and spontaneous activity of sensory afferents is an important generator of neuropathic pain (Millan 1999; Baron 2000). Clinical studies indicate that ongoing ectopic activities, in primary sensory afferents, contribute to the initiation of central sensitization and the maintenance of neuropathic pain (Gracely et al. 1992; Torebjork et al., 1992). Numerous experimental studies also demonstrate a close relationship between nerve-ligated ectopic discharges and neuropathic pain behaviors in both complete and partial sciatic nerve injury models. Thus, in the axotomy model, the ectopic discharge is correlated with autotomy, a self-mutilatory behavior (Abdulla and Smith 2001). In the partial injury model, the initiation of ectopic discharges is in a time frame closely resembling the on-set time of observed behaviors (Liu et al., 1999; Liu et al., 2000a; Liu et al., 2000b; Wu et al., 2001). Block of the ectopic discharge from Aβ fibers of the injured axons (Liu et al., 1999; Liu et al., 2000a; Liu et al., 2000b) and the

ectopic discharge from C fibers of uninjured neighboring axons (Boucher et al., 2000b; Li et al., 2000; Wu et al., 2001), with dorsal rhizotomy, eliminates or attenuates allodynia (Sheen and Chung, 1993; Yoon et al., 1996; Boucher et al., 2000b; Li et al., 2000). It is hypothesized that peripheral input from both *injured* and *uninjured* neighboring axons are necessary in maintaining allodynia and hyperalgesia (Gold, 2000; Wu et al., 2001). The spontaneous activity of the *uninjured C fiber* to generates central sensitization and the ectopic discharge from *injured Aβ* contributes to the ongoing pain.

Nerve injury induced changes VGSC likely plays a key role in generating electrical hyperexcitability and ectopic activities (Waxman, 1999; Waxman et al., 1999; Cummins, et al., 2000). Following nerve injury, Na^+ channels accumulate in the axonal membrane in neuroma, in the patches of demyelination and in the regenerating sprout, which renders the injured axons an ectopic source to generate discharge (Kocsis and Devor, 2000). The abnormal expression of TTX-s Na^+ channels (type III embryonic Na^+ channels), and the decreased expression of TTX-r Na^+ channels (SNS/PN, SNS-2/NaN) in the *injured axon* (Waxman, 1999; Waxman et al., 1999) induces an accelerated repriming of Na^+ current; therefore, the injured afferents become prone to generating more prolonged and higher frequency discharges.

Wallerian degeneration contributes to the spontaneous activity in uninjured neighboring axons and local inflammatory responses (Li et al., 2000; Wu et al., 2001). Wallerian degeneration is characterized by axon degeneration and myelin clearance by activated Schwann cells and invading macrophages. Schwann cells and macrophages produce a number of growth factors and cytokines (TNF-α, IL-6) to produce a local inflammatory response, which can directly act on the intact neighboring C fibers to

promote hyperalgesia (Ramer et al., 1997; Woolf et al., 1994; 1997). A massive inflammatory response has been observed in the sciatic nerve segment adjacent to the injured site, and the degree of infiltration of inflammatory cells (monocytes/macrophages), and the expression of proinflammatory cytokines are correlated with the degree of allodynia (Cui et al., 2000). Wallerian degeneration also *antidromically* stimulates sensory afferents to release SP and CGRP from the peripheral terminals (Daemen et al., 1998a;1998b), which retrogradely activates nociceptors to promote further sensitization.

Different phenotype changes occur in injured and uninjured axons, depending on the availability of neurotrophic factors (Table1.2). As for the *injured afferents*, the *decreased* provision of NGF usually results in *decreased* expression of neurochemical agents. For the *uninjured afferents*, an *increased* provision of NGF due to Wallerian degeneration induces the *increased* expression of neurochemical agents (Fukuoka et al. 2000). These phenotype changes in Aβ fibers are similar to that occurred inflammation, with an increased expression of SP, CGRP and BDNF. The phenotype changes in Aβ fibers (Table1.2) together with structure reorganization of Aβ fiber in spinal cord (Section 1.2.3) contributes to the development of allodynia.

Table 1.2: Phenotype changes induced by inflammation and nerve injury

	Normal		Inflammation		Never injury		
					Injured		Uninjured
Afferents	C	Aβ	C	Aβ	C	Aβ	C /Aβ *
SP	Y	N	↑	↑	↓↓	↑	↑
CGRP	Y	Y	↑	↑	↓↓	↑	↑
VR1	Y	N	↑	↔	↓↓	↔	↑
BDNF	Y	N	↑	↑	↓	↑	↑
TTX-r	Y	N	↑	↔	↓	↔	↑
TTX-s	Y	Y	↔	↔	↑	↑↑	↔
Galanin	Y	Y	↔	↔	↑	↑	↔
NPY	Y	Y	↔	↔	↑	↑↑	↔
VIP	Y	Y	↔	↔	↑	↑	↔
CCK	Y	Y	↔	↔	↑	↑	↔
α_{2A}-ADR	Y	Y	↔	↔	↑	↑↑	↑
α_{2C}-ADR	Y	Y	↔	↔	↔/↓	↔/↓	↔

Abbreviations are explained in List of Abbreviations.

Summarized from: Birder and Perl, 1999; Carlton and Coggeshall, 1999; Millan, 1999; Boucher et al., 2000a; Fukuoka et al., 2000; Xie et al., 2000.

*Most data about phenotype changes in uninjured sensory afferents is preliminary, and does not specify change in small or large diameter afferents separately (Fukuoka et al., 2000; Xie et al., 2000).

Y: normally expressed, N: normally not expressed; ↑: up-regulated, ↓ : down-regulated, ↔ : no change reported.

1.5.3 SPGNs and neuropathic pain

The involvement of abnormal coupling of SPGNs and sensory afferents in neuropathic pain has been demonstrated both clinically and experimentally. Pain maintained by sympathetic innervation or by circulating catecholamines has been termed sympathetically-maintained pain. In experimental models, the dependence on sympathetic mechanisms for maintenance of pain is variable dependent on the models, species and strains of animal used; and differences between individual animals within the same strain also occur (Kim et al., 1997; Lee et al., 1997; Park et al., 2000).

The coupling of SPGNs with sensory afferents develops at three sites: (a) in the neuroma of injured afferents, (b) the peripheral terminals of uninjured neighboring afferents, and (c) in the DRG of the injured afferents (Jänig et al., 1996; Perl; 1999; Michaelis, 2000; Fig. 5.1). In clinical studies, intradermal or perineuronal injection of noradrenaline aggravates pain, while local sympathetic block alleviates spontaneous pain or allodynia in some patients (Torebjörk et al., 1995; Raja, 1998; Ali et al., 2000). In experimental studies, activation of cutaneous nociceptors (Sato and Perl, 1991; Bossut and Perl, 1995), neuromas (Devor and Jänig, 1981; Chen et al., 1996; Rubin et al., 1997) and DRG neurons (Xie et al., 1995b; Leem et al., 1997; Zhang et al., 1997), by SPGN stimulation or noradrenaline infusion, has also been demonstrated. Morphological evidence also supports sympathetic-sensory coupling. Thus, there is an overt NGF-dependent sprouting of SPGNs, penetrating DRGs and forming a basket-like structure around some of the injured DRG neurons, preferentially large-diameter neurons (Chung et al., 1993; Chung et al., 1996; Ramer et al., 1999).

In contrast to the *indirect* coupling mechanism involved in *inflammatory* pain, most studies indicate a *direct* action of catecholamines activating adrenoreceptors on primary sensory afferents following *nerve injury* (Peterson et al., 1996; Rubin et al., 1997; Perl,1999; Michaelis, 2000). Thus, local infusion of noradrenaline or α_2-adrenoreceptor agonists can rekindle neuropathic pain following sympathectomy, indicating intact SPGN are not necessarily required (Torebjök et al., 1995, Rubin et al., 1997; Moon et al., 1999). *In vitro* activation by noradrenaline of cultured DRG neurons isolated from nerve-ligated rats further supports a direct action (Abdulla and Smith, 1997; 2001).

The adrenergic receptor mediating the sympathetic excitation following nerve injury is likely the α_2 subtype, as in most experimental studies, α_2-adrenoreceptor agonists can increase pain or sensory neuron activity, while α_2-adrenoreceptor antagonists can block pain or sensory neuron activity (Sato and Perl, 1991; Chen et al., 1996; Leem et al., 1997; Moon et al., 1999). However, α_1-adrenoreceptors may be involved in primates and some strains of rats (Ali et al., 1999; Lee et al., 1999). Specific subtypes of α_2-adrenoreceptors (α_{2A}, α_{2B}, α_{2C}) may produce either inhibitory or excitatory effects (MacDonald et al., 1997). The α_{2A}-adrenoreceptor subtype may contribute to the excitatory effect, as sciatic nerve transection produces an increase in expression of α_{2A}, but not α_{2C}-, adrenoreceptors in DRG neurons (Cho et al., 1997; Birder and Perl, 1999; Perl, 1999; Xie et al., 2000), and α_{2A}-adrenoreceptor-knockout animals expressed attenuated pain behaviors following nerve injury (Kingery et al., 2000).

1.6 FORMATION, METABOLISM AND RELEASE OF ADENOSINE

Purine nucleosides and nucleotides are emerging as physiological regulators of a number of cellular functions such as cell growth, differentiation, cell death, release of hormones, immune responses and neurotransmission. The potent extracellular actions of nucleosides and nucleotides are mediated by two families of cell surface receptors, namely P1 and P2, preferentially activated by adenosine and ATP, respectively (Abbracchio and Burnstock, 1998). P2 purinoceptors are subdivided into two subclasses, the ligand-gated ion channel-P2X family, and the G-protein-coupled ion channel-P2Y family (Abbracchio and Burnstock, 1998). Four subtypes of P1 purinoceptors have been cloned, namely adenosine A_1, A_{2A}, A_{2B} and A_3 receptors. All of them are seven-transmembrane-domain, G-protein-coupled receptors that are linked to a variety of transduction mechanisms and have multiple functions in modulating pain and inflammation (Table 1.3) (Ralevic and Burnstock, 1998). Adenosine A_1 and A_{2A} are two high affinity adenosine receptors predominantly mediating inhibitory and excitatory effects respectively in neuronal tissues (Ralevic and Burnstock, 1998). Under physiological circumstances, basal extracellular adenosine levels range from 0.03-0.3 μM in different tissues (Geiger et al., 1997; Dunwiddie and Masino, 2001). Given the affinity of adenosine for its receptors (Table 1.3), this would suggest a tonic activation of A_1 and A_{2A} might exist in some systems. The stimulatory effect of caffeine stems from its ability to antagonize the inhibitory effect by tonic adenosine A_1 receptor activation in the central nerve system (CNS), but little is known about this basal tone in the peripheral nervous system (Dunwiddie and Masino, 2001).

Table 1.3: Characteristics of adenosine receptor subtypes

Receptor	A_1	A_{2A}	A_{2B}	A_3
Adenosine (EC_{50})	~70nM	~150nM	~5100nM	~6500nM
G-protein	Gi and Go	Gs and G_{olf}	Gs	Gi and Gq
Transuction mechanisms	↓ adenylyl cyclase ↑ K+ channels ↓ Ca^{2+} influx ↑ PLC	↑ adenylyl cyclase ↑,↓ Ca^{2+} channels	↑ adenylyl cyclase ↑ PLC ↑ IP3	↑ PLC ↑ IP3 ↓ adenylyl cyclase ↑ intracellular Ca^{2+}
Agonist	CPA L-PIA	CGS-21680	NECA	IB-MECA
Antagonist	CPX, CPT, Caffeine	CSC Caffeine	Enprofylline Caffeine	MRS 1191
Pain transmission	Spinal, peripheral	Peripheral	Peripheral	Peripheral
	Inhibits neurotransmitter release	Promotes neurotransmitter release	Indirect excitation via mast cell degranulation	Indirect excitation via mast cell degranulation
	Hyperpolarizes membrane	Hypopolarizes membrane		
Inflammatory response	↑ Neutrophil accumulation	↓ Neutrophil accumulation ↓ cytokine production ↓ mast cell degranulation	↑ Mast cell degranulation	↑ Mast cell degranulation ↓ TNF α

Abbreviations are explained in List of abbreviations.

↑: Activating or increasing; ↓: inhibiting or decreasing

Summarized from Geiger et al., 1994; 1997; Sawynok, 1998; 1999; Sullivan and Linden 1998; Ralevic and Burnstock, 1998; Dunwiddie and Masino, 2001.

Although agonists and antagonists for specific adenosine receptor subtypes have been developed, the ubiquitous nature of adenosine receptors makes it extremely diffcult to modulate them in a tissue specific manner. Thus, site- and event- specific modulation of extracellular adenosine levels has been extensively explored as an alternative approach (Kowaluk et al., 1998; Kowaluk and Jarvis, 2000). Formation, degradation, release and uptake of adenosine determine extracellular levels, and various enzymes and membrane-bound transport proteins tightly regulate these processes (Fig. 1.2). Under conditions when oxygen and energy demands exceed supply, such as inflammation, tissue trauma, ischaemia, hypoxia and seizures, adenosine is released to function as a negative feedback signal between the level of metabolic activity and the level of cellular excitability (Abbracchio and Burnstock, 1998). For example, subcutaneous adenosine levels are around 0.1-0.3 μM (Fredholm and Sollevi, 1981; Lönnroth et al., 1989), while under inflammation, the levels are increased to 0.6-1.2 μM in inflammatory exudes (Cronstein et al., 1993; 1995; Gadangi et al., 1996).

1.6.1 *Adenosine formation*

Adenosine can be produced both intracellularly and extracellularly (Fig. 1.2). *Inside the cell*, the predominant pathway of adenosine formation is the dephosphorylation of adenine nucleotides (ATP, adenosine diphosphate ADP, cAMP, adenosine monophosphate AMP) via cytosolic 5'-nucleotidase. Because intracellular ATP levels are much higher than adenosine, even 1% conversion of ATP to adenosine would result in an approximate 100-fold increase in intracellular adenosine (Dunwiddie and Masino, 2001). Metabolism of S-adenosyl homocysteine (SAH) by SAH hydrolase also

contributes to intracellular adenosine formation in normoxic conditions (Dunwiddie and Masino, 2001).

Outside the cell, extracellular adenine nucleotides are converted into adenosine via ecto-5'-nucleotidase (Geiger et al., 1997). Most nucleotides are converted to adenosine in less than a second, and ecto-nucleotidase may be present in close physical proximity to presynaptic A_1 receptors (Dunwiddie et al., 1997). Ecto-nucleotidase is feed-forwardly inhibited by substrate levels of ATP and ADP (James and Richardson, 1993). Thus, the concentration of adenosine from adenine nucleotides is tightly regulated by ecto-5'-nucleotidase according to the amount of ATP released. If the amount of ATP released is small, the adenosine will be formed in a linear manner, but if the amount of ATP is great, extracellular AMP will accumulate until the levels of ATP and ADP fall below the values required to inhibit ecto-5'-nucleotidase. Therefore, a reduction of ecto-5'-nucleotidase inhibition will force a burst-like formation of adenosine, due to the accumulation of AMP (James and Richardson, 1993). A commonly used selective inhibitor of ecto-5'-nucleotidase is α,β-methylene ADP (α,β m ADP) (Geiger et al., 1997).

1.6.2 Adenosine metabolism

Adenosine can be catabolzied by deamination via adenosine deaminase, or phosphorylation via adenosine kinase. Incorporation into SAH via SAH hydrolase may also be involved, but not it is not as important as deamination and phosphorylation.

Adenosine kinase is a cytosolic enzyme that rapidly phosphorylates adenosine into AMP, thus maintaining intracellular adenosine concentration at low levels. One interesting characteristic of adenosine kinase is that higher levels of adenosine can inhibit

adenosine kinase activity (Arch and Newsholme, 1978). The mechanism of substrate inhibition is not clear, but increasing Mg^{2+} and lowering pH levels can facilitate substrate inhibition (Kowaluk et al., 1998). As adenosine uptake is driven by its concentration gradient, inhibition of adenosine kinase decreases cellular reuptake of adenosine and potentiates the levels of adenosine in the extracellular compartment (Kowaluk et al., 1998). Commonly used adenosine kinase inhibitors include 5-iodotubercidin and 5'-amino-5'-deoxyadenosine (NH_2dAD) (Fig. 1.2).

Adenosine deaminase is a member of a family of enzymes with purine nucleoside deaminase activity. It has two forms, one localized in the cytoplasm, the other localized on the cell surface (ecto-adenosine deaminase)(Franco et al., 1997). Apart from degradation of extracellular adenosine, ecto-adenosine deaminase is also able to transmit signals with two cell surface receptors, CD 26 and adenosine A_1 receptor (A_1R) (Franco et al., 1997). Ecto-adenosine deaminase plays a key role in the regulation of A_1 receptor desensitization and internalization. During the agonist-dependent internalization process, ecto-adenosine deaminase and A_1R follow a common endocytic pathway (Franco et al., 1997). It is still not clear how the coupling contributes to modulating extracellular adenosine levels (Franco et al., 1997), but it has been suggested that this co-internalization may contribute to the increased plasma adenosine levels following adenosine antagonist treatment (Franco et al., 1997). Compared with adenosine kinase, adenosine deaminase has relatively lower affinity, yet a higher capability, for adenosine (Arch and Newsholme, 1978; Phillips and Newsholme, 1979). Thus, it has been suggested that adenosine kinase is important in regulating adenosine levels under physiological circumstances, while adenosine deaminase becomes important when the

adenosine levels are largely increased (Lloyd and Fredholm, 1995; Kobayashi et al., 1998). Commonly used adenosine deaminase inhibitors are 2'-deoxycoformycin (DCF) and erythro-9-(2-hydroxy-3-nonyl) adenine (Fig. 1.2).

1.6.3 Adenosine transport and reuptake

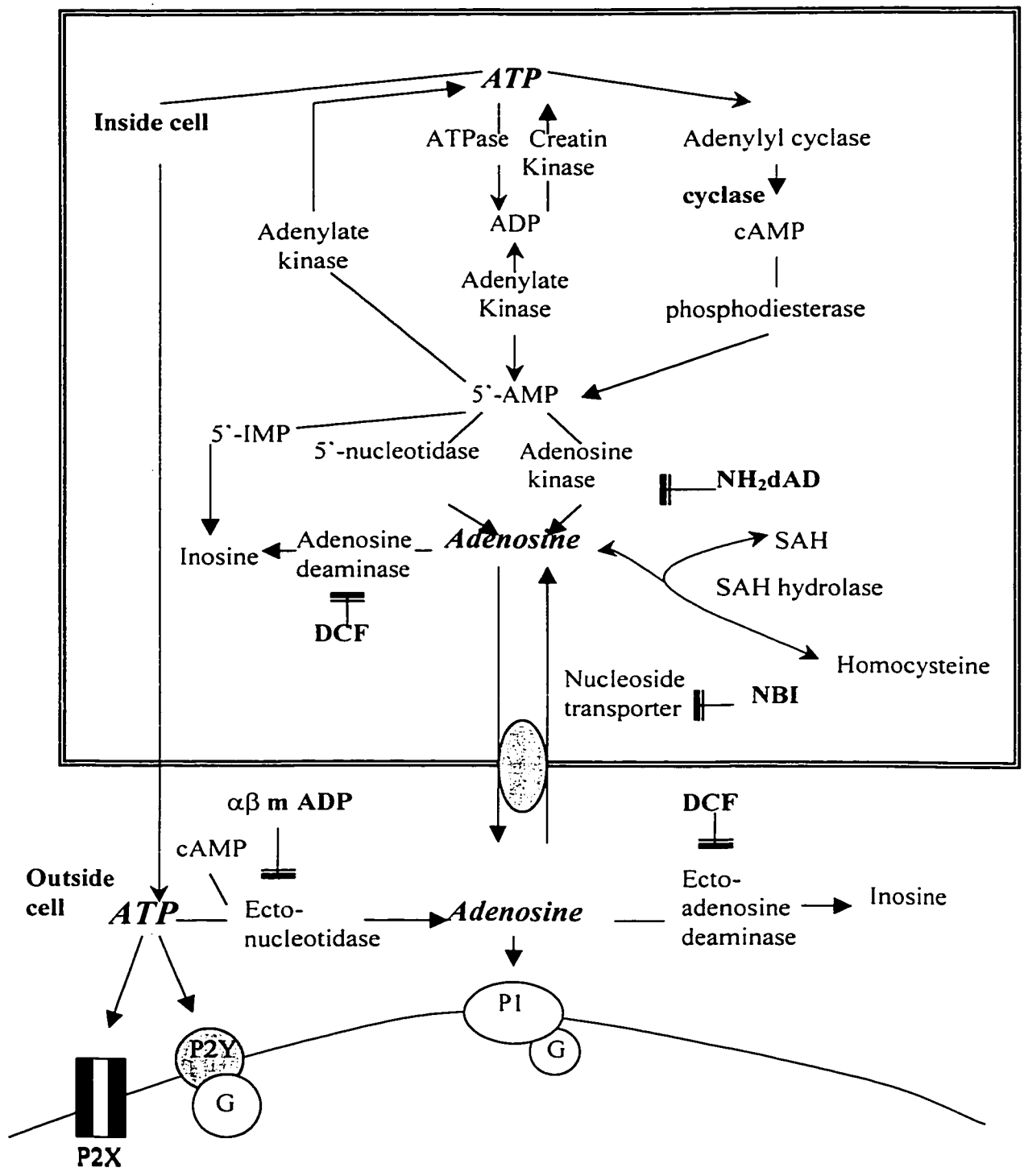
An important factor in termination of the extracellular actions of adenosine is its facilitated diffusion back into intracellular spaces (Fig. 1.2). Adenosine passes to and from the extracellular environment through nucleoside transporters (NT_S). There are two major classes of NT, *facilitated-diffusion nucleoside transporters* and *active transporters* driven by an inwardly directed transmembrane Na^+ gradient (Geiger et al., 1997; Thorn and Jarvis, 1996). The facilitated transporters do not depend on ATP or ionic gradients to transport adenosine, and they equilibrate the concentration of adenosine across the cellular membrane. As intracellular adenosine levels are relative low, the net influx through these transporters is inwardly directed. However, under conditions where intracellular adenosine is increased, these transporters can release adenosine (Thorn and Jarvis, 1996). Thus, inhibiting NT may decrease extracellular adenosine levels if adenosine is directly released from an intracellular pool. On the other hand, inhibition of NT can also increase extracellular adenosine levels if adenosine is largely formed extracellularly from ATP through ecto-nucleotidase. Commonly used adenosine transport inhibitors are dipyridamole, nitrobenzylthioinosine (NBI) and dilazep.

Na^+-dependent active transporters couple the transfer of nucleosides to inward movements of Na^+ across cell membranes and are capable of increasing nucleosides intracellularly. Their relative importance in regulating adenosine concentration is unclear yet, largely due to a lack of selective pharmacological inhibitors (Cass et al., 1998).

Fig. 1.2: Metabolic routes for adenosine and sites of action of various inhibitors. Metabolism by adenosine kinase is the major degrading pathway when adenosine levels are around physiological conditions, and can be blocked by NH2dAD. Adenosine deaminase exhibits actions both inside and outside the cell, which can be blocked by DCF. Nucleoside transporters mediate both release and reuptake of adenosine and can be blocked by NBI. Adenosine can also be formed extracellularly from released ATP through ecto-nucleotidase, which can be blocked by α,β m ADP. Extracellular adenosine and ATP act on P1 and P2 receptors respectively to produce receptor-mediated effects.

Based on Geiger et al., 1997; Dunwiddie and Masion, 2001. Abbreviations are explained in the list of abbreviations.

Figure 1.2: The primary intracellular and extracellular pathways of the formation and metabolism of adenosine



Cell membrane purinoceptors

1.6.4 Adenosine release

Adenosine and its precursor, ATP, can be released virtually from all cells and tissues. Adenosine *per se* can be released directly from the intracellular space or alternatively, adenine nucleotides can be released by a variety of mechanisms and then dephosphorylated extracellularly via ecto-nucleotidase to adenosine (Fig 1.2). Furthermore, adenosine or ATP can be passively released as a result of cell injury induced by nonspecific permeability changes, as well as from cells undergoing lysis with a release of intracellular purine stores (Hamilton and McMahon, 2000). Some adenosine released from non-neuronal cells, such as neutrophils (Cronstein et al., 1983; 1986), endothelial cells (Ager and Gordon, 1984; Bodin and Burnstock, 1998), mast cells (Marquardt et al., 1984) and platelets, may contribute to the increased adenosine levels in inflammatory exudates (Cronstein, 1997). ATP and adenosine can also be released from neuronal tissues in response to oxygen deprivation, ischemia, electrical stimulation, glutamate receptor activation and K^+ stimulation (Geiger et al., 1997). Peripherally, ATP can be released from sympathetic nerve terminals as a cotransmitter with NPY and noradrenaline, as well as from the parasympathetic nervous system as a cotransmitter with acetylcholine (Cunha et al., 1996; Msghina et al., 1999; Racchi et al., 1999). Importantly, sympathetic stimulation also simultaneously releases nucleotidases, so the released ATP is rapidly degraded into adenosine through both released nucleotidases and ecto-nucleotidase (Todorov et al., 1997). The conversion of extracellular cAMP also induces a large increase in adenosine levels, as observed following forskolin stimulation of adenylyl cyclase (Brundege et al., 1997) and following receptor-mediated activation of adenylyl cyclase (Gereau and Conn, 1994).

Several observations support a release of adenosine from the *central terminals* of sensory afferents. Neurochemical studies indicate that adenosine can be released from central terminals of both capsaicin-sensitive and capsaicin insensitive sensory afferents, and the release can be either calcium-dependent or calcium-independent (White et. al., 1985; Sweeney et al., 1988; 1989; 1990; Sawynok et al., 1993). Electrophysiological studies also indicate ATP can modulate sensory synaptic transmission between primary afferents and dorsal horn neurons (Salter and Henry, 1985; Gu and MacDermott, 1997; Li et al., 1998). It is not quite clear whether or not ATP or adenosine can be released *peripherally* following noxious stimulation to contribute to the *efferent* function of primary sensory afferents. Evidence supporting an efferent release includes (a) peripheral terminals of sensory afferents express both ATP and adenosine receptors (Abbracchio and Burnstock, 1998; Sawynok, 1999) and (b) antidromic stimulation of sensory nerves in the ear of rabbit releases ATP (Holton and Holton, 1954; Holton, 1959).

1.7 ADENOSINE AND ATP IN PAIN MODULATION

Classical analgesics are not very effective in pain following nerve injury, and many efforts have been made to investigate potential alternative treatments. Double-blind, placebo-controlled clinical studies indicate low dose (50-70μg/kg) intravenous administration of adenosine can reverse several aspects of neuropathic pain, such as spontaneous pain, ongoing pain (Belfrage et al., 1995) and tactile allodynia (Sollevi et al., 1995; Sjölund et al., 2000). Furthermore, spinal administration of an adenosine A_1 receptor agonist (Karlsten and Gordh, 1995), or adenosine itself (Belfrage et al., 1999),

can abolish or reverse allodynia and spontaneous pain, further indicating a clinically significant role for the modulation of the adenosine system in pain control. Although certain analgesic effects have also been observed in acute pain, adenosine produces more pronounced effects with pain inducing central sensitization (Segerdahl and Sollevi, 1998).

1.7.1 Adenosine in spinal pain transmission

The site of action of systemically infused adenosine in clinical studies is thought to be largely in the spinal cord (Karlsten and Gordh, 2000; Segerdahl and Sollevi, 1998). Indeed, experimental studies indicate adenosine, or adenosine analogs, produce pronounced antinociceptive effects at the spinal level (Sawynok, 1998; 1999). Both adenosine A_1 and A_{2A} receptors have been identified in the spinal cord dorsal horn, with the highest concentrations in the substantia gelatinosa (Geiger et al., 1984). The adenosine A_1 receptor is the predominant receptor subtype mediating the spinal antinociceptive effect of adenosine (Sawynok, 1998; 1999).

The antinociceptive effect of adenosine in the spinal cord is mediated both postsynaptically and presynaptically. As the adenosine A_1 receptors are predominantly expressed in interneurons (Choca et al., 1988), activation of A_1 receptors postsynaptically hyperpolarizes excitatory interneuron cell membranes and inhibits synaptic transmission through increased K^+ conductance via ATP sensitive K^+ channels (Salter and Henry, 1985; Salter et al., 1993; Li and Perl, 1994). Adenosine also presynaptically activates A_1 receptors on the central terminals of capsaicin-sensitive sensory afferents and inhibits SP and CGRP release by decreasing intracellular Ca^{2+} levels (Santicioli et al., 1992). The

electrophysiological outcome of presynaptic and postsynaptic inhibition is the reduction of noxious stimuli induced C fiber evoked post discharge and wind-up responses in dorsal horn neurons (Reeve and Dickenson, 1995; Nakamura, et al., 1997), thus reducing the synaptic transmission of noxious input.

Behaviorally, antinociceptive effects produced by spinal adenosine and adenosine analogs have been widely demonstrated in a wide range of experimental models, including nociceptive pain models (Delander and Hopkins, 1987), inflammatory pain models (Poon and Sawynok 1995; 1998; Mcgaraughty et al., 2001) and neuropathic pain models (Sosnowski and Yaksh, 1989; Lee and Yaksh, 1996; Sjölund et al., 1996; Lavand'homme and Eisenach, 1999). A recent preliminary study indicates there is an increase in the numbers of adenosine A_1 receptors in the spinal cord following nerve injury (Bantel et al., 2000), which could contribute to the enhanced antinociceptive effects of adenosine following nerve injury.

1.7.2 Adenosine receptors and peripheral pain transmission

Although spinal actions may account for the observed pain relieving effect of systemic adenosine infusion, a peripheral effect cannot be excluded. Adenosine produces complex effects in modulating nociception peripherally (Fig. 1.3). Generally speaking, activation of adenosine A_1 receptors produces antinociception via decreasing cAMP (Taiwo and Levine, 1991), while adenosine A_{2A} receptor activation produces nociception via increasing cAMP (Taiwo and Levine, 1991). Inhibition of neuropeptide release may contribute to the peripheral antinociceptive effect of adenosine. Thus, adenosine A_1 receptor activation inhibits CGRP release from the peripheral terminals of capsaicin-

sensitive sensory afferents and cultured trigeminal ganglional neurons, while activation of A_{2A} receptors has no effect on neurotransmitter release (Rubino et al., 1993). The antinociceptive effects of adenosine A_1 receptors are functionally linked with μ opioid receptors and α_{2C}-adrenoreceptors via coupling to Gi/Go proteins (Aley and Levine, 1997), which has been demonstrated by cross antagonism (Aley and Levine, 1997). The antinociceptive effect of A_1 receptors and the nociceptive effect of A_{2A} receptors have been demonstrated in both in a PG-induced mechanical hyperalgesic model (Taiwo and Levine, 1990) and the formalin model (Karlsten et al., 1992; Khasar et al., 1995). As extracellular adenosine levels are increased in inflamed tissues (Cronstein, 1997), endogenous adenosine can activate both A_1 and A_{2A} receptors to produce opposing tonic effects on peripheral pain modulation (Doak and Sawynok, 1995).

There is no evidence indicating that adenosine A_{2B} and A_3 receptors are localized on nerve terminals. However, these receptors may produce peripherally mediated *indirect* nociceptive effects (Sawynok, 1998). Both are localized on mast cells (Forsythe and Ennis, 1999), and can trigger mast cell degranulation upon activation, likely mediated by increased inositol 1,4,5-triphosphate (IP3) production and increased intracellular Ca^{2+} (Ramkumar et al., 1993). Activation of mast cell results in excytosis of histamine, 5-HT and ATP, which subsequently activate H_1, 5-HT_2 and $P2X_3$ receptors, located on nociceptive nerve terminals, to produce nociceptive effects (Fig. 1.3) (Sawynok, 1998). Subcutaneous injection of an adenosine A_3 receptor agonist produces an overt pain behaviors similar to that produced by low doses of formalin (Sawynok et al., 1997). Opposing the excitatory effect of A_{2B} and A_3 receptors, adenosine A_{2A} receptors inhibit mast cell degranulation via increasing cAMP (Suzuki et al., 1998). As adenosine has a

much higher affinity for A_{2A} than A_{2B} or A_3 (Table 1.3), under normal circumstances subcutaneous administration of adenosine *per se* is not likely to trigger mast cell degranulation. However, under conditions of hypoxia or ischemia, the levels of the adenosine metabolite, inosine, is increased. Inosine activates A_3 receptors, which may play an important physiological role in the regulation of mast cell degranulation (Jin et al., 1997). The regulatory effects of adenosine receptor subtypes in peripheral pain transmission are summarized in Figure 1.3.

Fig. 1.3: Schematic illustration of the role of adenosine receptors in peripheral pain transmission. The involvement of adenosine A_1, A_{2A}, A_{2B} and A_3 receptor subtypes, P2X3 purinoceptors, SPGNs and mast cells is depicted. Inset depicts the second messenger system activated by adenosine receptors on sensory nerve terminals. (Modified from Sawynok, 1998). Abbreviations are explained in the list of abbreviation.

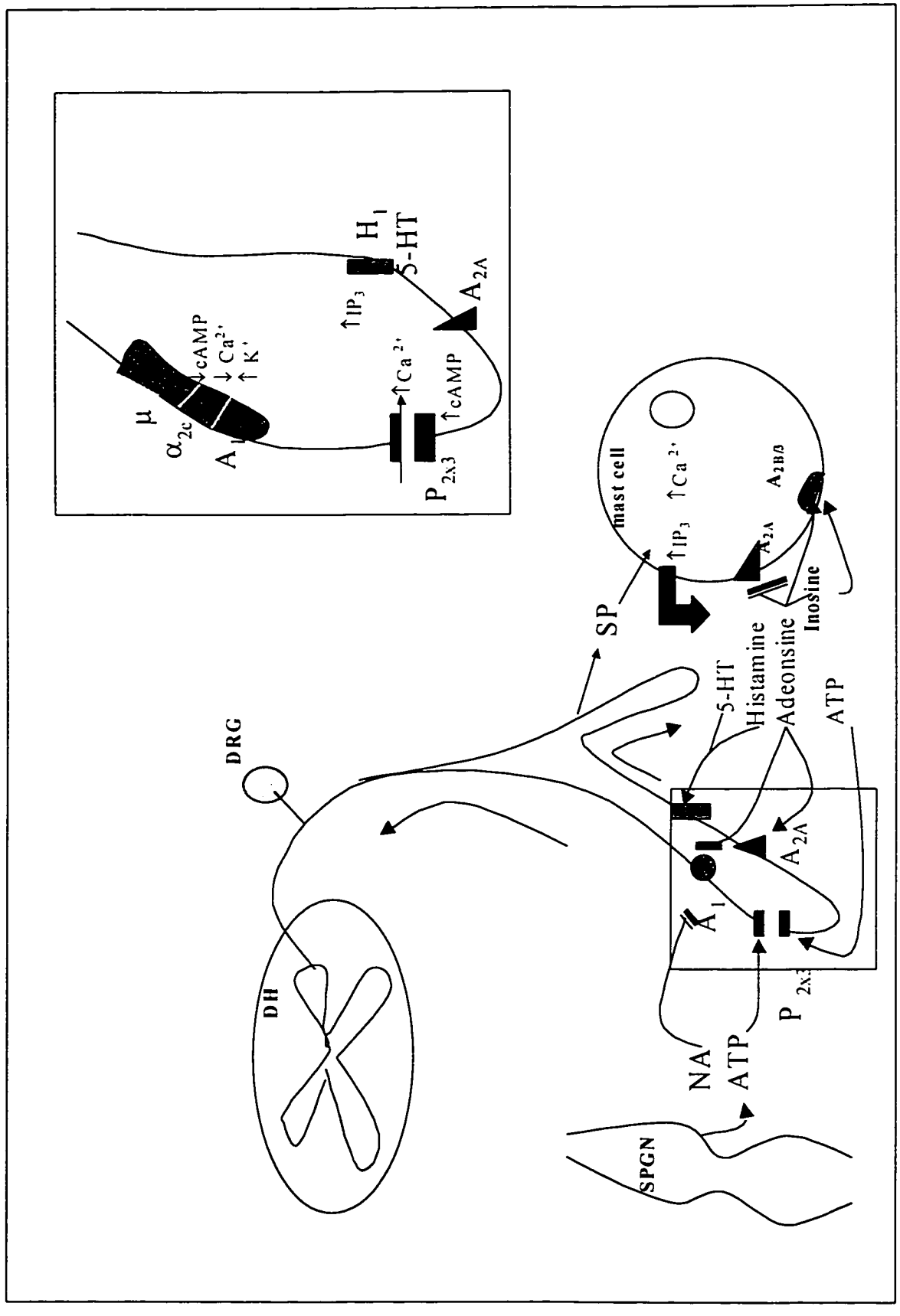

μ
α_{2c}
A_1
↓cAMP
↓ Ca^{2+}
↑ K^+
↑IP_3
H_1
5-HT
A_{2A}
↑Ca^{2+}
↑cAMP
P_{2X3}
DRG
DH
SPGN
SP
NA
ATP
5-HT
Histamine
Adeonsine
ATP
Inosine
mast cell
↑IP_3
↑Ca^{2+}
A_{2A}
A_{2BB}
A_1
A_{2A}
P_{2X3}

Fig. 1.3

1.7.3 Involvement of adenosine in antinociception by indirectly acting agents

The potential antinociceptive effects of inhibitors of adenosine metabolism have been extensively investigated (Kowaluk et al, 1998; Kowaluk and Jarvis, 2000). The antinociceptive effects of the adenosine kinase inhibitors NH_2dAD and 5-iodotubercidin, have been demonstrated in a diverse array of nociceptive models. Systemic, spinal and local, peripheral delivery of adenosine kinase inhibitors alleviated acute (Keil and DeLander, 1992; 1994), inflammatory (Poon and Sawynok, 1998; Kowaluk et al., 2000; Mcgaraughty et al., 2001) and neuropathic (Lee and Yaksh, 1996; Lavand'homme and Eisenach, 1999; Lynch et al., 1999; Kowaluk et al., 2000) pain. The spinal cord is likely to be the key site for adenosine kinase inhibitor-mediated antinociceptive effects (Poon and Sawynok, 1999; Mcgaraughty et al., 2001), but a local peripheral action also has been demonstrated in inflammatory pain models (Sawynok et al., 1998). Inhibitors of adenosine deaminase potentiate the antinociceptive effect of adenosine kinase inhibitors in several models (Keil and DeLander, 1992; 1996; Poon and Sawynok, 1998; 1999; Sawynok et al., 1998).

Adenosine may be involved in the antinociceptive effect produced by other agents. The antinociceptive effect of opioid in the spinal cord is partly mediated by endogenous adenosine release (DeLander and Hopkins, 1986; Sweeney et al., 1987; 1989). Thus, the antinociceptive effect produced by spinal opioids can be blocked by adenosine receptor antagonists (DeLander and Hopkins, 1986; Sweeney et al., 1987; Cahill et al., 1995), or be potentiated by increasing spinal cord adenosine levels (DeLander and Keil, 1994; Lavand'homme and Eisenach, 1999). Endogenous adenosine release may also be involved in the antinociceptive effect of spinally administered α-

adrenoreceptors (Sweeney et al., 1987; Yang et al., 1994) and 5-HT (DeLander and Hopkins, 1987; Sweeney et al., 1990) receptor agonists. Recent studies also demonstrate the peripheral antinociceptive effect of amitriptyline, a widely used antidepressant in the treatment of neuropathic pain, is partly mediated by the adenosine system (Sawynok et al., 1999a; Esser and Sawynok, 1999; 2000).

1.7.4 ATP and peripheral nociception

In the extracellular space, ATP can be converted to adenosine within seconds via ecto-nucleotidases (Section 1.6.1). However, ATP *per se* can also activate $P2X_3$ ionotropic receptors to produce peripherally mediated nociceptive effects (Hamilton and McMahon, 2000). Subcutaneous application of high dose ATP produces an overt pain behavior (Bland-Ward and Humphrey, 1997). Intraplantar injection of P2X receptor agonists also produces thermal hyperalgesia and mechanical allodynia (Tsuda et al., 2000), as well as potentiates pain behaviors induced by formalin (Sawynok and Reid, 1997; Jarvis et al., 2001). $P2X_3$ receptors are ATP-gated ion channels that selectively located on a subpopulation of C fibers, the IB4-positive C fibers (Bradbury et al., 1998). It is postulated that the $P2X_3$ receptors, expressed in C fibers, mediates the overt pain and thermal hyperalgesia, while the heteromeric $P2X_{2/3}$ receptors, expressed in large diameter sensory afferents, mediates allodynia (Tsuda et al., 2000). P2X receptor-mediated nociceptive effects are enhanced in inflamed tissues (Tusda et al., 2000). At a high concentration of formalin (5%), a tonic nociceptive effect of ATP has been demonstrated by P2X antagonists (Jarvis et al., 2001). ATP may also be important in the generation and maintenance of neuropathic, especially sympathetically maintained pain, as

ATP can be released from the hyperactive sympathetic nerve terminals (Burnstock and Wood, 1996) and the ectopic purinergic sensitivity develops at the site of injury in CCI model (Chen et al., 1999).

1.8 ADENOSINE AND INFLAMMATION

1.8.1 Anti-inflammatory and pro-inflammatory effects produced by adenosine receptors

Adenosine produces potent anti-inflammatory effects both *in vitro* and *in vivo* (Cronstein, 1994; 1997; Sullivan and Linden, 1998). Activation of adenosine A_{2A} receptors expressed on neutrophils, endothelial cells, monocytes, macrophages and mast cells, mediates most anti-inflammatory effects, via stimulating adenylyl cyclase and increasing cAMP. The most important effect is to modulate *neutrophil* function. Activation of adenosine A_{2A} receptors inhibits neutrophil adhesion and destruction of endothelial cells, inhibits superoxide anion generation, reduces neutrophil rolling and degranulation and decreases neutrophil migration into inflamed sites (Cronstein, 1997; Sullivan and Linden, 1998). Activation of A_{2A} receptors located on *endothelial cells* inhibits cytokine production, prevents TNFα- and endotoxin-evoked expression of adherence factors (Sullivan and Linden, 1998) and decreases vascular leakage (Rosengren et al., 1995). Activation of A_{2A} receptors expressed on *monocytes and macrophages* decreases endotoxin-induced proinflammatory cytokines, such as TNFα, IL-6, IL-8, release (Sullivan and Linden, 1998). Adenosine A_{2A} receptor activation also inhibits *mast cell* degranulation, thereby preventing inflammatory mediator release from mast cells (Sullivan and Linden, 1998).

It should be noted that other adenosine receptor subtypes are also involved in the modulation of inflammatory responses. In general, activation of adenosine A_1 receptors produces effects on neutrophils opposing A_{2A} receptors. Adenosine A_1 agonists increase neutrophil adherence to endothelial cells, promote neutrophil generation of superoxide anion, and increase neutrophil accumulation in the inflamed tissues (Cronstein, 1997; Sullivan and Linden, 1998). Thus, adenosine can produce a *proinflammatory* effect via the A_1 receptors. However, A_1 receptor agonists may also produce *anti-inflammatory* effects *in vivo*, which have been demonstrated in pleural and peritoneal inflammation models (Lesch et al., 1991).

Activation of adenosine A_{2B} and A_3 receptors expressed on mast cells also produces proinflammatory effects by degranulating mast cells (Sullivan and Linden, 1998). Upon activation, mast cells release inflammatory mediators such as histamine and trypases to produce a proinflammatory effect (Cronstein, 1997; Sullivan and Linden, 1998). However, activation of adenosine A_3 receptors, expressed on monocytes and macrophages, inhibits TNFα production, which may contribute to the anti-inflammatory effect produced by adenosine (Sajjadi et al., 1996). The involvement of different adenosine receptor subtypes in the inflammatory response is summarized in Table 1.3.

1.8.2 Involvement of adenosine in the anti-inflammatory effect by indirectly acting agents

Although different adenosine receptor subtypes produce complex effects in inflammation, most studies indicate increasing that extracellular adenosine levels produces an *anti-inflammatory* effect, predominantly mediated by A_{2A} receptors. It has been demonstrated that a suspension of neutrophils releases adenosine, and removal of

this endogenously released adenosine leads to a significant increase in neutrophil responses to chemoattractants (Cronstein et al., 1983) and a increase in endothelial cell injury by neutrophils (Cronstein et al., 1986). These *in vitro* studies indicate that following stimulation, neutrophils, and/or endothelials, endogenously release adenosine, and this release produces a protective effect on tissue injury. Thus, modulation of extracellular adenosine levels may be utilized to diminish inflammation. Indeed, the anti-inflammatory effects produced by systemic adenosine kinase inhibitors have been demonstrated in several *in vivo* models, including decreasing leukocyte accumulation in air pouch exudates in carrageenan-induced inflammation (Cronstein et al., 1995); decreasing carrageenan-induced paw edema (Rosengren et al., 1995; Poon and Sawynok, 1999; Kowaluk et al., 2000), decreasing mortality in a septic shock model (Firestein et al., 1994) and decreasing paw edema and tissue injury induced by adjuvant arthritis (Boyle et al., 2001). Furthermore, Cronstein has demonstrated that several commonly used agents in the treatment of rheumatoid arthritis produce their anti-inflammatory effects by increasing adenosine levels (Cronstein, 1994; 1997). Such agents include methotrexate (Cronstein et al., 1993) and sulfasalazine (Gadangi et al., 1996). Furthermore, recent studies demonstrate that salicylates and aspirin, whose anti-inflammatory effects are generally attributed to inhibition of cyclooxygenases, produce anti-inflammatory effects through increasing adenosine levels in a prostaglandin-independent manner (Cronstein et al., 1999). In the above studies, the adenosine kinase inhibitors, methotrexate, sulfasalazine and salicylates, decrease leukocyte accumulation by increasing adenosine levels in inflamed murine air pouch (Cronstein et al., 1993; 1995;1999; Gadangi et al., 1996; Morabito et al., 1998). The reduction in leukocyte

accumulation is largely reversed by injection of adenosine deaminase into the air pouch, as well as by selective adenosine A_2, but not A_1, receptor antagonists, confirming that the anti-inflammatory effect is due to an A_2 receptor mediated effect through a local increase in adenosine levels (Cronstein et al., 1993;1995; Gadangi et al., 1996).

1.9 RATIONALE, HYPOTHESIS AND OBJECTIVES

1.9.1 General rationale

Peripheral sensitization and ongoing input are necessary for initiating and maintaining central sensitization in neuropathic and inflammatory pain (Section 1.4; 1.5). Endogenous adenosine is released in inflammation, and can be modulated to produced local peripheral antinociceptive and anti-inflammatory effects (Section 1.7; 1.8); however, little is known about the involvement of peripheral adenosine systems in neuropathic pain. Development and use of adenosine-based topical analgesics may avoid the potential side effects produced by systemic adenosine receptor activation.

Both neuronal and non-neuronal origins may contribute to the peripheral release of adenosine in inflammation (Section 1.6.4). Electrical stimulation of primary sensory afferent nerves releases purines from the peripheral terminal of the axons. Purines released from SPGNs contribute to the SPGN-mediated inflammatory response and may also contribute to the peripheral sensitization in neuropathic pain (Section 1.6.4; 1.7.4). Mast cells are involved in both neurogenic and non-neurogenic inflammation and may release adenosine upon stimulation (Section 1.3.2).

1.9.2. General hypothesis

The inhibitory neuromodulator, adenosine, is released peripherally following noxious stimulation, inflammation and nerve injury. The released adenosine produces a protective feedback effect in modulating peripheral nociception and inflammation. Pharmacological modulation of such release with inhibitors of adenosine metabolism. or other indirectly acting agents, can reduce neuropathic pain and inflammatory pain by inhibiting peripheral sensitization and local inflammatory responses. Thus, primary sensory afferents, SPGNs and mast cells may contribute to peripheral adenosine release following inflammation and nerve injury.

1.9.3 Objectives

1. To examine the local peripheral antinociceptive effect of adenosine receptor agonists, an adenosine kinase inhibitor and an adenosine deaminase inhibitor in a rat neuropathic pain model. Behavioral studies will be conducted to determine the local effect of directly or indirectly acting adenosine agents on thermal hyperalgesia and mechanical allodynia produced by spinal nerve ligation. Paw volume studies will be conducted to determine the possible side effects produced by subcutaneous injection of adenosine analogs.

2. To determine the characteristics of subcutaneous adenosine release in an inflammatory pain model, the formalin model. Extracellular adenosine levels in the rat hind paw, at different concentrations of formalin, will be determined by subcutaneous microdialysis study and high performance liquid chromatography (HPLC). The

modulation of extracellular adenosine levels by an adenosine kinase inhibitor and an adenosine deaminase inhibitor will be determined. Involvement of capsaicin-sensitive primary sensory afferents, SPGNs and mast cells in peripheral adenosine release will be determined by systemic pretreatment with capsaicin, 6-OHDA and compound 48/80.

3. To determine the characteristics of subcutaneous adenosine release in a neuropathic pain model, the spinal nerve ligation model. Extracellular adenosine levels in the rat hind paw of both neuropathic and sham operated rats will be determined by microdialysis and HPLC. The modulation of extracellular adenosine levels by an adenosine kinase inhibitor and an adenosine deaminase inhibitor will be determined. Involvement of capsaicin-sensitive primary sensory afferents and SPGNs in peripheral adenosine release will be determined by systemic pretreatment with capsaicin and 6-OHDA.

4. To determine the effect of amitriptyline on peripheral adenosine release in both the formalin model and the neuropathic pain model. Behavioral studies have demonstrated that the peripheral antinociceptive effect of amitriptyline is partly mediated by adenosine mechanisms in both neuropathic and inflammatory pain models (Sawynok et al., 1999a; Esser and Sawynok, 1999; 2000). The effect of subcutaneous amitriptyline on peripheral adenosine release will be determined by microdialysis and HPLC in both models.

5. To determine the effects of subcutaneous glutamate on peripheral adenosine release and the involvement of ionotropic glutamate receptors in formalin-evoked peripheral adenosine release. In the CNS, glutamate evokes adenosine release to provide a negative feedback effect on glutamate-evoked stimulatory effects (Craig and White, 1992; White, 1996; Conway and Yaksh, 1998). As ionotropic glutamate receptors are expressed in both the peripheral terminals of sensory afferents and SPGNs (Carlton et al., 1995; 1998a), and glutamate is released in inflammation (Omote et al., 1998; Lawand et al., 2000), we hypothesize that a similar feedback mechanisms also exists in peripheral tissues to modulate peripheral sensitization and local inflammatory responses. Microdialysis studies and HPLC will be used to determine the peripheral adenosine levels evoked by subcutaneous injection of glutamate. Involvement of peripheral sensory terminals and SPGNs will be determined by capsaicin and 6-OHDA pretreatment. Involvement of ionotropic glutamate receptors in exogenous glutamate-evoked adenosine release and endogenous glutamate-evoked adenosine release in the formalin model will be determined by co-administering NMDA and non-NMDA receptor antagonists with glutamate and formalin.

CHAPTER 2

GENERAL METHODS

2.1 ANIMAL PREPARATION

2.1.1 Animals

Male Sprague-Dawley rats (Charles River Laboratories, Quebec), 100-120g (neuropathic surgery), 120-160g (formalin test) or 250-300g (microdialysis studies) were used for all experiments. Rats were housed in pairs, maintained on a 12/12 h light/dark cycle, and allowed free access to food and water. All experimental procedures were approved by the University Committee for Laboratory Animals of Dalhousie University.

2.1.2 Surgical procedures

For the neuropathic rat model, the surgical procedure was performed according to previous descriptions (Kim and Chung, 1992; Esser and Sawynok, 1999). Briefly, under halothane (1.2%-1.7%) inhalation anesthesia, the left L5 and L6 spinal nerves were exposed, isolated and then tightly ligated with sterilized 6-0 silk suture distal to the dorsal root ganglion and proximal to the formation of the sciatic nerve. After ensuring hemostasis, the wound was closed in layers. Rats were allowed to recover for 7 days after surgery. Animals that showed motor impairment during this period were excluded from the study and sacrificed immediately.

2.1.3 Chemical pretreatment procedures

Capsaicin pretreatment

To reduce the function of unmyelinated C fiber afferents, rats were pretreated with capsaicin for three consecutive days (30mg/kg on day 1, 50mg/kg on day 2, 70 mg/kg on day 3) (Zhang et al., 1998; Zhou et al., 1998) and microdialysis was performed on day 4. Capsaicin was prepared as a 10mg/ml solution in a solvent containing ethanol (10%), Tween 80 (10%) and saline (80%). Capsaicin and vehicle treatments were carried

out under pentobarbital anesthesia (45mg/kg i.p.) and drugs were injected into the subcutaneous space under the loose neck skin. Systemic pretreatment with capsaicin induces a reduction of 48% saphenous nerve C-fibers and a 17% reduction of DRG neurons (Holzer 1991). The present protocol results a reducation of 29% decrease of CGRP-IR staining in DRG neurons (Zhang et al., 1998; Zhou et al., 1998) and a reduction of 75% of c-Fos expression in dorsal horn evoked by 5% formalin (Zhou et al., 1998).

6-Hydroxydopamine pretreatment

To reduce the function of sympathetic efferents, 6-OHDA was injected at a dose of 75mg/kg i.p. for three consecutive days (Zhang et al., 1998; Zhou et al., 1998) and microdialysis was performed on day 4. 6-OHDA was dissolved in 0.1% sodium metabisulfite in distilled water. Systemic pretreatment with 6-OHDA produces a 60-90% reduction in peripheral noradrenaline (Green et al., 1993a). The present protocol almost completely depletes catecholamines staining of the perviscular plexus and sciatic nerve in subcutaneous paw tissues (Levine et al., 1986; Zhang et al., 1998; Zhou et al., 1998).

Compound 48/80 pretreatment

To degranulate mast cells, rats were pretreated with compound 48/80 over a two day period. Compound 48/80 was dissolved in saline and administered at 1mg/kg, i.p. four times at 3 h intervals on day 1 and at 1.5mg/kg two times at a 7 h interval on day 2 (Hannon et al., 1995). Microdialysis was performed on day 4. The protocol largely blocks adenosine A_3 receptor agonist induced mast cells degranulation in rat hind paw (Hannon et al., 1995; Sawynok et al., 2000).

2.2 BEHAVIORAL AND PAW VOLUME ASSESSMENTS

2.2.1 Behavioral assessment for the neuropathic pain model

Baseline assessment for thermal threshold and mechanical threshold testing

Behavioral tests were conducting on day 7, 14 and 21 following surgery. No rats were used more than 3 times. All behavioral tests were conducted between 8:00 and 16:00 h. Rats were first weighed and allowed to acclimatize to the testing environment for 30minutes and to the testing apparatus for 40 minutes before baseline measurements were performed. Two baseline measurements were determined at a 30min interval and the average value was considered as the baseline value.

Thermal threshold latency measurement

Thermal threshold latency was determined according to the method described by Hargeaves et al. (1988), using a commercially available paw thermal stimulator system (UARD, San Diego, CA). Rats were placed, in pairs, in clear plastic chambers on the top of a glass surface. The temperature of the surface was maintained at 30°C. The heat source consisted of a high intensity projector lamp bulb (50W, 8V) placed underneath the glass floor and projecting through a 5 mm diameter aperture. The voltage of the heat source was controlled by a constant voltage supply. The stimulus current was maintained at 4.9 amperes while a 20.4 second cut-off was used to limit possible tissue damage. Paw withdrawal latencies (PWLs, the time from the start of the light beam to the lifting of the paw from the glass plate) of both the nerve-ligated paw and non-ligated paw were measured. To ensure consistency, the radiant heat stimulus was always applied to the mid-plantar surface of the hind paw and care was taken not to focus the light source on the skin that was not in contact with the glass floor.

Static mechanical threshold measurement

The rats were placed in individual plastic cages with a wire mesh bottom. To test the tactile threshold required to evoke withdrawal of the stimulated paw, von Frey filaments 0.41-15.14g (Semmes-Weinstein monofilaments, Stoelting Co., Wood Dale, IL) were applied perpendicularly to the mid-plantar part of the hind paw (both nerve-ligated paw and non-ligated paw), avoiding the footpads. The 50% paw withdrawal threshold (50% PWT) was determined using the Dixons up-down method (Chaplan et al., 1994). Briefly, tests were carried out by starting with the 2.04g filament. If there was no response, the consecutive stronger stimulus was applied, while if there was a positive response, the consecutive weaker stimulus was applied. The test was continued until the responses of five more stimuli after the first change in response had been obtained. The resulting pattern of responses was tabulated and the 50% response thresholds were computed using the formula (Dixon, 1980): 50% PWT (g) = $10^{[X_f+kd]}/1000$. Where X_f is the final filament value (in log units), k represents the pattern of responses (Dixon, 1980), and d is the mean difference in log units between stimuli. In cases where continuous positive or negative responses were obtained untill the cut off filaments, values of 0.25 g and 15 g were assigned respectively (Chaplan et al., 1994). Chaplan et al., 1994

2.2.2 Behavioral assessment for the formalin model

The formalin test was performed as previously described (Sawynok et al., 1998). Rats were placed in a 28×28×28 cm plexiglass observation chamber for an initial 20 min to allow acclimatization to the testing environment. Formalin (0.5%-5%) or formalin/drug combinations were injected subcutaneously in a volume of 50 µl into the dorsal aspect of the hind paw. Following injections, rats were returned to the observation chamber and monitored for flinching behaviors (lifting, shaking of hind paw and overt flinching with a ripple over the haunch) and hind paw biting/licking time. Two rats in adjacent chambers were observed at one time, with observations occurring in alternate 2 min bins. Recorded episodes were not corrected, thus values represent about half of the total behaviors expressed.

2.2.3 Paw volume measurement

Paw volumes were measured as previously described (Sawynok et al., 1999b; 2000) using a commercially available plethysmometer (Ugo Basile). The hind paws were immersed to the junction of the hairy and non-hairy skin and volumes were read from a digital display. Measurements were performed in triplicate at 30min intervals for up to 180min following injections. Values were standardized by expression as a percentage of individual preinjection volumes to accommodate the variation in body weights.

2.3 MICRODIALYSIS AND ADENOSINE ANALYSIS

2.3.1 Subcutaneous microdialysis

Rats were anaesthetized with sodium pentobarbital (45mg/kg, i.p. for induction, 10 mg/kg i.p. per 30min for maintenance) and body temperature was maintained with an electric warming pad. The microdialysis probe (LM-5 linear probe, 5mm active membrane length, 320μm OD, 35kDa wt cut-off; BAS, USA) was inserted into the subcutaneous area of the plantar surface of the rat hind paw (Fig. 2.1). An introducer needle was inserted through the tissue and the microdialysis probe was pulled gently through the needle. After withdrawal of the needle, the probe was adjusted so that the entire membrane window was totally covered by tissue. The inlet and outlet tubing were then fixed at the entry and exit points using tissue glue. The inlet tubing was then connected to a microsyringe pump (Harvard/22 USA) through FEP Teflon tubing (26cm length, 650μm OD, BAS, USA). Following implantation, the probe was perfused with standard Krebs-Henseleit solution (mM NaCl, 111; $NaHCO_3$, 26.2; NaH_2PO_4, 1.2; KCl, 4.7; $CaCl_2$, 1.8; MgCl, 1.2, at pH 7.4) at a rate of 2μl/min and the dialysate was collected at 10min interval in a 1.5ml microtube containing 10μl $ZnSO_4$ (0.15M) at room temperature. The probe was flushed for at least 2 hours to achieve a steady basal level (Chapter 4). After two baseline samples were collected, a 50μl volume of drug was injected into the plantar subcutaneous area approximately 3mm parallel to the dialysis membrane using a 30-gauge needle (Fig. 2.1). Post injection dialysate collection was started with a 1.5min delay to compensate for the dead space of the outlet tubing.

Fig. 2.1: Schematic illustration of the microdialysis system in the rat hind paw. The probe was placed into the subcutaneous space of the rat hind paw through an introducer needle and then perfused with standard Krebs-Henseleit solution by a microsyringe pump. (1) Microsyringe pump, (2) inlet tubing, (3) active dialysis membrane, (4) injection site of formalin or formalin and drug combination (3 mm parallel to the probe), (5) outlet tubing, (6) collecting tube.

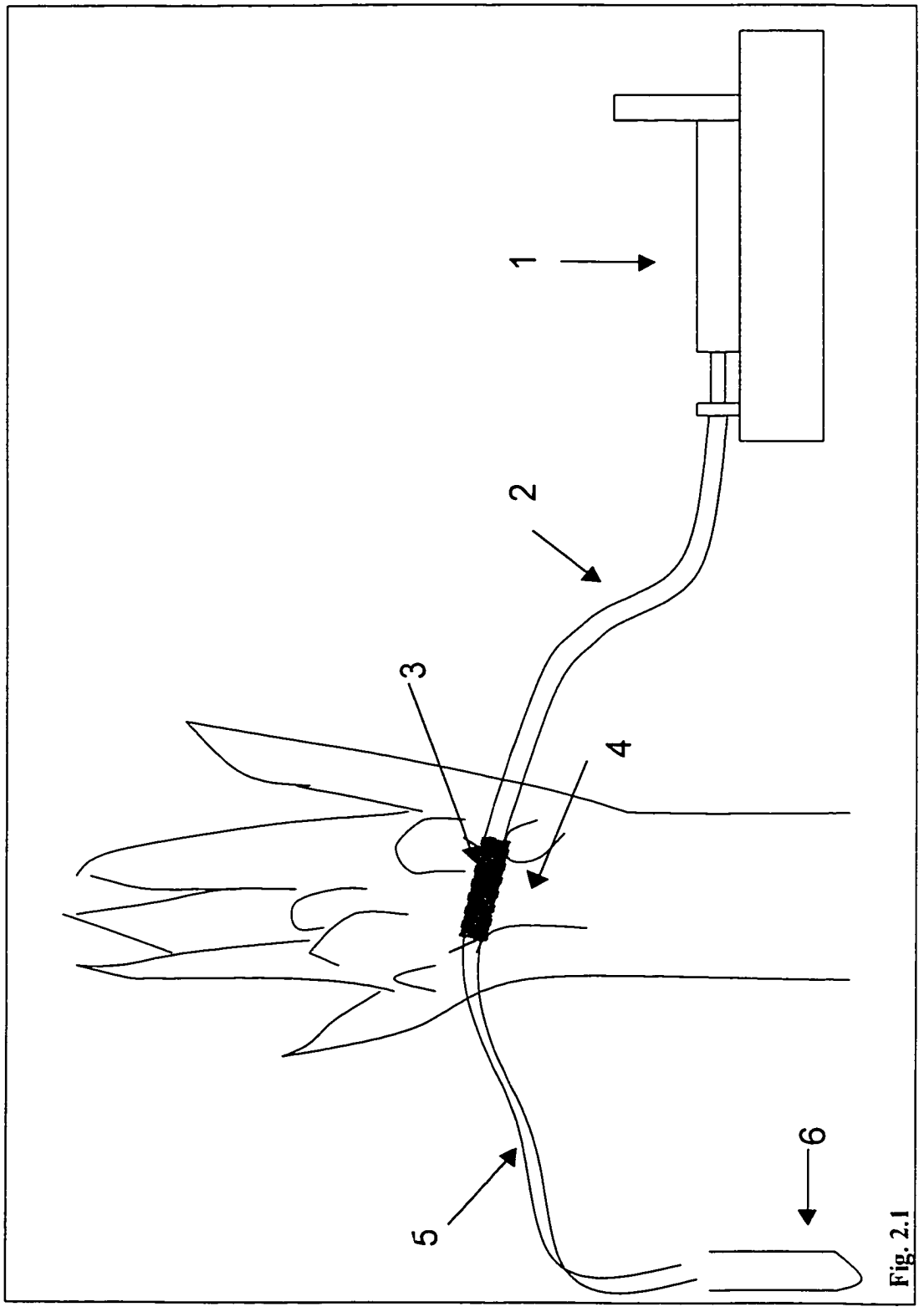


Fig. 2.1

2.3.2 Adenosine analysis

Adenosine was measured as described previously (Wang and White, 1998) with modification. Samples (20μl) were deproteinated with 10μl 0.15M $Ba(OH)_2$ and 10μl 0.15M $ZnSO_4$ immediately after collection, mixed and centrifuged at 11,600 g for 4 min in a Benchtop microcentrifuge (Beckman II). The supernatants (25μl) were carefully removed and added to 4μl of 4.5% chloroacetaldehyde in 1.5ml microcentrifuge tubes, which were then tightly sealed and boiled for 20 min to form 1-N^6-ethenoadenosine. Samples were allowed to cool to room temperature and then stored at 4°C no more than 72 hours before being assayed (stable for > 1 week, Wojcik and Neff, 1983). Adenosine standards were prepared from stock solutions made in millipore water and stored at −15°C. Standards were diluted with appropriate Krebs-Henseleit medium with or without drugs and processed simultaneously and identically to the dialysate sample.

Adenosine levels were determined by HPLC with fluorescence detection (Craig and White, 1992). Derivatized samples (20μl) were injected through a Water's 712 WISP autosampler into a pump solvent delivery system (Waters model 501) attached to a Water's 4μm C_{18} reversed-phase radial-pak cartrige (Nova-Pak C_{18}, 5mm I.D.) contained in a compressible Radial-Pak RCM 8x10 cartidge holder (Waters model 80100). The mobile phase consisted of 50mM acetate buffer (pH 4.5), 2.2mM 1-octanesulfonic acid and 18% acetonitrile, which was run at a flow rate of 0.7ml/min. The ethanol-adenosine peaks were detected with a fluorescence detector (Waters model 420 AC at a wavelegth of 290nm and a long pass emission filter at 420nm) and were quantitated by direct comparison of peak heights to those of known concentrations of adenosine standards. Adenosine peaks were identified by using standard adenosine to spike the peak or using

adenosine deaminase (10 unit/ml for 10min at room temperature) to eliminate the peak. Adenosine was expressed as pmol/20µl/10min. Total evoked adenosine release was determined as the cumulative amount of evoked release during a 60min interval (sum of the individual time point release minus the average basal release) and expressed as pmol/120µl/60min.

2.4 DRUGS AND CHEMICALS

Adenosine, N^6-cyclopentyladenosine (CPA), R-N^6-(2-phenylisopropyl)-adenosine (L-PIA), 2-*p*-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido adenosine hydrochloride (CGS 21680), 5'-amino-5'-deoxyadenosine (NH_2dAD), 2'deoxycoformycin (DCF), caffeine (CAF), amitriptyline, desipramine, L-glutamate, dizocipine maleate (MK 801), 6-cyano-7-nitroquinoxaline (CNQX), compound 48/80, mepyramine maleate, ketanserin tartrate, phentolamine hydrochloride, and formalin (37% formaldehyde) were dissolved in saline. 8-Cyclopentyl-1,3-dimethylxanthine (CPT) was dissolved in 10% dimethylsulfoxide (DMSO) or 0.02 N NaOH. Capsaicin was dissolved in 10% ethanol, 10% Tween 80, and 80% Saline. 6-Hydroxydopamine (6-OHDA) was dissolved in 0.1% sodium metabisulfite in distilled water. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA), except DCF from Parke-Davis Pharmaceutical Research Division of Warner-Lambert (Ann Arbor, MI, USA), and formalin from British Drug Houses (Toronto, Ontario, CAN).

2.5 DATA ANALYSIS

Data were analyzed with sigmaStat software. For mutiple treatments with normal distribution and equal variance, one way analysis of variance (ANOVA) was conducted when the effects of mutiple treatments at same time point (behavioral or microdialysis studies) and the cumulative or peak effects of mutiple treatments (behavioral and microdialysis studies) were investigated. One way repeated ANOVA was conducted when values from same group of indivadules over the time course were compared (eg. glutamate evoked adenosine release over time course). Two way repeated ANOVA was conducted when both the values from the same group of indivadules over the time course and and values from different treatments were compared (eg. for all neurochemical pretreatment groups in paw volume and microdialysis studies). All pairwise post-hoc was conducted with Student-Newman-Keuls test (SNK) test. Student t-test was conducted when only two groups were compared.

For the treatment effects that are not normally distributed (eg. 50% paw withdrawal threshold for mechanical threshold testing), Kruskal-Wallis one way ANOVA on rank was conducted if mutiple groups were compared, and the Mann-Whitney rank sum test were conducted if only two groups were compared. $P<0.05$ was considered significantly different from respective values for all tests.

CHAPTER 3

PERIPHERAL ANTIHYPERALGESIC EFFECTS PRODUCED BY ADENOSINE A_1 RECEPTOR AGONISTS AND INHIBITORS OF ADENOSINE METABOLISM IN A RAT NEUROPATHIC PAIN MODEL

3.1 INTRODUCTION

Neuropathic pain induced by injury or disease is characterized by spontaneous or ongoing pain, hyperalgesia and allodynia (Woolf and Doubell, 1994; Millan, 1999). Both central and peripheral mechanisms are believed to be involved in the initiation and maintenance of these symptoms (Coderre and Katz, 1997; Woolf and Salter, 2000). Central mechanisms include increased spontaneous neuronal activity in the spinal cord, functional loss of inhibitory control, reorganization of primary afferents in the spinal cord, and decreased threshold of activation of the neurons in the thalamus and cortex (Coderre et al., 1993; Woolf and Doubell, 1994; Woolf and Salter, 2000). Peripheral mechanisms include nerve-injury-induced ectopic activity in damaged nerves and in dorsal root ganglion neurons, development of abnormal sensory receptors and abnormal sympathetic-sensory couplings (Coderre and Katz, 1997).

While morphine is less effective in neuropathic pain than in acute pain, both in rodents and in humans (Lee et al., 1995; Ossipov et al., 1995; Benedetti et al., 1998; Dellemijn, 1999), the effectiveness of adenosine in neuropathic pain has been demonstrated in both animal and human studies (reviewed Segerdahl and Sollevi, 1998; Karlsten and Gordh, 2000). In rodents, spinal adenosine analogs reverse spontaneous pain (Sjölund et al., 1996), allodynia (Sosnowski and Yaksh, 1989; Lee and Yaksh, 1996; Cui et al., 1997; Sjölund et al., 1998; Kowaluk et al., 2000), and hyperalgesia (Yamamoto and Yaksh, 1991) in neuropathic pain models. Besides adenosine analogs, spinal or systemic administration of adenosine (Lavand'homme and Eisenach, 1999) or increasing adenosine levels by using inhibitors of adenosine metabolism (Lavand'homme and

Eisenach, 1999; Lynch et al., 1999; Chiari and Eisenach, 1999) also alleviates allodynia or hyperalgesia in several neuropathic pain models. In humans, spinal administration of an adenosine A_1 receptor agonist produces long lasting analgesia and abolishes allodynia in one instance (Karlsten and Gordh, 1995), and systemic infusion of adenosine alleviates or abolishes neuropathic pain syndromes in patients with neuropathy (Belfrage et al., 1995; 1999; Sollevi et al., 1995; Segerdahl and Sollevi, 1998; Sjölund et al., 2000). Although the effects of adenosine in neuropathic pain have been largely attributed to spinal actions, the actual amount of adenosine that enters the CNS is unclear, as adenosine has a very short half life in blood (seconds) (Sollevi, 1991), and there is a metabolic barrier within the endothelial cells which prevents adenosine from entering the CNS (Pardridge et al., 1994). A peripheral antinociceptive action of adenosine has been demonstrated in the rat formalin test (Karlsten et al., 1992; Doak and Sawynok, 1995; Sawynok et al., 1998) and prostaglandin E_2-induced hyperalgesia model (Taiwo and Levine, 1990; Aley and Levine, 1997), and it is possible that part of the pain alleviating effect of systemically infused adenosine in humans is due to peripheral actions.

The aim of the present study was to use the spinal nerve ligation model (Kim and Chung, 1992) to determine whether there is a peripherally mediated effect of adenosine receptors in a neuropathic pain model. Both electrophysiological (Na et al., 1993) and behavioral observations (Xie et al., 1995a; Moon et al., 1999) suggest peripherally mediated modulation occurs in this model. A spinal action of adenosine has already been demonstrated in this model (Lee and Yaksh, 1996; Lavand'homme and Eisenach, 1999). To determine peripheral actions of adenosine, adenosine analogs and inhibitors of adenosine metabolism were injected subcutaneously into the nerve-ligated paw.

Potential actions mediated by systemic absorption were evaluated by administering drugs into the non-ligated paw. The involvement of adenosine receptor activation was examined by using adenosine antagonists.

3.2 MATERIALS AND METHODS

Spinal nerve ligation surgery, thermal hyperalgesia and static mechanical allodynia measurements were performed as described in Chapter 2. All drugs were given in a blinded manner. Drugs were injected subcutaneously into the nerve-ligated paw or contralateral paw in a final volume of 50μl under brief halothane anesthesia, into the dorsal aspect of the rat paw to avoid a direct needle injury effect on the plantar aspect. If two drugs were used, they were mixed just before injection and coadministered in a 50μl final volume. Post-drug measurements were performed at 30, 60, 90 and 120min for all the groups except the 240min DCF groups in thermal threshold testing, in which post-drug measurements were performed at 60, 120, 180 and 240min. Rats were returned to their cages and allowed free access to food and water after each measurement in the long time course DCF groups to reduce possible stress, and were returned to testing boxes 20 min before the next measurement.

For the thermal hyperalgesia test, data were presented as a time course of PWLs or a cumulative change in PWLs during the time course and data were presented as means ± standard error. The cumulative change in PWLs was used to compare the general effect of different treatments as it reflects both the maximal effect and the duration of an action. This value was calculated according to the formula: Cumulative change in PWL=(PWL_1-PWL_0)+(PWL_2-PWL_0)+(PWL_3-PWL_0)+(PWL_4-PWL_0). PWL_0 was the baseline value

before drug administration. $PWLs_{1-4}$ were the PWLs values from the four post-drug time points. Statistical significance was established by one-way ANOVA followed by an all pairwise SNK test. If only two groups were compared, a student's *t*-test was used. For the mechanical threshold data, data were presented as the time course of the 50% PWT (g) and the Kruskal-Wallis one way ANOVA on ranks test was used. For paw volume studies, capsaicin pretreatment, 6-OHDA pretreatment and compound 48/80 pretreatment were conducted as described in Chapter 2. Data were expressed as a time course of the changes in paw volume as well as cumulative percentage increase, which was the sum of differences from the mean baseline. Comparisons of more than two groups were analyzed with ANOVA followed by SNK test. $P<0.05$ was considered significantly different from respective values for all tests.

3.3 RESULTS

3.3.1 Thermal hyperalgesia and static mechanical allodynia produced by spinal L5 and L6 nerve ligation

Spinal nerve ligation resulted in a significant reduction in PWL to radiant heat stimuli and a significant reduction in PWL to the von Frey hairs stimuli in the nerve-ligated paw as compared to the non-ligated paw. Both thermal hyperalgesia and mechanical allodynia were well maintained during the testing periods, day 7 to 21 post-surgery (Table 3.1). This result is consistent with the stable thermal hyperalgesia and mechanical allodynia observed in this model in previous studies (Kim and Chung, 1992; Esser and Sawynok, 1999).

Table 3.1: Basal thresholds to thermal and mechanical stimuli in rats following spinal nerve ligation

days after surgery	PWL (radiant heat, sec)		50% PWT (von Frey hair, g)	
	nerve-injured paw	non-injured paw	nerve-injured paw	non-injured paw
7	7.42 ± 0.11* (96)	9.45 ± 0.17	2.36 ± 0.11* (24)	13.04 ± 0.33
14	7.12 ± 0.09* (78)	8.91 ± 0.12	2.56 ± 0.10* (12)	13.56 ± 0.25
21	7.50 ± 0.11* (54)	9.50 ± 0.15	2.25 ± 0.05* (12)	13.58 ± 0.21

Data indicated that spinal nerve ligation (L5/L6) induces stable thermal hyperalgesia and mechanical allodynia from day 7 to day 21 after surgery. * $p < 0.05$ compared to non-injured paw. No difference detected between different values in the same column.
Numbers in parentheses represent the number of rats in each group.

3.3.2 Antihyperalgesic effect of peripheral administration of adenosine receptor agonists

Local injection of the adenosine A_1 receptor agonists, CPA (15-50nmol) and L-PIA (39nmol), produced an antihyperalgesic effect (Figs. 3.4). CPA, at 15nmol, reversed thermal hyperalgesia at 60min while at 50nmol, it reversed hyperalgesia from 30 to 60min after drug administration (Fig. 3.1A). L-PIA also completely reversed thermal hyperalgesia from 30 to 120min at 39 nmol (Fig. 3.1B) (39 nmol is the solubility limit of L-PIA in saline). The antihyperalgesic effect produced by CPA and L-PIA was due to a peripheral effect within the nerve-ligated paw, as when the active doses of CPA and L-PIA were administered into the non-ligated (contralateral) paw, no antihyperalgesic effect was observed (Fig. 3.2). CPA and L-PIA did not change PWLs in the non-ligated paw, either when drugs were injected into the nerve-ligated paw (Fig 3.4 for cumulative change), or the non-ligated paw (data not shown). No discernable sedative effect or motor effects were observed following CPA and L-PIA treatment.

The effect of the selective adenosine A_{2A} receptor agonist, CGS 21680, on thermal hyperalgesia also was examined. At 15 and 50nmol, peripheral injection of CGS 21680 neither alleviated nor exaggerated thermal hyperalgesia (Fig 3.4). A modest sedative or motor effect was observed with 50nmol CGS 21680 indicated by reduced exploration, reduced grooming, drooping eyelids and flattened body posture.

To determine if the effects of adenosine receptor agonists on nerve injury-induced thermal hyperalgesia were mediated by cell surface adenosine receptors, the non-selective adenosine receptor antagonist, caffeine, was used. When coadministered with CPA 50nmol, caffeine 1500nmol completely blocked the antihyperalgesia produced by

CPA (Fig. 3.3). Caffeine itself did not produce a significant effect (Fig. 3.3). We tried to use the selective adenosine A_1 receptor antagonist CPT to further investigate the receptor subtype, but no conclusive results were obtained due to vehicle effects.

3.3.3 *Antihyperalgesic effects of peripheral administration of an adenosine kinase inhibitor and an adenosine deaminase inhibitor*

To determine the effect of peripheral inhibition of adenosine metabolism on thermal hyperalgesia, the adenosine kinase inhibitor, NH_2dAD and the adenosine deaminase inhibitor, DCF were injected into the nerve-ligated paw. NH_2dAD (30-100nmol) and DCF (100nmol) produced antihyperalgesic effect (Fig. 3.8). NH_2dAD (100nmol) produced an early onset and short lasting antihyperalgesic effect (Fig. 3.5). A complete reversal of the thermal hyperalgesia in the nerve-ligated paw was observed only at the first testing time point (30min) but not at later time points. When DCF was injected into the nerve-ligated paw (100nmol), a reversal of hyperalgesia was observed with a later onset of action, from 90 to 120min (Fig. 3.7). Another group of rats was used to test the drug effect every 60min up to 240 min after drug administration to further examine this delayed effect. Using this protocol, the antihyperalgesic effect of DCF was observed from 120-240min following drug injection (Fig. 3.6). When drugs were administered into the non-ligated paw (contralateral paw), neither NH_2dAD nor DCF produced an antihyperalgesic effect in the nerve-ligated paw (Figs. 3.5 and 3.6), indicating a local peripheral mediated effect. Caffeine (1500nmol) completely reversed the antihyperalgesia produced by NH_2dAD (100nmol) and DCF (100nmol) when coadministered into the nerve-ligated paw (Fig. 3.7). No discernable sedative or motor effects were observed following NH_2dAD or DCF treatment.

Fig. 3.1: Increases in PWL by local injection of adenosine receptor agonists CPA (A) and L-PIA (B). Time course depicts the effect of CPA (A) and L-PIA (B) on PWLs in the nerve-ligated paw. Open symbols on the left represent the baseline threshold of respective non-ligated paws (post-drug PWLs of non-ligated paw were omitted, as no changes were observed). Drugs were administered into the nerve-ligated paw. *$p<0.05$, compared to saline group (one way ANOVA followed by SNK, means ± SEM, n=6-12).

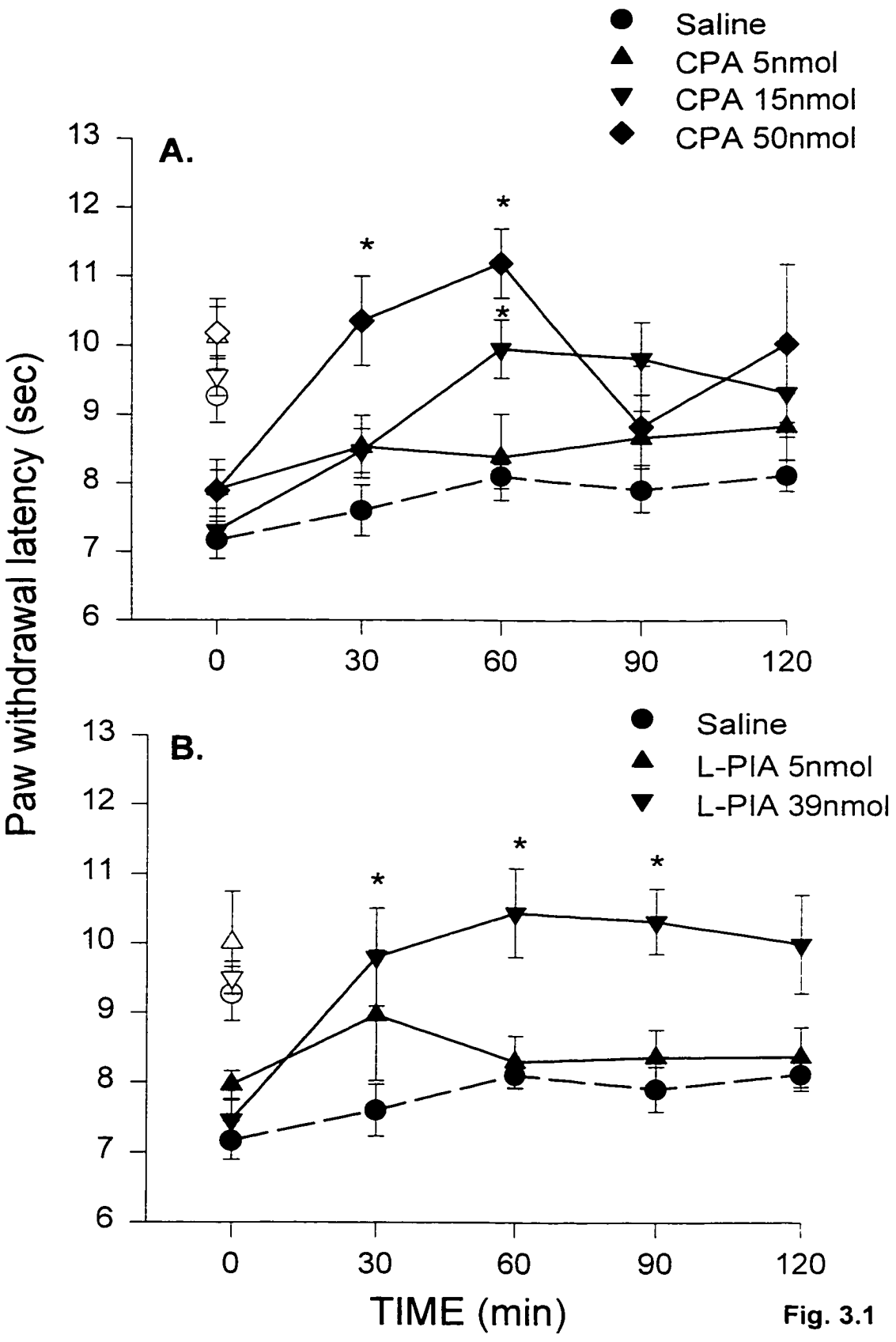

Saline
CPA 5nmol
CPA 15nmol
CPA 50nmol
A.
13
12
11
10
9
8
7
6
0
30
60
90
120
*
*
*
Saline
L-PIA 5nmol
L-PIA 39nmol
B.
13
12
11
10
9
8
7
6
0
30
60
90
120
*
*
*
Paw withdrawal latency (sec)
TIME (min)

Fig. 3.1

Fig. 3.2: Effects of CPA and L-PIA on PWLs when injected into the nerve-ligated paw (ipsilateral, ipsi) and non-ligated paw (contralateral, contr). (A) Time course of PWLs and (B) cumulative change in PWLs over 120min produced by CPA (15 and 50nmol) and L-PIA (39 nmol) in the nerve-ligated paw are depicted. Symbols on the left in panel (A) represent the baseline threshold of the respective non-ligated paw (post-drug contralateral paw PWL not shown). Drugs were administered into the nerve-ligated paw (solid symbols) or the non-ligated paw (open symbols) respectively. *$p<0.05$ compared to saline group, *t* $p<0.05$ compared to nerve-ligated group (one way ANOVA followed by SNK, means ± SEM, n=6-12).

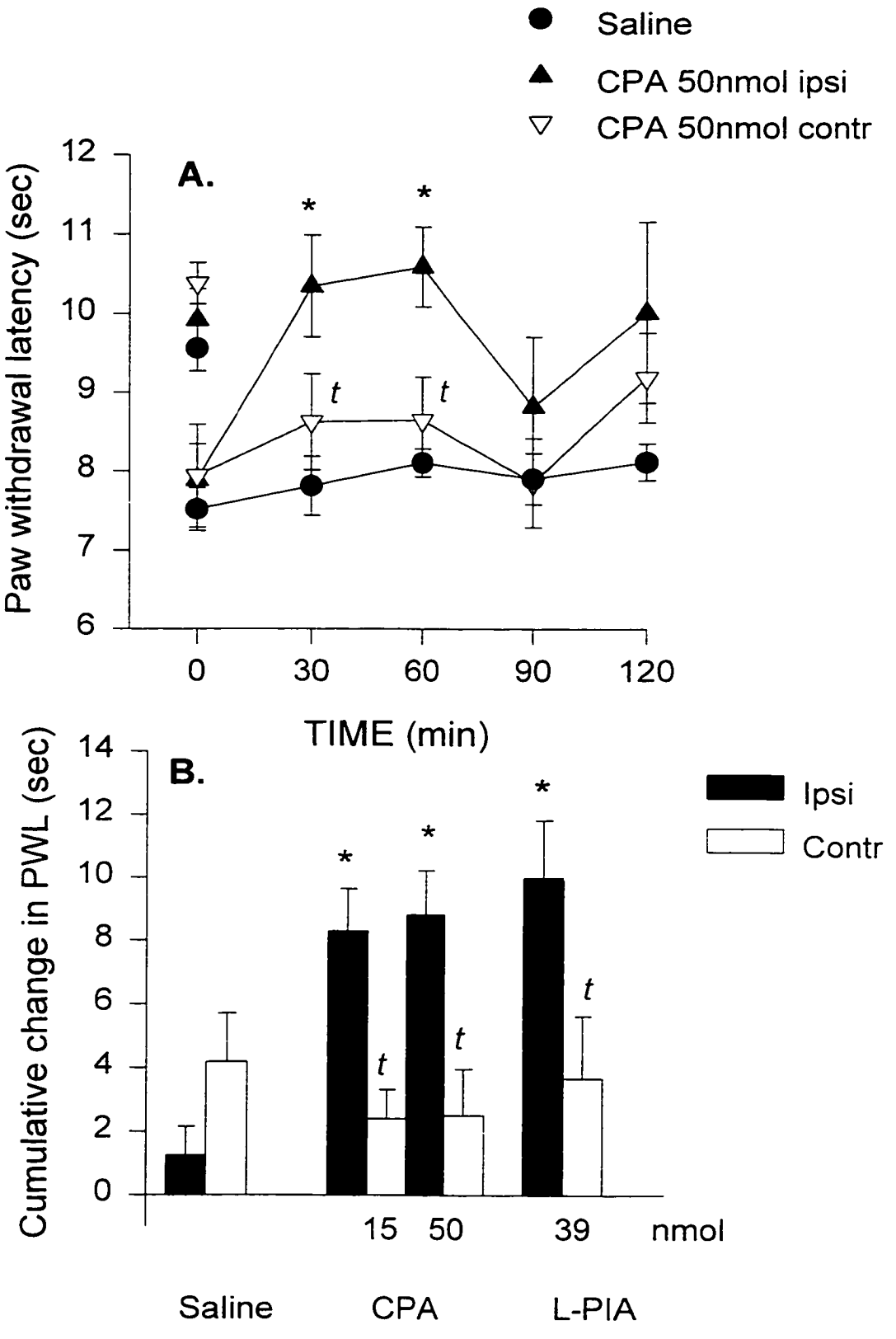

Saline
CPA 50nmol ipsi
CPA 50nmol contr
A.
Paw withdrawal latency (sec)
12
11
10
9
8
7
6
0
30
60
90
120
TIME (min)
B.
Cumulative change in PWL (sec)
14
12
10
8
6
4
2
0
Ipsi
Contr
15
50
39
nmol
Saline
CPA
L-PIA

Fig. 3.2

Fig. 3.3: Effects of the adenosine non-selective receptor antagonist caffeine on CPA induced PWLs changes. (A) Time course and (B) cumulative change over 120min of CPA 50nmol, with or without caffeine (CAF 1500 nmol), on PWLs in the nerve-ligated paw are depicted. Open symbols in panel (A) represent the baseline of respective non-ligated paws (post-drug contralateral paw PWL not shown). Drugs were administered into the nerve-ligated paw. *$p<0.05$, compared to saline group; *t* $p<0.05$, compared to CPA group (one way ANOVA followed by SNK, means ± SEM, n=8-9 per group except for caffeine group where n=5).

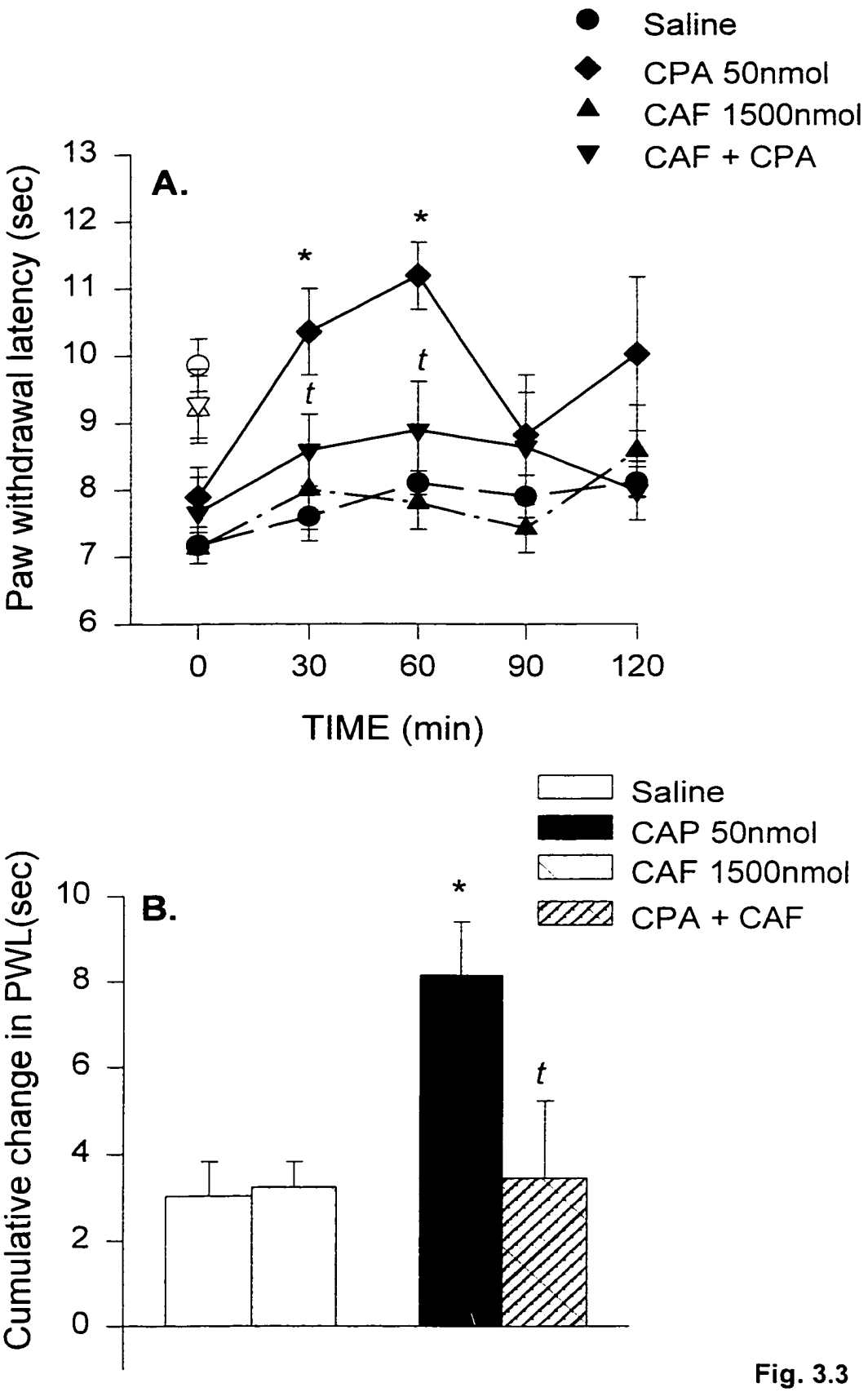

Saline
CPA 50nmol
CAF 1500nmol
CAF + CPA
A.
Paw withdrawal latency (sec)
13
12
11
10
9
8
7
6
0
30
60
90
120
TIME (min)
*
*
t
t
Saline
CAP 50nmol
CAF 1500nmol
CPA + CAF
B.
Cumulative change in PWL(sec)
10
8
6
4
2
0
*
t

Fig. 3.3

Fig. 3.4: Dose-response curve of cumulative changes in PWL in nerve-ligated paw induced by the adenosine A_1 receptor agonists CPA, L-PIA and adenosine A_2 receptor agonist, CGS21680. Filled symbols depict the cumulative changes in nerve-ligated paw. open symbols depict the cumulative changes in the contralateral paw. Drugs were administered into the nerve-ligated paw. * $p<0.05$ compared to saline (one way ANOVA followed by SNK, data from Figs. 3.1-3.3, CGS 21680 n=6).

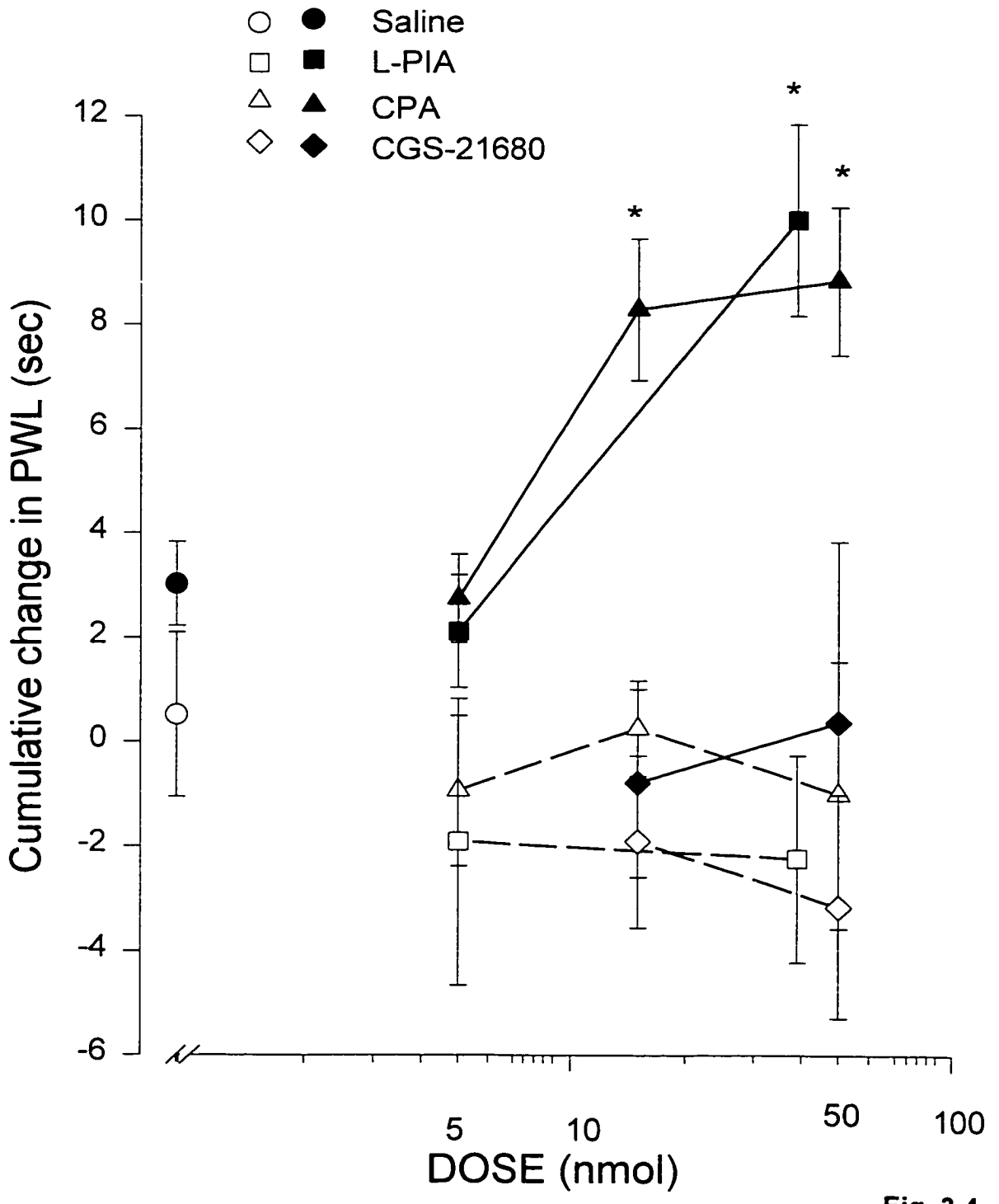

Saline
L-PIA
CPA
CGS-21680
Cumulative change in PWL (sec)
12
10
8
6
4
2
0
-2
-4
-6
*
*
*
5
10
50
100
DOSE (nmol)

Fig. 3.4

Fig. 3.5: Effects of the adenosine kinase inhibitor NH_2dAD 100nmol on PWL when injected into nerve-ligated paw (ipsi) or non-ligated paw (contr). (A) Time course and (B) cumulative change in PWLs in nerve-ligated paw over 120min are depicted. Symbols in left in panel (A) represent the baseline of respective non-ligated paws (post-drug contralateral paw PWL not shown). Drugs were administered into the nerve-injured paw (solid symbols) or non-ligated (open symbols) paw. *$p<0.05$, compared to saline group, *t* $p<0.05$, compared to nerve-ligated paw injection group (one way ANOVA followed by SNK, means ± SEM, n=6-8).

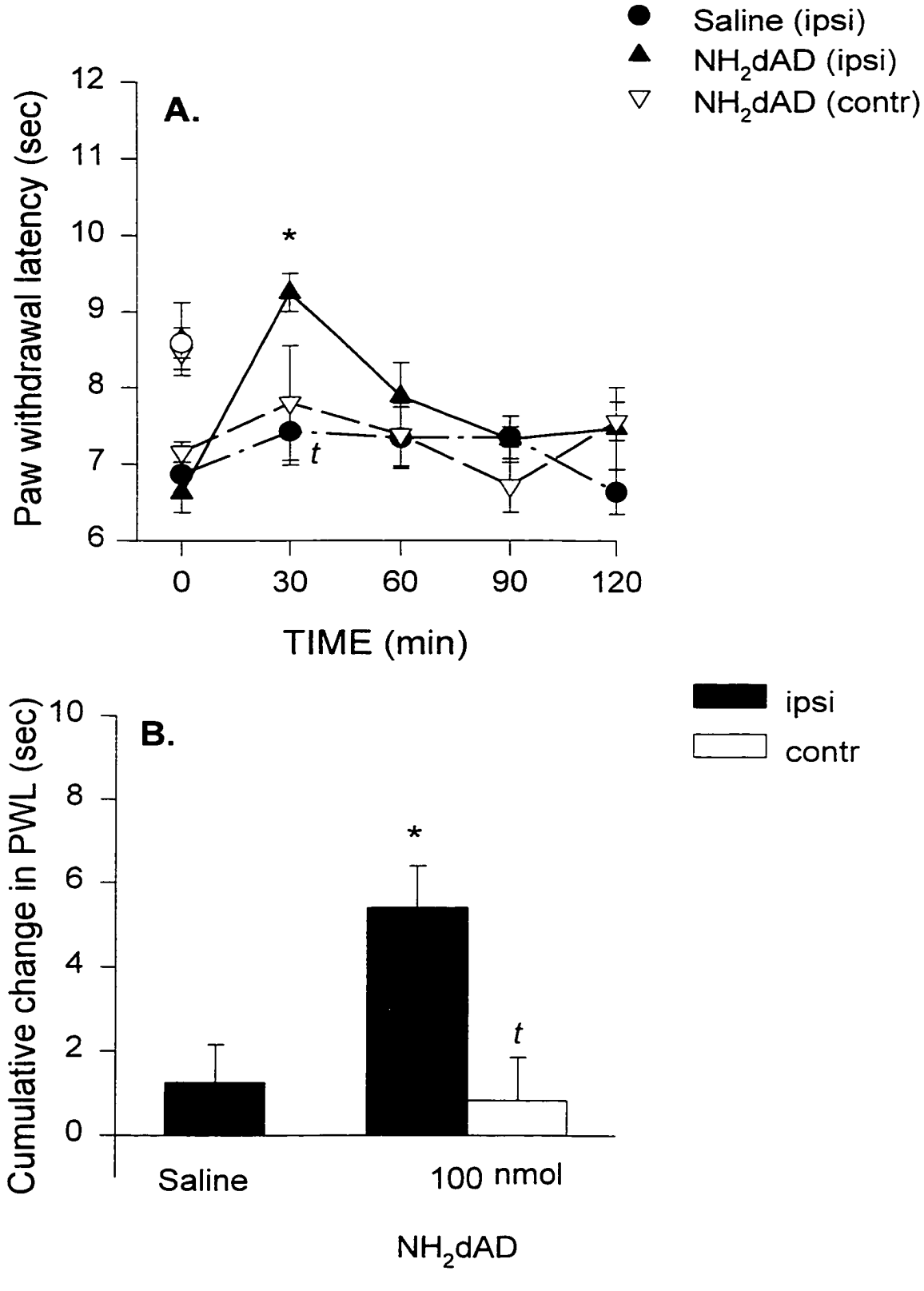
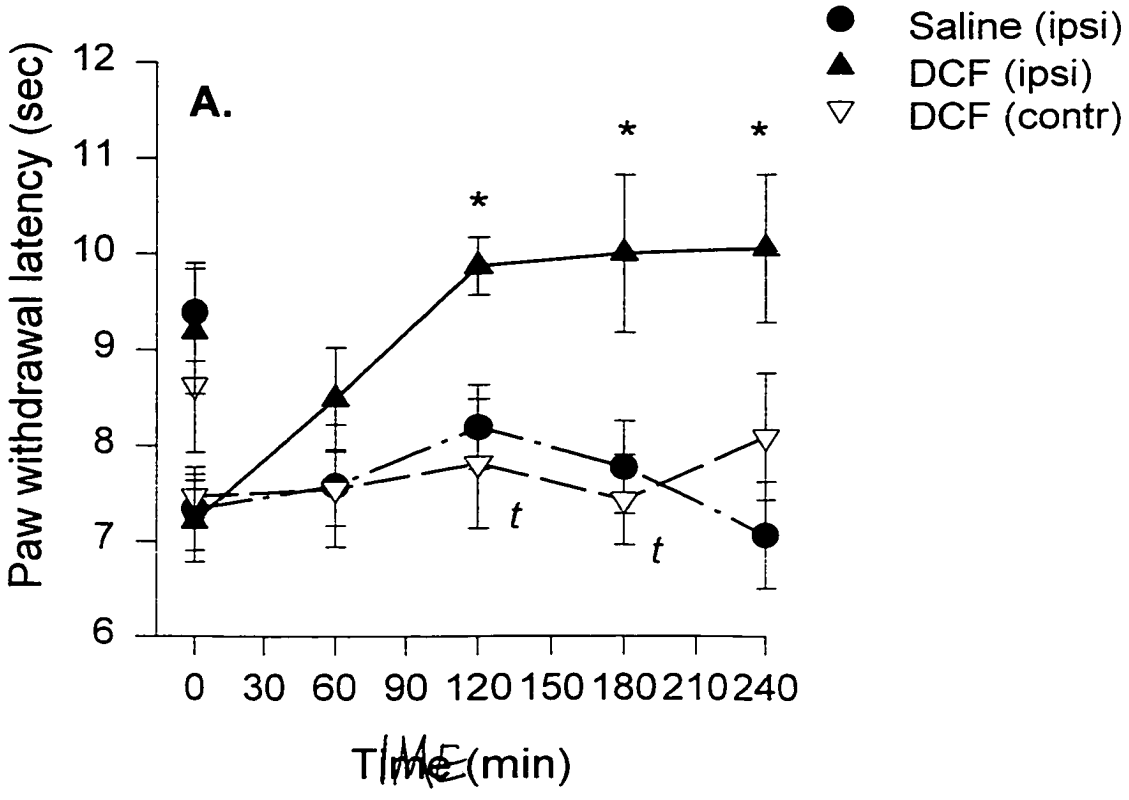

Saline (ipsi)
NH2dAD (ipsi)
NH2dAD (contr)
A.
Paw withdrawal latency (sec)
12
11
10
9
8
7
6
*
t
0
30
60
90
120
TIME (min)
B.
ipsi
contr
Cumulative change in PWL (sec)
10
8
6
4
2
0
*
t
Saline
100 nmol
NH2dAD

Fig. 3.5

Fig. 3.6: Effects of the adenosine deaminase inhibitor, DCF, on PWL when injected into nerve-ligated paw (ipsi) or non-ligated paw (contr). (A) Time course and (B) cumulative change in PWLs in nerve-ligated paw over *240min* are depicted. Symbols in left in panel (A) represent the baseline of respective non-ligated paws (post-drug contralateral paw PWL not shown). Drugs were administered into the nerve-ligated paw (solid symbols) or non-ligated (open symbols) paw. *NOTE: These three groups were the only groups that the PWL was measured at 60, 120, 180 and 240min post-drug.* *$p<0.05$, compared to saline, *t* $p<0.05$, compared to nerve-ligated paw injection group (one way ANOVA followed by SNK, means ± SEM, n=6).


Saline (ipsi)
DCF (ipsi)
DCF (contr)
A.
Paw withdrawal latency (sec)
12
11
10
9
8
7
6
0 30 60 90 120 150 180 210 240
Time (min)
*
*
*
t
t

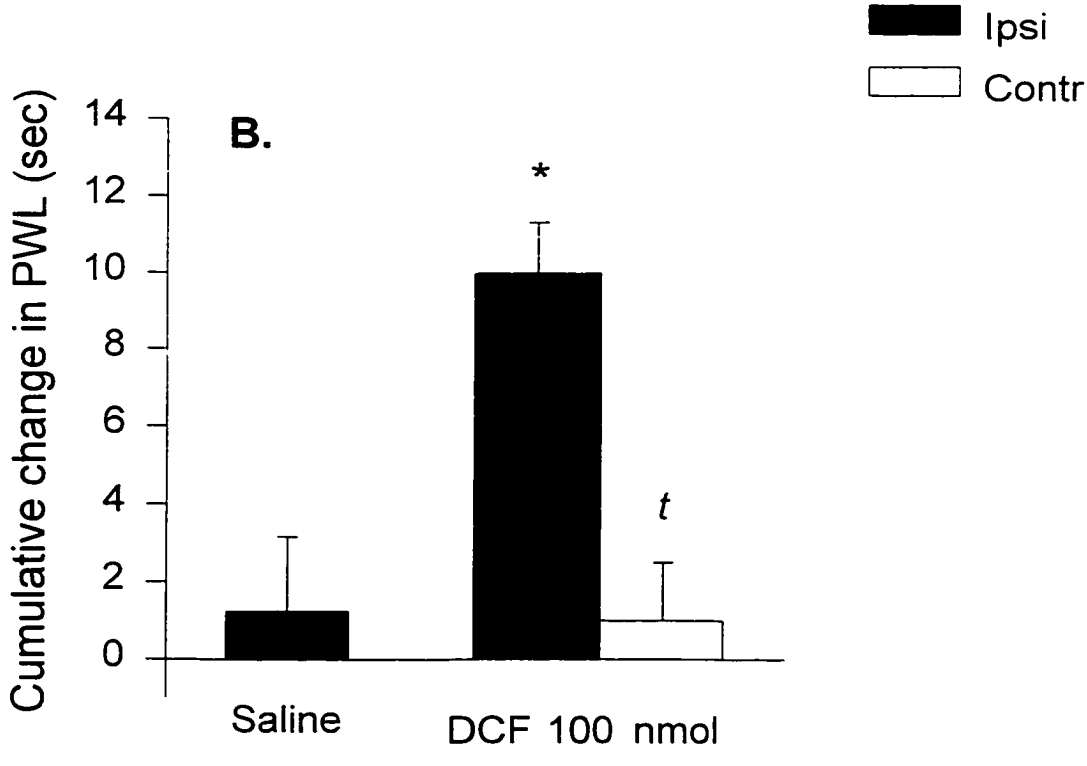

Ipsi
Contr
B.
Cumulative change in PWL (sec)
14
12
10
8
6
4
2
0
Saline
DCF 100 nmol
*
t

Fig 3.6

Fig. 3.7: The effect of the adenosine non-selective antagonist, caffeine (CAF) on DCF and NH_2dAD induced PWL changes. (A) Time course of PWL of DCF with or without caffeine, and (B) cumulative change in PWLs of both NH_2dAD 100nmol and DCF 100nmol without or with caffeine on PWL in the nerve-ligated paw are depicted. Symbols in the left in panel (A) represent the baseline of respective non-ligated paws (post-drug contralateral paw PWL not shown). Drugs were administered into the nerve-ligated paw. n=8 per group. *$p<0.05$ compared to saline group (one way ANOVA followed by SNK), *t* $p<0.05$, compared to respective without caffeine group (t-test, means ± SEM, n=8).

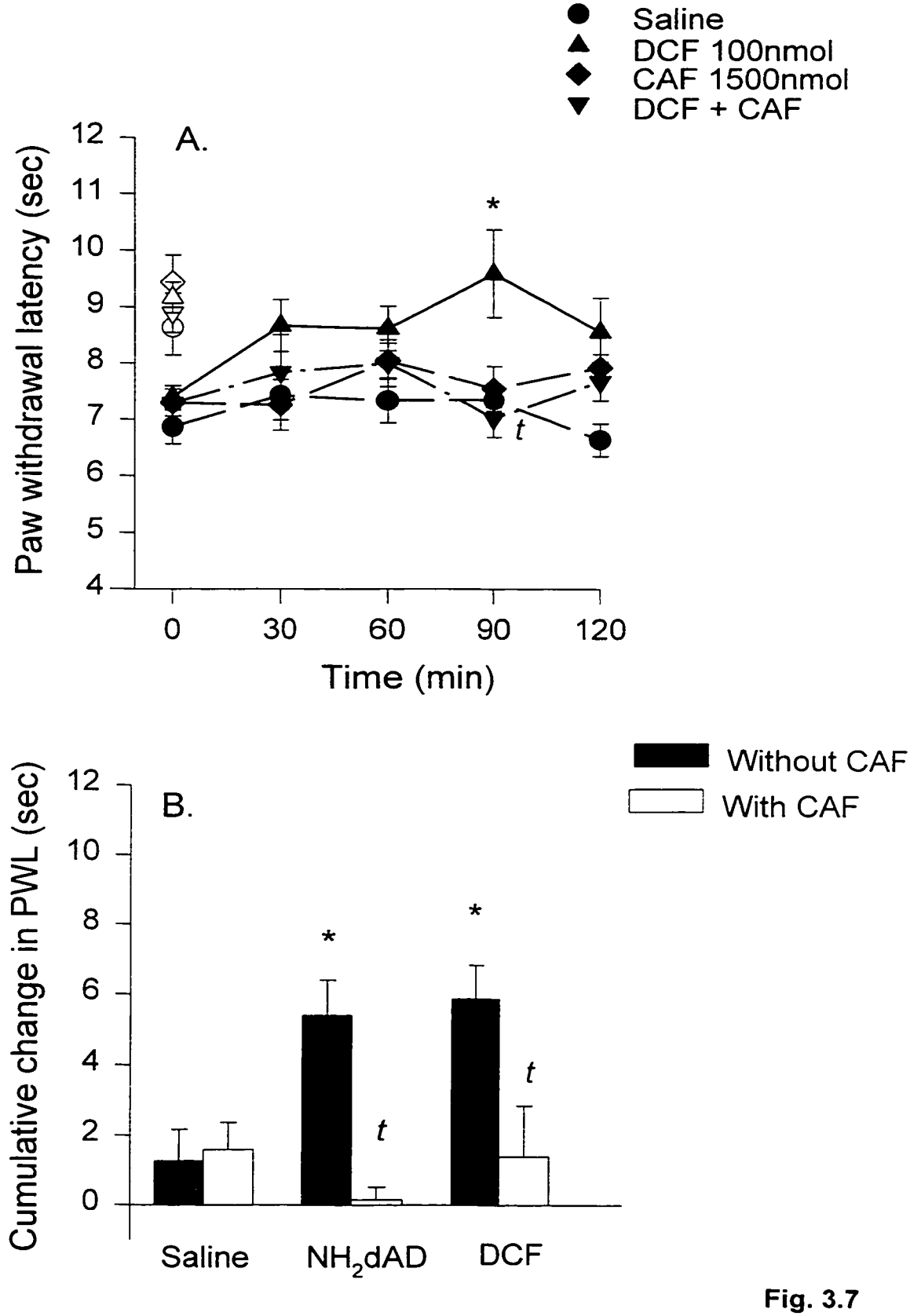

Saline
DCF 100nmol
CAF 1500nmol
DCF + CAF
A.
Paw withdrawal latency (sec)
12
11
10
9
8
7
6
5
4
0
30
60
90
120
Time (min)
*
t
Without CAF
With CAF
B.
Cumulative change in PWL (sec)
12
10
8
6
4
2
0
*
*
t
t
Saline
NH2dAD
DCF

Fig. 3.7

Fig. 3.8: Dose-response curve for cumulative changes in PWL induced by the adenosine kinase inhibitor, NH_2dAD and the adenosine deaminase inhibitor DCF. Values represent a time course over 120min for all groups. Filled symbols depict the cumulative changes in nerve-ligated paw, open symbols depict the cumulative changes in the contralateral paw. Drugs were administered into the nerve-ligated paw. $P<0.05$ compared to saline (means ± SEM, one way ANOVA followed by SNK, data from Fig. 3.5-3.7).

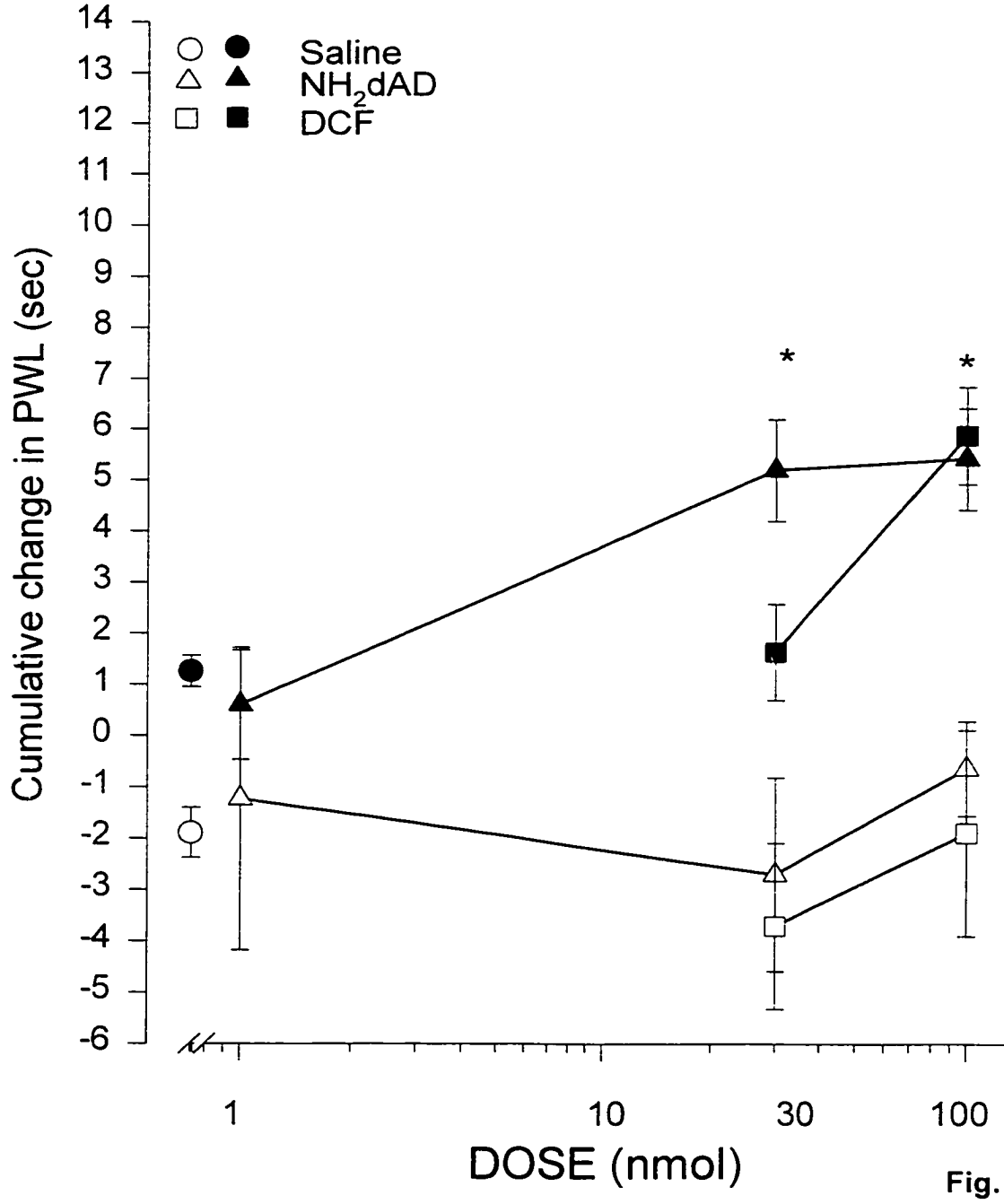

Saline
NH2dAD
DCF
Cumulative change in PWL (sec)
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
-2
-3
-4
-5
-6
*
*
1
10
30
100
DOSE (nmol)

Fig. 3.8

3.3.4 Lack of the effect of adenosine receptor agonists and inhibitors of adenosine metabolism on mechanical allodynia produced by nerve ligation

The adenosine A_1 receptor agonists CPA and L-PIA, the adenosine kinase inhibitor NH_2dAD, and the adenosine deaminase inhibitor DCF, at doses that completely reversed thermal hyperalgesia, did not exhibit a peripheral effect on mechanical allodynia (Fig. 3.9).

3.3.5 Paw volume effects produced by adenosine A_1 receptor agonists

During these experiments, we observed that CPA and L-PIA produced a significant paw edema when injected into the hind paw. The maximal paw edema produced by CPA is attained at 5nmol, and declines at higher doses (15-50nmol) (Fig. 3.10). When 50nmol CPA was injected into the contralateral paw, the paw volume effect produced by 5nmol CPA in the ipsilateral paw was attenuated (Fig. 3.10), indicating a systemic inhibitory effect on paw edema produced by CPA at higher doses. Mepyramine (histamine H_1 receptor antagonist), ketanserin (5-HT_2 receptor antagonist) and phentolamine (α_1 and α_2-adrenoreceptor antagonist) inhibited the increase in paw volume produced by CPA 5nmol (Fig. 3.11A). Mepyramine also inhibited the small increase in paw volume seen with saline (Fig 3.11A), suggesting involvement of histamine release in this response.

The adenosine A_2 receptor agonist CGS 21680 also produced a moderate paw volume effect, but less pronounced than CPA (data not shown). Local injection of NH_2dAD and DCF (10-100nmol) produced some increase in paw volume, but it was of much smaller magnitude compared with the increase produced by CPA and L-PIA (Fig 3.12).

The involvement of mast cells, primary sensory afferents and the sympathetic nervous systems were determined using compound 48/80, capsaicin and 6-OHDA respectively (Chapter 2). Systemic pretreatment with compound 48/80 markedly decreased, and systemic pretreatment with capsaicin partially decreased, the paw volume effect produced by CPA (5nmol) (Fig 3.13), while systemic pretreatment with 6-OHDA did not produce an effect (data not shown).

To investigate the possible influence of paw edema on the antihyperalgesia produced by CPA, the effect of mepyramine, a histamine H_1 receptor antagonist, on the effect of CPA on PWL was determined. At a dose that completely blocked the paw edema produced by CPA, mepyramine did not have an effect on PWLs, either when administered alone or in combination with 5nmol CPA (Fig. 3.11B). As maximal paw edema was produced at a dose that is insufficient to produce an antihyperalgesic effect, and blocking paw edema did not change the effect of CPA on PWL, it is likely that the paw edema effect by adenosine A_1 agonists does not contribute substantially to the antihyperalgesic effect.

Fig. 3.9: The lack of effect of L-PIA, CPA, NH_2dAD and DCF on mechanical allodynia. Time course of 50% PWT following drug administration is shown. Values represent means ± SEM. Open symbols: non-ligated paw, solid symbols: nerve-ligated paw. Drugs were administered into the nerve-ligated paw (Kruskal-Wallis one way ANOVA on rank, n=5-7 per group).

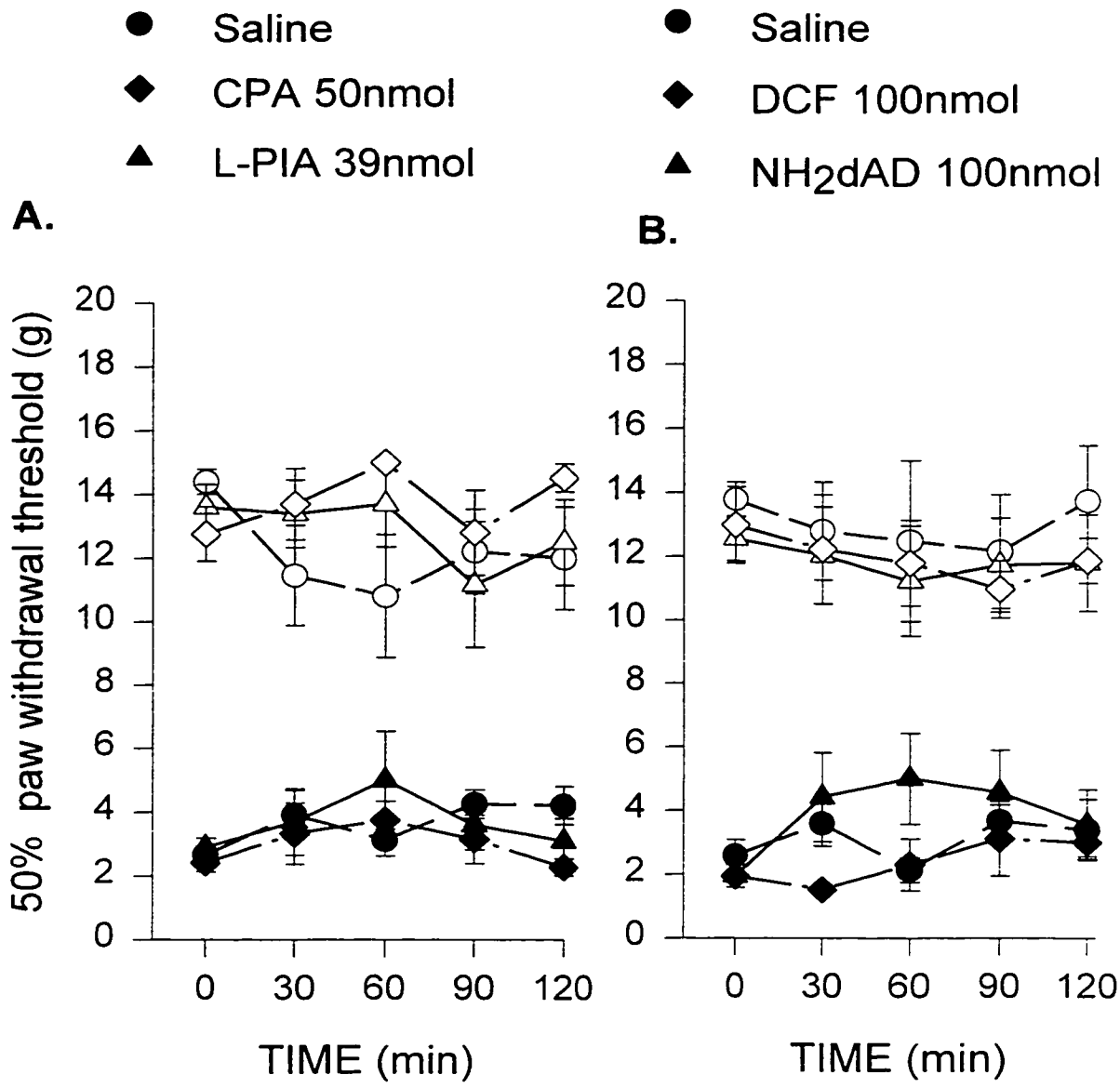

Saline
CPA 50nmol
L-PIA 39nmol
Saline
DCF 100nmol
NH2dAD 100nmol
A.
B.
50% paw withdrawal threshold (g)
20
18
16
14
12
10
8
6
4
2
0
0
30
60
90
120
TIME (min)
TIME (min)

Fig. 3.9

Fig. 3.10: Increase in paw volume induced by the local injection of the adenosine A_1 receptor agonists CPA and L-PIA. Cumulative response over 3 h is shown. The solid square represents the ipsilateral paw volume result for CPA 5nmol injected into the ipsilateral paw, and CPA 50nmol injected into the contralateral paw. * $p< 0.05$ compared to saline, *t* $p<0.05$ compared to CPA 5nmol (one way ANOVA followed by SNK, means ± SEM, n=6).

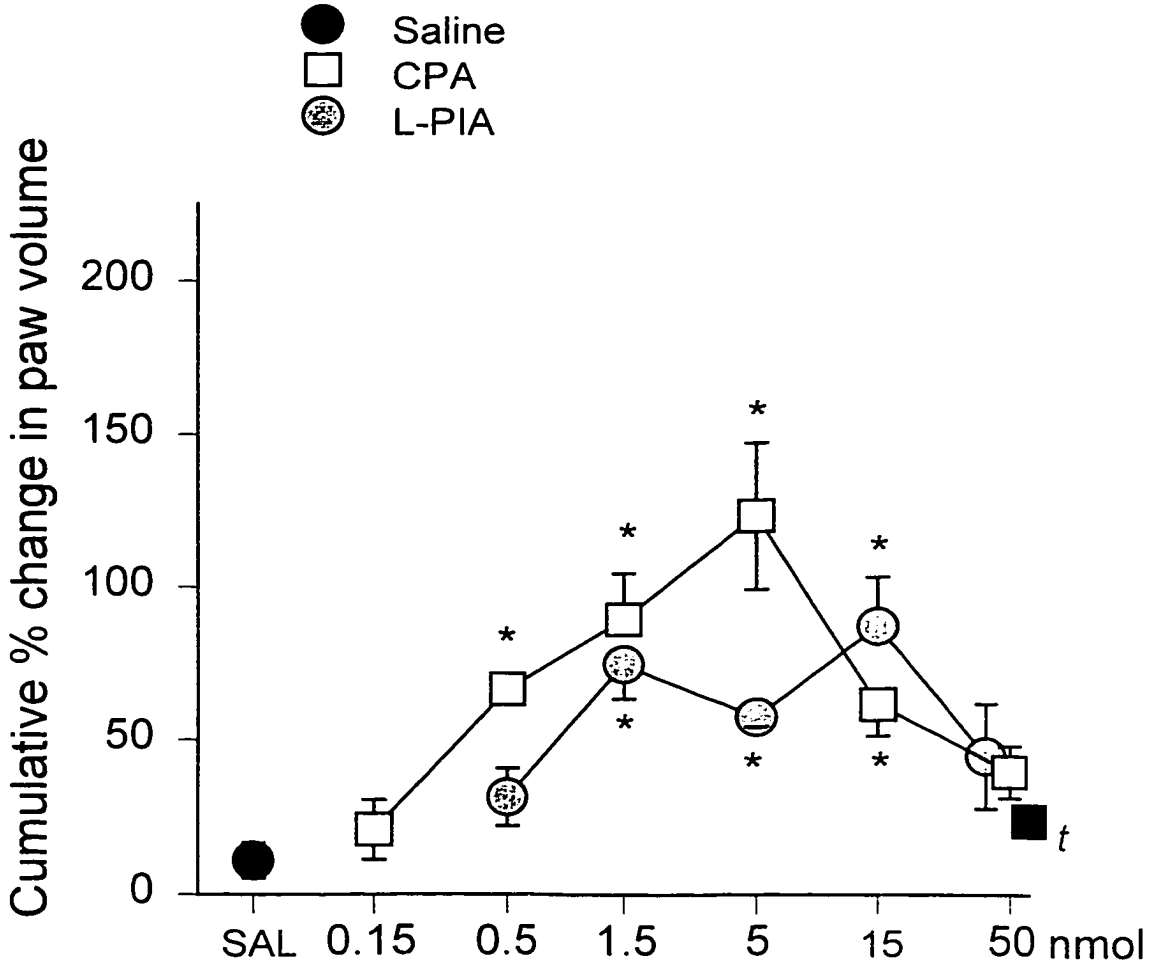

Saline
CPA
L-PIA
Cumulative % change in paw volume
200
150
100
50
0
SAL
0.15
0.5
1.5
5
15
50 nmol
t

Fig. 3.10

Fig. 3.11: Effects of histamine (HIST), 5-HT and an α-adrenoceptor antagonist (doses in nanomoles) on (A) the increase in paw volume produced by saline or CPA 5nmol, and (B) the effects of mepyramine (300 nmol) on PWL produced by CPA 5nmol. MEP mepyramine, KET ketanserin, PTA phentolamine, * $p<0.05$ compared to CPA , *t* $p < 0.05$ compared to saline (one way ANOVA followed by SNK, means ± SEM, n=6)

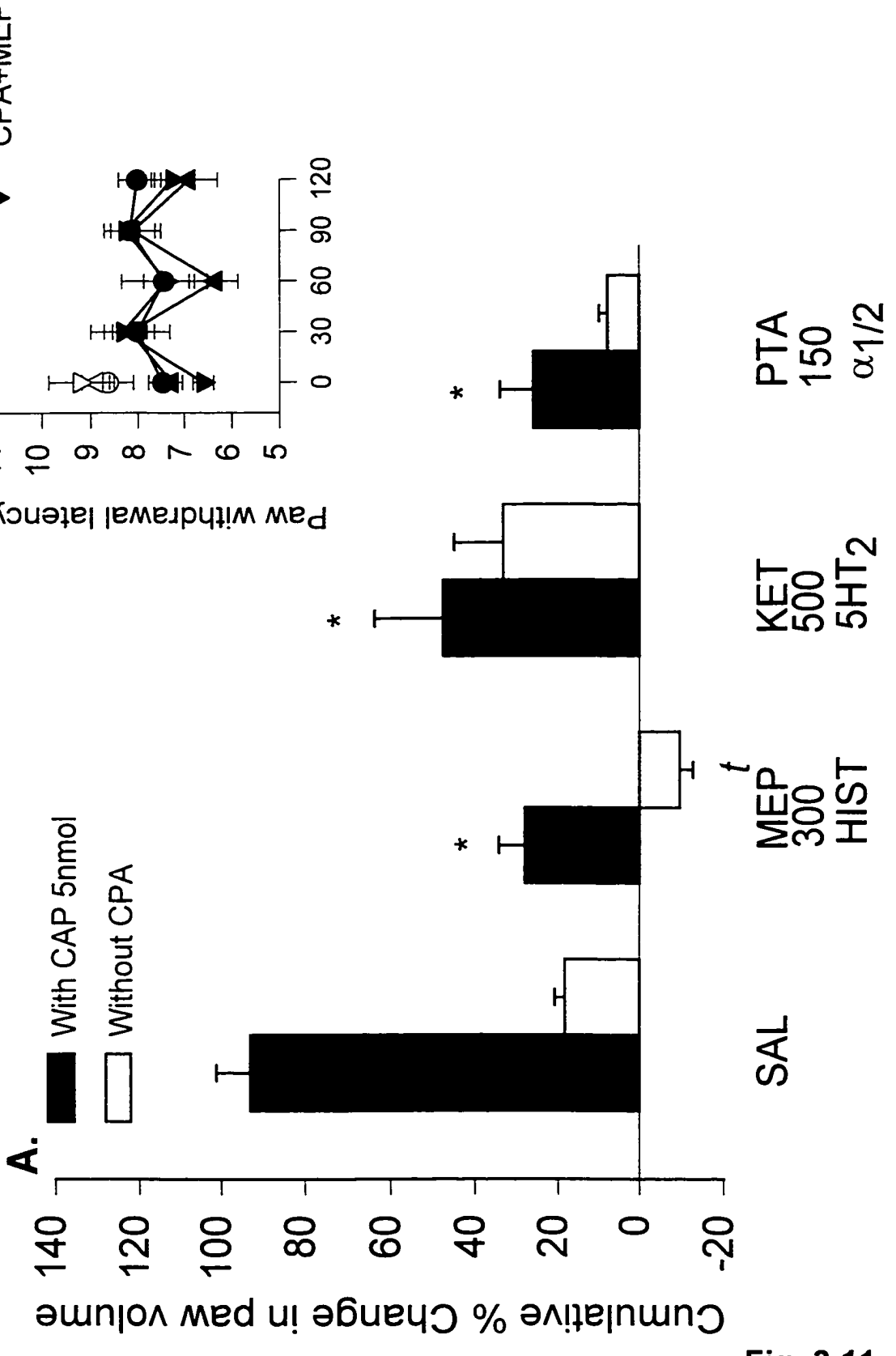

A.
With CAP 5nmol
Without CPA
Cumulative % Change in paw volume
140
120
100
80
60
40
20
0
-20
SAL
MEP 300 HIST
KET 500 5HT2
PTA 150 α1/2
*
*
*
t
B.
MEP
CPA
CPA+MEP
Paw withdrawal latency(sec)
12
11
10
9
8
7
6
5
0
30
60
90
120

Fig. 3.11

Fig. 3.12: Increase in paw volume induced by local injection of (A) the adenosine A_1 receptor CPA, (B) the adenosine kinase inhibitor NH_2dAD and (C) the adenosine deaminase inhibitor DCF. * $p<0.05$ compared to saline (one way ANOVA followed by SNK, means ± SEM, n=6).

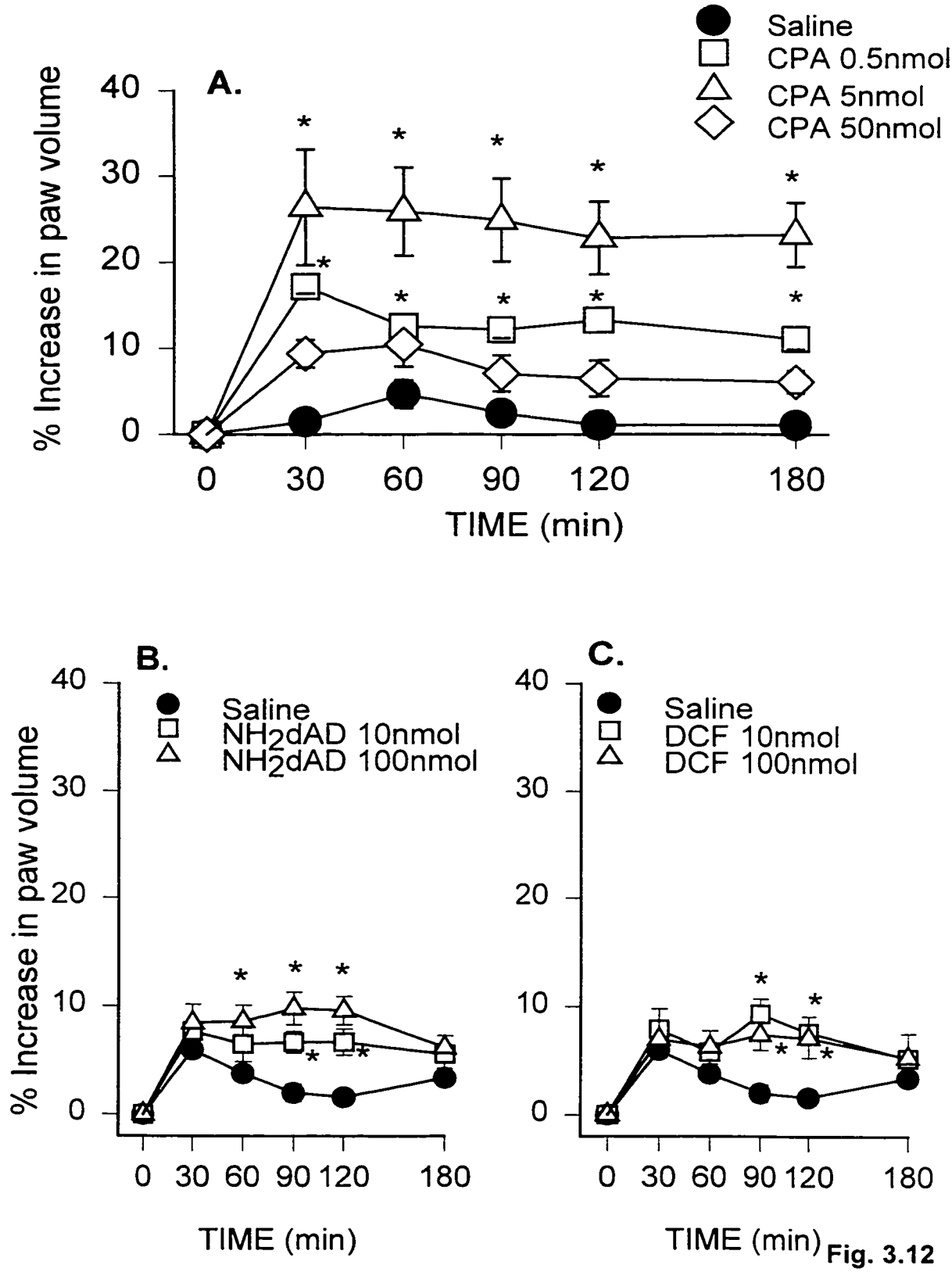

A.
Saline
CPA 0.5nmol
CPA 5nmol
CPA 50nmol
% Increase in paw volume
40
30
20
10
0
0
30
60
90
120
180
TIME (min)
B.
Saline
NH2dAD 10nmol
NH2dAD 100nmol
% Increase in paw volume
TIME (min)
C.
Saline
DCF 10nmol
DCF 100nmol
TIME (min)

Fig. 3.12

Fig. 3.13: Effects of systemic pretreatment with (A) compound 48/80, and (B) capsaicin on paw edema produced by CPA (5nmol). The time course of paw volume following CPA administration in drug pretreated and vehicle-pretreated rats is shown..

* $p < 0.05$ (two-way repeated ANOVA followed by SNK. means ± SEM, n=6).

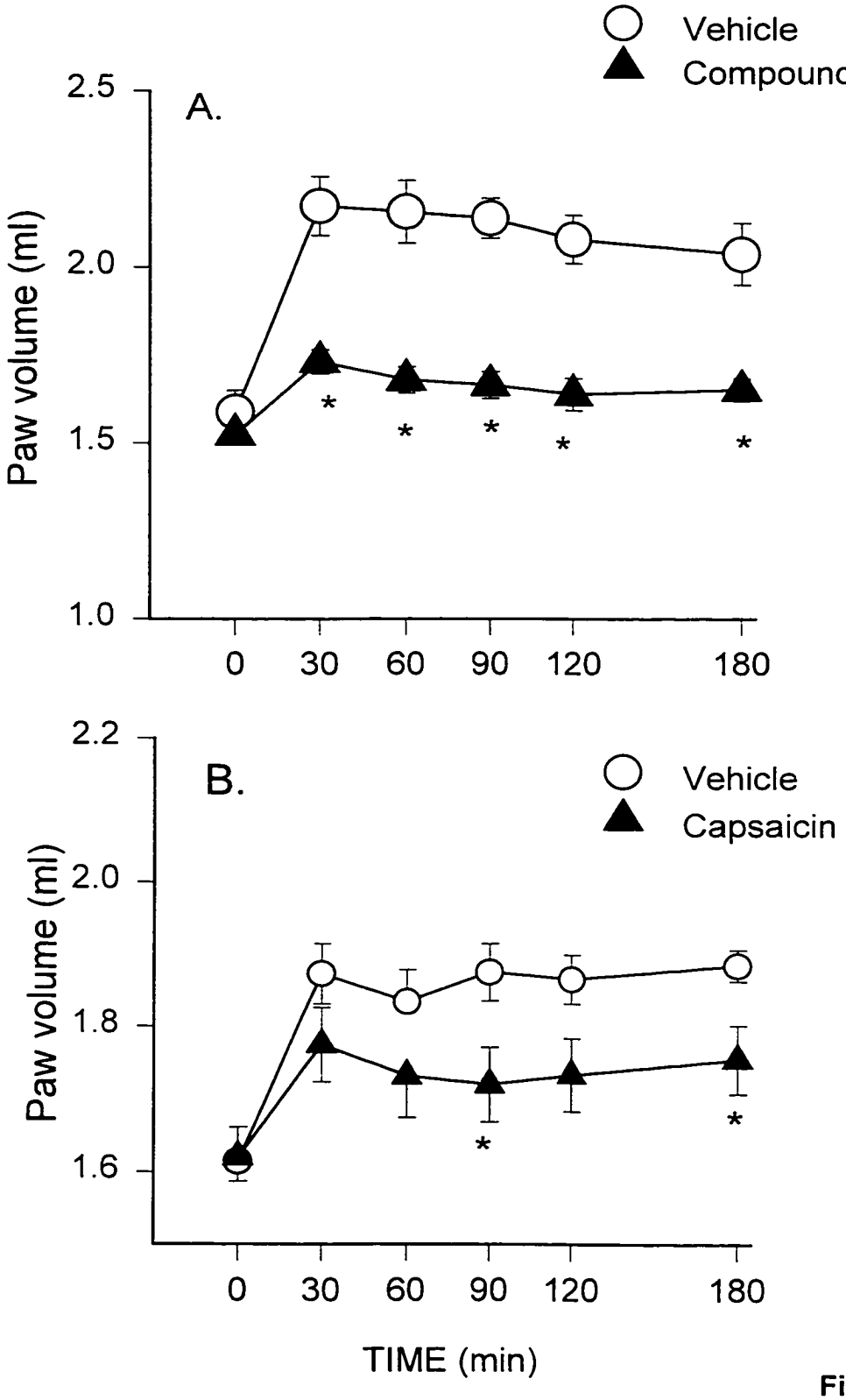

Vehicle
Compound 48/80
A.
Paw volume (ml)
2.5
2.0
1.5
1.0
0
30
60
90
120
180
*
B.
Vehicle
Capsaicin
Paw volume (ml)
2.2
2.0
1.8
1.6
TIME (min)

Fig. 3.13

3.4 DISCUSSION

The present study demonstrated that local administration of both adenosine A_1 receptor agonists (CPA, L-PIA) and inhibitors of adenosine metabolism (NH_2dAD, DCF) reverse thermal hyperalgesia, but not mechanical allodynia, in the rat spinal nerve ligation model. The local nature of the action was confirmed in each case by the demonstration of no effect following injection of active doses into the non-ligated paw. Caffeine, an adenosine A_1/A_{2A} receptor antagonist, completely blocked the antihyperalgesic effect produced by all agents, indicating that effects were mediated by cell surface adenosine receptors.

3.4.1 Adenosine A_1 receptor-mediated peripheral antihyperalgesic effect

Although we did not use selective receptor antagonist to block the antihyperalgesic effect because of the solubility and vehicle effects, the receptor subtype involved is likely the adenosine A_1 receptor. Several reasons support an adenosine A_1 receptor mediated effect: (1) a peripheral adenosine A_1 receptor-mediated antihyperalgesia has previously been demonstrated in the PGE_2-induced hyperalgesia (Taiwo and Levine, 1990; Aley et al., 1995; Aley and Levine, 1997) and formalin models (Karlsten et al., 1992; Doak and Sawynok, 1995; Sawynok et al., 1998), (2) CGS 21680, a selective adenosine A_{2A} receptor agonist (170 fold, van Galen et al., 1994), did not produce an antihyperalgesic effect, and (3) peripherally activation of adenosine A_{2A},A_{2B} and A_3 receptors produce nociceptive effect in previous studies (Taiwo and Levine, 1990; Karsten et al., 1992; Doak and Sawynok, 1995; Sawnok et al., 1997).

The antihyperalgesic effect of adenosine analogs and inhibitors of adenosine metabolism was likely due to actions on sensory nerve terminals. Functional adenosine

A_1 receptors have been demonstrated to exist on the cell bodies of dorsal root ganglia and the central nerve terminals of primary afferent neurons (Sawynok, 1998). On peripheral sensory nerve terminals, the antinociceptive effect of adenosine A_1 receptor activation is functionally linked with α_2-adrenoreceptors and μ-opioid receptors (Aley and Levine, 1997). These functionally linked receptors are coupled with G proteins and their activation results in inhibition of adenylyl cyclase activity which decreases levels of cyclic AMP resulting in antinociception (Taiwo and Levine, 1991; Khasar et al., 1995).

3.4.2 Peripheral antihyperalgesic effect produced by an adenosine kinase inhibitor and an adenosine deaminase inhibitor

The present study demonstrated that increasing local adenosine levels by the adenosine kinase inhibitor, NH_2dAD, produced a rapid onset and short lasting antihyperalgesic effect, while the adenosine deaminase inhibitor DCF produced a later onset, yet long lasting effect. The time dependent antihyperalgesic effect observed with NH_2dAD is likely due to the short half life of the drug, as a similar rapid and short lasting effect of NH_2dAD has been observed in previous spinal antinociceptive studies (Keil and DeLander, 1992, 1994; Poon and Sawynok, 1998). The later onset and long lasting effect of DCF is likely due to chemical properties of the drug, as cell membranes exhibit a poor transport of DCF (Newby, 1981), and this could account for the slow onset antihyperalgesia. The combination of DCF with adenosine deaminase is irreversible, with a single administration of DCF causing a long lasting reduction of adenosine deaminase levels (Padua et al., 1990). Thus, the long-term antihyperalgesia observed here is likely due to a long lasting inhibition of adenosine deaminase.

Adenosine kinase is an intracellular enzyme, phosphorylating adenosine to adenosine monophosphate (Geiger et al., 1997). Neurochemical studies have demonstrated that inhibition of adenosine kinase augments the extracellular adenosine levels in the spinal cord (Golembiowska et al., 1995; 1996), and increases subcutaneous adenosine levels in inflammation (Cronstein et al., 1995). Adenosine deaminase converts adenosine to inosine, and exists in cytosolic and extracellular forms (Meghji, 1991; Franco et al., 1997; Geiger et al., 1997). Inhibition of adenosine deaminase can also increase extracellular adenosine levels from the spinal cord *in vivo* (Golembiowska et al., 1995). It is likely that the antihyperalgesic effects observed in the current study by NH_2dAD and DCF are due to an increase in extracellular availability of adenosine acting on cell surface adenosine receptors caused by inhibiting enzymes involved in adenosine metabolism. The cellular sources of adenosine released locally in neuropathic pain will be discussed later (Chapter 6).

Analgesia produced by modulation of local adenosine levels by inhibiting adenosine kinase has been observed in previous studies. Thus, spinal analgesia by an adenosine kinase inhibitor has been observed in nociceptive threshold studies (Keil and DeLander, 1992; 1994) and inflammatory pain models (Poon and Sawynok, 1995;1998). In neuropathic pain models, an antiallodynic effect by adenosine kinase inhibitors following spinal or systemic administration has been reported (Lavand'homme and Eisenach, 1999; Lynch et al., 1999; Kowaluk et al., 2000). A peripheral analgesia produced by local inhibition of adenosine kinase has been reported in an inflammatory pain model (Sawynok et al., 1998). The present study extends those studies in

identifying a peripheral, local, anti-thermal hyperalgesic effect by an adenosine kinase inhibitor in a neuropathic pain model.

The peripheral antihyperalgesic effect produced by the adenosine deaminase inhibitor DCF in the current study was somewhat surprising. Thus, inhibition of adenosine deaminase does not produce any intrinsic antinociceptive effect at either spinal (Keil and DeLander, 1992; 1996; Poon and Sawynok, 1995;1998) or peripheral (Sawynok et al., 1998) site in normal animal or in the formalin-induced inflammatory pain model, although it is able to enhance the effect of adenosine kinase inhibitors in both instances. A possible explanation for the lack of efficacy of DCF in previous behavioral studies is that adenosine kinase plays a primary role in the modulation of adenosine metabolism under normal conditions. Only when the adenosine concentrations are higher than in physiological conditions, such as following exogenous administration of adenosine or when the tissue is under energy depletion, does adenosine deaminase begin to be important in regulating adenosine metabolism (Keil and DeLander, 1994; Lloyd and Fredholm, 1995). Thus, adenosine deaminase generally has a lower affinity but a higher maximal capability for adenosine metabolism than adenosine kinase (Arch and Newsholme, 1978). It may be that following nerve injury, subcutaneous adenosine levels are higher than in physiological conditions, allowing the adenosine deaminase inhibitor to produce a more significant effect. Another possible explanation is that the activity of adenosine deaminase may be altered in some way by the nerve injury. Extracellular adenosine deaminase is functionally coupled to adenosine A_1 receptors (Franco et al., 1997). As the peripheral antinociceptive effect of adenosine A_1 receptor activation is subject to down-regulation after repeated activation (Aley et al., 1995; Aley and Levine,

1997), there might be a change in adenosine A_1 receptor activity and extracellular adenosine deaminase activity following chronic nerve injury. Direct neurochemical studies are necessary to address the question of which of these possibilities accounts for the observed behavioral effects.

3.4.3 Lack of a peripheral effect by adenosine analogs and inhibitors of adenosine metabolism on mechanical threshold changes in neuropathic rats

The current study demonstrates a lack of a peripheral locally-mediated antiallodynic effect by adenosine agonists or inhibitors of adenosine metabolism. This suggests that thermal hyperalgesia and mechanical allodynia are mediated by different mechanisms and can be separately modulated by drug treatment. Thermal hyperalgesia induced by nerve injury is proposed to be mediated by C fiber afferents, while mechanical allodynia by Aβ fiber afferents (Woolf and Doubell, 1994), a concept supported by that a neurotoxin selective for C fibers reverses thermal hyperalgesia but has no effect on mechanical allodynia in the spinal nerve ligation model (Ossipov et al., 1999). The lack of a peripheral antiallodynic effect by local adenosine receptor agonists and inhibitors of metabolism may reflect a selective distribution of adenosine A_1 receptors on C but not on Aß fibers. This is consistent with the electrophysiological study that adenosine A_1 receptor agonist selectively inhibit C fiber, but not Aβ fiber-evoked responses and post-discharge of the dorsal horn neurons (Reeve and Dickenson, 1995; Nakamura et al., 1997). It is also consistent with the behavioral observation that at nerve terminals, adenosine A_1 receptors are functionally linked with μ-opioid receptors (Aley at al., 1995; Aley and Levine, 1997), and μ-opioid receptors appear to be

selectively located in small nociceptive neurons rather than Aβ afferent neurons (Taddese et al., 1995). Another possibility for this different profile is that peripheral input is more important for maintenance of thermal hyperalgesia, while spinal and supraspinal modulation are more important for maintenance of mechanical allodynia. Thus, spinalization significantly blocks the spinal withdrawal reflex response produced by mechanical stimulation but facilitates the reflex produced by noxious thermal stimulation (Bian et al., 1998; Kauppila et al., 1998).

3.4.4 Paw edema effects by adenosine analogs

During the behavioral study, we observed that the adenosine A_1 receptor agonists, CPA and L-PIA produce paw edema at doses that are not effective in producing antinociceptive effects. The mechanisms mediating this effect were determined in normal rats. The paw volume effect can be blocked by the selective adenosine A_1 receptor antagonist CPT (Sawynok et al., 1999b), the histamine H_1 receptor antagonist mepyramine, a 5-HT_2 receptor antagonist ketanserin, as well as the mast cell degranulating agent compound 48/80, indicating the involvement of adenosine A_1 receptors and mast cell degranulation. Although adenosine A_{2B} and A_3 receptors are expressed in mast cells and can trigger mast cell degranulation upon activation (Chapter 1), there is no evidence indicating adenosine A_1 receptors on mast cells (Ramkumar et al., 1993). A CPA mediated non-selective activation of A_{2B} and A_3 receptor is unlikely involved in the paw edema effect, because (a) CPA is one hundred-times more selective for A_1 receptors, and (b) selective activation of adenosine A_{2B} and A_3 receptors produces edema with different properties (Sawynok et al., 1999b; 2000). An indirect activation of mast cells via activation of primary sensory afferents may be an alternative explanation,

as capsaicin pretreatment partially blocked the paw volume effect, and neurogenic inflammation can trigger mast cell degranulation by release of SP (Chapter 1). However, given only the partial block by capsaicin and the more complete blockade by compound 48/80, it is unclear as to whether or not the primary sensory afferent activation is a necessary prelude to mast cell degranulation. As adenosine A_1 receptors can activate neutrophils (Chapter 1), a non-neurogenic inflammatory response may be another explanation. Regardless of the mechanism mediating this effect, the results here suggest that nociception and inflammation can be separately modulated by adenosine A_1 receptor agonists. Interestingly, opioids also produce similar opposing effects. Thus, intraplantar injection of morphine (Perrot et al., 1999) or a δ-opioid receptor agonist (Hong and Abbott, 1995) significantly blocks nociception, while producing an increase in paw volume and plasma extravasation.

3.4.5 Significance of the observations

The peripheral antihyperalgesic effects observed in the present study may at least partly contribute to the pain alleviating effects observed with systemic adenosine infusion in humans (Belfrage et al., 1995; 1999; Sollevi, et al., 1995; Sjölund et al., 2000). They also raise the possibility that peripherally administered adenosine agents may be a useful strategy to explore in neuropathic pain. Thus, peripheral adenosine A_1 agonists may potentially reduce possible side effects such as hypotension and bradycardia commonly seen with systemic administration of such agents (Katims et al., 1983; Kuan et al., 1992). While topical analgesics based on adenosine may be worthy of further examination, there could also be some limitations to this approach. (1) Mechanical allodynia was not

alleviated by these agents administered peripherally, so a combination with other drugs may be needed to reverse all symptoms. (2) Tolerance can occur to the peripheral analgesic effect of adenosine A_1 receptor agonists in acute pain models (Aley et al., 1995; Aley and Levine, 1997), and if this also occurs in a chronic pain situation, it could be a limitation. (3) Peripheral adenosine A_1 receptor agonists produce significant local edema when injected subcutaneously; if edema also occurs when applied topically, this could be another limitation. Local administration of NH_2dAD or DCF induces a much more limited edema compared to CPA or L-PIA, so inhibitors of adenosine metabolism may have an advantage compared to agonists in this respect. The long lasting effect of DCF could be a particular advantage for clinical usage.

3.5 CONCLUSION

The present study demonstrates that local injection of adenosine A_1 receptor agonists, an adenosine kinase inhibitor and an adenosine deaminase inhibitor reversed thermal hyperalgesia but not mechanical allodynia in the neuropathic pain model. This action was peripherally mediated and was blocked by the adenosine receptor antagonist caffeine. Adenosine A_1 receptor agonists, but not the inhibitors of adenosine metabolism also produced an overt paw edema effect. Mast cells and primary sensory afferents are involved in this effect.

CHAPTER 4

FORMALIN-EVOKED ADENOSINE RELEASE IN THE RAT HIND PAW: A MICRODIALYSIS STUDY

4.1 INTRODUCTION

Adenosine is an endogenous neuromodulator producing complex effects on nociceptive signaling (reviewed Sawynok, 1998; 1999; Section 1.7). Peripherally, activation of adenosine A_1 receptors produces antinociceptive effects, while activation of adenosine A_{2B} and A_3 receptors produces nociceptive effects (Section 1.7). The antinociceptive effects of both adenosine directly (agonists), or indirectly acting agents (inhibitors of adenosine metabolisms or other adenosine based agents) have been demonstrated in various inflammatory pain models (Karlsten et al., 1992; Doak and Sawynok, 1995; Khasar et al., 1995; Sawynok et al., 1998; Kowaluk et al., 2000; Mcgaraughty et al., 2001). Besides its effects on pain, adenosine also has been suggested to be an autocoid modulating inflammation, primarily acting on adenosine A_{2A} receptors (Cronstein, 1994; Sullivan and Linden, 1998; Section 1.8). The anti-inflammatory effects of certain agents used in the treatment of rheumatoid arthritis, such as methotrexate, sulfasalazine and aspirin are mediated by an increase in extracellular adenosine concentrations (Cronstein et al., 1993; 1995; 1999; Gadangi et al., 1996; Morabito et al., 1998). Thus, recruitment of peripheral adenosine systems is important in the management of inflammatory pain.

There are at least four key factors that are important in adenosine metabolism. Adenosine is formed from ATP via 5'-nucleotidase, metabolized by adenosine kinase and adenosine deaminase to 5'-AMP and inosine, respectively, and transported bidirectionally by adenosine transporters on the cell surface (Fig. 1.2). The net effect of formation, degradation and transportation determines intracellular and extracellular levels of adenosine. The extracellular level of adenosine is increased in the exudates of

inflamed tissues (Cronstein et al., 1993; 1995). Increasing endogenous levels of adenosine by inhibiting enzymes involved in adenosine metabolism produces both antinociceptive and anti-inflammatory effects in inflammatory pain models involving both peripheral and central sites (Sawynok et al., 1998; Poon and Sawynok, 1999; Kowaluk et al., 2000; Mcgaraughty et al., 2001).

The formalin model is a widely used model of persistent pain involving tissue injury. Injection of formalin produces a biphasic response consisting of an initial phase lasting about 10min followed, after a short quiescent interphase, by a longer period of sustained behavior lasting 45-60min, and this is observed both electrophysiologically (Dickenson and Sullivan, 1987; Puig and Sorkin, 1995) and behaviorally (Dubuisson and Dennis, 1977; Tjølsen et al., 1992). The first phase is thought to result from direct activation of nociceptive nerve terminals, while the second phase is mediated by a combination of peripheral input and spinal cord sensitization (Tjølsen et al., 1992; Coderre et al., 1993; Dallel et al., 1995). Formalin-evoked behaviors are dependent on the concentration of formalin (Coderre et al., 1993; Abbott et al., 1995).

Formalin injection also induces a local inflammatory response involving paw edema, plasma extravasation, blood vessel dilation, and tissue ulceration (Rosland et al., 1990; Damas and Liégeois, 1999; Fu et al., 2000; Taylor et al., 2000). The inflammatory response in the early phase is neurogenic, resulting from neuropeptides released from nociceptive nerve terminals through a local axon reflex, while in the later phase, a tissue injury, non-neurogenic inflammation is primarily involved (Hunskaar and Hole, 1987; Wheeler-Aceto and Cowan, 1991; Damas and Liégeois, 1999).

Although there is no direct evidence indicating that adenosine levels are increased by formalin, behavioral studies suggest there is a tonic antinociceptive effect of endogenous adenosine (Doak and Sawynok, 1995) and that this effect can be modulated by an adenosine kinase inhibitor (Sawynok et al., 1998). These studies suggest that there is a local release of adenosine by formalin. In the air pouch exudates of carrageenan-induced inflammation, adenosine levels are increased (Cronstein et al., 1993; 1995). Non-specific release of adenosine from cell necrosis, from stressed neutrophils, endothelial cells or platelet aggregation is believed to be the major source of released adenosine following inflammation (Cronstein, 1994; 1997), but the sources have not been experimentally verified. Furthermore, little is known about the correlation between local adenosine levels and inflammatory pain behavior. Although *systemic* adenosine kinase inhibitors produce an *anti-inflammatory* response by modulating local adenosine levels (Cronstein et al., 1995), there is no direct report on the *local peripheral* effect of inhibitors of adenosine metabolism on extracellular adenosine levels *in vivo*. Microdialysis provides a method to continuously measure the extracellular levels of endogenous substances *in vivo*. Microdialysis has been used to measure adenosine levels in both brain and peripheral tissues (Lönnroth et al., 1989; Blay et al., 1997; Britton et al., 1999; Richter et al., 1999). In formalin-induced inflammation, an increase of excitatory amino acids and nitric oxide in the rat hind paw have been demonstrated by *in vivo* subcutaneous microdialysis studies (Omote et al., 1998; 2000).

The aim of the present study was to use the microdialysis technique to examine peripheral adenosine levels in the formalin test, and the possible neuronal and non-neuronal origin of peripheral adenosine evoked by formalin. The following issues were addressed in the present study: (1) whether or not adenosine is released subcutaneously after formalin injection, (2) whether or not capsaicin-sensitive sensory afferents, sympathetic postganglionic neurons, or mast cells are involved in such release, (3) whether or not such release can be modulated by an adenosine kinase inhibitor and an adenosine deaminase inhibitor, and (4)whether or not the released adenosine and the modulation of adenosine levels by enzyme inhibitors correlates with previously reported antinociceptive effects and anti-inflammatory effects produced by adenosine indirectly acting agents.

4.2 MATERIALS AND METHODS

Subcutaneous microdialysis was performed in pentobarbital anaesthetized rats (Chapter 2) to determine formalin-evoked adenosine release. In naïve rats, formalin (0.5-5%), the adenosine kinase inhibitor, NH_2dAD 100nmol, and the adenosine deaminase inhibitor, DCF 100nmol, were studied to determine formalin-evoked adenosine release and the modulatory effect of inhibitors of adenosine metabolism on adenosine release evoked by inflammation. Capsaicin (1%) and vehicle were injected to determine capsaicin-evoked adenosine release. All drugs and drug combinations were injected in a final volume of 50µl into the subcutaneous tissue in the plantar aspect of the rat hind paw. When two drugs were co-administered, they were mixed just before administration.

Neurochemical pretreatments (Chapter 2) were performed to determined the involvement of capsaicin-sensitive primary sensory afferents (systemic pretreatment with the nociceptive afferent-selective neurotoxin, capsaicin), sympathetic efferents (systemic pretreatment with the sympathetic efferent-selective neurotoxin 6-OHDA) and mast cells (systemic pretreatment with the mast cell-degranulating agent, compound 48/80) (Fig. 4.1). Formalin (1.5% or 5%, 50 μl) was injected s.c. into the hind paw of both drug treated and vehicle treated rats. In all experiments, drug treated rats and vehicle treated rats were always performed at the same time for microdialysis, adenosine analysis, and behavioral studies.

Behavioral effects of the formalin test (Chapter 2) and paw volume (Chapter 2) were measured in both naïve rats and rats following neurochemical pretreatments to determine the correlation between neurochemical observations and nociceptive behaviors and inflammation.

Microdialysis data were expressed as a time course of adenosine levels in individual dialysate (10min) or total evoked release (60min). Formalin test data were expressed as the number of episodes (flinches) or total time (biting/licking) in 2min bins or as a cumulative score over phase 1 (0-12min) or phase 2 (16-60min). Data were analyzed using ANOVA followed by SNK, or student's *t* test if only two groups were compared. The level of significance was set at 0.05.

Fig. 4.1: Schematic illustration of potential sources of formalin-evoked adenosine. Formalin induces a massive local inflammatory response which could induce non-neuronal release of purines via cell lysis, neutrophil and endothelial cell activation, platelet aggression and mast cell degranulation. Formalin also activates primary sensory afferents and SPGN to induce a neuronal release. Capsaicin, 6-OHDA and compound 48/80 were used to determine the involvement of these elements in formalin-evoked subcutaneous adenosine release. Receptors located on sensory terminals and their activation by mediators released from sensory afferents, SPGNs and mast cells are depicted. Abbreviations are explained in List of Abbreviations.

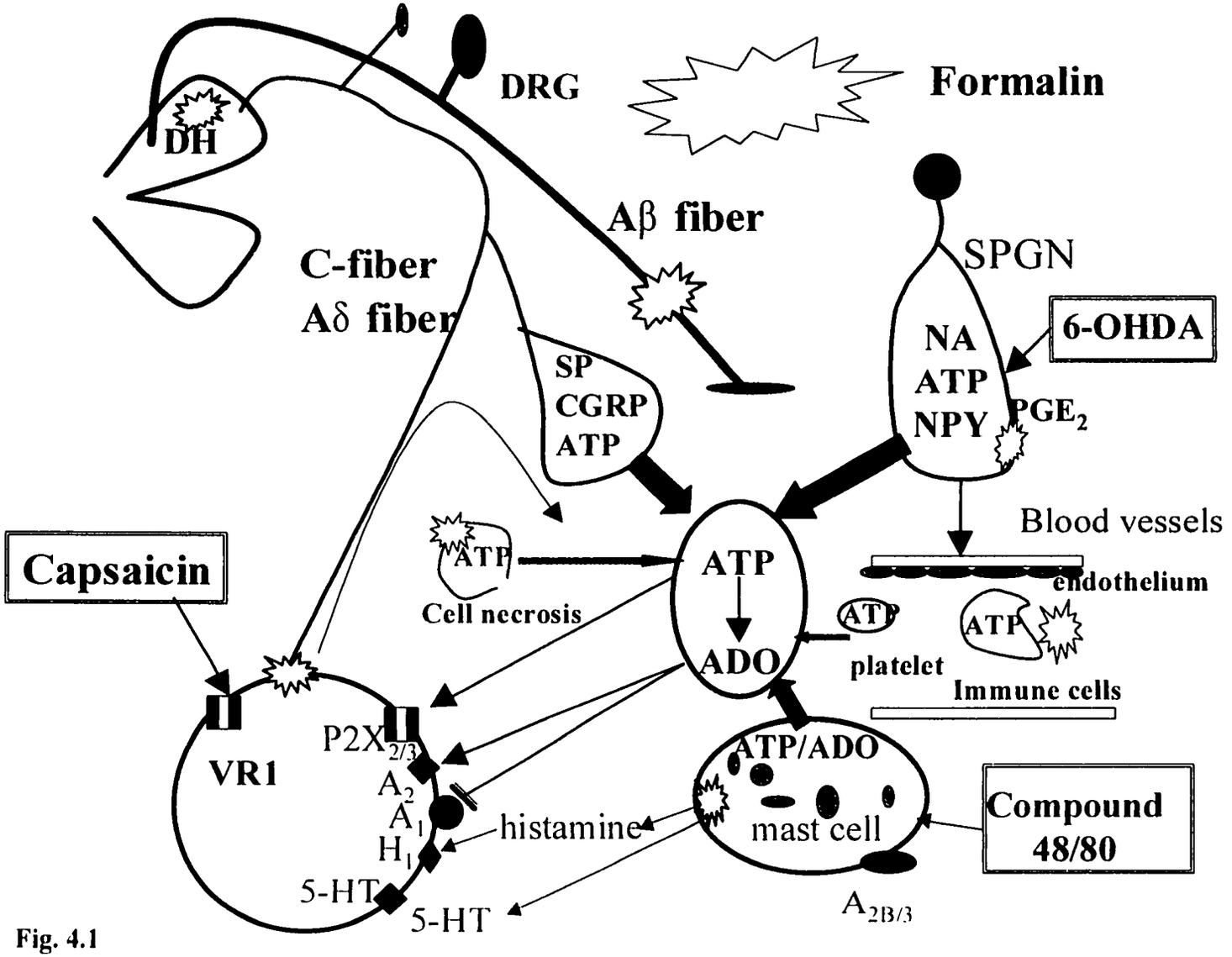

DRG
Formalin
DH
Aβ fiber
C-fiber
Aδ fiber
SPGN
6-OHDA
NA
ATP
NPY
PGE2
SP
CGRP
ATP
Blood vessels
Capsaicin
ATP
Cell necrosis
ATP
ADO
endothelium
ATP
platelet
ATP
Immune cells
VR1
P2X2/3
A2
A1
H1
5-HT
histamine
5-HT
ATP/ADO
mast cell
Compound
48/80
A2B/3

Fig. 4.1

4.3 RESULTS

4.3.1 Basal adenosine levels in the subcutaneous space of the rat hind paw

Figure 4.2A shows the time course of changes in adenosine levels in the subcutaneous space after implantation of the microdialysis probe. Samples were taken with a flow rate of 2μl/min from the start of perfusion and collected at 10min intervals. Adenosine concentrations were initially high but fell to a steady state level within 80-100 min after probe implantation (Fig 4.2A). Basal adenosine levels were thus measured 120min after probe implantation. The basal subcutaneous adenosine level was 1.50±0.10 pmol/20μl/10min (means ± SEM, n=76). To estimate the adenosine concentration *in vivo* and to find out if formalin or different concentrations of adenosine had an effect on recovery, the *in vitro* recovery rate was determined for the dialysis probes. There was no significant difference in probe recovery rate at different adenosine concentrations (Fig. 4.2B), and formalin (5%) did not significantly change the recovery rate (Fig. 4.2B). The average recovery of adenosine from the microdialysis probes was 32.7±1.4% (Fig. 4.2B). After correction for the *in vitro* recovery rate, the *in vivo* basal adenosine concentration in rat hind paw subcutaneous tissues was estimated to be 0.23±0.02μM (means ± SEM).

Fig. 4.2 (A) Time course of changes in measured adenosine levels following the microdialysis probe implantation at time zero. Sample collection began immediately after probe implantation. **(B) *In vitro* recovery rate of adenosine at different concentrations of adenosine in the absence and presence of 5% formalin.** (means ± SEM., n=8).

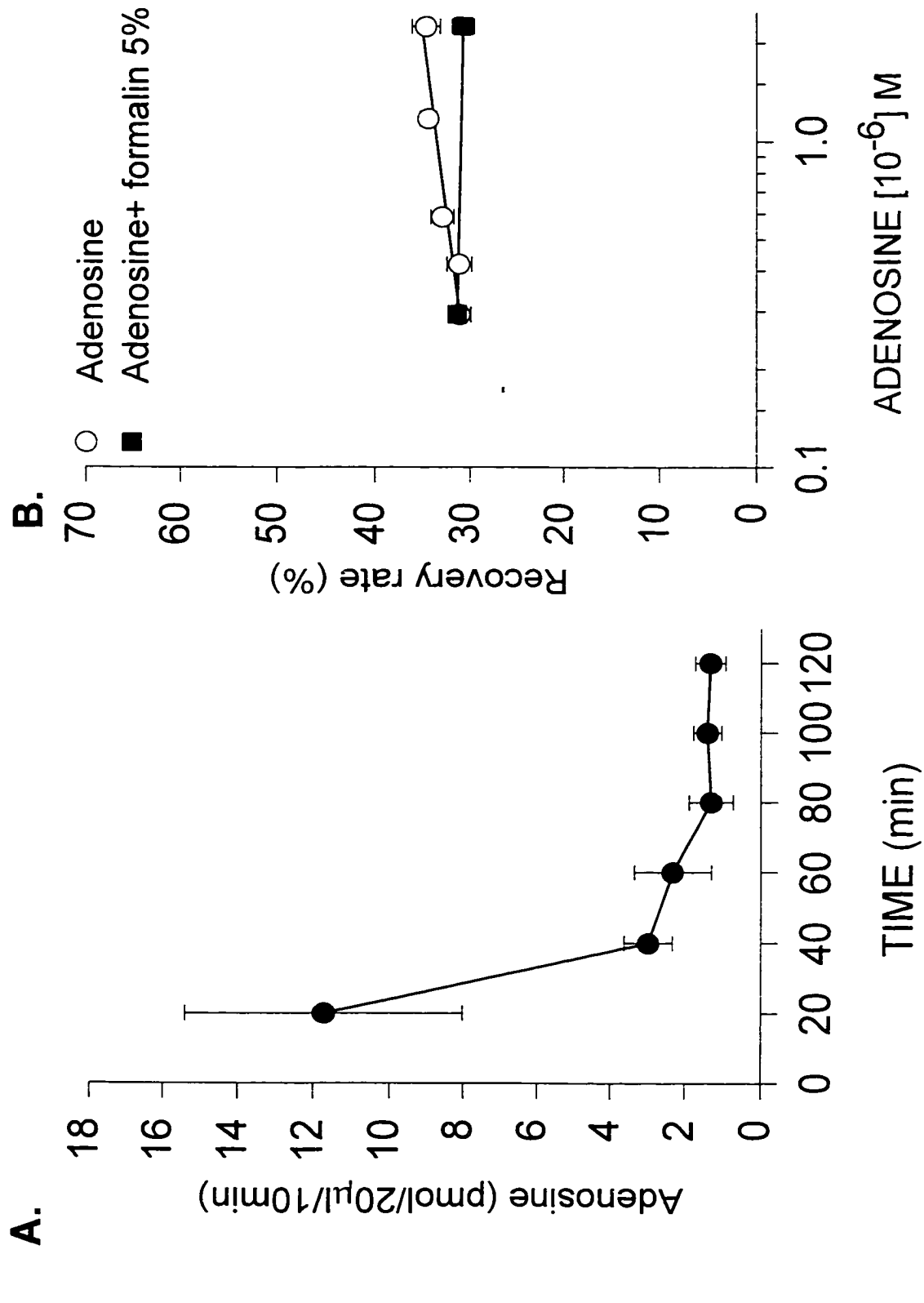

A.
Adenosine (pmol/20µl/10min)
18
16
14
12
10
8
6
4
2
0
0 20 40 60 80 100 120
TIME (min)
B.
Adenosine
Adenosine+ formalin 5%
Recovery rate (%)
70
60
50
40
30
20
10
0
0.1
1.0
ADENOSINE [10-6] M

Fig. 4.2

4.3.2 Effects of formalin on adenosine release

Injection of saline (50µl) did not alter adenosine levels (Fig. 4.3). Injection of formalin (0.5%-5%) increased adenosine release (Figs. 4.3 and 4.5). During the first 10min after injection, all concentrations of formalin increased adenosine release; 1.5%-5% formalin values were significantly different from saline with the highest level of 22.52±4.32pmol/20µl/10min being evoked by 5% formalin. The increase in adenosine produced by 0.5-2.5% formalin was transient, with no significant increase being measured after the first 10min, while the increase evoked by 5% formalin was long lasting, persisting for up to 60min after injection (Fig. 4.3).

4.3.3 Effects of the adenosine kinase inhibitor NH_2dAD on formalin-evoked adenosine release

Injection of the adenosine kinase inhibitor NH_2dAD at 100nmol did not alter basal adenosine levels (Fig. 4.4A). Co-administration of NH_2dAD with 0.5% formalin increased adenosine release during the initial 20min (Fig. 4.4B). The total evoked adenosine release produced by the combination of NH_2dAD with 0.5% formalin was 67.8±18.9pmol/120µl/60min, which was significantly higher than 0.5% formalin alone (11.8±4.7pmol/120µl/60min), and comparable to release evoked by 5% formalin (Fig. 4.5). With a higher concentration of formalin (1.5%), a significant increase in total evoked adenosine release was observed but this increase was not greater than the NH_2dAD plus 0.5% formalin group (Fig. 4.5). There was no significant increase in evoked adenosine release when NH_2dAD was co-administered with 5% formalin (Figs. 4.4C and 4.5).

4.3.4 Effects of the adenosine deaminase inhibitor DCF on formalin-evoked adenosine release

Injection of the adenosine deaminase inhibitor DCF at 100nmol did not alter basal adenosine levels (Fig. 4.4A), and co-administration of DCF with 0.5-1.5% formalin did not increase formalin-evoked adenosine release (Figs. 4.4B and 4.5). However, co-administration of DCF with 5% formalin markedly increased evoked adenosine levels during the entire 60min time course (Fig. 4.4C). Under these conditions, adenosine levels increased to a maximum of 46.30±4.77pmol/20μl/10min, which is much higher than 5% formalin alone (18.31±6.91pmol/20μl/10min) and NH_2dAD plus 5% formalin (18.19±2.14pmol/20μl/10min, Fig. 4.4C). The total amount of change in extracellular adenosine levels by the combination of DCF with 5% formalin was 193.88±31.22pmol/120μl/60min, which is much higher than 5% formalin alone (73.6±22.9pmol/120μl/60min) or the combination of NH_2dAD with 5% formalin (62.18±11.59pmol/120μl/60min, Fig. 4.5).

4.3.5 Pain behaviors and increase in paw volume induced by different concentrations of formalin

Subcutaneous injection of 0.5-5% formalin produced a dose-related biphasic behavioral response (Fig. 4.6). Phase 2 (16-60min) flinches and biting/licking time induced by 5% formalin were significantly higher than those induced by lower concentrations (0.5-1.5%), but there was no significant difference between 2.5% and 5% formalin (Fig. 4.6, A-D). Phase 1 (0-12 min) behaviors exhibited more variability and there were no significant differences in cumulative changes in flinches and biting/licking times at different concentrations of formalin (data not shown). Formalin (0.5-5%) increased paw volume in a dose-related manner, and the increase was significantly higher at 5% formalin than at 0.5% (Fig. 4.7).

Fig. 4.3: Time course of measured adenosine levels in microdialysate samples following saline or formalin 0.5-5% injection. Formalin was injected at time zero.

* $P<0.05$ compared to saline group (one-way ANOVA followed by SNK, means ± SEM, n=6-8).

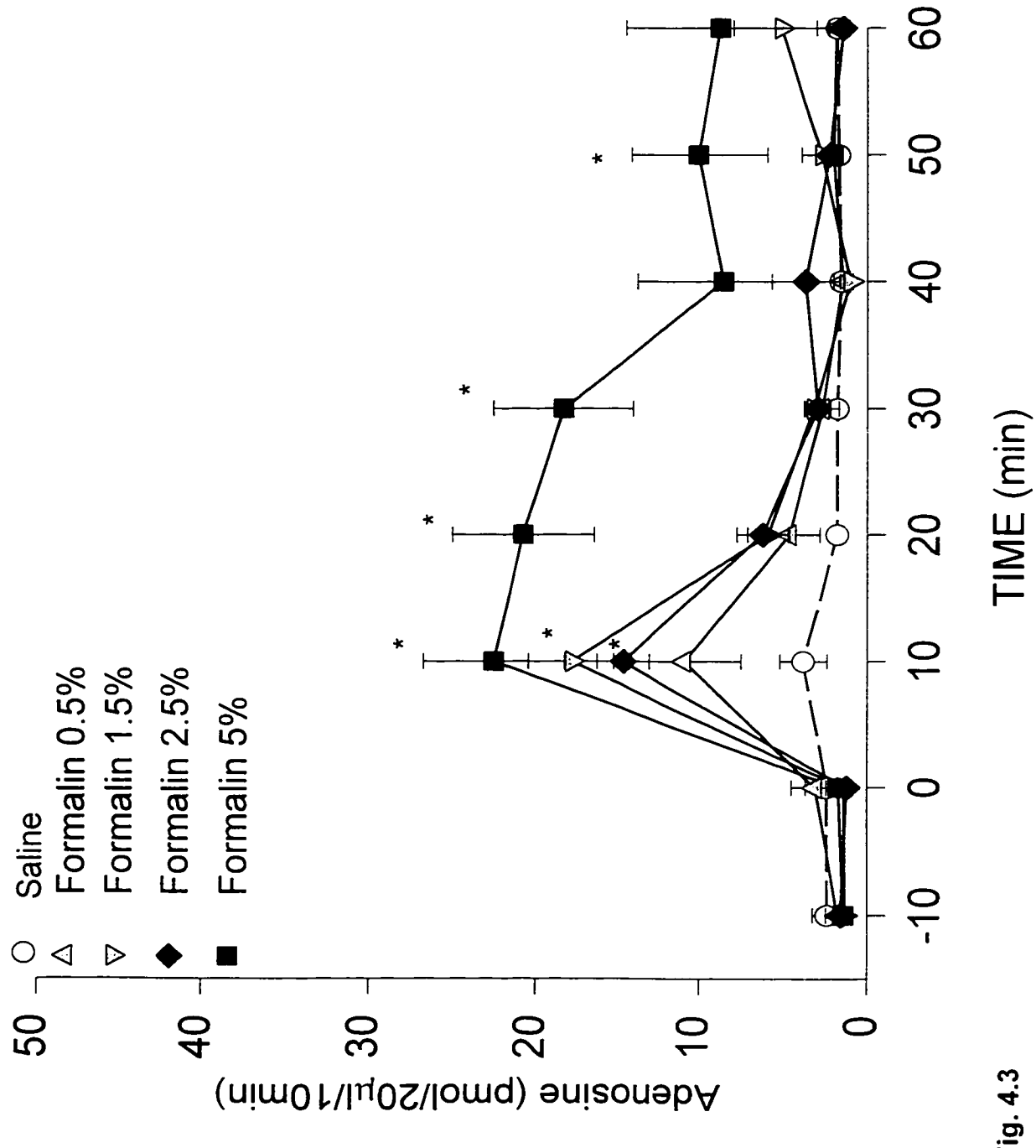

Saline
Formalin 0.5%
Formalin 1.5%
Formalin 2.5%
Formalin 5%
Adenosine (pmol/20µl/10min)
0
10
20
30
40
50
-10
0
10
20
30
40
50
60
TIME (min)
*
*
*
*
*
*

Fig. 4.3

Fig. 4.4: Time course of adenosine levels in microdialysate samples following injection of the adenosine kinase inhibitor, NH_2dAD 100nmol, and the adenosine deaminase inhibitor, DCF 100nmol with (A) saline, (B) 0.5% formalin (FOR) or (C) 5% formalin. Formalin or formalin-drug combinations were injected at time zero (50μl). * $P<0.05$ compared to corresponding control group (one-way ANOVA followed by SNK, means ± SEM, n=6-8).

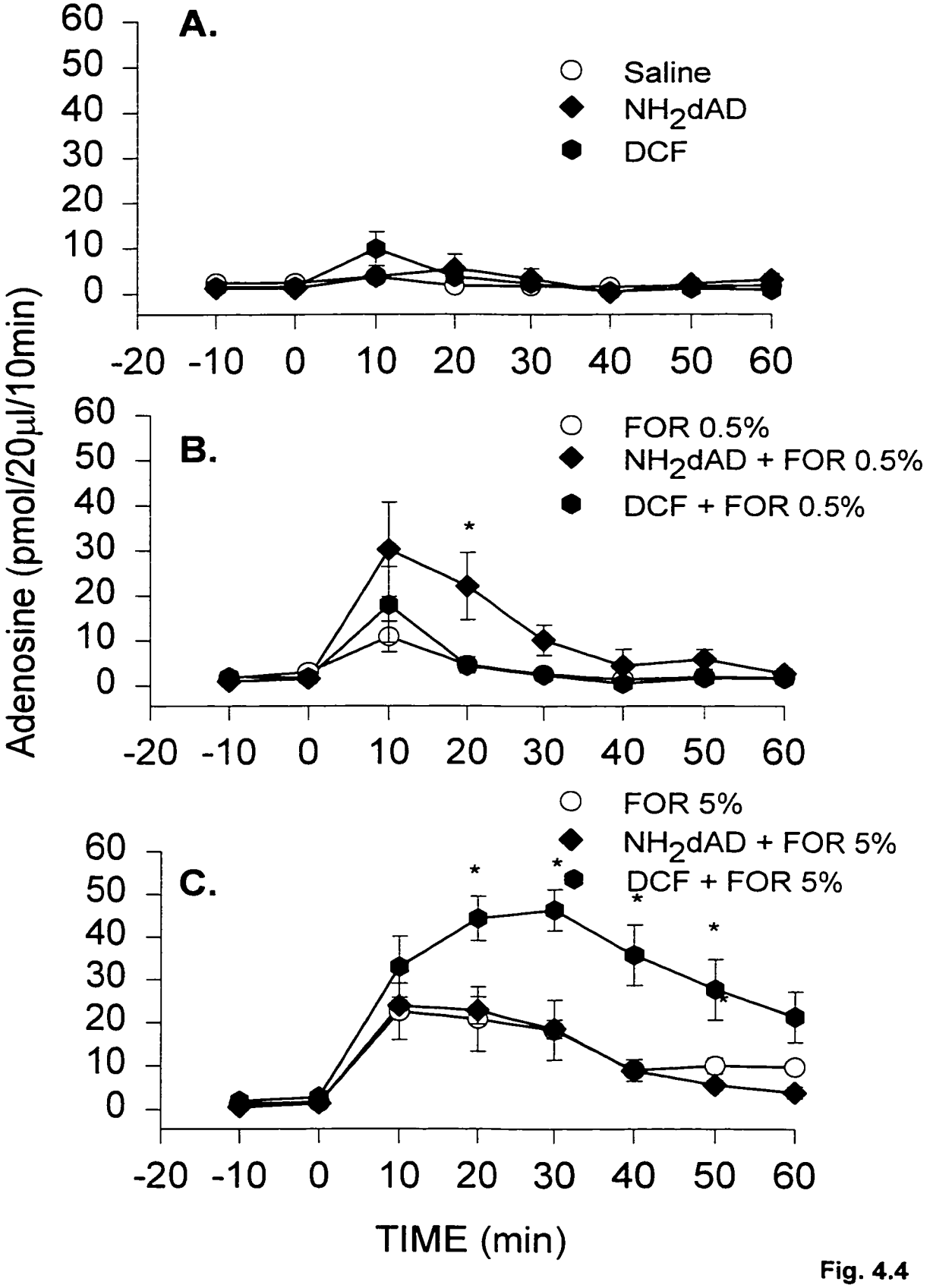

A.
Saline
NH2dAD
DCF
B.
FOR 0.5%
NH2dAD + FOR 0.5%
DCF + FOR 0.5%
C.
FOR 5%
NH2dAD + FOR 5%
DCF + FOR 5%
Adenosine (pmol/20µl/10min)
TIME (min)
60
50
40
30
20
10
0
-20 -10 0 10 20 30 40 50 60

Fig. 4.4

Fig. 4.5: Total evoked adenosine release over 60min interval following formalin, formalin with NH_2dAD, and formalin with DCF. Formalin or formalin and drugs combinations (50μl) were injected at time zero. *P <0.05 compared to corresponding formalin group. (One-way ANOVA followed by SNK, data from Fig. 4.4).

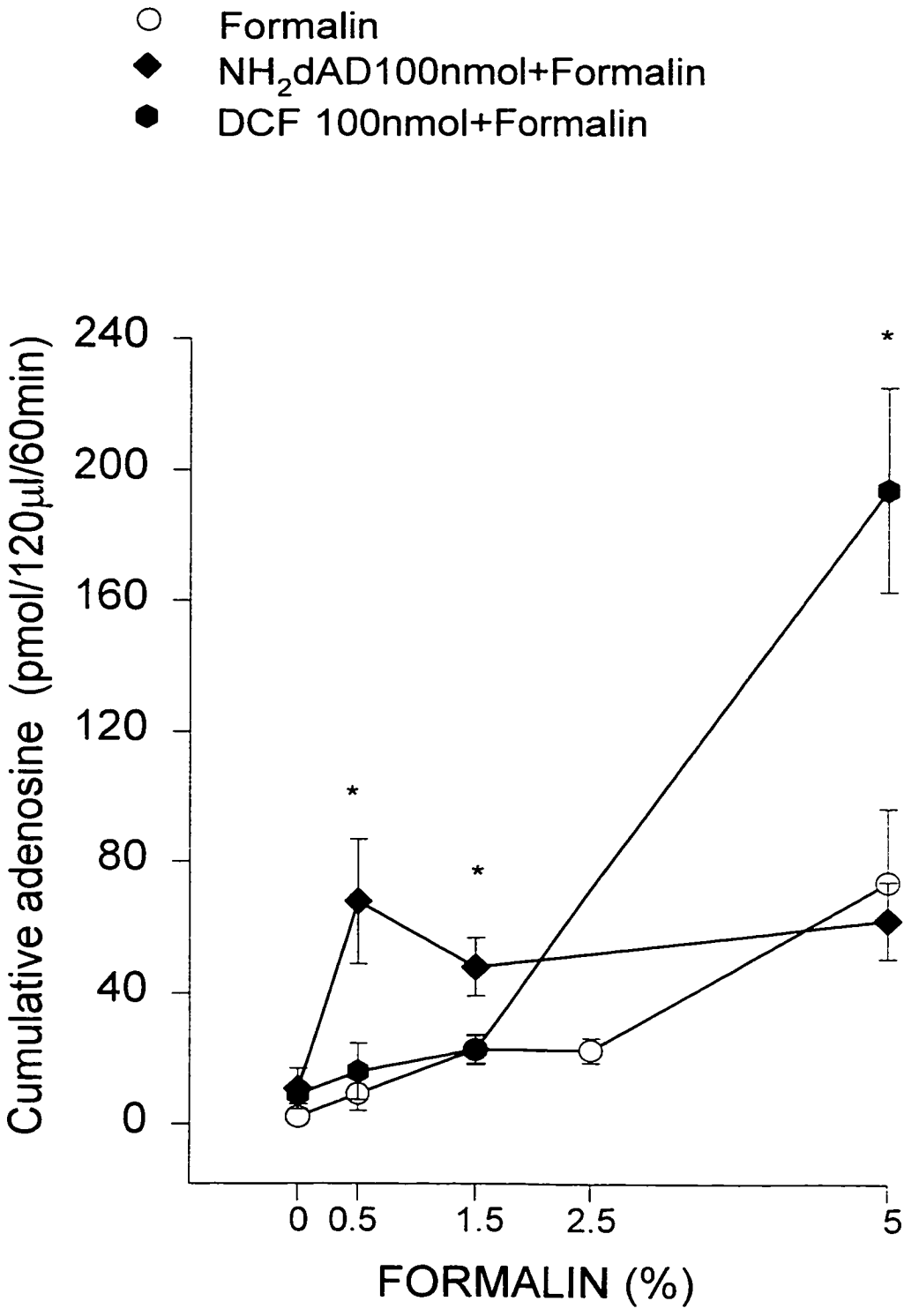

Formalin
NH2dAD100nmol+Formalin
DCF 100nmol+Formalin
Cumulative adenosine (pmol/120µl/60min)
240
200
160
120
80
40
0
0
0.5
1.5
2.5
5
FORMALIN (%)
*
*
*

Fig. 4.5

Fig. 4.6: Time course and cumulative changes over 60min produced by different concentrations of formalin (FOR) with respect to flinches (A and B) or biting/licking (C and D) behaviors. Formalin was injected at time zero. * $P<0.05$ compared to 5% formalin group (one-way ANOVA followed by SNK, means ± SEM, n=5-7).

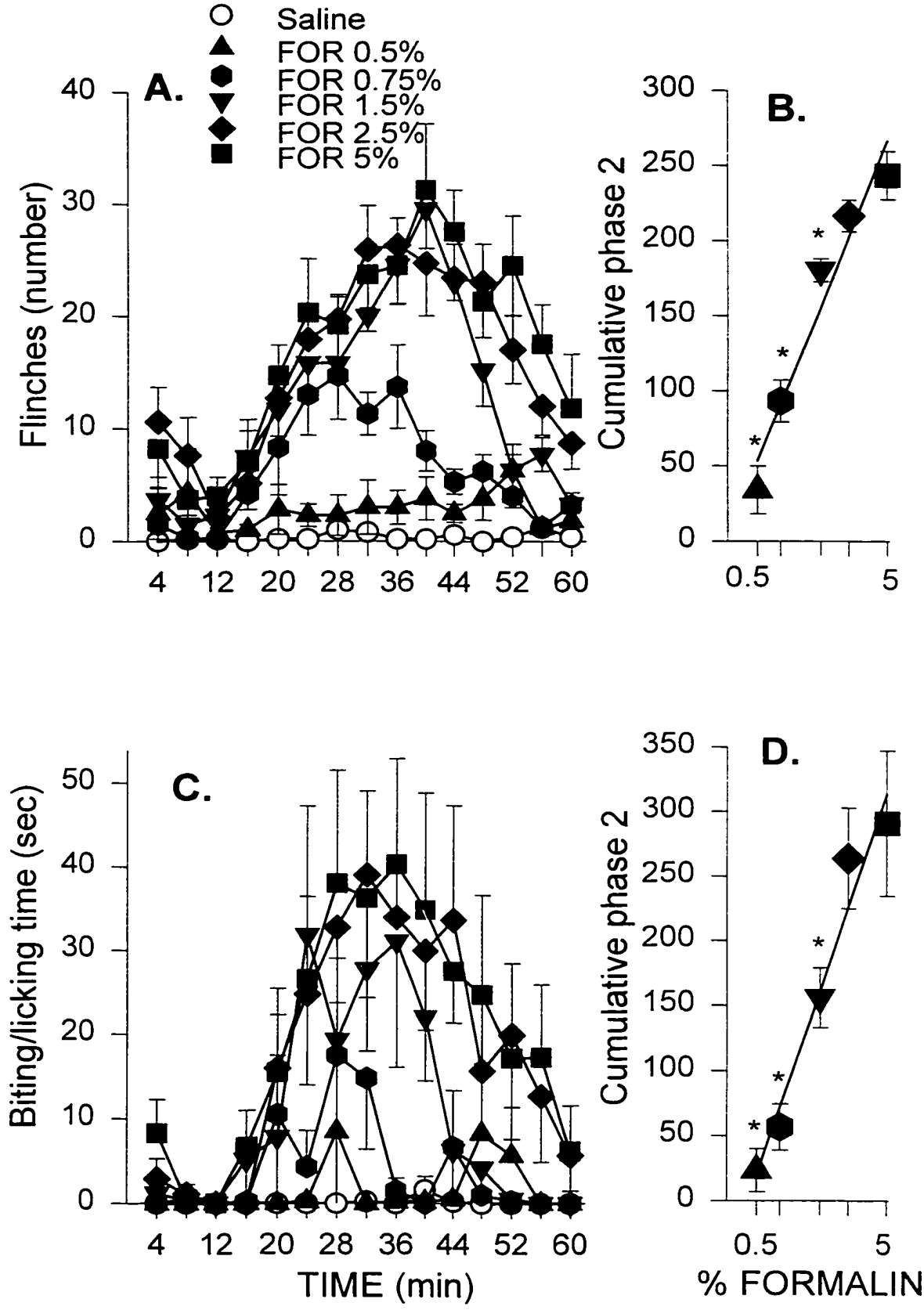

Saline
FOR 0.5%
FOR 0.75%
FOR 1.5%
FOR 2.5%
FOR 5%
A.
B.
C.
D.
Flinches (number)
Cumulative phase 2
Biting/licking time (sec)
TIME (min)
% FORMALIN
0
10
20
30
40
50
60
100
150
200
250
300
350
0.5
5
4
12
20
28
36
44
52
60
*

Fig. 4.6

Fig. 4.7: Time course and cumulative changes over 180min produced by different concentrations of formalin (FOR) with respect to paw volume. Formalin was injected at time zero. * $P<0.05$ compared to 5% formalin group (one-way ANOVA followed by SNK, means ± SEM, n=5-7).

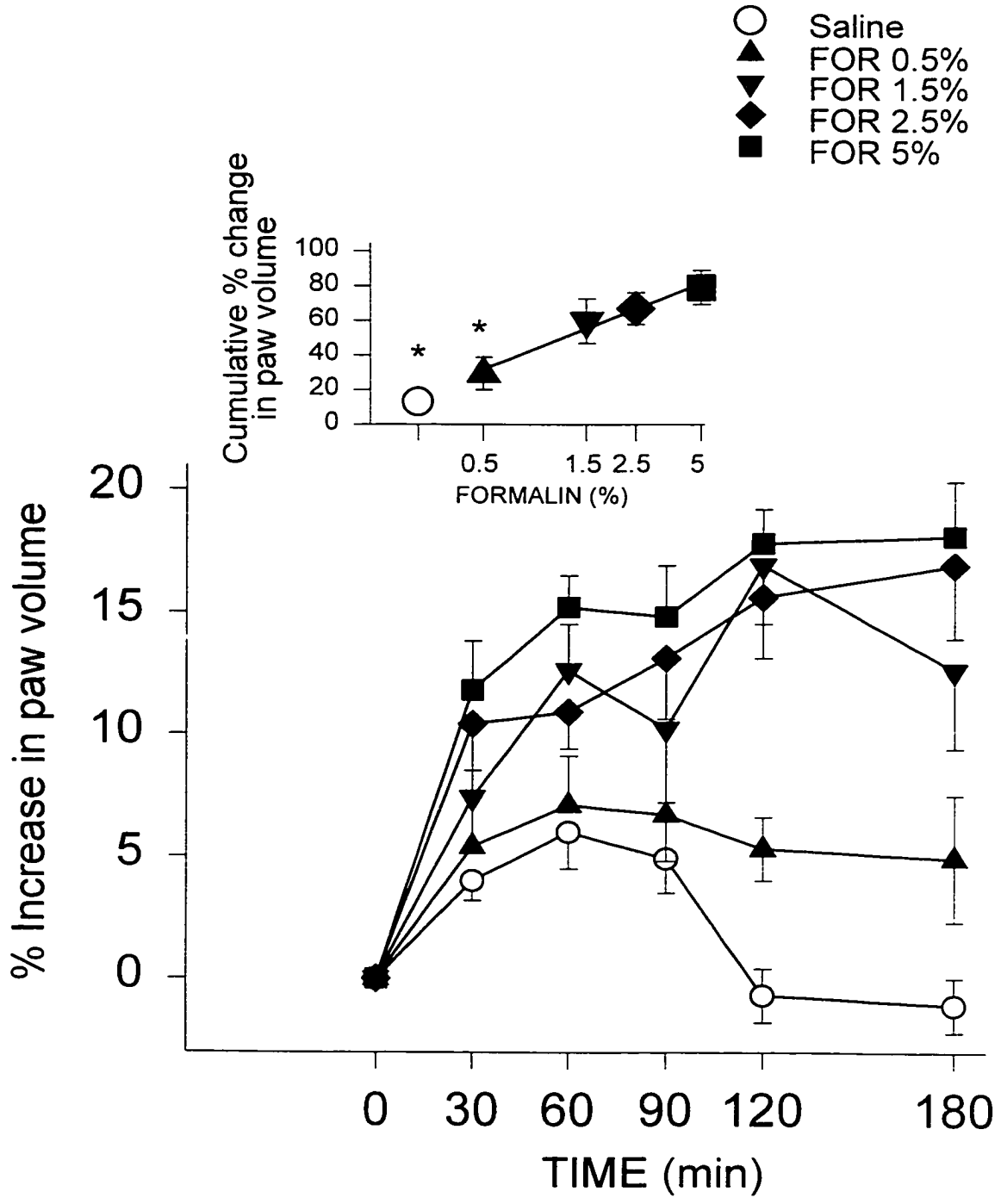

Saline
FOR 0.5%
FOR 1.5%
FOR 2.5%
FOR 5%
Cumulative % change in paw volume
100
80
60
40
20
0
*
*
0.5
1.5
2.5
5
FORMALIN (%)
% Increase in paw volume
20
15
10
5
0
0
30
60
90
120
180
TIME (min)

Fig. 4.7

4.3.6 Effects of capsaicin pretreatment on formalin-evoked adenosine release, flinches, and paw volume

Injection of formalin 1.5% induced a rapid increase in adenosine levels within the first 10min following formalin injection in vehicle-pretreated rats (Fig. 4.8A). Adenosine levels returned to baseline by the second sample and there were no significant increases in adenosine levels over the remaining collection time. Pretreatment with capsaicin significantly decreased 1.5% formalin-evoked release of adenosine (Fig 4.8A). Injection of 5% formalin induced a marked release of adenosine in vehicle-pretreated rats, and the increase was significantly higher than basal levels for the time period from the 10-50min following injection (Fig 4.8B). Capsaicin pretreatment reduced 5% formalin-induced adenosine release, with the significant decrease being observed within the first 20min following formalin injection (Fig 4.8B).

Pretreatment with capsaicin significantly reduces 1.5% formalin-evoked phase 2 flinches compared to the vehicle-pretreated rats, but did not produce a significant change on phase 1 (Fig 4.12A). Capsaicin pretreatment also significantly decreased 1.5% formalin-evoked increase in paw volume (vehicle-pretreated vs capsaicin-pretreated: 13±1.8% vs 3.5±0.9%, $p < 0.05$, *t*-test). Capsaicin pretreatment did not produce an effect either on flinches (Fig. 4.12 B) or on the paw volume effect (vehicle-pretreated vs capsaicin-pretreated: 18.8±3.0% vs 13.0±3.3%, $p=0.24$, *t*-test) evoked by 5% formalin. There was more variability in biting/lickig time and there was no significant difference in biting/licking time following capsaicin pretreatment at either concentrations of formalin (data not shown).

4.3.7 Acute capsaicin injection and adenosine release

Capsaicin (1%, 50µl) injected locally into the hind paw induced a sustained increase in adenosine levels. The increase was significantly elevated from baseline from the first sample until the fifth sample (at 50min), with the peak release at 10-20 min after injection (Fig. 4.9). Vehicle injection did not induce a significant increase in adenosine levels over baseline although values appeared somewhat elevated initially (Fig 4.9).

4.3.8 Effects of 6-OHDA pretreatment on formalin-evoked adenosine release, flinches and paw volume

At 1.5% formalin, both the vehicle and 6-OHDA-pretreated rats exhibited a rapid adenosine release within the first 10min following formalin injection, and no difference was observed between groups (Fig. 4.10A). Injection of 5% formalin induced a sustained increase in adenosine levels in vehicle-pretreated rats (Fig. 4.10B). Pretreatment with 6-OHDA markedly reduced 5% formalin-evoked release of adenosine compared to vehicle-pretreated rats at 20-40 min following injection (Fig. 4.10B).

Pretreatment with 6-OHDA did not produce an effect on either phase of flinching behaviors evoked by 1.5% formalin (Fig 4.12C). However, pretreatment with 6-OHDA significantly decreased phase 2, but not phase 1 flinches evoked by 5% formalin (Fig. 4.12 D). There was more variability in biting/licking time and there was no significant difference in biting/licking time following 6-OHDA pretreatment at either concentrations

4.3.9 Effects of compound 48/80 pretreatment on formalin-evoked adenosine release

Pretreatment with compound 48/80 did not produce a significant change in formalin-evoked adenosine release at either 1.5% or 5% over the entire time course (Figs. 4.11A and 4.11B).

Fig. 4.8: The effect of pretreatment with capsaicin on (A) 1.5% and (B) 5% formalin-evoked release of adenosine. Formalin was injected at time zero. * $p<0.05$ compared to vehicle-pretreated controls, *t* $p<0.05$ compared to respective basal levels (two-way repeated ANOVA followed by SNK, means ± SEM, n=7-8).

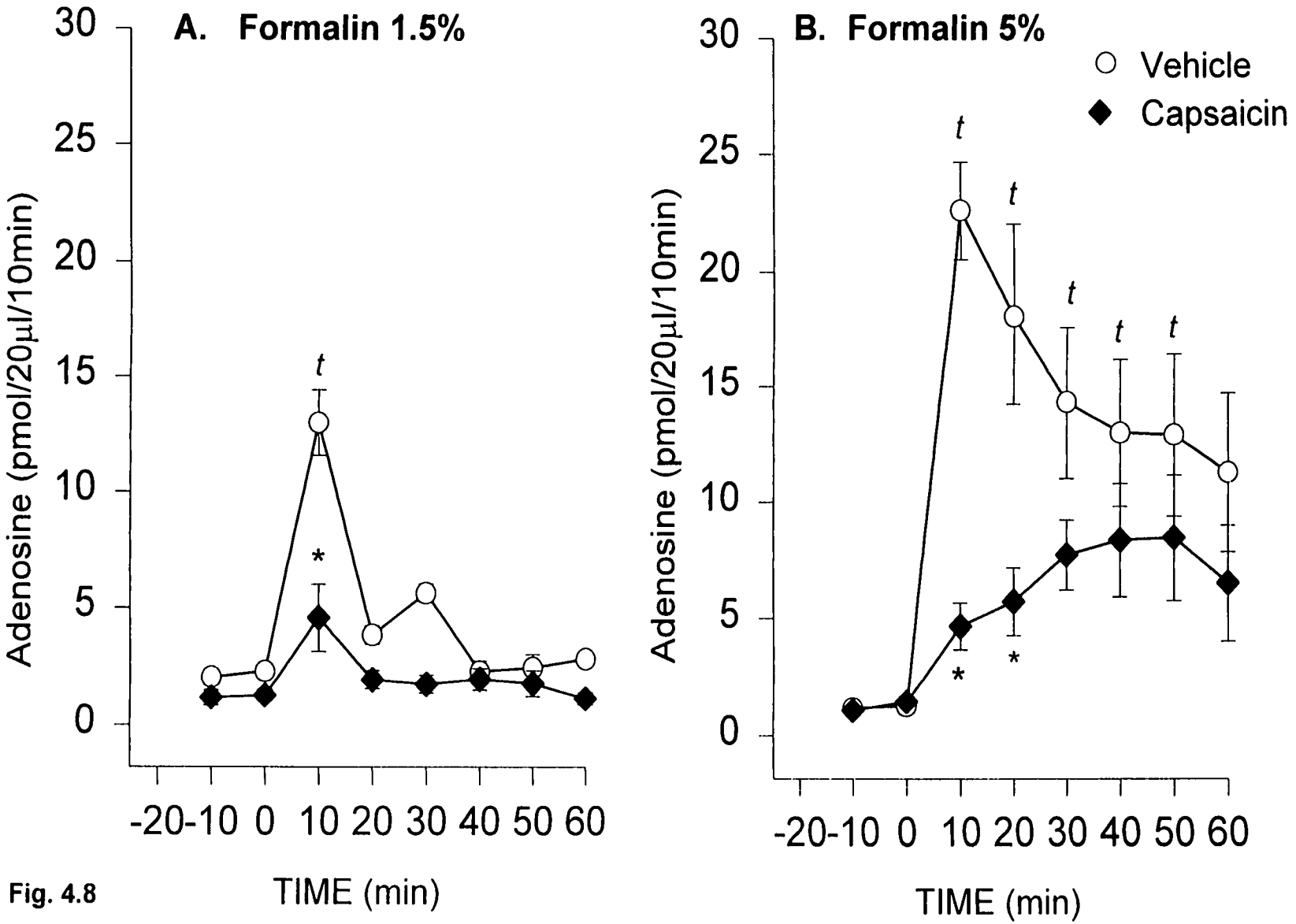

A. Formalin 1.5%
B. Formalin 5%
Vehicle
Capsaicin
Adenosine (pmol/20µl/10min)
TIME (min)
30
25
20
15
10
5
0
-20 -10 0 10 20 30 40 50 60
t
*

Fig. 4.8

Fig. 4.9: The effect of acute injection of capsaicin (1%) into the rat hind paw on local adenosine levels. Time course and cumulative change (inset) are depicted. Capsaicin or vehicle were injected at time zero. * $p<0.05$ compared to vehicle-pretreated controls, *t* $p<0.05$ compared to respective basal levels (two-way repeated ANOVA followed by SNK for time course, student's *t*-test for cumulative release, means ± SEM, n=9).

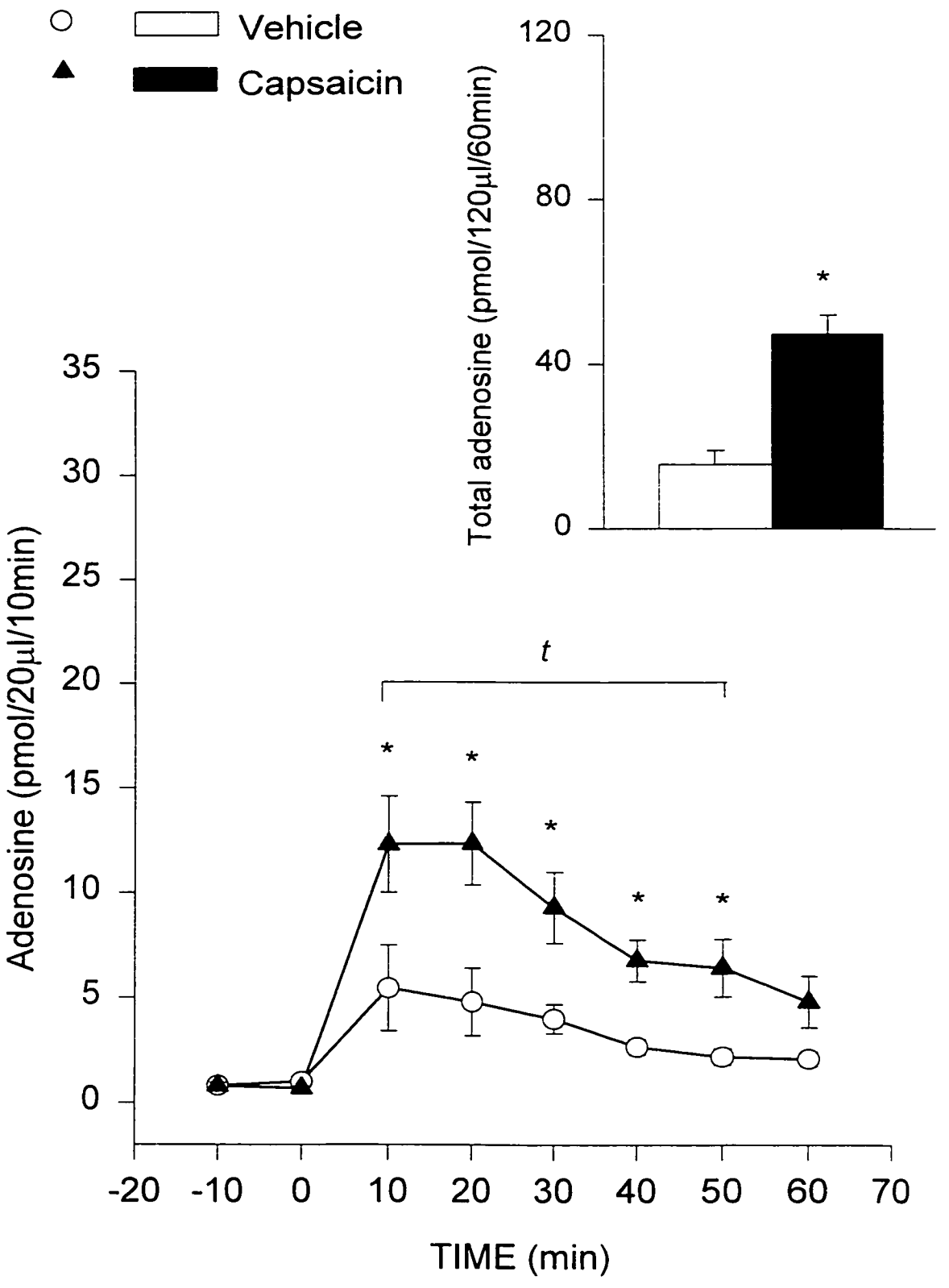

Vehicle
Capsaicin
Total adenosine (pmol/120µl/60min)
120
80
40
0
*
Adenosine (pmol/20µl/10min)
35
30
25
20
15
10
5
0
t
*
*
*
*
*
-20
-10
0
10
20
30
40
50
60
70
TIME (min)

Fig. 4.9

Fig. 4.10: The effect of pretreatment with 6-OHDA on (A) 1.5% and (B) 5% formalin-evoked adenosine release. Formalin was injected at time zero.

* $p<0.05$ compared to vehicle-pretreated controls, *t* $p<0.05$ compared to respective basal levels (two-way repeated ANOVA followed by SNK, means ± SEM, n=7-8).

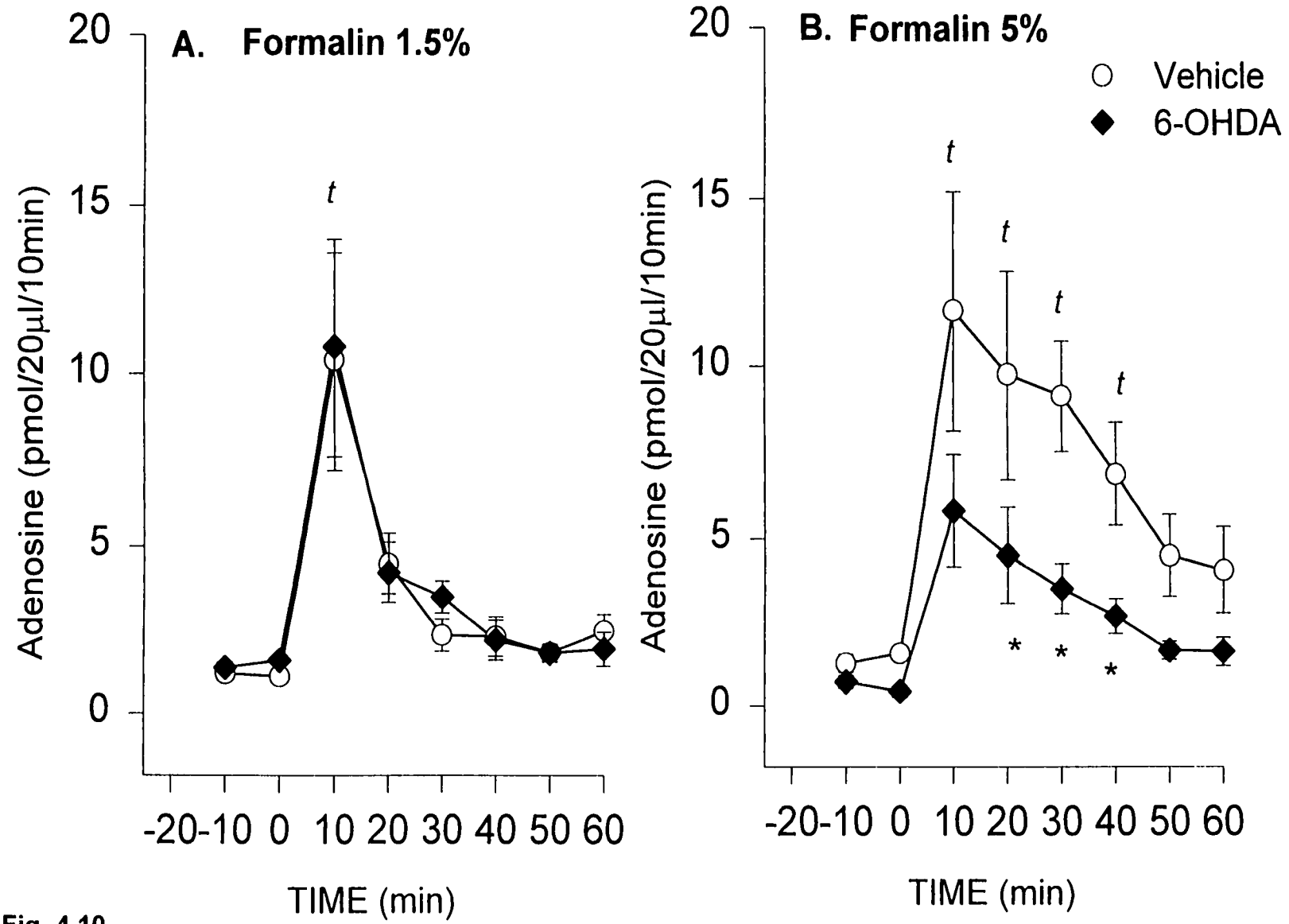

A. Formalin 1.5%
B. Formalin 5%
Vehicle
6-OHDA
Adenosine (pmol/20µl/10min)
TIME (min)
20
15
10
5
0
-20 -10 0 10 20 30 40 50 60
t
*

Fig. 4.10

Fig. 4.11: The effect of pretreatment with compound 48/80 on (A) 1.5% and (B) 5% formalin-evoked adenosine release. Formalin was injected at time zero. *t* $p<0.05$ compared to respective basal levels (two-way repeated ANOVA followed by SNK, means ± SEM, n=8).

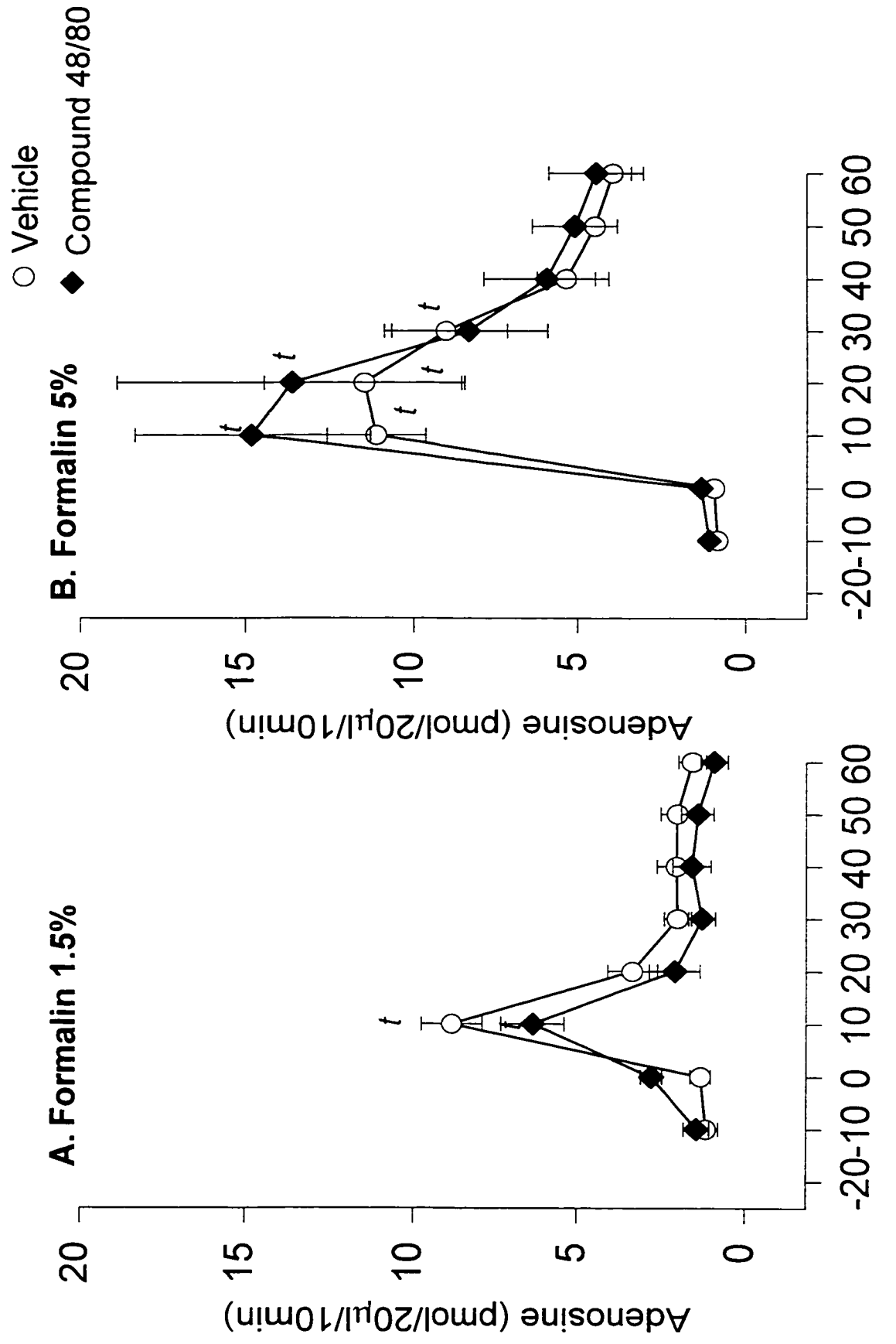

A. Formalin 1.5%
B. Formalin 5%
Vehicle
Compound 48/80
Adenosine (pmol/20µl/10min)
0
5
10
15
20
-20-10 0 10 20 30 40 50 60
TIME (min)
t

Fig. 4.11

Fig. 4.12: Effects of pretreatment with (A,B) capsaicin and (C,D) 6-OHDA on formalin-evoked behaviors. Cumulative scores of formalin-evoked phase 1 (P1, 0-12 min) and phase 2 (P2, 16-60min) flinches are depicted. * $p<0.05$ compared to respective vehicle-pretreated group. Open column, vehicle-pretreated group, filled column, drug-pretreated group (student's *t*-test, means ± SEM, n=5-6).

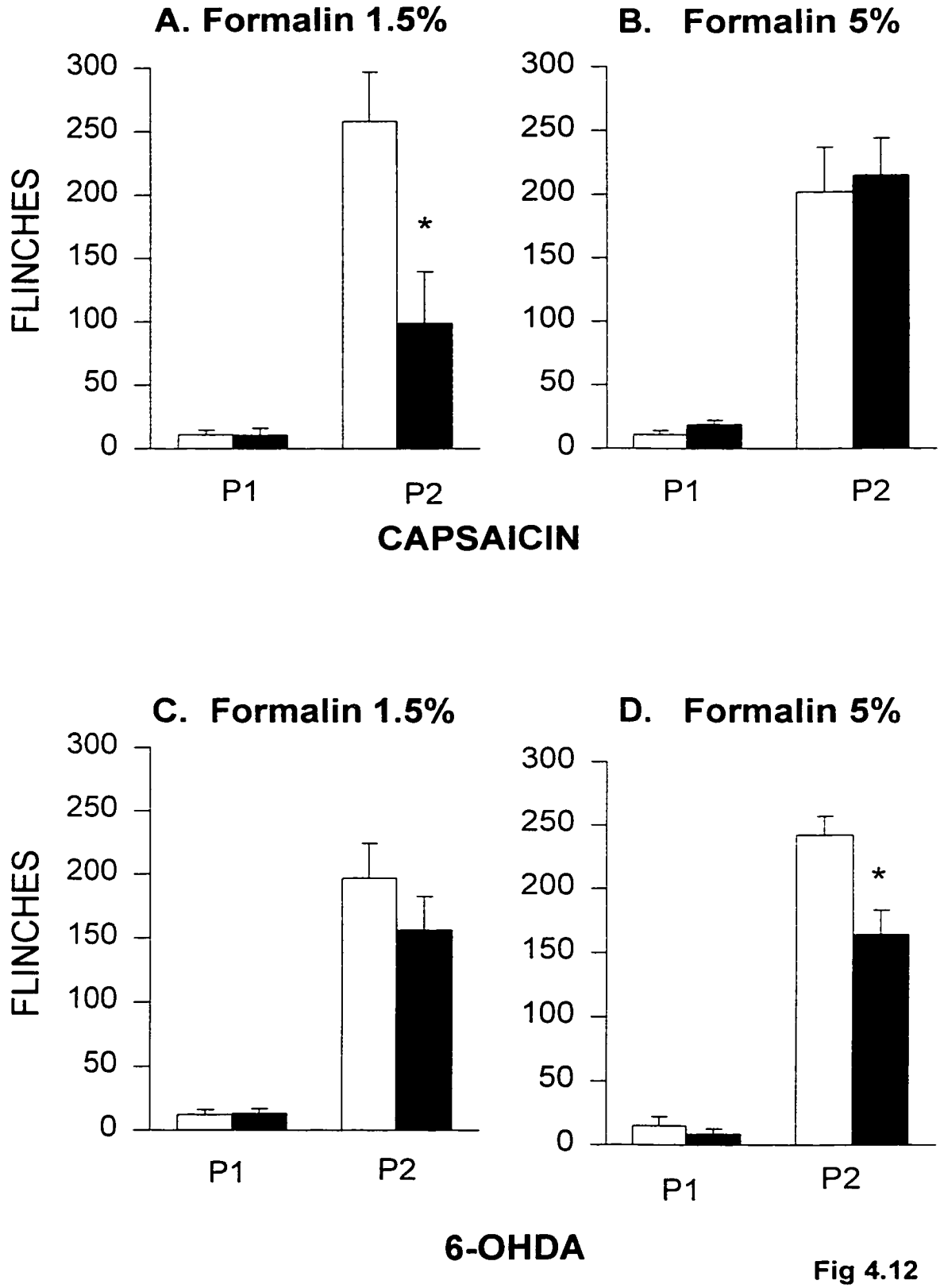

A. Formalin 1.5%
B. Formalin 5%
FLINCHES
300
250
200
150
100
50
0
*
P1
P2
CAPSAICIN
C. Formalin 1.5%
D. Formalin 5%
6-OHDA

Fig 4.12

4.4 DISCUSSION

Microdialysis has previously been used to measure purine release in both brain (Porkka-Heiskanen et al., 1997; Bell et al., 1998; Britton et al., 1999) and peripheral tissues (Lönnroth et al., 1989; Blay et al., 1997; MacLean et al., 1998; Manthei et al., 1998). In the present study, using this technique, we demonstrated that the basal adenosine concentrations in the subcutaneous area of the rat hind paw are about 0.23±0.02μM. This is comparable to basal adenosine concentrations in subcutaneous tissue of canine (0.32±0.04μM, Fredholm and Sollevi, 1981) and human (0.13±0.03μM, Lönnroth et al., 1989) subjects. The present study also demonstrates that the adenosine recovery rate is relatively constant and independent of the surrounding adenosine concentration gradient or the presence of formalin. Thus, subcutaneous microdialysis appears to be a valid method to measure local adenosine levels in the presence of formalin.

Although it has been demonstrated that adenosine is released peripherally during inflammation, and that this released adenosine can be pharmacologically modulated to produce anti-inflammatory (Cronstein et al., 1995; Rosengren et al., 1995; Firestein, 1996; Poon and Sawynok, 1999) and antinociceptive effects (Doak and Sawynok, 1995; Sawynok et al., 1998), little is known about the origin of such release. Adenosine, as a metabolite of ATP, a cellular energy source, can potentially be released from all cell types, either by transport of adenosine *per se* by plasma membrane transporters, or by degradation of extracellular ATP or other nucleotides by 5'-ectonucleotidase (Geiger et al., 1997; Section 1.6). Subcutaneous tissues of the rat hind paw exhibit marked activity

of enzymes degrading ATP (Bland-Ward and Humphrey, 1997), so released nucleotides can be rapidly broken down to adenosine by enzymes at this site.

It is generally believed that the non-specific release of adenosine or nucleotides from stressed cells, especially from neutrophils (Cronstein et al., 1983), endothelial cells (Ager and Gordon 1984) and mast cells (Marquardt et al., 1984), is the major source of extracellular adenosine in inflamed tissues. Although the present study does not exclude the involvement of non-specific adenosine release due to cell damage, the blockade by capsaicin and 6-OHDA pretreatment suggests a neuronal origin for substantial amounts of the adenosine released by formalin-induced inflammation. It should be noted that depleting nerve terminals might also reduce purine release secondarily from non-neuronal tissues that are innervated by these nerve terminals. For example, blocking primary sensory afferents (Taylor et al., 2000) and sympathetic nerve terminals (Fuchs et al., 1999) can decrease local blood flow and potentially decrease adenosine or ATP release from endothelial cells (Bodin and Burnstock, 1998).

4.4.1 Concentration-related adenosine release evoked by formalin

Injection of formalin into the rat hind paw produces two distinct phases of pain behaviors. Phase 1 (0-12 min) behaviors are generally considered to result from direct nociceptor activation, while phase 2 (16-60min) behaviors result from the combination of peripheral inflammation with central sensitization (Tjølsen et al., 1992; Puig and Sorkin, 1996). Nociceptive behaviors and inflammation produced by formalin are dependent on formalin concentrations. High concentrations of formalin produced more prominent nociceptive behaviors and paw edema than lower concentrations. Formalin-evoked

adenosine release was also dependent on formalin concentration. Lower concentrations of formalin (1.5-2.5%) produced an increase in adenosine release, which was predominantly observed during the phase 1 period (0-10min). There was a plateau between 1.5% and 2.5% formalin, and 5% formalin-evoked a release that was both higher in peak release and longer in duration (observed in both phase 1 and phase 2).

At lower concentrations (1.5-2.5%), as the released adenosine was observed primarily during the first 10min, it is likely that neuronal activation plays a major role in this release, although a non-specific release resulting from tissue injury cannot be excluded. It is interesting to note that the pattern of adenosine release evoked by 5% formalin was quite different from that evoked by 2.5% formalin, although the nociceptive behaviors produced by the two concentrations were comparable. It is likely that 1.5-2.5% formalin produces a maximal activation of neuronal activity. At 5% formalin, an additional release of adenosine due to tissue injury, cell necrosis and platelet aggregation may occur, and this non-neuronal release may contribute a major source of adenosine in the later phase of the formalin test. 5% Formalin produces a more pronounced tissue damage (Rosland et al., 1990) with an involvement of multiple inflammatory mediators (Damas and Liégeois, 1999). With 5% formalin, combination of both neurogenic and non-neurogenic inflammatory mediators including neuropeptides, prostanoids, 5-HT, histamine (Damas and Liégeois, 1999) glutamate (Omote et al., 1998; 2000) and nitric oxide (Omote et al., 2000) is involved. The various inflammatory mediators may act on neuronal tissues to promote adenosine release (Sweeney et al., 1990; Fallahi et al., 1996; Cahill et al., 1997; Conway et al., 1997; Conway and Yaksh, 1998). Non-neuronal tissues such as endothelial cells (Ager and Gordon, 1984), neutrophils (Cronstein et al.,

1983), mast cells (Marquardt et al., 1984) and subcutaneous adipose tissue (Fredholm and Sollevi, 1981) can also release adenosine secondary to neuronal activity or inflammatory mediator stimulation. The release of adenosine by these inflammatory mediators may contribute substantially to the adenosine release evoked by 5% formalin, especially in the later phase when inflammation has developed. This hypothesis was further investigated by determining the involvement of capsaicin-sensitive sensory afferents, the sympathetic nervous system and mast cells in a low dose (1.5%) and a high dose (5%) formalin-evoked adenosine release.

4.4.2 Involvement of capsaicin-sensitive sensory afferents in formalin-evoked adenosine release

Capsaicin, by activating non-selective cation channel receptors on small diameter unmyelinated C fibers and thinly myelinated Aδ fibers, induces an acute release of pro-inflammatory neuropeptides such as SP and CGRP from both the central terminal and the peripheral terminals of these afferents, while large-dose repeated treatment of capsaicin selectively destroys these small diameter primary afferents (Holzer, 1991). Pretreatment with capsaicin, in the present study, almost eliminated the 1.5% formalin-evoked spike of adenosine release, and markedly reduced the adenosine release evoked by 5% formalin in the first 20min, indicating the involvement of C fibers and Aδ fibers in such release. It is consistent with electrophysiological studies that show that formalin induces a predominant activity of Aβ, Aδ and C fibers in phase 1, and that while the activity of Aδ and C fibers persists into phase two, the magnitude is much lower (Puig and Sorkin, 1995; McCall et al., 1996).

Acute injection of 1% capsaicin into the untreated rat hind paw induced a significant release of adenosine, providing further support for the involvement of capsaicin sensitive peripheral terminals of sensory afferents in such release. In previous studies, capsaicin-sensitive release of adenosine and nucleotides from the central terminals of primary sensory afferents has been reported (Sweeney et al., 1989; 1990; Cahill et al., 1997), and antidromic stimulation of sensory nerves induced ATP release into rabbit ear artery indicating a peripheral release of purines (Holton and Holton, 1954; Holton, 1959). The present study extends those studies, identifying an invlovement of capsaicin-sensitive primary sensory afferent terminals in peripheral adenosine release in upon chemical stimulation.

Consistent with the microdialysis study, capsaicin pretreatment also significantly blocked the flinch behavior and paw volume changes evoked by 1.5% formalin. The marked block of low dose formalin-evoked adenosine release, behavioral responses and paw volume changes by capsaicin pretreatment suggests a predominant small diameter sensory afferent activation following low dose formalin injection. This is consistent with previous studies that showed that block of capsaicin-sensitive sensory afferents reduces the nociceptive behaviors (Shibata et al., 1989; Peterson et al., 1997) and the inflammatory response (Damas and Liégeois, 1999). At 5% formalin, capsaicin pretreatment only blocked the early phase but not the late phase of adenosine release, indicating that nociceptive afferent activity is predominant in the early phase, while other inputs contribute to the later phase of adenosine release. Our capsaicin studies did not detect any changes in flinch behaviors and paw volume evoked by 5% formalin, suggesting the role of direct nociceptor excitation in modulating nociceptive behaviors

and inflammation evoked by the high concentration formalin is less important compared with that evoked by lower concentrations of formalin. However, previous studies indicate neonatal pretreatment (Peterson et al., 1997) or local capsaicin desensitization (Wheeler-Aceto and Cowan, 1991) is able to block both phases of the nociceptive response evoked by 5% formalin. The difference may be due to the different capsaicin pretreatment protocol, as it has been demonstrated that neonatal pretreatment with capsaicin produces a more complete depletion of nociceptive afferents compared with capsaicin pretreatment in adult rats (Holzer, 1991). Using plasma extravasation as an indicator, a previous study also demonstrates nociceptive sensory afferents play a role in the 5% formalin-evoked local inflammatory response (Damas and Liégeois, 1999).

4.4.3 Involvement of SPGNs in formalin-evoked adenosine release

The inhibition of 5% formalin-evoked release of adenosine by 6-OHDA pretreatment indicates the involvement of sympathetic postganglionic nerve terminals in adenosine release in the late phase following a high dose of formalin. As 6-OHDA does not cross the blood brain barrier in adult rats, systemic administration selectively depletes sympathetic postganglionic nerve terminals rather than central aminergic nerves (Kostrzewa and Jacobowitz, 1974). ATP is a well-known neurotransmitter in sympathetic nerve terminals, and activation of such terminals can release ATP as a cotransmitter with noradrenaline and neuropeptide Y (Racchi et all, 1999). Importantly, sympathetic stimulation also simultaneously releases nucleotidases, so the released ATP is rapidly degraded into adenosine through both released nucleotidases and ecto-nucleotidase (Todorov et al., 1997).

Besides neurotransmitters, inflammatory prostaglandins can be synthesized *de novo* by sympathetic postganglionic neurons, and subsequently released to induce plasma extravasation and sensitization of nociceptors (Cooper and Malik, 1985; Coderre et al., 1989; Gonzales, et al., 1991). Interestingly, inhibition of prostaglandin production by NSAIDs also produces a late phase, but not an early phase, inhibition of formalin-induced nociceptive behaviors (Hunskaar and Hole 1987; Shibata et al., 1989; Rosland et al., 1990; Yashpal and Coderre, 1998).

Chemical sympathectomy with 6-OHDA pretreatment only blocked the *late phase* responses evoked by 5% but not 1.5% formalin in adenosine release and nociceptive behaviors. Previous behavioral studies with both pharmacological blockade of adrenoreceptors (Hong and Abbott, 1996), or surgical (Fuchs et al., 1999) and chemical sympathectomy (Coderre et al., 1984ab) also showed a greater reduction in formalin-induced nociceptive behaviors in phase 2, but at lower formalin concentrations (1%-2.5%). Different pretreatment procedures, different strains of animals and different recording methods may contribute to the discrepancy. However, consistent with our study, the involvement of SPGNs in the inflammatory response was only observed at high dose formalin (Coderre et al., 1984b; Lam and Ferrell, 1991). These results indicate that SPGNs are selectively activated when overt inflammation and tissue injury are present (Yashpal and Coderre, 1998; Damas and Liégeois, 1999; Fu et al., 2000).

4.4.4 The lack of involvement of mast cells in formalin-evoked adenosine release

Pretreatment with compound 48/80 failed to demonstrate an involvement of mast cells in adenosine release by both concentrations of formalin, even though it has been shown that acute challenge of mast cells *in vitro* with compound 48/80 releases adenosine (Marquardt et al., 1984). It is unlikely that the negative observation in the present study reflects an ineffective mast cell degranulation, as we used the same protocol to block the inflammation induced by an adenosine A_3 receptor agonist which acts on mast cells (Sawynok et al., 2000). Although it has been suggested that C fiber activation can release substance P, which consequently degranulates mast cells to release histamine and other substances (Coderre et al., 1989; Suzuki et al., 1999), there is little evidence implicating mast cell degranulation in the early phase of formalin-induced responses (Damas and Liégeois, 1999; Shibita et al., 1989). However, in the late phase, there is histological evidence for mast cell degranulation even at very low doses of formalin (Rosland et al., 1990), and mast cells appear to mediate late phase low dose (0.5%) formalin behavioral responses (Shibita et al., 1989) as well as high dose (5%) formalin inflammatory responses (Damas and Liégeois, 1999). The lack of effect of compound 48/80 in the present study suggests either that adenosine released from mast cells is not substantial enough to be detectable by the current method, or that mast cells are not as important as primary sensory neurons or SPGNs in formalin-induced responses. Consistent with our study, mast cell degranulation is much less effective than capsaicin pretreatment in blocking plasma extravasation induced by a wide range of concentrations of formalin (Arvier et al., 1977).

4.4.5 Qualitatively different mechanisms mediating low and high dose formalin-evoked neurochemical and nociceptive responses

Behavioral responses to formalin are clearly dose related (Rosland et al., 1990; Abbott et al., 1995). Many previous studies considered the different concentrations of formalin to reflect only a quantitative difference in intensity of responses, rather than qualitative differences in mediating these responses. However, an emerging body of data indicate the responses at low and high concentrations of formalin are qualitatively quite distinct. (1) Yashpal and Coderre (1998) have demonstrated a selective dependence on prostaglandin synthesize in nociceptive behaviors and inflammatory responses evoked by higher but not lower concentrations of formalin. (2) Damas and Liégeois (1999) have demonstrated that mechanisms mediating peripheral inflammation and neurochemical responses are quite different at low and high concentrations of formalin, as there is a predominant capsaicin-sensitive afferent activity at low doses, with the additional involvement of a more complex inflammatory component at the higher dose. (3) Fu and colleagues (1999; 2000) have demonstrated that only high doses of formalin produce chronic effects such as spinal cord microglial activation, severe tissue ulceration, and long term hyperalgesia, which are not observed at low doses of formalin. The results in those studies, together with observations in the present studies, collectively demonstrate that distinct mechanisms are involved following low and high doses of formalin. Thus, when interpreting information obtained from the formalin model, it is important to consider the concentration used, and in order to obtain comprehensive information about drug actions, both low and high doses of formalin should be used.

4.4.6 Modulation of extracellular adenosine levels by NH_2dAD and DCF

The present study directly demonstrates that inhibition of adenosine kinase and adenosine deaminase have no effect on basal adenosine release, but can increase extracellular adenosine levels in presence of formalin, in a manner that correlates with the kinetics of the two enzymes. In the absence of formalin, neither the adenosine kinase inhibitor NH_2dAD nor the adenosine deaminase inhibitor DCF increased basal extracellular adenosine levels. This accords to the previous studies in brain (White, 1996; Britton et al., 1999) demonstrating that the augmentation of adenosine levels by inhibiting these enzymes is event specific, and that the enhancement becomes substantial only in conditions where basal adenosine levels are increased.

In the present study, the adenosine kinase inhibitor NH_2dAD increased adenosine levels at lower concentrations of formalin but not at 5% formalin, while the adenosine deaminase inhibitor DCF increased adenosine levels at 5% but not lower concentrations of formalin. These results reflect the different kinetics of the two enzymes. Adenosine kinase has a higher affinity (K_m: 0.8-2.0 vs 17-47μM; Arch and Newsholme, 1978; Phillips and Newsholme, 1979) and a lower maximum activity (V_{max}: 60-136 vs 174-1430nmol/min/g fresh tissue; Arch and Newsholme, 1978) than adenosine deaminase for metabolizing adenosine in most tissues. The most significant increase in extracellular adenosine levels by the adenosine kinase inhibitor was observed 10-20min after 0.5% formalin injection. This indicates the optimal concentration of adenosine f6+or adenosine kinase in rat paw subcutaneous tissues is around 0.93μM *in vivo* (after correction for the recovery rate). In the presence of 5% formalin, when more adenosine is released, there is no increase in adenosine levels by inhibiting adenosine kinase. It is

inhibition occurs under this condition, as adenosine kinase exhibits substrate inhibition at high adenosine levels (Arch and Newsholme, 1978).

On the other hand, at lower concentrations of formalin, inhibition of adenosine deaminase did not increase adenosine levels, as adenosine levels were lower than the required concentration for metabolism by this lower affinity enzyme. In the presence of 5% formalin, when more adenosine is released at all time intervals, the modulation by adenosine deaminase became significant, and there was an increase in adenosine levels by DCF throughout the entire 60min. Previous neurochemical studies in brain (Lloyd and Fredholm, 1995; White, 1996; Hebb and White, 1998) and spinal cord (Golembiowska et al., 1996) also demonstrate that adenosine kinase exerts a predominant role in adenosine metabolism in most tissues. When adenosine concentrations are elevated substantially, such as in tissues that are under severe energy depletion (Lloyd and Fredholm, 1995), adenosine deaminase inhibition produces a substantial effect. The current study clearly demonstrates that adenosine kinase is the more important enzyme modulating adenosine levels at low to moderate levels of inflammation, while adenosine deaminase becomes more important at higher levels of inflammation when large amounts of adenosine are released.

4.4.7 Correlation of formalin-evoked adenosine release with behavioral and paw volume studies

The concentration-related adenosine release correlates with the tonic adenosine receptor activity revealed in previous behavioral studies. Thus, at 2.5% formalin, an adenosine A_1 receptor antagonist augments, while an A_{2A} receptor antagonist reduces, the

early part of phase 2 flinching behaviors, but no such modulation occurs at 0.5% formalin (Doak and Sawynok, 1995; Sawynok et al., 1998). These studies indicate that adenosine released by 0.5% formalin is not sufficient to activate peripheral adenosine receptors, but at higher concentrations, such as 2.5% formalin, the released adenosine can produce a tonic activation of both adenosine A_1 and A_{2A} receptors which mediate antinociceptive and nociceptive effects, respectively (Karlsten et al., 1992; Sawynok, 1998). In the present study, the increase in adenosine levels caused by low concentrations of formalin is significant only within 10min after injection, which corresponds to phase 1 behaviors, but the tonic nociceptive effect revealed by adenosine receptor antagonists is predominately observed in the early part of phase 2 behaviors (14-36min, Doak and Sawynok, 1995). There is thus a delayed expression of adenosine effects on behavior. A dissociation of neurochemical and behavioral effects also has been demonstrated for formalin-induced glutamate release. Thus, while formalin evokes spinal glutamate and aspartate release predominately in phase 1 (Malmberg and Yaksh, 1995), spinal antagonists acting on glutamate receptors have a predominant antinociceptive effect on phase 2 behaviors (Haley et al., 1990; Yamamoto and Yaksh, 1992).

The different abilities of adenosine kinase inhibitors and adenosine deaminase inhibitors to modulate adenosine levels also accord with previous behavioral studies. Thus, while the antinociceptive effect of adenosine kinase inhibitors has been demonstrated both peripherally (Sawynok et al., 1998) and spinally (Keil and DeLander, 1992; 1996; Poon and Sawynok, 1995; 1998), inhibition of adenosine deaminase does not produce an intrinsic antinociceptive effect. However, when substrate adenosine levels are substantially elevated, such as in the presence of exogenous adenosine (Keil and

DeLander, 1994), inhibition of adenosine deaminase produces enhanced antinociceptive effects.

In view of the marked enhancement of extracellular adenosine levels by local co-administration of DCF with 5% formalin, we investigated if DCF could alter nociceptive responses or the change in paw volume produced by 5% formalin. However, we did not observe any antinociceptive effect of DCF on either flinching or biting/licking behaviors at 5% formalin (data not shown). One explanation is that the adenosine amounts released under these conditions was high enough to activate not only adenosine A_1 receptors producing antinociceptive effects, but also adenosine A_{2A} and A_3 receptors producing nociceptive effects (Sawynok, 1998) and thus the antinociceptive effects were obscured. Another possibility is that, at 5% formalin, so many nociceptive mediators were released (cf. Tjølsen et al., 1992; Damas and Liegéois, 1999) that the effect of adenosine was obscured amongst the multiple effects.

The lack of effect of DCF on paw volume at 5% formalin over the 3 hour time course (data not shown) reflects a similar involvement of multiple mediators in generating this effect at 5% formalin (Damas and Liégeois, 1999). Curiously, no modulatory effect on paw volume was observed with NH_2dAD at either 0.5% or 1.5% formalin over this time course (data not shown). (Earlier time intervals at 10 and 20min, considered more relevant to the neurochemical observations, could not be examined, as the stress of repeated handling at the short time intervals prevented formalin from increasing the paw volume). In other paradigms of inflammation, inhibition of adenosine kinase produced anti-inflammatory actions (Cronstein et al., 1994; Firestein et al., 1994; Rosengren et al., 1995). The lack of effect of the inhibition of adenosine kinase in this

study may indicate (a) that paw volume alone reflects only a limited aspect of the inflammatory response, and that other anti-inflammatory endpoints are not reflected in this paradigm, or (b) that when adenosine kinase inhibitors are administered systemically, a component of their activities is mediated at spinal, rather than at peripheral, sites (cf. Bong et al., 1996). It is likely that the influence of inhibiting adenosine kinase on inflammation is dependent on the degree of inflammation and substrate adenosine concentration.

4.5 CONCLUSION

In summary, this study demonstrates that subcutaneous formalin evokes adenosine release in the rat hind paw. The pattern of adenosine release is dependent on formalin concentration, which suggests that, at different levels of inflammation, different mechanisms mediate this release. Only small diameter capsaicin-sensitive afferents are involved at a low dose formalin, while both capsaicin-sensitive primary afferents (during the early phase) and SPGN terminals (during the late phase) are involved in a high dose formalin-evoked adenosine release. Mast cell degranulation is not involved in such release. The ability of inhibitors of adenosine kinase and adenosine deaminase to modulate extracellular adenosine level in the presence of formalin accords with the kinetics of the two enzymes.

CHAPTER 5

ENHANCED PERIPHERAL ADENOSINE RELEASE IN THE RAT HIND PAW FOLLOWING SPINAL NERVE LIGATION: INVOLVEMENT OF THE CAPSAICIN-SENSITIVE SENSORY AFFERENTS

5.1 INTRODUCTION

A large body of clinical and animal data suggest activation of endogenous adenosine receptors by exogenously administered adenosine, or altering endogenous adenosine availability, may be a potentially effective pharmacological approach in managing neuropathic pain (Sawynok, 1999; Karlsten and Gordh, 2000). Clinically, systemic infusion of adenosine alleviates tactile allodynia and ongoing pain in patients with peripheral neuropathy (Sollevi et al., 1995; Sjölund et al., 2000). In animal studies, increasing endogenous adenosine levels by inhibiting enzymes involved in adenosine metabolism through systemic (Lynch et al., 1999; Kowaluk et al., 2000), spinal (Lavand'homme and Eisenach, 1999) and local peripheral (Chapter 3) administration of inhibitors of adenosine kinase can produce antiallodynic or antihyperalgesic effects in rat models of neuropathic pain.

Inhibition of adenosine kinase or adenosine deaminase selectively increases adenosine levels at tissue sites where pathological changes result in an increased adenosine production (Britton et al., 1999; Chapter 4). As the adenosine kinase inhibitor NH_2dAD and the adenosine deaminase inhibitor DCF both can produce a locally mediated antihyperalgesic effect in neuropathic rats (Chapter 3), we hypothesized that nerve injury results in an increase in peripheral adenosine levels which can be further modulated by inhibiting adenosine breakdown. This increase may be due to hyperactivity in sensory primary afferent neurons and/or sympathetic postganglionic neurons induced by nerve injury. Thus, spontaneous activity of primary sensory fibers, and hypersensitivity of SPGNs have been widely demonstrated in various neuropathic

models (Fig. 5.1; Section 1.5). Furthermore, adenosine and ATP can be released from the peripheral terminals of SPGNs (Burnstock and Wood 1996; Burnstock, 2000) and primary afferent neurons (Holton and Holton, 1954; Holton, 1959).

Spinal nerve ligation (SNL) is a sciatic nerve partial injury model produced by tight ligation of the L5 and L6 spinal nerves (Kim and Chung, 1992; Fig. 5.1). In this model, neuropathic pain behaviors can be reduced by modulating endogenous adenosine concentrations with peripheral (Chapter 3), systemic (Kowaluk et al., 2000) and spinal (Lavand'homme and Eisenach 1999) administration of inhibitors of enzymes involved in adenosine metabolism. The sympathetic hypersensitivity (Kim et al., 1993; Leem et al., 1997) and primary sensory afferent neuron hyperactivity (Sheen and Chung, 1993; Han et al., 2000) have been observed in this model.

The aim of the current study was to use subcutaneous microdialysis to determine: (1) whether or not spinal nerve ligation induces a change in peripheral adenosine levels, (2) whether or not primary afferent neurons (by capsaicin pretreatment) or SPGNs (by 6-OHDA pretreatment) are involved in this change, and (3) whether or not such release can be modulated by the adenosine kinase inhibitor NH_2dAD or the adenosine deaminase inhibitor DCF.

Fig. 5.1: Schematic illustration of the potential involvement of capsaicin-sensitive sensory afferents and sympathetic postganglionic nervous (SPGN) efferents in peripheral adenosine release induced by nerve injury. The sciatic nerve is formed by the L4-L6 spinal nerves. In the spinal nerve ligation model, L5 and L6 are tightly ligated and degenerated, while L4 is left intact. Following spinal nerve ligation, spontaneous activity develops in C and Aδ fibers from the intact L4 axon, which may induce the release of SP, CGRP and ATP or adenosine via axon reflex. Nerve injury also induces abnormal sympathetic-sensory coupling, at DRG neurons, neuroma and peripheral terminal of sensory afferents. Hyperactivity of SPGN may induce the release of noradrenaline, NPY, PGE_2, ATP, and adenosine. By activating respective receptors located on the peripheral terminal of sensory afferents, the released neurotransmitters further sensitize nociceptors. Adenosine, by activating A_1 receptors, may produce an antinociceptive effect. Receptors for ATP ($P2X_3$, $P2X_2/_3$), noradrenaline (α_2), and ADO (A_1) are depicted. (Summarized from section 1.5; abbreviations are explained in the List of Abbreviations).

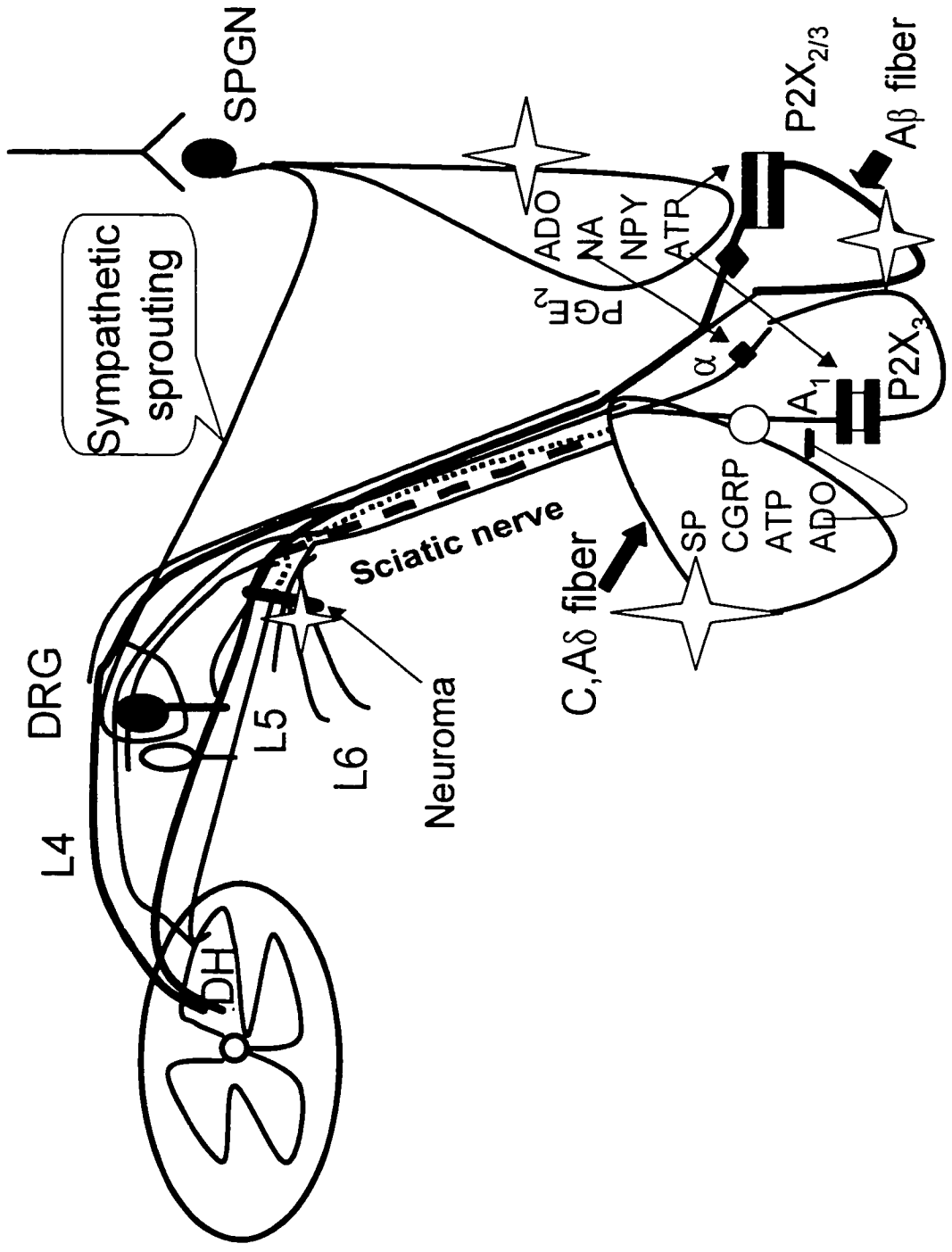

SPGN
Sympathetic sprouting
ADO
NA
NPY
ATR
PGE2
α
P2X2/3
Aβ fiber
P2X3
A1
SP
CGRP
ATP
ADO
C,Aδ fiber
Sciatic nerve
DRG
L4
L5
L6
Neuroma
DH

Fig. 5.1

5.2 MATERIALS AND METHODS

Spinal nerve ligation surgery was performed as described in Chapter 2. For the sham operated group, the surgical procedure was the same, except that the spinal nerves were only isolated but not ligated. Static mechanical thresholds were determined 8-9 days following surgery in both nerve-ligated rats and sham-operated rats (Chapter 2). Capsaicin pretreatment and 6-OHDA pretreatment were performed 9-11 days following surgery (Chapter 2). Microdialysis was performed three days later (eg. day 12-14 post surgery) and saline, NH_2dAD or DCF (50µl) was injected s.c. into the plantar aspect of the rat hind paw *ipsilateral* to the nerve injury or sham operation. Microdialysis and adenosine analysis were performed as described in Chapter 2.

5.3 RESULTS

Behavioral tests demonstrated that tight ligation of the L5/L6 spinal nerves produced mechanical allodynia in the nerve-ligated paw (50% PWT = 2.65±0.1g, n=8), while sham operated rats did not exhibit allodynia (50% PWT >15 g, n=8, $p<0.01$) (data from rats used in Fig. 5.2).

5.3.1 Saline-evoked subcutaneous adenosine release in the ipsilateral hind paw following spinal nerve ligation

In the microdialysis study, basal subcutaneous adenosine levels in neuropathic rats did not differ from the sham operated or untreated rats (Fig. 5.2). Injection of 50µl saline into the subcutaneous tissues under the glabrous skin of the rat hind paw following ipsilateral spinal nerve ligation induced a significant release of adenosine during the first

10min after injection (9.30±1.47pmol/20μl), which is significantly higher than the effect elicited in sham operated rats (4.21±1.2pmol/20μl, $p<0.05$) and untreated rats (3.73±1.4 pmol/20 μl, $p<0.05$) (Fig. 5.2).

5.3.2 Effects of NH_2dAD and DCF on saline-evoked adenosine release in the nerve-ligated paw

Previously, we demonstrated that s.c. injection of the adenosine kinase inhibitor, NH_2dAD, and the adenosine deaminase inhibitor DCF produce a peripherally-mediated thermal antihyperalgesia in the SNL model and this effect can be locally blocked by caffeine, suggesting an involvement of adenosine receptors (Chapter 3). The present study determined whether inhibitors of adenosine metabolism increased local extracellular adenosine levels in neuropathic rats, which could mediate their peripheral antinociceptive effect. At a behaviorally active dose, injection of the adenosine kinase inhibitor, NH_2dAD (100nmol, 50μl) and the adenosine deaminase inhibitor, DCF (100nmol, 50μl) into the nerve-ligated paw of neuropathic rats *did not* produce a further increase in saline-evoked adenosine release (Fig. 5.3).

Fig. 5.2: Effects of intraplantar injection of saline on subcutaneous extracellular adenosine levels in the rat hind paw of naïve rats, or following spinal nerve ligation, or sham operation. Saline (50µl) injection into the subcutaneous tissue under the glabrous skin of the hind paw induces adenosine release in the ipsilateral paw of neuropathic rats following SNL, but not in sham operated rats or naïve rats. * $p< 0.05$ compared to sham operated rats, *t* $p<0.05$ compared to naïve rats (one way ANOVA followed by SNK, means ± SEM, n=7-8).

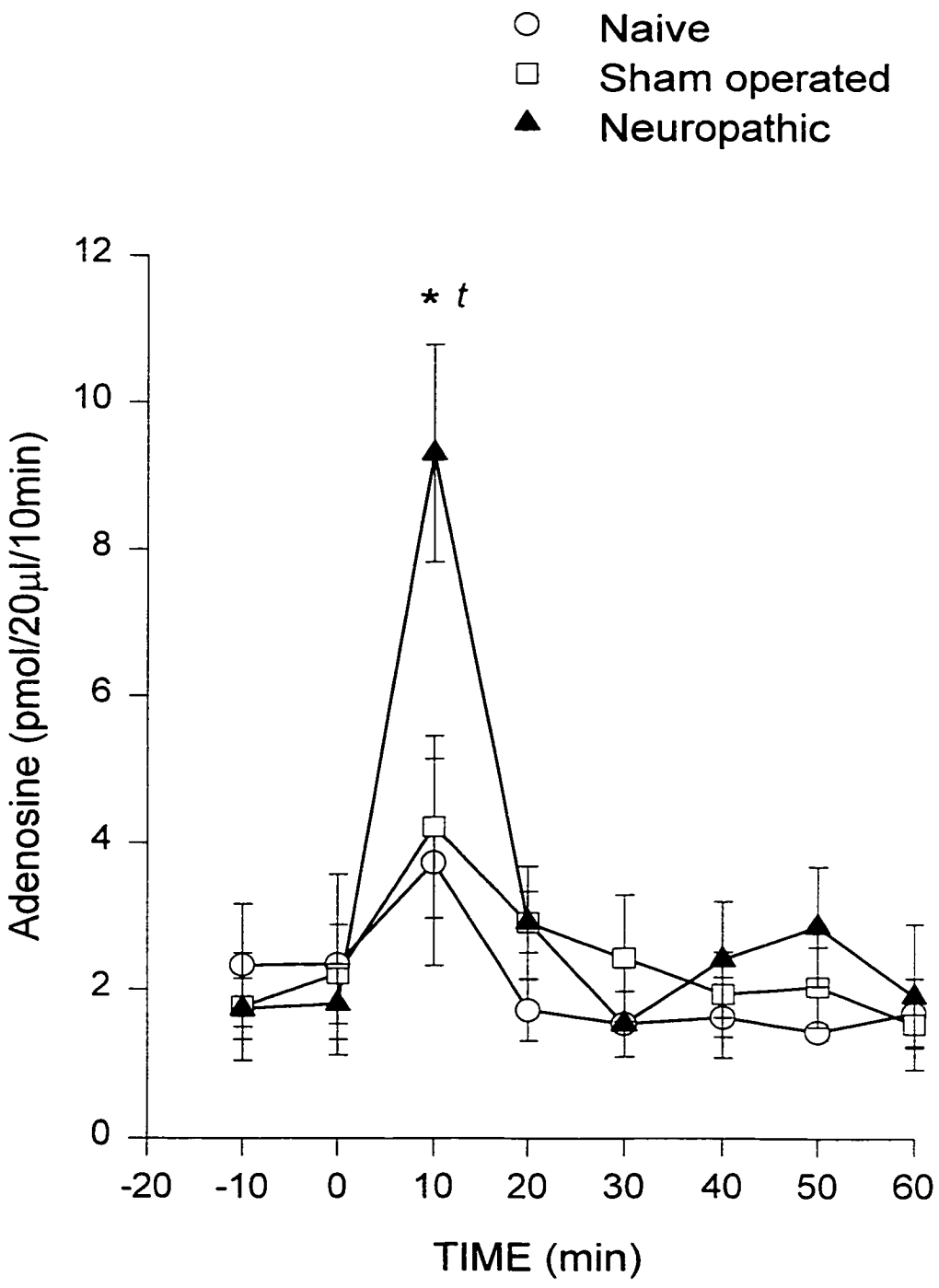

Naive
Sham operated
Neuropathic
* t
Adenosine (pmol/20µl/10min)
12
10
8
6
4
2
0
-20
-10
0
10
20
30
40
50
60
TIME (min)

Fig. 5.2

Fig. 5.3: Effects of the adenosine kinase inhibitor, NH_2dAD, and the adenosine deaminase inhibitor, DCF, on saline-evoked adenosine release in the nerve-ligated paw. Saline or drugs were injected at time zero into the nerve-ligated paw. The adenosine kinase inhibitor NH_2dAD 100nmol, and the adenosine deaminase inhibitor, DCF 100nmol, did not further increase adenosine release in neuropathic rats (means ± SEM , n=8-10).

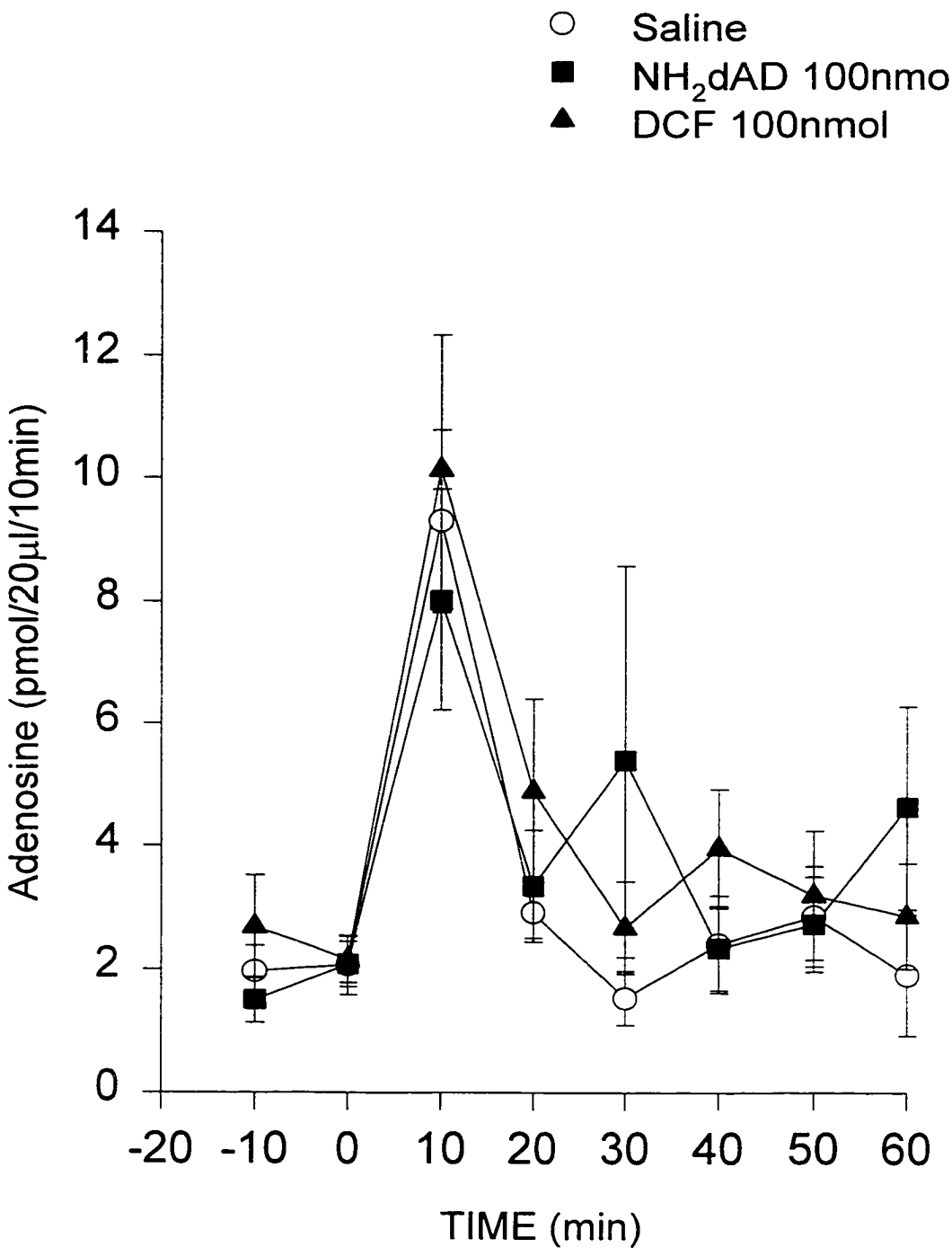

Saline
NH2dAD 100nmol
DCF 100nmol
Adenosine (pmol/20µl/10min)
14
12
10
8
6
4
2
0
-20
-10
0
10
20
30
40
50
60
TIME (min)

Fig. 5.3

5.3.3 Effect of pretreatment with capsaicin and 6-OHDA on saline-evoked adenosine release in the hind paw following spinal nerve ligation

As saline does not induce adenosine release in normal rats and sham operated rats, the abnormal adenosine release following saline injection into the nerve-ligated paw suggests pathological changes induced by nerve injury. Nerve injury induces spontaneous activity and ectopic discharges of primary sensory afferents, as well as abnormal coupling with SPGN (Fig. 5.1). Our previous study demonstrates adenosine can be released from both capsaicin-sensitive sensory afferents and SPGNs upon noxious stimulation (Chapter 4). Thus, we investigated the involvement of these systems in saline-evoked adenosine release in the nerve-ligated paw. Systemic pretreatment with capsaicin, a neurotoxin which selectively destroys small diameter unmyelinated C fibers and thinly myelinated Aδ fibers, significantly reduced the saline-induced release of adenosine in neuropathic rats (Fig. 5.4A). Systemic pretreatment with 6-OHDA, a neurotoxin selective for sympathetic nerve terminals, did not have any effect on such release (Fig. 5.4B).

Fig. 5.4: Effects of pretreatment with (A) capsaicin and (B) 6-OHDA with respective vehicles, on the saline-induced release of adenosine in the ipsilateral hind paw following spinal nerve ligation. Saline was injected at time zero into the nerve-ligated paw. * $p< 0.05$ compared to respective vehicle-pretreated group, *t* $p<0.05$ compared to baseline levels (two-way repeated ANOVA followed by SNK, means ± S.E.M, n=8).

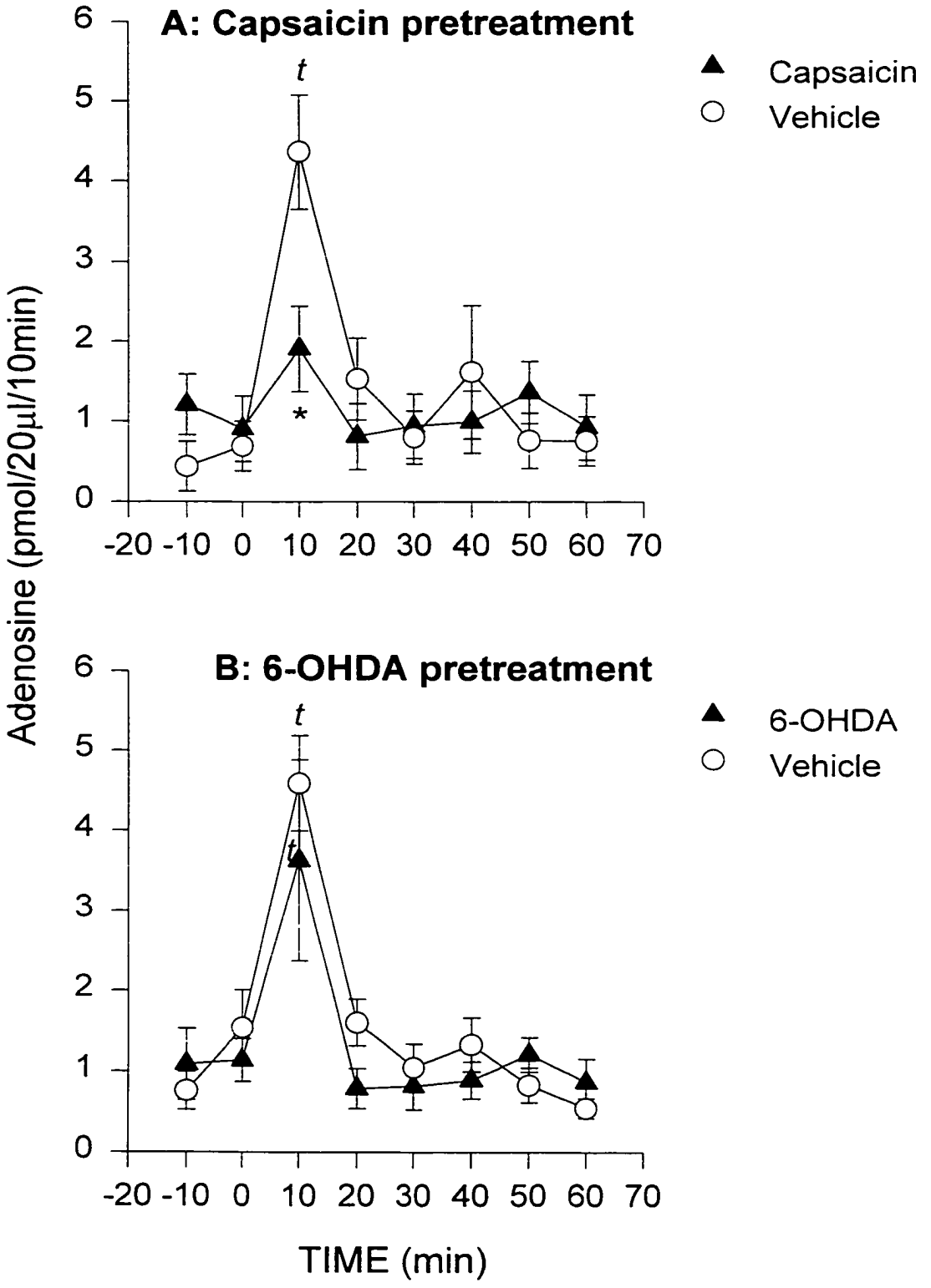

A: Capsaicin pretreatment
Capsaicin
Vehicle
t
*
B: 6-OHDA pretreatment
6-OHDA
Vehicle
t
Adenosine (pmol/20µl/10min)
TIME (min)
0
1
2
3
4
5
6
-20 -10 0 10 20 30 40 50 60 70

Fig. 5.4

5.4 DISCUSSION

The present study demonstrates that tight ligation of the L5 and L6 spinal nerves results in hypersensitivity of capsaicin-sensitive primary afferent neurons such that these afferents now release adenosine in response to stimulation, such as saline, that usually does not evoked adenosine release in normal conditions.

5.4.1 Involvement of capsaicin-sensitive primary sensory afferents in saline-evoked adenosine release following nerve injury

Injection of 50μl saline induced a significant release of adenosine only in the hind paw of rats with spinal nerve ligation, but not when the nerves were intact. Pretreatment with capsaicin, a neurotoxin selective for small-diameter unmyelinated C fibers and thinly myelinated Aδ fibers, markedly reduced the release of adenosine in the nerve-ligated paw in neuropathic rats, indicating an involvement of these fibers in such release. As saline injection did not induce adenosine release in sham-operated or untreated rats, the release observed in neuropathic rats is due to peripheral nerve sensitization. Electrophysiological studies demonstrate the predominant population of injured axons exhibiting spontaneous activity following nerve injury is Aβ fibers (Han et al., 2000; Liu et al., 2000a). Although ATP may be released from the central terminals of myelinated large diameter afferents (Fyffe and Perl, 1984; Salter and Henry, 1987; Sawynok et al., 1993), a direct release of adenosine or nucleotides from Aβ fibers is unlikely to contribute to the major portion of the released adenosine observed here, as capsaicin pretreatment almost eliminates such release. We suggest that this adenosine release is mediated by hypersensitive C fibers or Aδ fibers. Although in the spinal nerve ligation model, Aβ rather than C fiber spontaneous activity from *injured* axons was observed

(Han et al., 2000; Liu et al., 2000a; Liu et al., 2000b), C fiber from *intact* axons such as L4 also develop spontaneous activity and hypersensitivity, likely due to Wallerian degeneration (Ali et al., 1999; Boucher et al., 2000a; Wu et al., 2001). The nerve injury also may antidromically activate residual capsaicin-sensitive sensory afferents to release neuropeptides from the peripheral terminals (Daemen et al., 1998a; 1998b), and these then activate mechanisms that subsequently sensitize cutaneous nociceptors. A recent clinical study demonstrates an increased axon reflex in complex regional pain syndrome (Weber et al., 2001), indicating a hyperactive C fiber-mediated peripheral neurochemical release also occurs in humans following nerve injury.

Injection of saline could act as mechanical stimuli by the prick with the needle or the volume effect to activate the hypersensitized C-polymodal nociceptor (Meyer et al., 1994), or it could lead to the release of factors that act as a chemical stimulus on nociceptors. Thus, the intraplantar injection of saline can release histamine (Guo et al., 1997), and this initiates a histamine-mediated paw edema (Guo et al., 1997; Sawynok et al., 1999b). As histamine is able to activate C-nociceptors, the increased adenosine release observed here could result from a local histamine release. ATP or adenosine may be released directly from C fibers as a cotransmitter with substance P or CGRP reflecting a direct activation of sensory afferents, or it could be released indirectly from adjacent tissues, such as endothelium, stimulated by a local axon reflex (Burnstock, 2000). In support of the direct release is the observation that a capsaicin-sensitive release of adenosine and nucleotides from the central terminals of primary sensory afferents (Sweeney et al., 1989), and it is reasonable to assume that such release also can occur at the peripheral terminal (Holton and Holton, 1954; Holton, 1959).

5.4.2 Lack of involvement of SPGNs in saline-evoked adenosine release following nerve injury

Systemic pretreatment with 6-OHDA, which selectively depletes sympathetic nerve terminals, did not produce any effect on the saline-evoked release of adenosine, suggesting SPGNs are not involved in such release. It is well known that ATP can be released from sympathetic nerve terminals as a cotransmitter with noradrenaline and NPY (Burnstock, 2000), and it has been hypothesized that ATP, released from sympathetic nerve terminals, acts on P2X purinergic receptors contributing to the initiation and maintenance of sympathetically maintained pain (Burnstock, 2000). However, we did not observe a SPGN involvement in the saline-evoked release of adenosine following nerve injury. Incomplete chemical sympathectomy is not a likely explanation, as using the same protocol, we clearly demonstrate a SPGN involvement in adenosine release in an inflammatory pain model (Chapter 4). Thus, the present study suggests a nociceptive C fiber-mediated, sympathetic-independent mechanism for the abnormal adenosine release following nerve injury. It should be noted that although previous studies demonstrate the SNL model is dependent on sympathetic activity (Kim et al., 1993; 1997; Moon et al., 1999), there are some recent studies that question this dependency (Lavand'homme et al., 1998; Ringkamp et al., 1999a; 1999b). The inconsistency may result from differences between strains of rats, or in individual differences between individual rats within the same strain (Lee et al., 1997; Park et al., 2000).

5.4.3 Effects of the adenosine kinase inhibitor, NH_2dAD, and the adenosine deaminase inhibitor, DCF, on saline-evoked adenosine release in neuropathic rats

Under the present study conditions, we did not observe a further increase in adenosine levels following administration of the adenosine kinase inhibitor, NH_2dAD, and the adenosine deaminase inhibitor, DCF, at the doses that produce a locally mediated antihyperalgesic effect in the SNL model (Chapter 3). This may reflect a highly localized release of adenosine that is not detected by microdialysis. Thus, due to the negative feedback control of ATP on ecto-nucleotidase activity (Section 1.6.1), a burst like formation of adenosine from neuronal ATP may occur at nerve terminals, as seen at neuromuscular synapses (Cunha et al., 1996), and this may not necessarily be detected by the present methodology. Consistent with this hypothesis is the observation that in the formalin model, no significant *augmentation* in adenosine release by NH_2dAD and DCF is measured within the first 10min following formalin (Chapter 4, Fig. 4.4), when the adenosine release is from a neuronal source. On the other hand, when inflammation has developed and non-neuronal tissue contributes to adenosine release, a significant increase in adenosine levels was observed with both agents (Chapter 4, Fig. 4.4).

5.5 CONCLUSION

The present study demonstrates that there is an abnormal, local, peripheral release of adenosine following spinal nerve ligation. This release is mediated by hypersensitive primary afferent C fibers, and is independent of SPGNs. Modulation of this release by inhibition of enzymes involved in adenosine metabolism is not readily observed using this model, but may produce a highly localized effect on primary nerve terminals.

CHAPTER 6

AMITRIPTYLINE ENHANCES PERIPHERAL EXTRACELLULAR ADENOSINE LEVELS IN THE RAT HIND PAW IN AN INFLAMMATORY PAIN MODEL AND A NEUROPATHIC PAIN MODEL

6.1 INTRODUCTION

Amitriptyline is used widely to treat chronic pain in humans (Reviewed McQuay et al., 1996; Sindrup and Jensen, 1999). Amitriptyline is a complex drug exerting a number of pharmacological actions, including inhibition of noradrenaline and 5-HT reuptake, binding to opioid receptors, blockade of α-adrenergic, histaminergic, cholinergic and NMDA receptors and blockade of certain ion channels. A number of these actions have been implicated in the antinociceptive properties of amitriptyline (Sindrup and Jensen, 1999; Eschalier et al., 1999).

The antinociceptive effects of amitriptyline may also involve endogenous adenosine systems. Thus, methylxanthine non-selective adenosine A_1/A_{2A} antagonists (caffeine, theophylline) block the antinociceptive effect of *systemically* administered antidepressants in several nociceptive models (Pareek et al., 1994; Sierralta et al., 1995). Recently, in an inflammatory pain model, the formalin test, and a neuropathic pain model, SNL model, the *peripheral* antinociceptive effect by amitriptyline was shown to be blocked by caffeine (Sawynok et al., 1999a; Esser and Sawynok, 2000), suggesting the involvement of peripheral adenosine systems in this effect. The mechanisms mediating this adenosine involvement in the antinociceptive effect produced by amitriptyline are not entirely clear. Although it has been demonstrated that antidepressants inhibit adenosine uptake *in vitro* (Phillis and Wu, 1982), the affinity of antidepressants for adenosine uptake is generally low, such that it may not indeed form the basis for the interaction with the adenosine system *in vivo*.

We have demonstrated that s.c. injection of formalin increases extracellular adenosine levels in the rat hind paw (Chapter 4). In rats following spinal nerve ligation, saline injection can induce an abnormal peripheral adenosine release (Chapter 5). As amitriptyline produces an adenosine-mediated local, peripheral antinociceptive effect in both models, we hypothesized that this effect is achieved by potentiation of local extracellular adenosine levels.

The aim of the current study was to use subcutaneous microdialysis to determine: (1) whether or not intraplantar injection of amitriptyline modulates formalin-evoked local adenosine release; (2) whether or not intraplantar injection of amitriptyline modulates saline-evoked adenosine release in the nerve-ligated paw following spinal nerve ligation; and (3) whether or not desipramine, another antidepressant, producing a peripheral antinociceptive effect without the involvement of the local adenosine system, modulates local adenosine levels.

6.2 MATERIALS AND METHODS

Spinal nerve ligation surgery, microdialysis and adenosine analysis were performed as described in Chapter 2. In naïve rats, formalin, amitriptyline, desipramine, or a formalin and drug combination was injected in a volume of 50μl into the plantar aspect of rat hind paw. When two drugs were co-administered, they were mixed just before administration. In spinal nerve-ligated or sham operated rats, microdialysis was performed on 8-10 days post-surgery and saline or amitriptyline were injected into the ipsilateral paw. The cumulative adenosine release was determined by subtracting the

mean of two baseline values from subsequent value of samples collected over 60min following drug administration.

6.3 RESULTS

6.3.1 Effects of amitriptyline and desipramine on formalin-evoked peripheral adenosine release in the rat hind paw

Consistent with our previous studies, in naïve rats, injection of saline 50µl into the plantar surface of the rat hind paw did not produce an increase in the extracellular levels of adenosine (Figs. 6.1A and 6.3A). Local injection of formalin 1.5% and 5% produced a concentration related adenosine release, with a rapid, short lasting release at 1.5% and a persistent release at 5% (Figs. 6.1B and 6.1C). Amitriptyline 100nmol, a dose that produced a caffeine-*sensitive* inhibition of nociceptive behaviors produced by formalin (Sawynok et al., 1999a), produced some increase in adenosine when administered alone. This was not significant in the time course, but significantly different from saline in the cumulative release score (Figs. 6.1A, 6.2). Amitriptyline significantly augmented 1.5% formalin-evoked adenosine release, both in the time course and in cumulative change (Figs. 6.1B, 6.2), yet did not produce an effect on 5% formalin-evoked adenosine release in rat hind paw (Figs. 6.1C, 6.2). Desipramine 100nmol, a dose that produced a caffeine-*insensitive* peripheral antinociception, had no effect on formalin evoked adenosine release when coadministered with 1.5% formalin (Fig. 6.2).

Fig. 6.1: Effects of intraplantar s.c. injection of amitriptyline 100nmol to the rat hind paw on adenosine release evoked by formalin. Amitriptyline (AMI) was injected at time zero with saline (SAL) (A), 1.5% formalin (FOR) (B), or 5% formalin (C). * $p < 0.05$ compared to saline or formalin alone (student's *t*-test, means ± SEM, n=7-8).

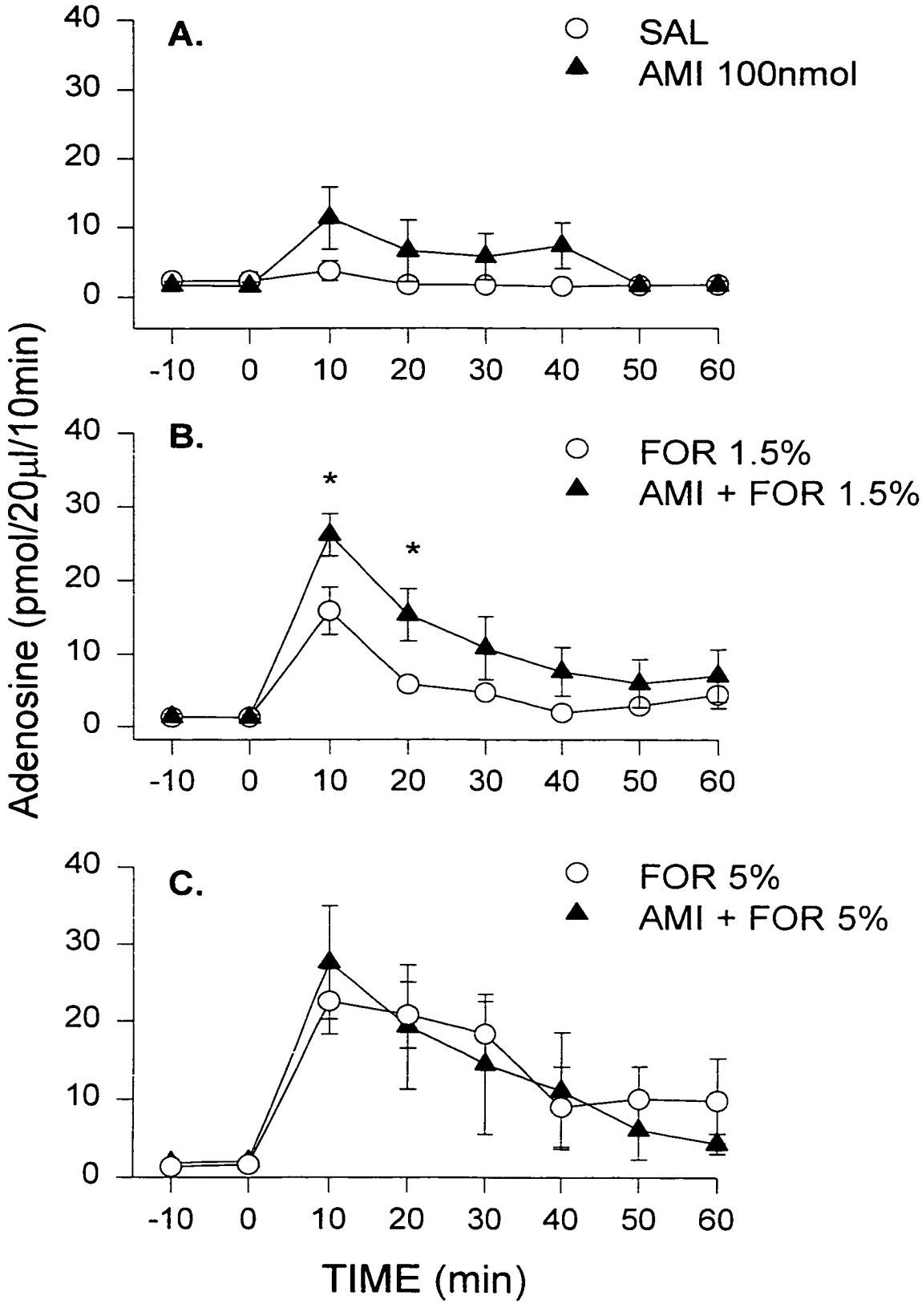

A.
SAL
AMI 100nmol
B.
FOR 1.5%
AMI + FOR 1.5%
C.
FOR 5%
AMI + FOR 5%
Adenosine (pmol/20µl/10min)
TIME (min)
40
30
20
10
0
-10
0
10
20
30
40
50
60
*
*

Fig. 6.1

Fig. 6.2: Cumulative release of adenosine by formalin (FOR) or formalin co-administered with 100nmol amitriptyline (AMI) or desipramine (DES) during 60 min following formalin or formalin/drug administeration. Formalin 0=saline * $p<0.05$ compared to formalin (one way ANOVA followed by SNK, data from Fig. 6.1, DES n=4).

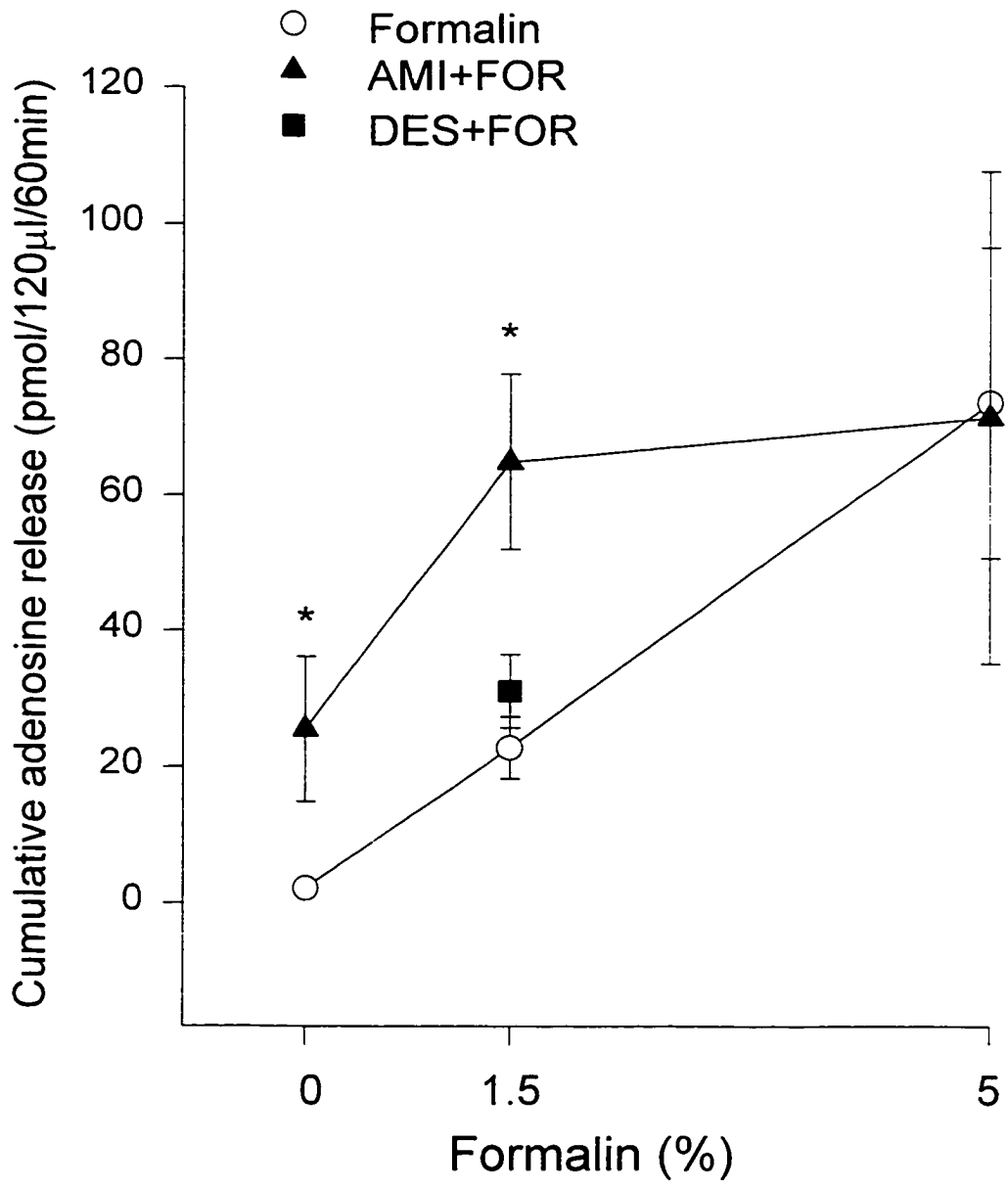

Formalin
AMI+FOR
DES+FOR
Cumulative adenosine release (pmol/120µl/60min)
120
100
80
60
40
20
0
*
*
0
1.5
5
Formalin (%)

Fig. 6.2

6.3.2 Effects of amitriptyline on saline-evoked adenosine release in the nerve-ligated paw or sham operated paw

In *sham operated* rats, saline had no effect on adenosine levels, and amitriptyline (100nmol) significantly induced a rapid increase in adenosine levels, observed the first 10min after amitriptyline (100nmol) administration (Fig. 6.3A). Saline *per se* induced a significant adenosine release in the *spinal nerve ligated* paw (Fig. 6.3B). Amitriptyline (100nmol) further increased saline-evoked adenosine release in spinal nerve ligated paw (Fig. 6.3B). Local administration of this dose of amitriptyline produces a caffeine-sensitive local peripheral antihyperalgesic effect in the SNL model (Esser and Sawynok, 1999; 2000)

Fig. 6.3: Effects of amitriptyline (AMI) 100nmol on extracellular adenosine levels in the ipsilateral hind paw of the rat following (A) sham operation or (B) spinal nerve ligation. Amitriptyline or saline were injected at time zero. * $p < 0.05$ compared to the release by saline, *t* $p<0.05$ compared to pre-injection levels (two-way repeated ANOVA followed by SNK, means ± SEM, n=6-9).

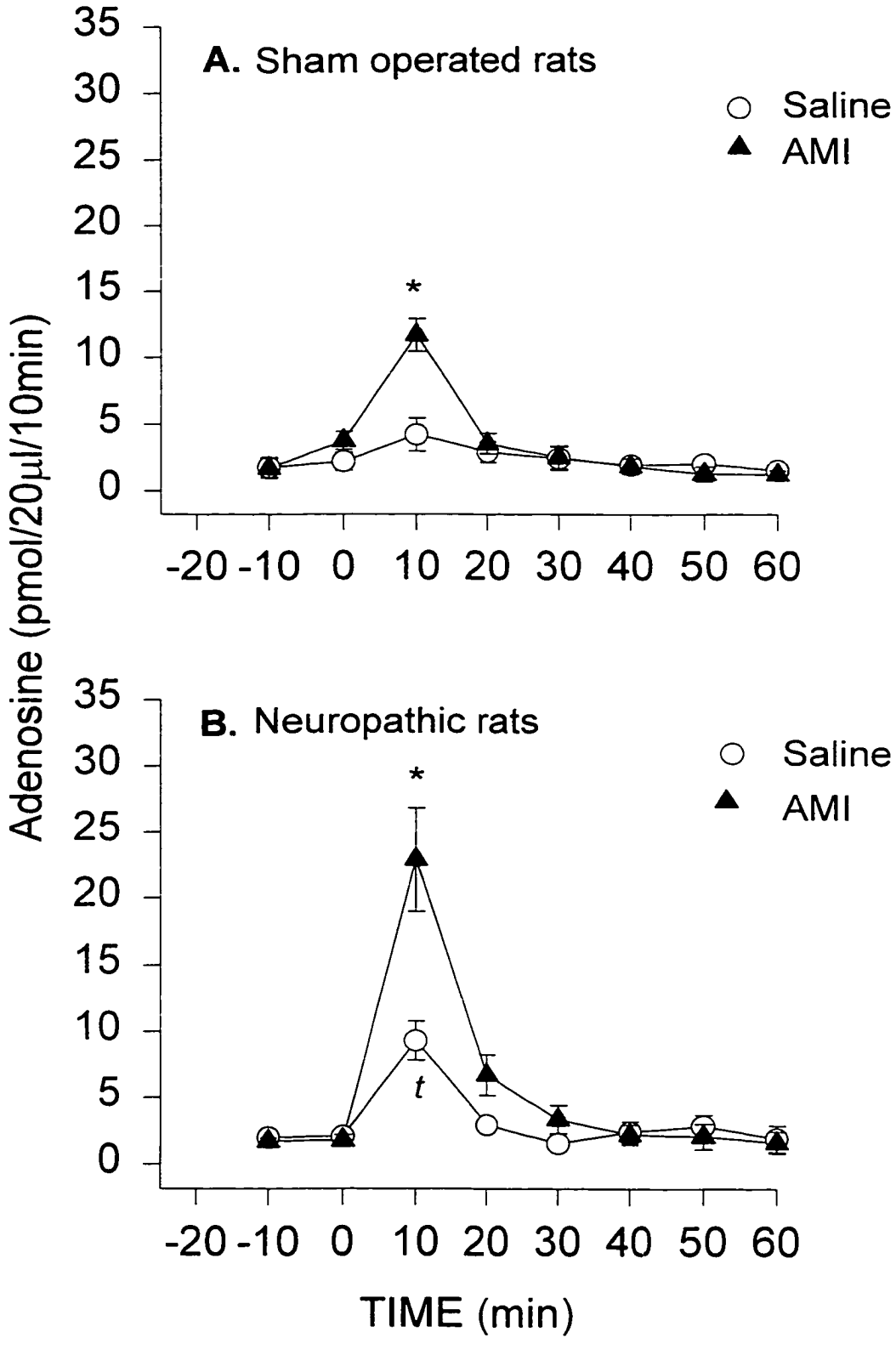

A. Sham operated rats
Saline
AMI
*
B. Neuropathic rats
Saline
AMI
*
t
Adenosine (pmol/20µl/10min)
35
30
25
20
15
10
5
0
-20 -10 0 10 20 30 40 50 60
TIME (min)

Fig. 6.3

6.4 DISCUSSION

6.4.1 Correlation of increase in local adenosine levels and the peripheral antinociceptive effect produced by amitriptyline

The present study indicates that local peripheral administration of amitriptyline, at a dose that produces a marked suppression of nociceptive behaviors produced by formalin (Sawynok et al., 1999a) and a reduction of thermal hyperalgesia produced by spinal nerve ligation (Esser and Sawynok, 1999; 2000), can enhance local extracellular adenosine levels. The enhanced adenosine levels appear to contribute the local antinociceptive effect produced by amitriptyline as this action is inhibited by coadministration of caffeine, a non-selective adenosine A_1 and A_2 receptor antagonist (Sawynok et al., 1999a; Esser and Sawynok, 2000), as well as by coadministration of an adenosine A_1 receptor antagonist (Sawynok et al., 1999a). The effect of desipramine on peripheral adenosine release was also determined. Desipramine, an antidepressant that selectively inhibits noradrenaline uptake, also produces a local peripheral antinociceptive effect in the formalin and neuropathic pain models (Sawynok et al., 1999c). However, co-administering caffeine does not alter the peripheral antinociceptive effect by desipramine, suggesting a lack of involvement of the peripheral adenosine systems (Sawynok et al., 1999c). Consistent with the behavioral studies, desipramine, at a dose producing a local antinociceptive effect, did not have an effect on 1.5% formalin-evoked adenosine release in the rat hind paw. Thus, the enhancement of peripheral adenosine levels is unique to amitriptyline, which recruits adenosine systems to produce peripheral antinociception.

6.4.2 Comparison of the neurochemical and antinociception profiles of amitriptyline and inhibitors of adenosine metabolism

The mechanism by which amitriptyline enhances extracellular adenosine levels is not entirely clear. The profile of amitriptyline in modulating adenosine levels is different from the adenosine kinase inhibitor, NH_2dAD, or the adenosine deaminase inhibitor, DCF. Thus, neither NH_2dAD nor DCF enhances basal extracellular adenosine levels in naïve rats and sham-operated rats (Chapter 4, 5), while amitriptyline enhances basal levels in both cases. Amitriptyline is also different from the adenosine deaminase inhibitor in that it only increased 1.5% formalin-evoked adenosine release, but had no effect on adenosine release by 5% formalin, while DCF only increases 5% formalin-evoked adenosine release but has no effect in other circumstances (Chapter 4). Furthermore, microdialysis does not detect an enhancement of saline evoked adenosine release, in the neuropathic rats, by inhibitors of adenosine metabolism (Chapter 5), while amitriptyline significantly increased such release.

The peripheral behavioral profile of amitriptyline is also different from the adenosine kinase inhibitor and the adenosine deaminase inhibitor. Thus, the peripheral antinociceptive effect of NH_2dAD is observed only at 0.5-1.5% formalin (Sawynok et al. 1998), not at higher concentrations, while the antinociceptive effect of amitriptyline is observed at higher concentrations but not at 0.5% formalin (Sawynok et al., 1999a). As the peripheral antinociceptive effect of both amitriptyline (Sawynok et al. 1999a) and NH_2dAD (Sawynok et al., 1998) can be blocked by adenosine non-selective receptor antagonist, caffeine, and the A_1 receptor antagonist, CPT, the different profiles of these

agents modulating formalin-evoked nociceptive behavior indicates a different involvement of peripheral adenosine systems in these actions.

6.4.3 Possible mechanisms mediating the effect of amitriptyline on extracellular adenosine levels

The mechanisms underlying the increase in extracellular adenosine levels by amitriptyline is not entirely clear yet. A possible mechanism of amitriptyline in modulating peripheral adenosine levels is to inhibit adenosine uptake, as antidepressants increase adenosine levels in brain cortical slices by inhibiting adenosine uptake (Phillis and Wu, 1982). Amitriptyline, and other antidepressants, also increase cAMP production in cortical slices, and this action is blocked by methylxanthines (Kodama et al., 1971; Sattin et al., 1978). Enhanced production of cAMP could lead to an increase in the formation of adenosine inside the cell via nucleotidase, or outside the cell when the release of cyclic AMP is increased, with subsequent breakdown into adenosine via ecto-nucleotidase (Fig. 1.2; Section 1.6). However, the inhibition of adenosine uptake or the enhancement of cAMP levels is not unique to amitriptyline. Desipramine also inhibits adenosine uptake and increases cAMP production (Kodama et al., 1971; Sattin et al., 1978). Thus, other mechanisms may contribute to the unique enhancement of extracellular adenosine levels by amitriptyline in the inflammatory pain and neuropathic pain models, observed in the present study, and the recruitment of the adenosine system in peripheral antinociception by amitriptyline in such models, reported in previous studies (Sawynok et al., 1999a; Esser and Sawynok, 2000).

6.5 CONCLUSION

Intraplantar administration of amitriptyline enhances local adenosine levels in the rat hind paw following administration of formalin. Amitriptyline also enhances saline-evoked subcutaneous adenosine release in the rat hind paw following spinal nerve ligation. The local modulation of extracellular adenosine levels likely contributes to the peripheral antinociceptive effect of amitriptyline in both models. Desipramine does not produce an increase in extracellular adenosine levels, which is consistent with a lack of adenosine involvement in the antinociceptive effect produced by this agent.

CHAPTER 7

INTRAPLANTAR INJECTION OF GLUTAMATE-EVOKED ADENOSINE RELEASE IN THE RAT HIND PAW: INVOLVEMENT OF THE PERIPHERAL IONOTROPIC GLUTAMATE RECEPTORS AND THE CAPSAICIN SENSITIVE SENSORY AFFERENTS

7.1 INTRODUCTION

Accumulating evidence indicates that excitatory amino acids and their receptors play a key role in pain transmission and the plastic changes that underlie central sensitization in the spinal cord (Section 1. 2.3). Recent studies indicate excitatory amino acids may also play an important role in peripheral nociceptor excitation and sensitization. Thus, chronic inflammation increases excitatory amino acid levels in synovial fluid from patients with arthritis (McNearnery et al., 2000). The levels of excitatory amino acids are also increased by formalin (Omote et al., 1998) and carrageenan (Lawand et al., 2000) in experimental models of inflammation. Furthermore, local injection of glutamate receptor antagonists attenuates pain behaviors induced by formalin and carrageenan (Jackson et al., 1995; Davidson et al., 1997; Lawand et al., 1997; Davidson and Carlton 1998). These studies indicate that endogenous excitatory amino acids, released in inflamed tissues, activate or sensitize their receptors located on peripheral terminals of sensory afferents to produce tonic nociceptive effects. Indeed, NMDA receptors and non-NMDA receptors, (AMPA or kainate KA) have been localized on the peripheral terminals of *sensory afferents* both in humans (Kinkelin et al., 2000) and in rats (Carlton et al., 1995; Coggeshall and Carlton, 1998). On the other hand, endogenous excitatory amino acids could also act on nociceptors *indirectly*, via activation of SPGNs, as both NMDA and AMPA receptors are expressed in postganglionic sympathetic efferent fibers (Carlton et al., 1998a). Given the prominent role of SPGNs in the modulation of hyperalgesia and inflammation (Chapter 1), it is reasonable to hypothesize that endogenous excitatory amino acids activate glutamate receptors on SPGNs to release noradrenaline, ATP and PGs, which subsequently activate nociceptors. Concurrently, SPGN activation would

also activate blood vessels and inflammatory cells to modulate the local inflammatory response (Chapter 1). In a chronic inflammatory model, the complete Freund's adjuvant model, there is an increased expression of ionotropic glutamate receptors in both the peripheral terminals of sensory afferents (Carlton and Coggeshall, 1999) and sympathetic postganglionic efferents (Coggeshall and Carlton, 1999), which further suggests that peripheral glutamate receptors contribute to inflammatory pain through both sensory and sympathetic systems.

Recently, we demonstrated that the intraplantar s.c. injection of formalin evokes peripheral release of adenosine (Chapter 4). Both capsaicin-sensitive nociceptive sensory afferents and sympathetic postganglionic efferents contribute to the origin of this release (Chapter 4). We also demonstrated that selective activation of VR1 receptors, expressed on C and Aδ fibers, by acutely administering capsaicin, evokes peripheral adenosine release (Chapter 4). The expression of glutamate receptors on unmyelinated sensory afferents and the endogenous release of EAAs in formalin-induced inflammation raises the issue as to whether or not EAAs can evoke adenosine release peripherally. This is an especially intriguing possibility since in the CNS, activation of both NMDA or non-NMDA receptors increases extracellular adenosine levels in rat cortex (Hoehn and White, 1990; White, 1996), hippocampus (Arens et al., 1992; Latini et al., 1999), striatum (Delaney et al., 1998) and spinal cord (Conway et al., 1997; Conway and Yaksh, 1998). Thus, it is reasonable to hypothesize that activation of peripheral NMDA and non-NMDA receptors could also induce adenosine release from the peripheral terminal of sensory nerve afferents and SPGNs.

The aim of the current study was to address the following issues: (1) whether or not exogenous glutamate can induce peripheral adenosine release, (2) whether or not such release can be modulated by glutamate receptor antagonists, (3) whether or not nociceptive sensory afferents and SPGNs contribute to the origin of adenosine and (4) whether or not endogenous excitatory amino acids contribute to the peripheral adenosine release in the formalin-induced inflammatory pain model.

7.2 MATERIALS AND METHODS

In all experiments, drug treated rats and vehicle treated rats were always performed at the same time for microdialysis and sample analysis. Capsaicin pretreatment, 6-OHDA pretreatment, microdialysis and adenosine analysis were performed described in Chapter 2. Drugs or drug combinations were injected s.c. into the plantar aspect of rat hind paw in a final volume of 50μl. When two drugs were combined, they are mixed just before administration.

7.3 RESULTS

7.3.1 Glutamate-induced peripheral release of adenosine in the rat hind paw

Subcutaneous injection of L-glutamate (0.03μmol-100μmol in 50μl) induced release of adenosine (Fig. 7.1). The significant increase in extracellular adenosine levels (0.3μmol-100μmol) was only observed within the first 10min after glutamate injection (i.e. first post-injection collection sample). Even at the highest dose (100μmol), adenosine levels after first 10min were not significantly different from basal levels (Fig. 7.1). To avoid the influence of the variability amongst the baseline levels, peak evoked release was used to determine the dose-response relationship curve. Peak evoked release was calculated as adenosine levels at time 10min minus basal adenosine levels. Basal adenosine levels were determined by the means value of the two baseline measurements. Glutamate dose-dependently increased peak evoked adenosine levels (Fig. 7.2).

7.3.2 Role of NMDA and non-NMDA ionotropic receptors in glutamate-evoked peripheral release of adenosine in the rat hind paw

The involvement of peripheral ionotropic glutamate receptors in glutamate-induced adenosine release was evaluated by using the non-competitive NMDA receptor antagonist, MK-801 (1-10nmol) and the non-selective AMPA /KA receptor antagonist, CNQX (10nmol). Coadministration of either MK-801 or CNQX with glutamate significantly blocked glutamate (1μmol) induced peripheral adenosine release (Fig. 7.3).

7.3.3 Effect of capsaicin pretreatment on glutamate-evoked adenosine release

The involvement of primary sensory afferents in glutamate-evoked peripheral adenosine release was evaluated with systemic capsaicin pretreatment. Glutamate (1 μmol) evoked a rapid adenosine release in vehicle-pretreated rats within 10min following glutamate administration at a magnitude comparable to untreated rats, but did not evoke an adenosine release in the capsaicin-pretreated rats (Fig. 7.4A). Thus, capsaicin-sensitive primary sensory afferents are necessary for glutamate to induce a peripheral adenosine release.

7.3.4 Effect of 6-OHDA pretreatment on glutamate-evoked adenosine release

The involvement of SPGNs in glutamate-evoked peripheral adenosine release was evaluated with systemic 6-OHDA pretreatment. Glutamate (1μmol) injection induced a similar adenosine release in both vehicle-pretreated rats and 6-OHDA-pretreated rats (Fig. 7.4B). There was no significant difference between the two treatments. Thus, SPGNs are not necessary to glutamate-evoked peripheral adenosine release.

7.3.5 Role of NMDA and non-NMDA ionotropic receptors in 5% formalin-evoked peripheral release of adenosine in the rat hind paw

Omote and colleagues have demonstrated that 5% formalin evokes a 2-3 fold increase in peripheral subcutaneous glutamate and aspartate levels in anesthetized rats (Omote et al., 1998). Furthermore, Davidson and colleagues demonstrated that pretreatment or posttreatment with NMDA and non-NMDA receptor antagonists produces a moderate reduction in 5% formalin-evoked pain behaviors in awake rats

(Davidson et al., 1997). Thus, we evaluated the involvement of endogenous glutamate, released by formalin, and the subsequent activation of NMDA and non-NMDA receptors in 5% formalin-evoked peripheral adenosine release. Consistent with our previous studies (Chapter 4), 5% formalin-evoked a persistent release of adenosine, lasting around 60min. Coadministration of the non-competitive NMDA receptor antagonist, MK-801 (1-10nmol) and the non-selective AMPA /KA receptor antagonist CNQX (100nmol) with 5% formalin did not produce a significant effect on 5% formalin-evoked adenosine release (Fig. 7.5). Thus, at the doses equal to, and 10 times higher than, that sufficient to block the effect of exogenous glutamate on adenosine release, the NMDA and non-NMDA receptor antagonists did not alter formalin-evoked peripheral adenosine release.

Fig. 7.1: Time course of intraplantar s.c. injection of L-glutamate on peripheral adenosine release in rat hind paw. L-Glutamate (L-Glu, 0.03μmol-100μmol) was injected at time zero in a volume of 50μl. * $p < 0.05$ compared to basal level at time zero (one way repeated ANOVA followed by SNK, means ± SEM, n=4-5 per group except for L-Glu 1μmol group, where n=12).

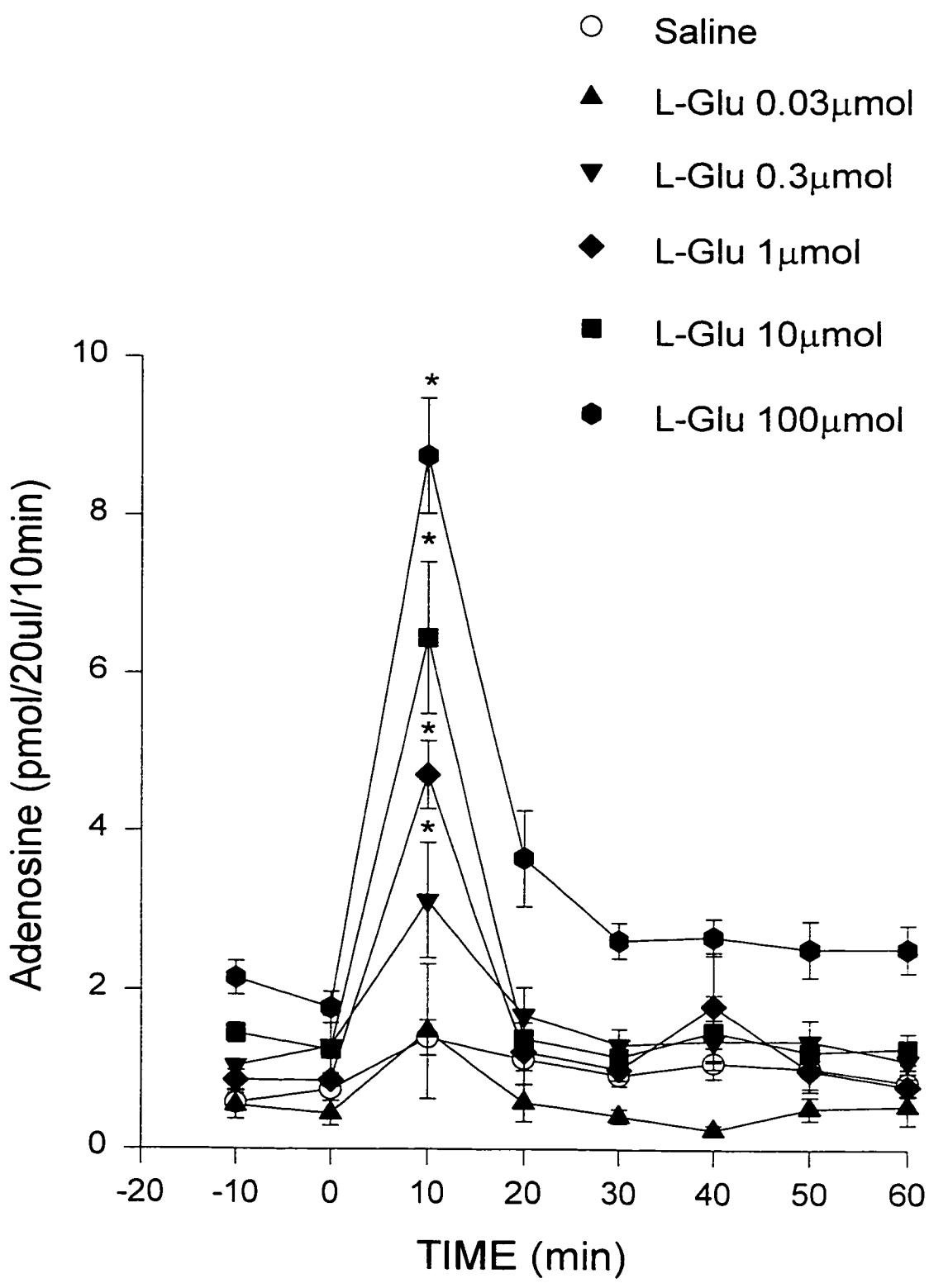

Saline
L-Glu 0.03μmol
L-Glu 0.3μmol
L-Glu 1μmol
L-Glu 10μmol
L-Glu 100μmol
Adenosine (pmol/20ul/10min)
0
2
4
6
8
10
-20
-10
0
10
20
30
40
50
60
TIME (min)
*
*
*
*

Fig. 7.1

Fig. 7.2: Dose-response curve for glutamate-evoked release of adenosine in rat hind paw. Peak evoked release is determined by adenosine levels at time 10min minus the means of basal adenosine levels. * $p<0.05$ compared to saline group (one way ANOVA followed by SNK, means ± SEM, data from Fig. 7.1).

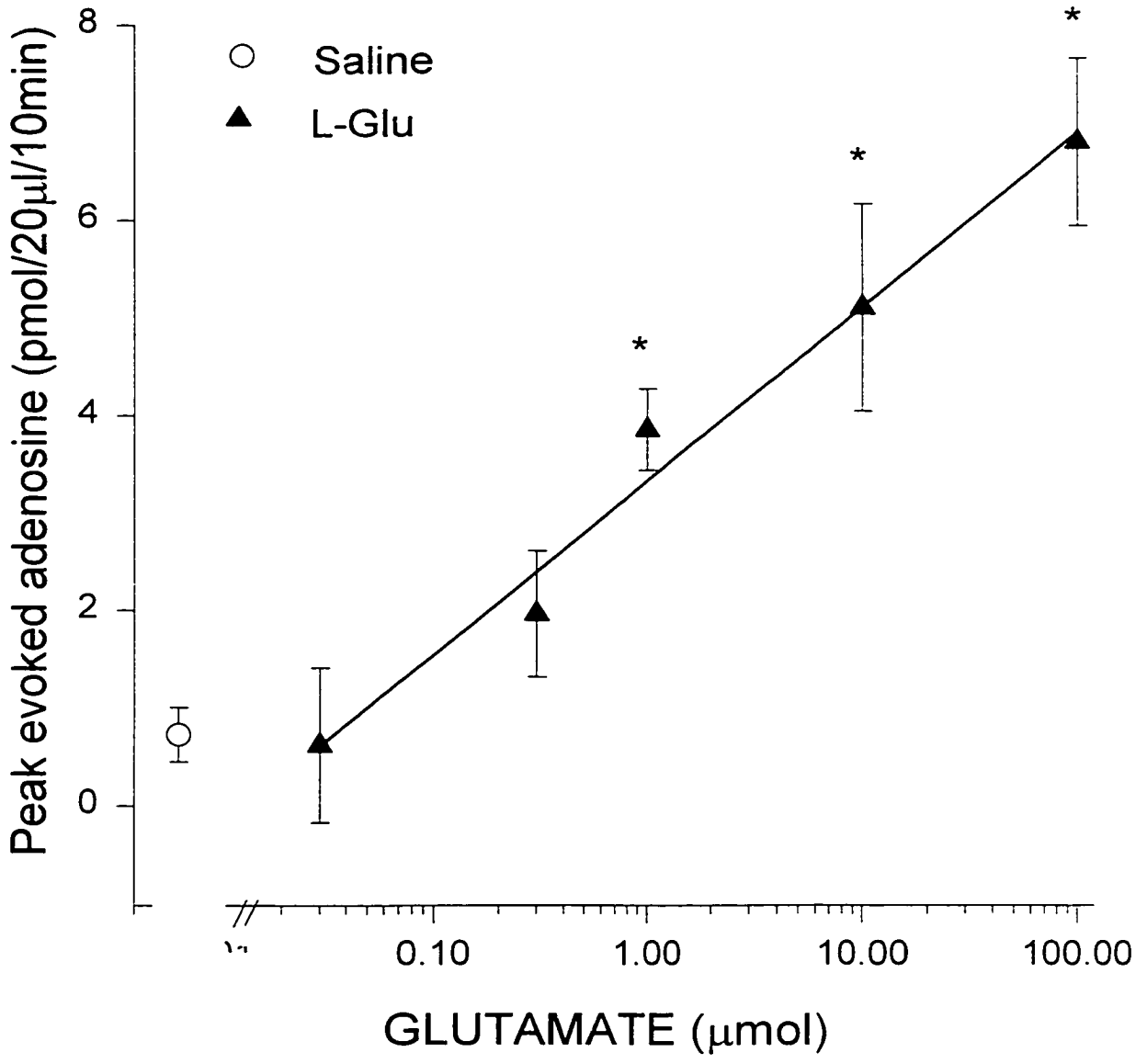

Peak evoked adenosine (pmol/20µl/10min)
8
6
4
2
0
Saline
L-Glu
*
*
*
0.10
1.00
10.00
100.00
GLUTAMATE (µmol)

Fig. 7.2

Fig. 7.3: Effects of the non-competitive NMDA receptor antagonist MK 801 (1-10 nmol) and the non-selective AMPA/KA receptor antagonist CNQX (10nmol) on L-glutamate (1μmol) evoked adenosine release in the rat hind paw. L-glutamate and antagonists were mixed just before injection in a final volume of 50μl, and administered at time zero. * $p < 0.05$ compared to glutamate 1μmol group (one way ANOVA followed by SNK, means ± SEM, n=6-7).

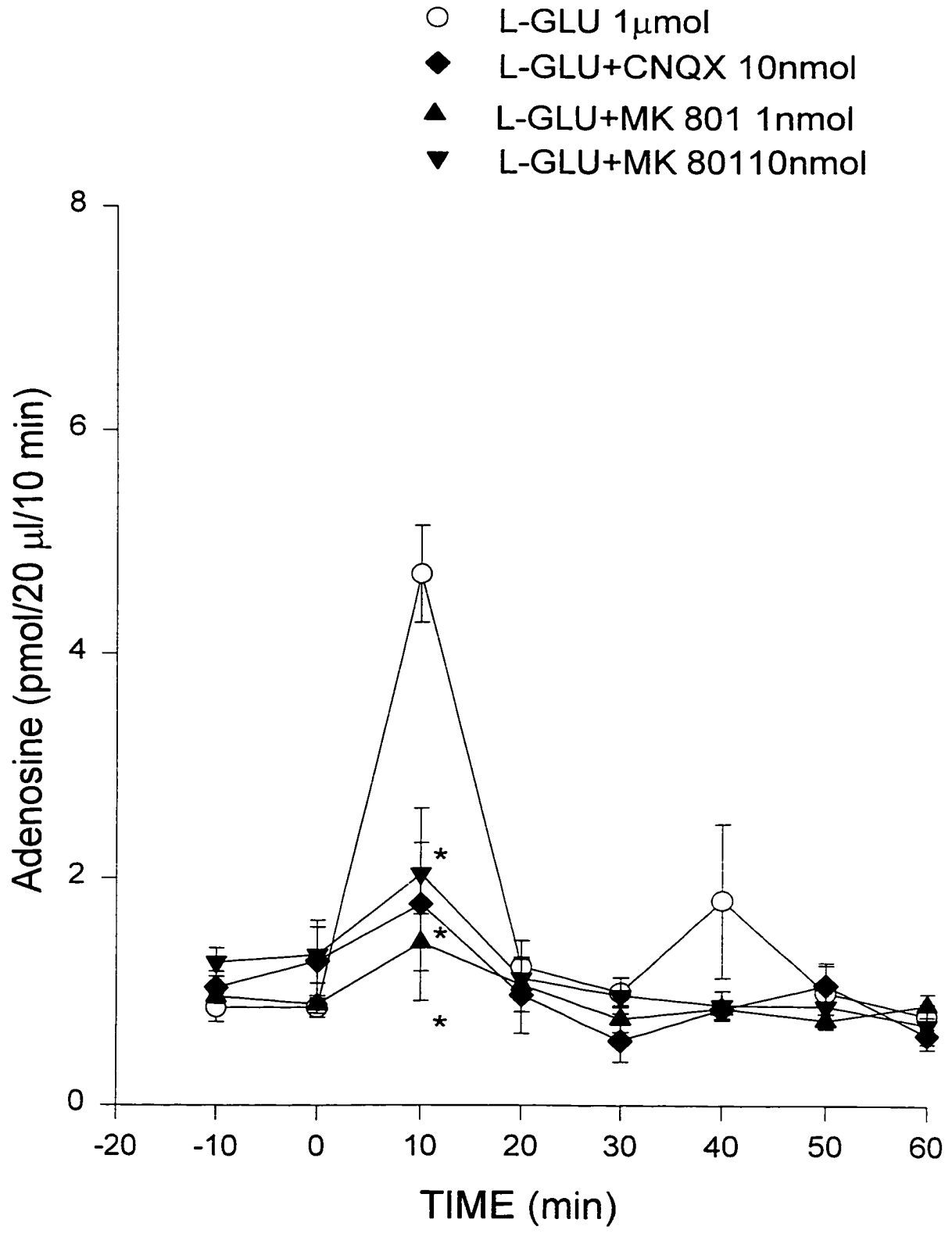

L-GLU 1μmol
L-GLU+CNQX 10nmol
L-GLU+MK 801 1nmol
L-GLU+MK 80110nmol
Adenosine (pmol/20 μl/10 min)
0
2
4
6
8
-20
-10
0
10
20
30
40
50
60
TIME (min)
*
*
*

Fig. 7.3

Fig. 7.4: Effects of pretreatment with (A) capsaicin and (B) 6-OHDA with respective vehicles, on L-glutamate (1μmol) evoked peripheral adenosine release in rat hind paw. L-glutamate was injected at time zero. * $p<0.05$ compared to vehicle-pretreated group (two- way repeated ANOVA followed by SNK, means ± SEM, n=7-8)

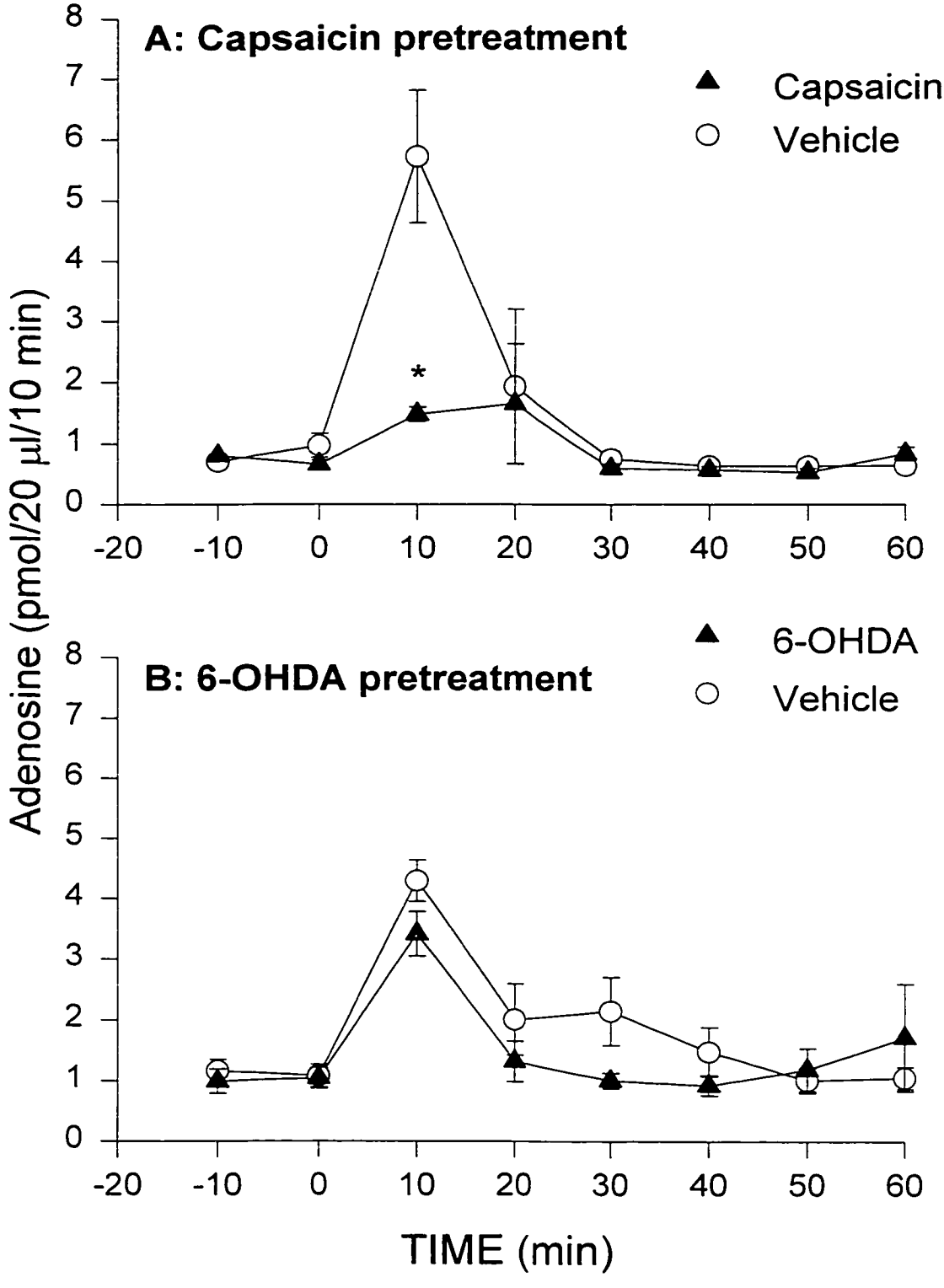

A: Capsaicin pretreatment
Capsaicin
Vehicle
*
B: 6-OHDA pretreatment
6-OHDA
Vehicle
Adenosine (pmol/20 µl/10 min)
TIME (min)
0
1
2
3
4
5
6
7
8
-20
-10
0
10
20
30
40
50
60

Fig. 7.4

Fig. 7.5: Effects of the non-competitive NMDA receptor antagonist MK 801 (1-10 nmol) and the non-selective AMPA/KA receptor antagonist CNQX (100nmol) on 5% formalin-evoked adenosine release in rat hind paw. Antagonists and formalin (FOR) were mixed just before injection in a final volume of 50μl . all drugs were injected at time zero. Neither MK 801 nor CNQX had an effect on 5% formalin-evoked adenosine release (means ± S.E.M. n=6-9).

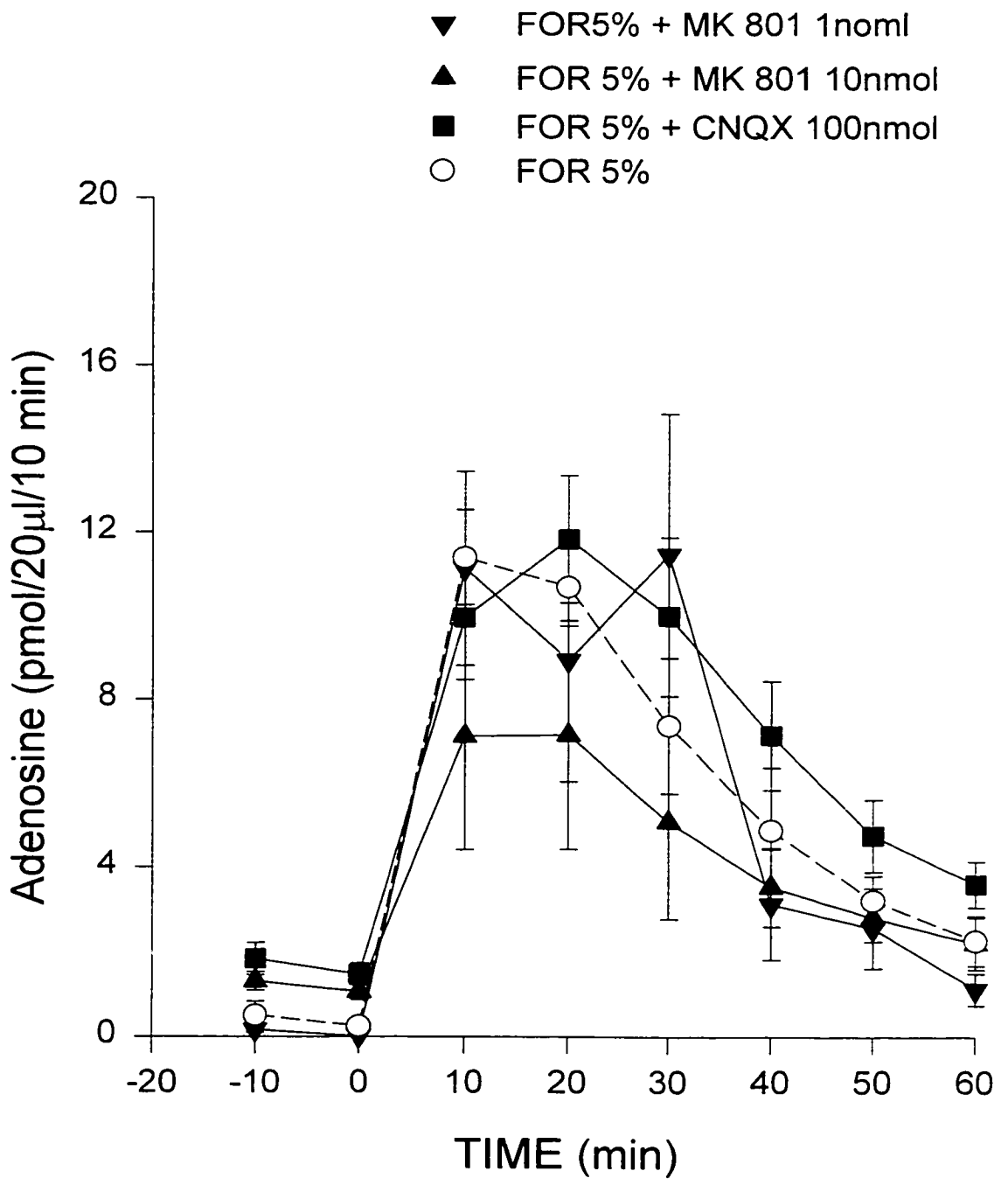

FOR5% + MK 801 1noml
FOR 5% + MK 801 10nmol
FOR 5% + CNQX 100nmol
FOR 5%
Adenosine (pmol/20µl/10 min)
20
16
12
8
4
0
-20
-10
0
10
20
30
40
50
60
TIME (min)

Fig. 7.5

7.4 DISCUSSION

The present study demonstrates that injection of glutamate intraplantarly into the rat hind paw produces a dose-dependent increase in subcutaneous extracellular levels of adenosine. This increase is blocked by both NMDA and non-NMDA receptor antagonists. Systemic pretreatment with capsaicin significantly inhibits glutamate-evoked peripheral adenosine release, while pretreatment with 6-OHDA does not affect such release. Furthermore, co-administration of NMDA and non-NMDA receptor antagonists fails to block 5% formalin-evoked adenosine release.

7.4.1 Exogenous glutamate induced peripheral adenosine release in the rat hind paw

Intraplantar injection of glutamate induces a rapid onset and short lasting release of adenosine. The only significant increase was observed within the first 10min following glutamate injection. Our previous studies indicate the pattern of subcutaneous adenosine release from the hind paw depends on the character of the stimulus. Thus, formalin, at lower concentrations, induces a spike of release, while formalin at 5% induces a release lasting ~60min (Chapter 4). Glutamate evokes only a transient adenosine release even at the highest concentration used, which is near the maximal solubility of glutamate, indicating there is no qualitative difference in release at different concentrations of glutamate. Interestingly, the transient nature of the release observed here is consistent with that following the intrathecal administration of NMDA. Thus, in spinal cord, the peak release of adenosine occurs within 5min following NMDA injection and returns to baseline within 10min (Conway et al., 1997; Conway and Yaksh, 1998). The resemblance in the time pattern of adenosine release following central and peripheral

glutamate receptor activation (both sites used microdialysis methods) might imply a similar underlying mechanism. Furthermore, this rapid onset transient release of adenosine argues against a non-specific release of adenosine because of cell damage.

7.4.2 Involvement of both NMDA and non-NMDA receptors in glutamate-evoked adenosine release

Coadministering the non-competitive NMDA receptor antagonist MK 801 and the AMPA/KA receptor antagonist CNQX with glutamate largely blocked glutamate-evoked adenosine release. The doses of antagonists used in the current study are comparable to those effective in blocking glutamate-induced hyperalgesia peripherally (Jackson et al., 1995). The antagonist blockade studies indicate the involvement of both NMDA and non-NMDA ionotropic glutamate receptors in peripheral adenosine release. This is consistent with both anatomical and functional profiles of peripheral glutamate receptor subtypes. Thus, anatomical studies demonstrate that there are approximately equal numbers (17-20%) of unmyelinated cutaneous sensory axon profiles in the glabrous skin of the rat hind paw expressing NMDA, AMPA and KA receptors (Coggeshall and Carlton, 1998). Furthermore, behavioral studies demonstrate that intraplantar injection of the specific glutamate receptor agonists NMDA, AMPA and KA, induces a comparable degree of mechanical hyperalgesia (Zhou et al., 1996) and that both NMDA and non-NMDA receptor antagonists attenuate glutamatergic receptor mediated hyperalgesia (Jackson et al., 1995; Lawand et al., 1997). Electrophysiological studies also demonstrate the approximately equal magnitude of excitation induced by NMDA and non-NMDA receptor agonists in the temporomandibular joint (Cairns et al., 1998).

Adenosine release by activation of NMDA and KA receptors has also been demonstrated in cortical slices (Hoehn and White, 1990; Craig and White, 1993a; 1993b; White, 1996), hippocampal slices (Pedata et al., 1991) and striatum (Delaney et al., 1998). Activation of AMPA receptors also contributes to adenosine release in cortical slices (Craig and White, 1993 a; 1993b, White 1996). The mechanisms mediating adenosine release by activation of NMDA and non-NMDA receptors could be different. Thus, in cortical slices, the adenosine released by activation of NMDA receptors can be blocked by an ecto-nucleotidase inhibitor as well as be promoted by an inhibitor of nucleoside transport, indicating that this adenosine originates from released adenine nucleotides, likely ATP, which is subsequently converted to adenosine via ecto-nucleotidase (Craig and White, 1993a). In contrast, activation of either AMPA or KA receptors releases adenosine *per se* into the extracellular space (Craig and White 1993a; 1993b). Whether NMDA and non-NMDA receptors mediate *peripheral* adenosine release by different mechanisms remained to be determined.

7.4.3 Involvement of capsaicin-sensitive sensory afferents in glutamate-evoked peripheral adenosine release

Pretreatment with systemic capsaicin almost completely inhibited glutamate-evoked peripheral adenosine release, indicating this release is largely dependent on capsaicin-sensitive C-and Aδ fiber activation. Previous studies demonstrated that ionotropic glutamate receptors are expressed in unmyelinated sensory fibers (Carlton et al., 1995; Coggeshall and Carlton, 1998; Carlton and Coggeshall, 1999). Furthermore, application of glutamate to the receptive fields induces significant spontaneous

discharges of nociceptors, and also sensitizes nociceptors to subsequent thermal stimulation (Du et al., 2001). As the transducer for noxious heat is the capsaicin receptor VR1, which is selectively expressed in small diameter sensory afferents (Tominaga et al., 1998; Caterina and Julius, 1999), and activation of VR1 receptors by acute capsaicin injection induces adenosine release (Chapter 4), it is not surprising that glutamate evokes adenosine release by activation of nociceptive sensory afferents.

As glutamate receptors are also expressed in large diameter myelinated Aβ afferents (Coggeshall and Carlton, 1998), and adenosine could be released from the central terminal of myelinated sensory afferents (Fyffe and Perl, 1984; Salter and Henry, 1987; Sawynok et al., 1993), it is possible that myelinated sensory afferents might also contribute to glutamate-evoked peripheral adenosine. However, in the current study, a nearly complete blockade of adenosine release by systemic capsaicin pretreatment argues against a significant role of myelinated Aβ fibers in peripheral adenosine release. Although not as well documented as with small diameter sensory afferents, Aβ fibers could also exhibit *efferent* function. For example, glutamate itself can be released from Aβ fibers by antidromic electrical stimulation (deGroot et al., 2000). Therefore, the lack of apparent involvement of Aβ fibers, in the current study, might simply reflect the selective excitability of nociceptive and non-nociceptive sensory afferents to glutamate, as it has been shown that application of glutamate to the receptive field selectively induced spontaneous discharge in C and Aδ fibers but not Aβ fibers (Du et al., 2001). Another possibility is that Aβ fiber-mediated ATP release occurs predominantly at central terminals rather than peripheral terminals, while in nociceptive sensory afferents, such release occurs both peripherally and centrally.

7.4.4 Lack of involvement of sympathetic postganglionic nerves in glutamate-evoked peripheral adenosine release

Pretreatment with systemic 6-OHDA to selectively deplete SPGN did not produce an effect on glutamate-evoked peripheral adenosine release in the rat hind paw. Both NMDA and AMPA receptors have been localized on postganglionic sympathetic axon in gray rami (Carlton et al., 1998a; Coggeshall and Carlton, 1999). If these receptors are functionally active, glutamate injection could induce the release of mediators, such as noradrenaline, NPY, ATP and adenosine from sympathetic neurons, which may contribute to nociception and inflammation (Chapter 1). However, the present study demonstrates that even if glutamate did activate receptors on SPGNs and promote neurotransmitter release, the released purine does not significantly contribute to the total release detected. Thus, although Carlton and colleagues suggest SPGNs contribute to the glutamate-induced axon reflex (Carlton et al., 1998a), our data suggest that the peripheral glutamate-induced axon reflex was predominantly mediated by nociceptive sensory afferents rather than through SPGNs.

7.4.5 Lack of contribution of endogenous excitatory amino acids and the activation of ionotropic glutamate receptors in formalin-evoked adenosine release

Formalin, at 5%, evokes endogenous release of glutamate and aspartate from the rat hind paw (Omote et al., 1998). Theoretically, the endogenously released excitatory amino acids could activate receptors located on primary sensory afferents to produce membrane depolarization and adenosine release. However, co-administration of both

MK 801 and CNQX with formalin failed to reveal an ionotropic glutamate receptor-mediated mechanism involved in the release of adenosine, at this concentration of formalin. The doses of the antagonists used here are equivalent or higher than those used to block release of adenosine evoked by exogenous glutamate. Previous studies also demonstrated that comparable doses of MK 801 and CNQX are able to block the pain behaviors induced by exogenous and endogenous excitatory amino acids (Jackson et al., 1995; Zhou et al., 1996; Davidson et al., 1997; Lawand et al., 1997; Carlton et al., 1998). Thus, although a secondary release of adenosine due to endogenous NMDA and non-NMDA receptors could not be totally excluded, it is clear that such release does not significantly contribute to the total adenosine release evoked by 5% formalin.

Electrophysiological studies demonstrate that the intensity of nociceptor excitation evoked by glutamate is ten to hundred-times weaker than that induced by formalin and capsaicin (Kenins, 1982; Puig and Sorkin, 1996; Du et al., 2001). Therefore, even though endogenous glutamate could evoke an axon reflex, it would be expected to be masked by the massive axon activation induced directly by formalin or capsaicin. Indeed, in the present microdialysis study, although the exogenous glutamate concentration added was much higher than that of the glutamate released by formalin (Omote et al., 1998), or by electrical stimulation of the sensory afferents (deGroot et al., 2000), the magnitude of the peak adenosine release is much less than that induced by 5% formalin. The current study suggests that peripheral adenosine release is concurrent with, rather than secondary to, the release of glutamate in formalin-induced inflammation.

7.5 CONCLUSION

The present study demonstrates that subcutaneous glutamate injection evokes a rapid-onset, short-lasting peripheral adenosine release. Both NMDA and non-NMDA receptors are involved in such release. Capsaicin-sensitive small diameter primary sensory afferents are predominantly involved in peripheral adenosine release by glutamate, while SPGN efferents do not contribute to such release. Although endogenous glutamate is released by 5% formalin, ionotropic glutamate activation does not significantly contribute to formalin-evoked adenosine release. Thus, adenosine may be release concurrently with glutamate, rather than secondarily to glutamate release.

CHAPTER 8

SUMMARY, SIGNIFICANCE AND FUTURE STUDIES

8.1 SUMMARY OF THE RESULTS

8.1.1 Microdialysis studies

The present study demonstrates an increase in peripheral extracellular adenosine levels occurring in both an inflammatory pain model (formalin test), and a neuropathic pain model (SNL model). In normal rats, subcutaneous saline injection does not release adenosine, but noxious stimuli such as formalin, capsaicin and glutamate injection induces peripheral adenosine release. In nerve-ligated rats, saline injection induces a peripheral release of adenosine. Peripheral adenosine release evoked by formalin or glutamate in normal rats, or saline injection in SNL rats exhibits different characteristics (Table 8.1).

(1) The time course of evoked peripheral adenosine release is different in different models. A low dose formalin or glutamate in normal rats, and saline in neuropathic rats produced a rapid-onset short-lasting adenosine release, while 5% formalin and 1% capsaicin produced a persistent adenosine release.

(2) The involvement of capsaicin-sensitive small diameter sensory afferent and SPGNs differs in the various models. Thus, capsaicin pretreatment blocks all the rapid-onset, short-lasting adenosine release (1.5% formalin, glutamate in normal rats, saline in neuropathic rats), but only blocks the early phase of the persistent release induced by 5% formalin. The 6-OHDA pretreatment blocks only the late phase of 5% formalin-evoked adenosine, but does not have an effect in other groups, indicating a selective involvement of SPGN in peripheral adenosine release only under some conditions. Degranulation of mast cells by compound 48/80 does not produce an effect at either

concentration of formalin, so mast cell degranulation was not further evaluated in other groups.

(3) *The potentiation of extracellular release of adenosine by the adenosine kinase inhibitor NH_2dAD, the adenosine deaminase inhibitor DCF, and the antidepressant AMI is also different in various models.* Thus, NH_2dAD only increases lower concentrations of formalin-evoked adenosine release, DCF only increases 5% formalin-evoked release, and AMI increases basal and 1.5% formalin-evoked adenosine release in normal rats as well as saline-evoked adenosine release in neuropathic rats.

(4) *Excitatory amino acids can evoke a peripheral release of adenosine.* Capsaicin-sensitive afferents but not sympathetic nerve terminals are involved in such release. Ionotropic glutamate receptors are involved in glutamate-evoked adenosine release, but not in 5% formalin-evoked adenosine release.

Table 8.1 Characteristics of peripheral adenosine release in the rat hind paw

Model		***Formalin***		***SNL***	***L-Glutamate***
		1.5%	5%	Saline	(0.03-100μmol)
Time course		< 10min	~ 60min	< 10min	<10min
Pre-treatment	Capsaicin	↓	↓ (early)	↓	↓
	6-OHDA	↔	↓ (late)	↔	↔
	Compound 48/80	↔	↔	Not tested	Not tested
Potentiation	NH_2dAD	↑	↔	↔	Not tested
	DCF	↔	↑	↔	Not tested
	AMI	↑	↔	↑	Not tested
IGlu R Blockade	MK 801	Not tested	↔	Not tested	↓
	CNQX	Not tested	↔	Not tested	↓

Summarized from the results of microdialysis studies (Chapter 4; Chapter5; Chapter6; Chapter7). ↑: Increased, ↓: decreased, ↔: no change.

8.1.2 Behavioral studies and paw volume studies

Behavioral studies in neuropathic rats demonstrated that adenosine A_1 receptor agonists, an adenosine kinase inhibitor and an adenosine deaminase inhibitor all produce local peripheral effects to reduce thermal hyperalgesia induced by nerve injury. In all cases, antihyperalgesic effects can be blocked by caffeine, indicating an adenosine receptor mediated effect. Local injection of low-dose adenosine A_1 receptor agonists produces an increase in paw volume, which is mediated partially by primary sensory afferents and mast cells. Inhibitors of adenosine metabolism do not produce overt paw edema, and might have an advantage for development as topical analgesics.

8.2 SIGNIFICANCE OF THE STUDY

8.2.1 Neurochemical considerations

To our knowledge, the present study is the first study demonstrating directly that ***noxious chemical stimuli*** *such as formalin, capsaicin and glutamate, as well as* ***spinal nerve injury****, can induce peripheral adenosine release via capsaicin-sensitive sensory afferents.* Although increase in extracellular adenosine levels in inflammatory exudes have been reported previously (Cronstein et al., 1993; 1995; Gadangi et al., 1996), the origin of the adenosine has been largely attributed to neutrophils (Cronstein et al., 1983;

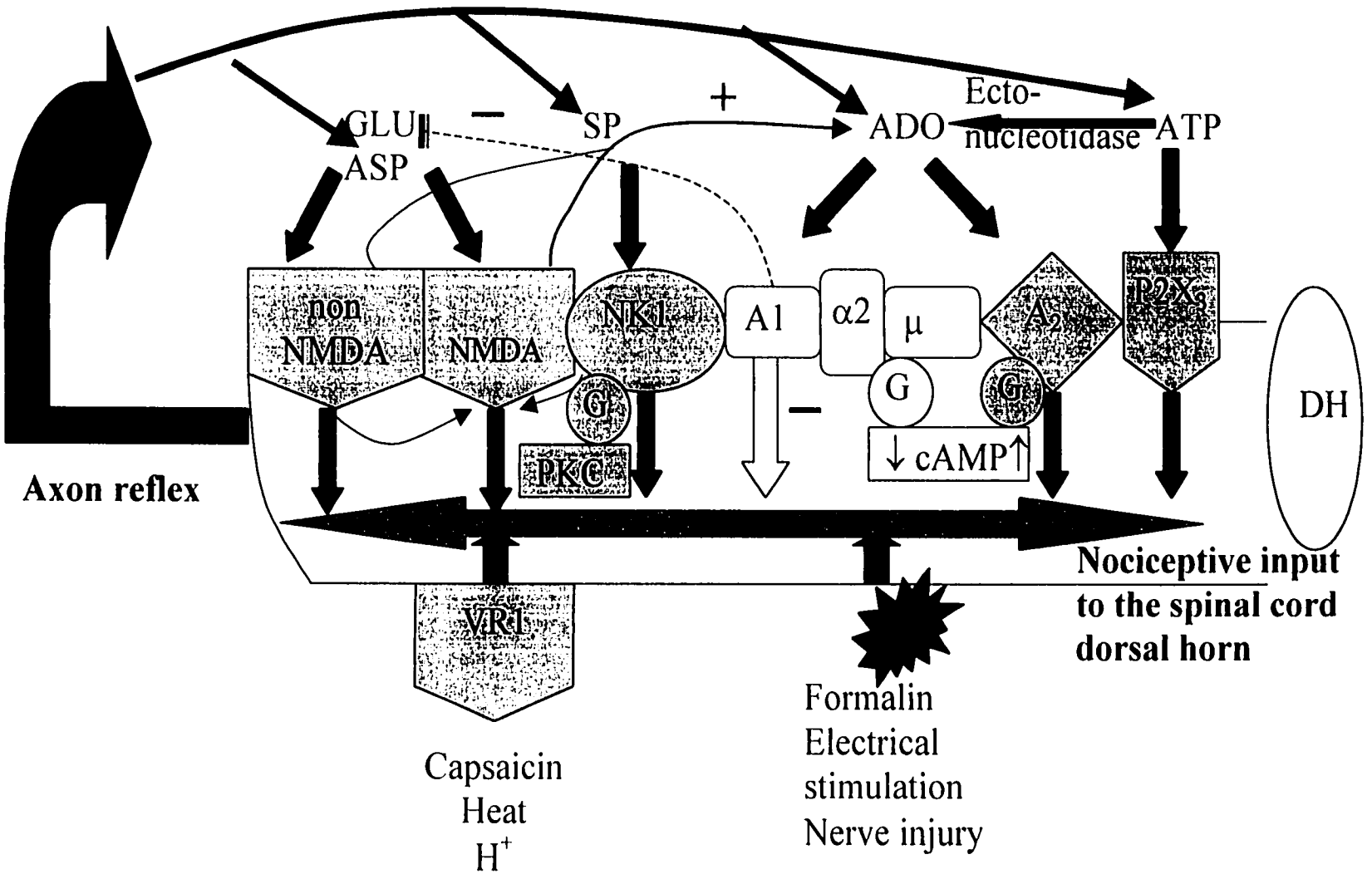
1986), endothelial cells (Ager and Gordon, 1984; Bodin and Burnstock, 1998) other non-specific cellular sources. Glutamate is known to release adenosine in brain and spinal cord (Section 7.1), yet no peripheral release has been reported. The only sensory afferent mediated peripheral release of adenosine comes from electrical stimulation of sensory afferents in rabbit ear releasing purines into circulation (Holton and Holton, 1954; Holton, 1959), but those studies did not identify whether nociceptive sensory afferents or non-nociceptive sensory afferents contribute to such release. Using *in vivo* microdialysis, we clearly demonstrate that noxious stimuli produced by chemical agents and nerve injury evoke peripheral adenosine through capsaicin-sensitive nociceptive sensory afferent mediated mechanisms. Although a secondary release of adenosine from adjacent tissues could not be excluded (see Chapter 4; 5), a direct release of adenosine from the peripheral terminal of nociceptive sensory afferents is consistent with the previous studies (Holton and Holton, 1954; Holton, 1959).

8.2.2 Functional considerations

Nociceptive sensory afferent-mediated peripheral adenosine release evoked by noxious stimuli or nerve injury suggests that during the axon reflex, an inhibitory neuromodulator (adenosine) is released concurrently with excitatory neurotransmitters. Previous studies demonstrate that SP, CGRP (Section 1.3.1) and excitatory amino acids (deGroot et al., 2000) are released from nociceptive sensory afferents during the axon reflex. By retrograde activation of sensory afferents, these excitatory neurotransmitters will further sensitize sensory terminals and function as a positive feedback on the axon reflex. The present study suggests that besides excitatory neurotransmitters, the

inhibitory modulator, adenosine, is also released through the axon reflex, and inhibitors of adenosine metabolism can modulate such release (Chapter 4). Other indirectly acting agents, such as amitriptyline can also modulate noxious stimuli or nerve injury induced peripheral adenosine release (Chapter 5; 6). Thus, *we hypothesize that the released adenosine may function as a negative feedback to modulate the axon reflex and peripheral sensitization by activating A_1 receptors* (Fig. 8.1). This hypothesis is supported by some behavioral studies. Thus, in the neuropathic pain model, we demonstrated that inhibitors of adenosine metabolism produce local, peripherally mediated antihyperalgesic effects (Chapter 3). In the formalin model, both peripheral, tonic antinociceptive effects mediated by adenosine A_1 receptors (Doak and Sawynok, 1995) and an antinociceptive effect produced by inhibitors of adenosine metabolism (Sawynok et al., 1998) have been demonstrated at certain (primarily low) concentrations of formalin. Amitriptyline produces peripherally mediated antinociceptive effects in both the formalin model (Sawynok et al., 1999a) and the neuropathic pain model (Esser and Sawynok, 1999; 2000), and this may also be partly due to its ability to increase extracellular adenosine levels in these models. However, an opposing effect produced by adenosine A_{2A} and A_3 receptors in peripheral nociception (Section 1.7.2) may partly limit the A_1 receptor mediated antinociceptive effect if adenosine levels are sufficiently raised (Fig. 8.1). Thus, when the modulation induces a huge amount of adenosine release into extracellular space (eg. DCF plus 5% formalin), no adenosine-mediated antinociceptive effect was observed (Chapter 4).

Fig. 8.1: Schematic illustration for the possible physiological functions of noxious stimuli-evoked peripheral adenosine release. Glutamate, capsaicin, formalin, electrical stimulation and nerve injury all can activate an axon reflex in capsaicin-sensitive primary sensory afferents. Glutamate, aspartate (ASP), SP and adenosine or ATP are released peripherally through the axon reflex. Excitatory receptors located on sensory terminals, such as NMDA, AMPA, KA, NK_1, $P2X_3$, VR1 are activated and sensitized by the released substances to enhance neurotransmitter release as well as to increase the nociceptive input into the dorsal horn neurons (DH) in spinal cord, potentially generating an amplified signal. Concurrently, adenosine (ADO) is released to activate adenosine A_1 receptors, which are functionally coupled to α_2-adrenoreceptors and μ-opioid receptors and will decrease cAMP, decrease calcium influx and hyperpolarize the sensory membrane. Neurotransmitter release (including glutamate) will be decreased, and the nociceptive information transmission will be inhibited upon adenosine A_1 receptor activation. However, nociceptive adenosine A_2 receptors can be activated at higher adenosine levels, which may mask the A_1 receptor mediated antinociceptive effects. Recruitment of particular aspects of this mechanism will depend on the particular pain condition. Rest abbreviations are explained in the List of Abbreviations.



Peripheral terminal of nociceptive primary sensory afferents

Fig. 8.1

8.2.3 About the models

The present study demonstrates the different involvement of capsaicin-sensitive sensory afferents and SPGNs in adenosine release and nociceptive behavioral-evoked effects by different concentrations of formalin (Chapter 4). Up to now, only a few studies have investigated the qualitatively different mechanisms mediating responses evoked by the low and high concentrations of formalin. These indicate that 5% formalin produces more pronounced inflammation and more prolonged behavioral and morphological changes (Yashpal and Coderre, 1998; Damas and Liégeois, 1999; Fu et al., 2000). Our results extend those studies in identifying the different involvement of neurogenic and sympathetic components in neurochemical changes and behavioral responses evoked by a low and a high dose of formalin.

The present study also demonstrates an abnormal response of capsaicin-sensitive sensory afferents in saline-evoked adenosine release in the SNL model, which indicates an increased axon reflex induced by nerve injury (Chapter 5). Spinal nerve injury induces spontaneous activity in neighboring, intact C fibers (Section 1.5.2). Local neurogenic responses mediated by capsaicin-sensitive sensory afferents, have also been demonstrated in CCI models (Daemen et al., 1998a; 1998b). Thus, it is likely that nerve injury increases the excitability of nociceptive afferents, which generate an axon reflex and the release of adenosine as well as SP and CGRP following stimuli that do not produce an effect under normal circumstances. A recent clinical study also reports a facilitated neurogenic plasma extravasation and vasodilatation in patients with complex regional pain syndrome (Weber et al., 2001). Thus, our results, together with the above

studies, suggest nerve injury induces an increased peripheral neurogenic response, which may be an important factor in initiating and maintaining neuropathic pain.

8.3 FUTURE STUDIES

8.3.1 To further investigate excitatory amino acid-evoked peripheral adenosine release

Are aspartate and nitric oxide involved in glutamate-evoked adenosine release from primary sensory afferents? Noxious stimuli can simultaneously release glutamate, aspartate and NO (Omote et al., 1998; 2000; deGroot et al., 1999), and complex interactions among these agents have been reported. Thus, (1) aspartate produces synergistic, peripheral nociceptive effects with glutamate and L-arginine (an NO donor) (Lawand et al., 1997), (2) inhibiting NMDA receptors reduces formalin-evoked, peripheral NO release (Omote et al., 2000), and (3) an NO synthesis inhibitor peripherally reduces nociception produced by NMDA and formalin (Wang et al., 1999). In brain and cardiovascular tissues, NO increases extracellular adenosine concentrations by facilitating ATP hydrolysis and inhibiting adenosine kinase (Obata et al., 1998; Rosenberg et al., 2000). Given the importance of NO in peripheral pain transmission (Chen and Levine, 1999), inflammatory responses (Salvemini et al., 1996), and its interaction with EAAs, it will be interesting to investigate the effect of NO on peripheral adenosine release and its interaction with EAAs mediated adenosine release. Our preliminary data show at 1μmol, L-aspartate and L-arginine did not evoke significant peripheral adenosine release. However, exploring different doses, different drug combinations, and cross antagonism is necessary to answer the question.

Do metabotropic glutamate receptors (mGluR) produce an effect on peripheral adenosine release? Our studies indicate that iGluRs mediates peripheral adenosine release by exogenous glutamate, but does not contribute to formalin-evoked adenosine release. A very recent study indicates mGluRs are also expressed in the peripheral terminal of sensory afferents and are involved in inflammatory hyperalgesia (Walker et al., 2001). This raises the question of whether or not mGluRs contribute to glutamate-induced adenosine release. Specific mGluR receptor antagonists will be used to address the question.

Do adenosine A_1 receptor agonists, or indirectly acting adenosine agents inhibit glutamate-induced peripheral sensitization? We hypothesized that the released adenosine can potentially generate a negative feedback effect in peripheral sensitization induced by noxious stimuli or nerve injury. Behavioral studies were performed in the neuropathic pain model and the formalin model to test this hypothesis (Section 8.2.2). Local peripheral injection of glutamate induces mechanical allodynia and hyperalgesia (Zhou et al., 1996), and co-injection with SP produces longer duration pain behaviors (Carlton et al., 1998b). To determine if the peripheral adenosine system plays a role in glutamate induced sensitization, several studies are necessary: (1) use of a selective adenosine A_1 receptor antagonist to determine if there is a tonic effect produced by endogenous adenosine; (2) use of an adenosine kinase inhibitor or an adenosine deaminase inhibitor to determine if modulating extracellular adenosine levels can produce an effect; (3) use of a selective adenosine A_1 receptor agonist to determine if direct activation of A_1 receptors can inhibit glutamate induced pain behavior. Besides

behavioral studies, neurochemical studies should also be explored to determine if peripheral adenosine A_1 receptor activation can produce an inhibitory effect on the axon reflex. If activation of adenosine A_1 receptors produces an inhibitory effect on the axon reflex, the adenosine, along with other neurotransmitters released through axon reflex, should also be decreased (Fig. 8.1). In the spinal cord, pre-injection of an adenosine A_1 receptor agonist suppresses NMDA-evoked release of both adenosine and EAAs (Conway and Yaksh, 1998). Whether such inhibitory effects also occur peripherally will be of great interest.

8.3.2 To investigate whether or not adenosine per se is released through nucleoside transporters or formed extracellularly through ecto-nucleotidase.

The current studies use extracellular adenosine levels as the end point to study noxious stimuli and nerve-injury induced purine release. The extracellular adenosine detected can be released either *per se* through nucleoside transporters or be formed from extracellular adenine nucleotides via ecto-nucleotidase (Fig 1.2; Section 1.6.4). ATP also modulates peripheral nociceptive transmission by activating $P2X_3$ or $P2X_{2/3}$ receptors, which may play an important role in inflammatory pain and neuropathic pain (Section 1. 7.4). Although extracellular ATP can be converted into adenosine within seconds, in some circumstances, the tonic antinociceptive effect mediated by adenosine through A_1 receptors (Doak and Sawynok, 1995; Sawynok et al., 1998) and the pro-nociceptive mediated by ATP, through the P2X receptors (Sawynok and Reid, 1997; Jarvis et al., 2001) may simultaneously occur. Thus, it would be interesting to identify whether it is ATP rather than adenosine *per se* released by noxious stimuli or nerve

whether it is ATP rather than adenosine *per se* released by noxious stimuli or nerve injury. The effect of inhibitors of ecto-nucleotidase and nucleoside transporters (NT) on noxious stimuli-evoked peripheral adenosine release will be studied. If adenosine is directly released from the intracellular space, inhibiting NT should *decrease*, while ecto-nucleotidase inhibitors should have little effect, on such release. On the other hand, if the adenosine is largely formed outside the cell, inhibiting NT should *increase* the release (re-uptake is inhibited), while inhibiting ecto-nucleotidase should *decrease* such release.

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