

**BEHAVIORAL AND NEUROANATOMICAL ASSESSMENT OF
AMITRIPTYLINE IN THE TREATMENT OF CHRONIC PAIN FOLLOWING
PERIPHERAL NERVE INJURY IN THE RAT**

by

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for the degree of Doctor of Philosophy**

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DEDICATION

This thesis is dedicated to my parents Hans and Julia Esser. While I am sure they didn't understand the motivation behind some of my life choices, they never wavered in their belief in me. Although I don't often say so, their support and encouragement is very important to me, and I greatly appreciate everything they have done for me.

I also wish to dedicate this thesis to the many sufferers of chronic pain. I sincerely hope that in some small way the research in this thesis helps to further advance our understanding of neuropathic pain, and ultimately in the development of more effective treatment strategies.

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ABSTRACT

The present study was designed to determine whether amitriptyline, a prototypical tricyclic antidepressant, could produce pain-alleviating effects in a rat model of neuropathic pain. Nerve injury was produced by unilateral spinal nerve ligation and this resulted in persistent stimulus-evoked neuropathic pain symptoms (tactile allodynia and thermal hyperalgesia). Following acute systemic administration, amitriptyline and to a lesser extent desipramine, reversed thermal hyperalgesia in the injured paw. The anti-hyperalgesic effect of systemic amitriptyline, but not desipramine, was blocked by acute caffeine. Spinal administration of amitriptyline produced an anti-hyperalgesic effect that was not blocked by caffeine. An immediate anti-hyperalgesic effect was observed following local peripheral administration of both amitriptyline and desipramine. The local administration of caffeine blocked the actions of amitriptyline but not desipramine. Neither amitriptyline nor desipramine exerted any anti-allodynic effect, but amitriptyline produced hyperaesthesia in the contralateral paw. A time course analysis was made of the immunoreactive expression of the 27 kDa heat shock protein (Hsp27-IR), neuropeptide Y (NPY-IR), and substance P (SP-IR) following nerve injury. Changes in Hsp27-IR were first apparent at 4 days in laminae I through III in the L5 to S2 segmental region of the ipsilateral dorsal horn, were most intense by 12 days, and declined by 180 days. An increase in NPY-IR in the same regions paralleled that of Hsp27-IR, but was offset by a 2-3 day delay. Decreased SP-IR was observed in the superficial laminae of the ipsilateral spinal dorsal horn by 7 days, was greatest after 12 days, and was still evident after 180 days. Both Hsp27-IR and NPY-IR were increased in the dorsal columns and the gracile nucleus by 17 days and persisted to 180 days, but no changes were observed in SP-IR in these regions. A non-invasive chronic drug paradigm (in drinking water) was used to study the behavioral and immunohistochemical effects of chronic amitriptyline alone, and in combination with chronic caffeine. Chronic amitriptyline decreased the expression of thermal hyperalgesia, an effect that was blocked by the concomitant consumption of chronic caffeine. While having no effect on static tactile allodynia of the ipsilateral paw, chronic amitriptyline caused tactile hyperaesthesia in the contralateral paw, an effect that was exacerbated by concomitant chronic caffeine. These behavioral effects were reflected in decreases in Hsp27-IR and NPY-IR in the deeper laminae of ipsilateral spinal dorsal horn.

The results of this thesis suggest that acute and chronic amitriptyline are effective against stimulus-evoked thermal hyperalgesia, and this effect is partially achieved through manipulation of endogenous adenosine levels. The symptom-specific action, and adenosine link in the effect of amitriptyline may be important clinical considerations governing its use in neuropathic pain. Also, the mirrored effects on Hsp27-IR and NPY-IR in the spinal dorsal horn suggest that these two markers may be sensitive indicators of chronic drug manipulation of the mechanisms underlying the development and maintenance of neuropathic pain.

LIST OF ABBREVIATIONS

4V	fourth ventricle
5-HT	5-hydroxytryptamine
A_{1,2,3}	adenosine A1, 2, 3 receptor
ADA	adenosine deaminase
ADP	adenosine 5'-diphosphate
ADs	antidepressants
AK	adenosine kinase
ami	amitriptyline
AMP	adenosine 5'-monophosphate
AMPA	(RS)-α-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid
ATP	adenosine 5'-triphosphate
Ca MK II	calcium calmodulin kinase
caff	caffeine
cAMP	cyclic adenosine monophosphate
CCI	chronic constriction injury
CCK	cholecystokinin
contra	contralateral (non injured) paw
CRE	cyclic AMP response element
CREBs	cyclic AMP response element DNA binding proteins
CRF	corticotrophin releasing factor
DAG	diacylglycerol
des	desipramine

DRG	dorsal root ganglion
EAA	excitatory amino acid
ER	endoplasmic reticulum
flx	fluoxetine
GABA	γ-aminobutyric acid
GABA_A-R	γ-aminobutyric acid A receptor
G_i	inhibitory G proteins
Glu	glutamate
Gr	gracile nucleus
GR	glucocorticoid receptor
Hsp27	27 kDa heat shock protein
i.m.	intramuscular
i.p.	intraperitoneal
i.t.	intrathecal
IFN-γ	interferon gamma
IL-1	interleukin 1
IL-6	interleukin 6
IP₃	1,4,5-inositol trisphosphate
ipsi	ipsilateral (nerve-injured) paw
L-NAME	L- N^g -nitro arginine methyl ester
mGluR	metabotropic glutamate receptor
MK-801	dizocilpine
MPE	maximum possible effect
NA	noradrenaline

NBI	nitrobenzyl thioinosine
NGF	nerve growth factor
NK1(-R)	neurokinin 1 (receptor)
NK2(-R)	neurokinin 2 (receptor)
NKA(-R)	neurokinin A
NKB	neurokinin B receptor
NMDA	N-methyl-D-aspartate
NMDA-R	N-methyl-D-aspartate receptor
NO	nitric oxide
NPY	neuropeptide Y
NTS	nucleus tractus solitarius
PGs	prostaglandins
PIP₂	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PL-A₂	phospholipase A₂
PLC	phospholipase C
PNL	partial nerve ligation
R-PIA	R-N⁶-phenylisopropyladenosine
s.c.	subcutaneous
SAH	s-adenosyl homocysteine
sal	saline
SNL	spinal nerve ligation
SP	substance P
SSR1	selective serotonin reuptake inhibitor

TCA	tricyclic antidepressant
TNF-α	tumor necrosis factor-alpha
VDCC	voltage dependent calcium channel
Y1	neuropeptide Y1 receptor
Y2	neuropeptide Y2 receptor

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PUBLICATIONS

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Papers:

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Sawynok J, Reid A, Esser MJ (1999) Peripheral antinociceptive action of amitriptyline in the rat formalin test: involvement of adenosine. *Pain* 80: 45-55.

Esser MJ, Plumier J-C.L, Allen GV, Sawynok J (1999) Spinal and supraspinal correlation of the temporal expression of heat shock protein immunoreactivity (Hsp27), neuropeptide Y (NPY) with mechanical allodynia in the rat following unilateral spinal nerve ligation. *Neuroscience* (submitted).

Esser MJ, Sawynok J (1999) Caffeine block of the antihyperalgesic action of amitriptyline in a rat model of neuropathic pain. *Eur J Pharmacol* (in press).

Sawynok J, Esser MJ, Reid A (1999) Peripheral antinociceptive actions of desipramine and fluoxetine in an inflammatory and neuropathic pain test in the rat. *Pain* (in press).

Abstracts:

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Allen GV, Esser MJ, Plumier J-CL, Sawynok J (1998) Spinal cord expression of heat shock protein (HSP27) in a rat model of neuropathic pain following tight ligation of L5 and L6 dorsal roots. Neuroscience Abstracts, 24(2), 547.5, pp 1387.

Esser MJ, Sawynok J (1998) Caffeine inhibits the thermal antihyperalgesic effect of amitriptyline in a rat model of neuropathic pain. Drug Development Res.,43(1), 202, 55.

Esser MJ, Sawynok J (1998) Local peripheral action of antidepressants in rat models of neuropathic pain and inflammation. Neuroscience Abstracts, 24(2), 641.20, pp 1631.

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INTRODUCTION

Pain is generally thought to be an adaptive mechanism that allows an organism to survive and prevent further damage to the injured area. While this may be true in the case of acute nociceptive pain, it is difficult to understand the benefit of chronic pain especially when it persists long after the injured area has healed. Yet this phenomenon does exist, and treatments for it are less than optimal. While considerable research effort has led to a greater understanding of the mechanisms involved in acute nociceptive pain, more needs to be known about more persistent and chronic types of pain such as nerve injury-induced neuropathic pain.

Neuropathic pain is a complex pathophysiological phenomenon that may arise from various etiologies such as trauma, compression injury, disease states, viral infection, amputation, or metabolic disorders such as diabetes (Portenoy, 1991; Elliott, 1994). While specific symptoms of neuropathic pain may vary widely among patients, the hallmark symptoms include thermal and mechanical hyperalgesia (exaggerated response to a noxious stimulus), thermal and mechanical allodynia (a noxious response to normally innocuous stimuli), paroxysmal and spontaneous pain (Elliott, 1994; Woolf and Doubell, 1994). The variation in presenting symptoms may be a result of differing patterns of the neural pathophysiology so that patients present not with a unitary symptom, but more often with a composite of symptoms (Max, 1990). While the exact pathophysiological mechanisms involved in the development and maintenance of neuropathic pain are disease/injury dependent and are not yet fully understood,

some common attributes do exist. For the purpose of this thesis, the focus of the pathophysiological description will be the events following peripheral nerve injury.

1.1 Pathophysiology of nerve injury-induced neuropathic pain

Following nerve injury, there are extensive pathophysiological changes at various levels along the neuroaxis that contribute to the development and maintenance of the neuropathic pain state (Fig. 1). While not intended to be an exhaustive analysis of the events, this thesis will highlight some of the salient features that are especially relevant to the antidepressant pharmacotherapy of neuropathic pain.

1.1.1 Peripheral sensitization and changes in dorsal root ganglion neurons

In the periphery, there is an alteration in the sensory dynamics of peripheral nociceptors and mechanoreceptors. While the exact mechanisms leading to the changes in peripheral sensory transduction are not fully known, studies have determined that the hyperresponsiveness is not due to an alteration in the activation threshold (reviewed Tanner et al., 1997). The functional changes that lead to this hyperresponsiveness have instead been attributed to many factors including changes in the phenotype of the nociceptor, inappropriate sympathetic–sensory transduction, novel expression of proteins, and abnormal coupling of biochemical mechanisms (reviewed Coderre et al., 1997; Tanner et al., 1997). Maladaptive reorganization of peripheral sympathetic fibers in the periphery (reviewed Devor, 1994; Coderre and Katz, 1997) and in the dorsal root ganglion (DRG) are also thought to contribute to the generation of ectopic foci of

Figure 1

Schematic depiction of the potential pathophysiological alterations in the peripheral (dorsal root ganglion, nerve fibers and peripheral receptors) and central (spinal cord) nervous system following peripheral nerve injury. Identified in the drawing is the site of nerve injury used in this study indicating involvement of large myelinated A β fibers as well as smaller thinly myelinated A δ fibers and unmyelinated C fibers. (Adapted from Hökfelt et al., 1997)

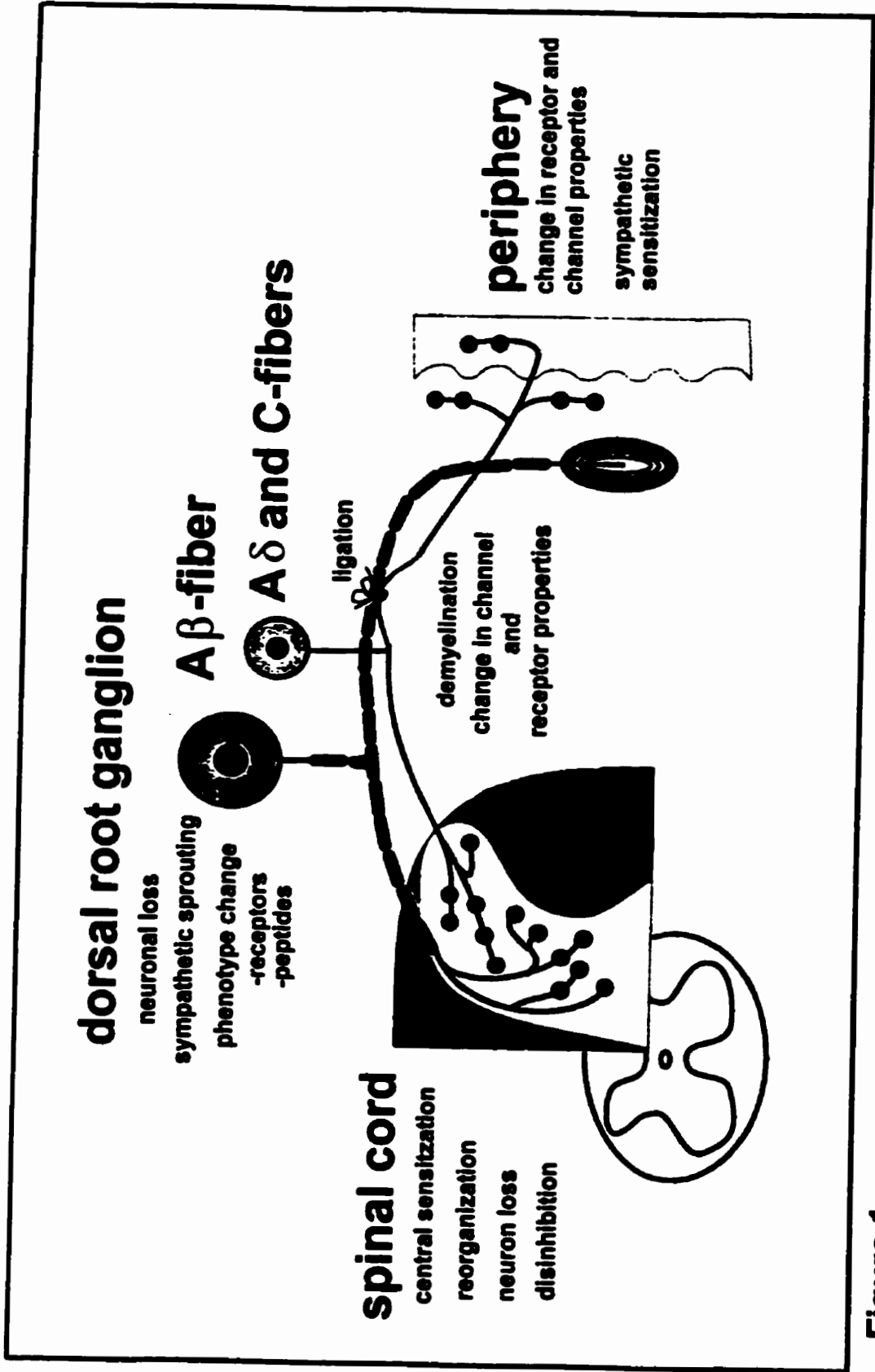


Figure 1

nerve impulse generation (Chung et al., 1996; Abbadie and Basbam, 1998; Ramer and Bisby, 1998). Factors potentially contributing to this ectopic activity include changes in the Na⁺ channel receptors that have been demonstrated in inflammatory (Gould et al., 1998) and neuropathic (Kral et al., 1999) pain. Na⁺ channel blockers are more effective when the channel is in the activated state, and this makes drug-induced alterations in Na⁺ channel dynamics especially applicable in neuropathic pain where spontaneous ectopic foci are prevalent (reviewed Tanner et al., 1997). While more common to inflammatory pain, peripheral nerve injury also initiates recruitment of macrophages from the bloodstream, as well as inducing local reactive gliosis (Kobierski, 1997). The release of several factors from these cells (cytokines, peptides, and growth factors) can sensitize the peripheral nerve endings resulting in hyperresponsive nociceptors.

At the site of the injury, depending on the extent of the damage, there may be regenerative sprouting and the formation of a nerve entanglement or neuroma. There may also be expression of novel Na⁺ channels that contribute to the generation of ectopic foci in the nerve stump region (reviewed Devor, 1994).

1.1.2 Central sensitization

In the spinal cord, multiple factors lead to a hyperactive and hyper-responsive state. These include central sensitization, that is manifested by a change in the response properties of dorsal horn neurons (Fig. 2), a loss of inhibitory interneurons in the spinal cord, and reorganization of primary afferents such that the tactile A β afferents now project to a region of the cord previously

Figure 2

Schematic representation of some of the events involved in central sensitization of spinal dorsal horn transmission neurons. Of emphasis is the intracellular events following release of glutamate (Glu) and substance P (SP) from primary afferent terminals, and their interaction with other cellular proteins (cell surface receptors and others). Also indicated is the potential liberation of positive feedback mediators (NO, PGs) and involvement of release of other mediators from glial cells. Abbreviations: AMPA, (RS)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDA, N-methyl-D-aspartate; PIP₂, phosphoinositol biphosphate; PLC, phospholipase C; PKC, protein kinase C; DAG, diacylglycerol; IP₃, 1,4,5-inositol triphosphate; mGluR, metabotropic glutamate receptor; NK₁, neurokinin 1 receptor; NO, nitric oxide; PL-A₂, phospholipase A₂; PGs, prostaglandins; TNF- α , tumor necrosis factor-alpha; IL, interleukins; P, phosphorylation; ER, endoplasmic reticulum (intracellular calcium stores); IFN γ , interferon gamma. (Adapted from Wilcox and Seybold, 1997).

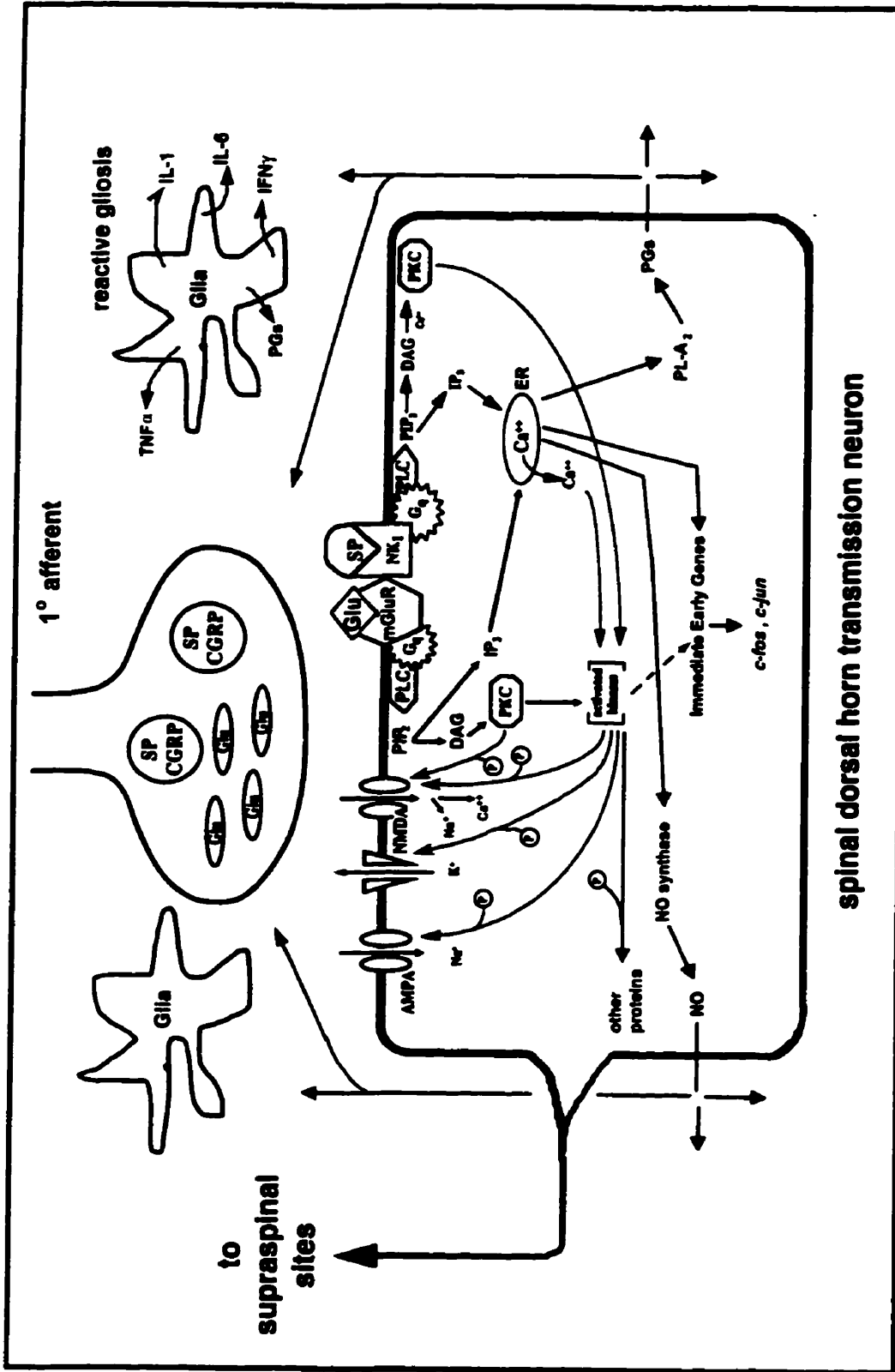


Figure 2

spinal dorsal horn transmission neuron

exclusively innervated by nociceptive A δ and C fibers (reviewed Coderre et al., 1993; Elliott, 1994; Ochoa, 1994).

1.1.2.1 Excitatory amino acids and the N-methyl-D-aspartate receptor

The involvement of excitatory amino acids (EAA) in the pathophysiology of chronic pain is supported by studies showing the release of EAAs in the spinal cord following nociceptive stimuli (reviewed Coderre et al., 1993, Coderre and Katz, 1997). The behavioral hyperalgesia generated following intrathecal administration of EAAs (Aanonsen et al., 1990; Aanonsen and Wicox, 1986, 1987; Dougherty and Willis, 1991) is further evidence in support of the role of EAAs in nociception. Release of EAAs from primary afferents leads to one of the most critical events in the generation of central sensitization, increased sensitivity of NMDA receptors (NMDA-R) to glutamate (Woolf, 1997). While the net effect of NMDA-R activation by glutamate is an influx of Ca²⁺ and Na⁺, this only occurs after alleviation of a Mg²⁺ block inside the channel. At normal resting membrane potentials, glutamate fails to generate an inward current upon binding to the NMDA-R (Mayer et al., 1984). Depolarization of the cell membrane relieves the Mg²⁺ block and allows the influx of Na⁺ and Ca²⁺. The Mg²⁺ block can be relieved through repetitive C-fiber stimulation (Woolf and Wall, 1986; reviewed Coderre et al., 1993). Substance P (SP), neurokinin A (NKA) and glutamate, which are released by noxious sensory stimulation, can act at the NK1, NK2, and metabotropic glutamate receptors (mGluR) respectively to activate a G-protein coupled transduction systems. The second messenger systems involved include activation of phospholipase C (PLC) that catalyzes the hydrolysis of

phosphatidylinositol to the intracellular messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG); IP_3 then acts to stimulate the release of intracellular Ca^{2+} , while DAG stimulates the translocation and activation of protein kinase C (PKC). Activation of PKC in turn can phosphorylate the NMDA-R leading to enhancement of Ca^{2+} currents (DeReim et al., 1985) and creation of a positive feedforward loop. Phosphorylation of the NMDA-R increases the functional sensitivity to glutamate such that glutamate then generates an inward current at resting membrane potentials (Chen and Huang, 1992). PKC is also involved in the phosphorylation of other proteins (channels or receptors) and this alters their response characteristics and makes a considerable contribution to neuron excitability (reviewed Firestone and Firestone, 1997). The elevated intracellular Ca^{2+} levels, as well as the phosphorylation of other receptors and ion channels, creates a sensitized neuron that generates suprathreshold responses to subthreshold inputs, has a decreased response threshold, an expanded receptive field, and may exhibit prolonged after discharges (Woolf, 1997).

1.2.2.2 Neuropeptides and facilitation of central sensitization

As mentioned above, SP and NKA, acting at NK1 and NK2 receptors, contribute to the sensitization of the NMDA-R through common second messenger systems. Thus, activation of NK1 receptors by SP, and NK2 receptors by NKA, results in an increase in intracellular Ca^{2+} levels and activation of PKC through the PLC- IP_3 pathway (Mantyh et al., 1984). SP and NMDA produce a synergistic effect on nociceptive processing and on the generation of central sensitization. This hypothesis is supported by studies showing co-

localization of SP and EAAs in primary afferents (DeBiasi and Rustioni, 1988), as well a hyperalgesic and allodynic effect of combined spinal SP and NMDA application (Willis and Dougherty, 1991). The facilitative actions of these neuropeptides are thought to occur presynaptically by increasing EAA neurotransmitter release (Kangrga and Randic, 1991; Smullin et al., 1990), and postsynaptically by potentiating NMDA-induced currents (Randic et al., 1990). It is interesting to note that peripheral neuropathy reduces the amount of SP required to increase EAA release (Skilling et al., 1992).

1.1.2.3 Cellular and molecular mechanisms of neuroplasticity in the spinal dorsal horn

While Ca^{2+} influx is important to dorsal horn neuron excitability and therefore nociception in general, studies suggest that it is even more important in models of persistent pain where sensitization is involved (reviewed Coderre et al., 1993, 1997). Increases in intracellular Ca^{2+} may result from a NMDA-R operated Ca^{2+} -channel influx, a SP facilitated mobilization of Ca^{2+} from internal stores (Womack et al., 1988), or a calcitonin gene-related protein (CGRP)-induced increase through voltage gated Ca^{2+} channels (Oku et al., 1987; Womack et al., 1989). As alluded to above, the NMDA-R linked Ca^{2+} influx appears to be particularly important in central sensitization. This importance is highlighted by studies demonstrating that the augmenting effect of intrathecal EAA administration in a model of persistent pain (formalin) is prevented by agents that block NMDA-R operated Ca^{2+} -channels (Dougherty and Willis, 1992; Dougherty et al., 1992). Thus increases in intracellular Ca^{2+} are important in

facilitating the heightened responsiveness of the post synaptic dorsal horn neurons (Fig. 2).

Similarly, PKC has been shown to be an integral component of central sensitization, as suggested by the role in NMDA-R sensitization discussed above. This conclusion is supported by studies using inhibitors of PKC to suppress the expression of neuropathic pain behaviors (Hayes et al., 1992). The role of PKC in spinal cord neuron sensitization is also consistent with the importance of PKC in long term potentiation (Malenka et al., 1986; Hu et al., 1987) as the two phenomena are somewhat analogous. While the principal effect of PKC is exerted through the modification of membrane ion channels (e.g. Ca^{2+} , Na^+ , K^+ , and Cl^-), it is also involved in the phosphorylation of other intracellular enzymes as well as G-proteins (reviewed Firestone and Firestone, 1997). The net result of the actions of PKC is an increase in synaptic efficacy.

A third important intracellular mediator of central sensitization is nitric oxide (NO). This diffusible gas is thought to be generated by the Ca^{2+} -induced activation of nitric oxide synthase (NOS), which catalyses the conversion of L-arginine to L-citrulline and in the process, liberates NO (reviewed Meller and Gebhart, 1993). The facilitated Ca^{2+} influx that occurs with sensitization of the NMDA-R, as well increased release from intracellular stores, can create a greater intracellular concentration of Ca^{2+} ultimately leading to an increased generation of NO. The role of NO in persistent pain states is thought to be primarily one of a retrograde messenger that acts to further increase neurotransmitter release (reviewed Meller and Gebhart, 1993; Sorkin, 1993). Evidence supporting the involvement of NO comes from studies using inhibitors of NOS such as

L-N6-nitro arginine methyl ester (L-NAME)) that inhibit the generation of NO and thermal hyperalgesia (reviewed Meller and Gebhart, 1993).

Further downstream, the increases in Ca^{2+} and PKC may lead to altered expression of protooncogenes and ultimately to the regulation of gene transcription of a variety of neuropeptides including enkephalins and neurokinins (reviewed Finkbeiner and Greenberg, 1998).

1.1.3 Disinhibition

In the normal state of the dorsal horn, there is an endogenous inhibitory tone that helps to regulate post-synaptic responses to afferent traffic. Without it, we would be constantly detecting stimuli that would bombard higher cortical structures. Indeed, this may in part be the case in neuropathic pain where loss of inhibition contributes to the altered sensory perception. This endogenous inhibitory tone is exerted through two main systems, γ -aminobutyric acid (GABA) and opioids.

The involvement of opioid systems in inhibition is indicated by their key role in analgesia (reviewed Dickenson, 1995). Actions of opioids and enkephalins are complex and depend on the site and type (μ , δ , or κ) of receptor involved. The spinal analgesic effect of opioids is thought to largely result from presynaptic inhibition of neurotransmitter release through activation of μ receptors, as well as hyperpolarization of the presynaptic cell membrane (Dickenson and Sullivan, 1986; Besson and Chaouch, 1989; Besse et al., 1990; Kangra and Randic, 1991). Importantly, the presynaptic distribution of opioid receptors on C and small diameter A fibers (A δ), but not on larger diameter A

fibers (A β), suggests a selective role in noxious stimulation (Dickenson, 1990). This selective distribution may also account for the lack, or reduced effect, of spinal opioids on allodynia which is putatively mediated by A β fibers. The reduced potency of spinal opioids in neuropathic pain has been demonstrated both behaviorally (reviewed Dray et al., 1994) and clinically (Dickenson, 1990) (but see DelleMijn, 1999). A loss of presynaptic opioid receptors on damaged primary afferents as well as an up-regulation of cholecystinin (CCK), an endogenous opioid antagonist, have been suggested as mechanisms for the reduced potency of spinal opioids (Stanfa et al., 1992; Xu et al., 1993).

The inhibitory tone within the spinal cord is also regulated by GABA and glycine. The intrathecal administration of bicuculline (GABA_A antagonist) and strychnine (glycine antagonist) results in the expression of allodynia, and this system has been proposed to also serve as a model of neuropathic pain (Yaksh, 1989; Sivilotti and Woolf, 1994). The general consensus in the literature is that the loss of this inhibitory component in the dorsal horn is a significant contributor to the maintenance of neuropathic pain (Woolf and Doubell, 1994; Woolf, 1997). Disinhibition may result from pre- and postsynaptic down regulation of respective inhibitory receptors (Hokfelt et al., 1994), a down regulation of GABA_A-R in DRG neurons and subsequently at the central terminals (Fukuoka et al., 1998), or a down-regulation of inhibitory neurotransmitters in intrinsic inhibitory interneurons (Castro-Lopes et al., 1993). Another possibility is a transsynaptic degeneration of inhibitory interneurons secondary to nerve injury-induced excitotoxicity (Bennett, 1991). While this still remains mostly speculative and is in need of more direct evidence (Woolf, 1997), the possibility is nonetheless intriguing.

The final component of inhibitory tone in the spinal cord is a descending modulation arising from bulbospinal projections containing noradrenaline (NA) and serotonin (5-HT). Acting through the α_2 -adrenoreceptor, noradrenergic efferents from the midbrain and brainstem produce mild antinociception and potentiate the actions of morphine (Dickenson and Sullivan, 1993; Stanfa and Dickenson, 1994). However, it is not fully understood to what extent this aspect of synaptic modulation of dorsal horn transmission neurons is inhibited following nerve injury.

1.1.4 Structural reorganization

In addition to the increased excitability and the decreased inhibition described above, the third component of central sensitization is structural reorganization. Following nerve injury, both degenerative and regenerative changes can occur in the spinal dorsal horn. The exact mechanisms that facilitate these changes are complex, but atrophic degenerative changes are thought to partly arise from deprivation of target-derived growth factors (e.g. nerve growth factor) which leads to breakdown and death of DRG neurons (Gold et al., 1991; Hökfelt et al., 1994; Zhang et al., 1994). Central axons of the DRG neurons from the injured fibers may subsequently atrophy and withdraw from the terminal region (Castro-Lopes et al., 1990). Conversely, regenerative changes also occur as indicated by the expression of the growth associated protein-43 (GAP-43) in growth cone-like structures in the superficial laminae of the dorsal horn following nerve injury (Woolf et al., 1990; Coggeshall et al., 1991). It is thought that it is the combination of factors from damaged terminals and the

vacancy created by atrophying central terminals that facilitates the sprouting of collateral fibers (Woolf et al., 1992; Shortland and Woolf, 1993). Of special relevance to neuropathic pain and allodynia is the reorganization of A β afferents from their central terminal field in laminae III/IV into laminae I and II regions normally restricted to A δ and C fiber terminals (Molander et al; 1992; Woolf et al., 1992; Shortland and Woolf, 1993). As a result of this altered connectivity, tactile information conveyed through A β afferents has the ability to excite wide dynamic and nociceptive-specific spinal dorsal horn neurons (Molander et al., 1992), and in so doing light innocuous tactile information may be interpreted as noxious. Another important aspect of reorganization is the aberrant sympathetic sprouting that occurs in the DRG following nerve injury (see above). As a result of the reorganization of A β afferents in the dorsal horn, ectopic DRG impulses can now be generated by sympathetic input (see above).

In light of the multiple contributing mechanisms to the development and maintenance of neuropathic pain, it is not surprising that it is a difficult clinical entity to treat. Many medical interventions have been utilized to treat neuropathic pain, but have met with limited success (reviewed MacFarlane et al., 1997). In an effort to avoid invasive surgical measures, various classes of pharmacotherapeutic agents have been used in the treatment of neuropathic pains (Ollat and Cesaro, 1995). Some success has been experienced with tricyclic antidepressants (e.g. amitriptyline, imipramine, desipramine, clomipramine), anticonvulsants (carbamazepine, valproate, gabapentin), and sympathomimetic agents (clonidine). Neuropathic pain is still considered to be relatively refractory to opioid treatment (Ollat and Cesaro, 1995; MacFarlane et

al., 1997; but see DelleMijn, 1999). Tricyclic antidepressants are considered front-line therapy for neuropathic pain (Onghena and Van Houdenhove, 1992), however conclusions from recent meta-analytical studies are equivocal as to whether there is a significant difference in the efficacy of antidepressants and anticonvulsants in the treatment of chronic pain (Onghena and van Houdehove, 1992; McQuay et al., 1995; 1996).

1.2 Antidepressant pharmacotherapy of neuropathic pain

Antidepressants (ADs), as the name suggests, are more commonly used in the treatment of mood disorders. However, as early as the 1960s (Paoli et al., 1960; Lance and Curran, 1964), reports arose suggesting an analgesic efficacy of antidepressants. The analgesic effect was initially attributed to alleviation of the depression often associated with chronic pain. However studies have shown that the analgesic effect of antidepressants occurs irrespective of mood altering effects (reviewed Sindrup, 1997; Eschalier et al., 1999). Support for this premise comes from the fact that the dose required for the analgesic effect is well below that required for the antidepressant effect (Sindrup, 1997). In clinical studies, as with treatment of depression, the analgesic effect of antidepressants is not immediate but exhibits a delay period (Sindrup, 1997). However, the delay period for the analgesic effect is typically one week while that for the antidepressant effect is approximately one month (Walsh, 1983; and reviewed Sindrup, 1997).

Two recent meta-analyses (Onghena and Van Houdenhove, 1992; McQuay et al., 1996) attempted to systematically evaluate the clinical evidence

for using ADs to treat chronic pain. While slightly different in terms of their evaluation criteria (McQuay et al. focussed on neuropathic pain) the two studies came to very similar conclusions. Both found that ADs were effective in treating chronic pain but only provided partial relief. In fact, McQuay et al. concluded that only 30% of patients achieved a greater than 50% reduction in pain state. Both studies also determined that tricyclic antidepressants (TCAs) were the most effective, followed by the heterocyclics, while the serotonin selective reuptake inhibitors (SSRIs) had minimal to no effect. Finally, both studies found that neuropathic pain of peripheral or central origin was the most responsive to AD treatment, and this effect was irrespective of alterations in mood. The clinical evidence therefore supports the use of ADs for the treatment of chronic pain in general, and neuropathic pain in particular. It still remains to be determined however, just where and how these agents work to exert their analgesic effect.

1.2.1 Analgesic mechanisms following acute administration

When ADs are taken systemically, they have the potential to have peripheral, spinal and supraspinal effects. Initial studies focussed on supraspinal and spinal sites of action for the analgesic effect of ADs (Spiegel et al., 1983; Dirksen et al., 1993; reviewed Eschalier, 1999). More recently, studies have also suggested a peripheral site of action for ADs (Jett et al., 1997; Sawynok et al., 1999a and b). Interestingly, the class of ADs that provide the greatest relief in the treatment of neuropathic pain are the TCAs, a group of drugs that have been termed 'dirty' because of their ability to interact with many systems. In particular, one of the most commonly used TCA in the treatment of neuropathic pain is

amitriptyline, an agent which has multiple actions some of which are still incompletely understood (Baldessarini, 1995). Perhaps it is this ability to interact with many systems that affords the TCAs the greatest analgesic effect.

Unfortunately, the same multiplicity of action that may lead to its success, is also a major dose-limiting factor in its use, as these multiple actions can cause intolerable side effects (MacFarlane et al., 1997). For the purpose of this dissertation, the neurochemical mechanisms of action will be discussed from the perspective of both acute and chronic administration experiments and will highlight the evidence supporting each of the respective mechanisms.

1.2.1.1 Amine hypothesis

The most widely known mechanism of action of ADs is inhibition of monoamine reuptake (noradrenaline and serotonin) with a consequent change in central amine receptor systems. This mechanism is thought to largely underlie the means by which mood is improved (reviewed Leonard, 1993; 1996). Most of the emphasis of this hypothesis is centered on the inhibition of serotonin reuptake (reviewed Blier and de Montigny, 1995). However the minimal efficacy of SSRIs in the treatment of chronic pain (Onghena and Van Houdenhove, 1992; McQuay et al., 1996) argues against the primary involvement of this mechanism in alleviating pain. Similar findings with respect to minimal effects of SSRIs as compared to TCAs in animal studies further suggest that this is not the main mechanism of action. Rather, it appears that a combination of serotonin and noradrenaline reuptake inhibition is important. This conclusion is supported by preclinical studies demonstrating that inhibition of serotonin or noradrenaline

synthesis inhibits the analgesic effects of the mixed (noradrenaline and serotonin) as well as both types of selective (noradrenaline or serotonin) reuptake inhibitors (reviewed Eschalier et al., 1999). Similarly, serotonin and α_2 -adrenoreceptor antagonists are effective in inhibiting the analgesia induced from all types ADs (mixed or selective)(Eschalier et al., 1994). This finding is in line with the known synergistic effect of noradrenergic and serotonergic descending modulation systems on pain transmission (Post and Archer, 1990; reviewed Millan, 1999).

1.2.1.2 NMDA receptor antagonism

There is compelling evidence suggesting an NMDA antagonistic action of ADs (Svensson et al., 1994; reviewed Leonard 1996). TCAs in general, and amitriptyline in particular, are structurally similar to well established NMDA antagonists like dizocilpine (MK-801) (Iwamoto and Marion, 1994), and bind to the NMDA receptor with an IC_{50} in the low μ M range (Reynolds and Miller, 1988; Sills and Loo, 1989). It is interesting to note that NMDA antagonists are being developed for use as antidepressants, further confirming the structural and mechanistic similarities to antidepressants (Trullas and Skolnick, 1990; Paul et al., 1992; Papp and Moryl, 1993). Behavioral studies indicate that spinally delivered ADs are effective in reducing hyperalgesia following intraplantar carrageenan (Eisenach and Gebhart, 1995a) and spinal NMDA-induced behaviors (Mjellum et al., 1993; Eisenach and Gebhart, 1995a). Antidepressants have also been shown to be protective against NMDA-induced toxicity (Leander, 1989; McCaslin et al., 1992; Mjellum et al., 1993). The exact

means through which acute ADs inhibit NMDA induced elevations of intracellular Ca^{2+} is not fully known. Studies suggest the action is similar to that observed with MK-801, and does not reflect binding at regulatory sites for glycine, Mg^{2+} , or Zn^{2+} (Reynolds and Miller, 1988; Sernagor et al., 1989; Kitamura et al., 1991). (The same may not be true following chronic AD administration, as will be discussed below). While acute ADs antagonize NMDA-induced nociceptive behaviors, they do not have any effect on NMDA, kainate or quisqualate evoked glutamate release (Sernagor et al., 1989; McCaslin et al., 1992). Furthermore, the effect of ADs appears to require an open channel (Sernagor et al., 1989; Tohda et al., 1995) which may make this action especially applicable in situations involving wind-up and central sensitization, where the Mg^{2+} block is removed. All of the above described evidence points to a possible NMDA antagonistic effect of ADs that may contribute to the antinociceptive effect observed in acute pain paradigms.

1.2.1.3 Interactions with opioids

Preclinical data suggests that ADs interact with opioid systems. Although they have a low affinity for the opioid receptor (high μM ; Hall and Ogren, 1981; Isenberg and Cicero, 1984), a number of animal studies have demonstrated naloxone inhibition of the analgesic effect of ADs (Eshalier et al., 1981; Ardid and Guilbaud, 1992; Gray et al., 1998). The ability of naloxone to partially reverse the inhibitory effect of desipramine and clomipramine on mechanosensitive afferent activation (Su and Gebhart, 1998) provides support for an opioid link in the peripheral actions of ADs. Moreover, ADs increase the levels of leucine and

methione-enkephalin in several regions of the rat brain after daily administration (De Filipe et al., 1985; Hamon et al., 1987), and enhance morphine antinociception after single administration (Fialip et al., 1989). However, clinical studies of the effect of naloxone on ADs-evoked analgesia are still equivocal (see Eschalier et al., 1999 and references therein).

1.2.1.4 Neurokinin antagonism

The most compelling evidence for a putative AD antagonism of SP receptors comes from a study demonstrating inhibition of SP-induced nociceptive behaviors with intrathecal imipramine (Imahita and Shimizu, 1992). In the study by Imahita and Shimizu (1992), it was unclear whether imipramine exerted its effect through direct interaction with the NK1 receptor. Indeed, it has been shown that ADs have a low affinity for NK1 receptors (see Kramer et al., 1998). Therefore, the effect of ADs on SP-evoked behaviors appears to be indirect and may involve actions at other receptor sites (e.g NMDA) that are affected by NK1 receptor activation. In a similar manner, peripheral clomipramine has been shown to reduce SP production in the carrageenan-induced exudate (Bianchi et al., 1995), which also suggests an indirect SP-antagonistic effect.

1.2.1.5 Interactions with cation (Ca^{2+} , K^+ , and Na^+) channels

The ability of ADs to indirectly affect Ca^{2+} conductance was previously described with respect to their interactions with NMDA receptors. However, ADs have also been shown to inhibit Ca^{2+} conductance through voltage dependant calcium channels (VDCC)(Aronstam and Hoss, 1985; Lavoie et al., 1990; Choi et

al., 1992). Choi et al. (1992) suggested that the effect of imipramine on Ca^{2+} conductance in cultured murine dorsal root ganglia was exerted through L-type channels, and that the effect may involve an interaction with pertussis-sensitive G proteins (G_i and/or G_o). Conversely, Kamatchi and Ticku (1991) determined that the inhibition of Ca^{2+} -activated K^+ efflux in cultured spinal cord neurons was not a result of interaction at a VDCC or interaction with a G-protein. Therefore the actual mechanism through which ADs modify Ca^{2+} conductance still remains to be fully determined, but the action in itself is a potentially important contributor to the antinociceptive effect of ADs.

The quinidine-like (class Ib anti-arrhythmic) antagonistic effect of some ADs on Na^+ channels is another mechanism putatively contributing to their antinociceptive effect. This postulate was recently substantiated in a recent study that showed similar efficacy between desipramine and mexelitine, which is a class Ib anti-arrhythmic agent (Jett et al., 1997). That there are beneficial effects with peripheral Na^+ channel blockers in treating some types of neuropathic pain is well established (Galer, 1995), and this effect may be related to an inhibition of primary afferent traffic. Ardid et al. (1991) showed no effect of peripheral clomipramine on carrageenan-evoked hyperalgesia, but recent studies (Sawynok et al., 1999a and b) determined that peripheral amitriptyline and desipramine were effective in alleviating formalin induced nociceptive behaviors. Thus, it may be that the potential quinidine-like effects of ADs are dependent on the test and the particular AD used.

1.2.1.6 Other potential mechanisms

Other evidence supports a potential contribution of the anti-histaminergic action of ADs contributing in the antinociceptive effect. While histamine has been shown to be involved in modulation of nociceptive responses (Thoburn et al., 1994) and to play a role in clomipramine analgesia (Arrigo-Reina and Chiechio, 1998), the exact nature of AD-histamine interaction in nerve-injury induced neuropathic pain remains to be determined. However studies suggest a role in stress induced analgesia (Arrigo-Reina et al., 1988; Barke and Hough, 1993) and β -endorphin release (Arrigo-Reina et al., 1987) following long term ADs administration.

Antidepressants are also widely known to interact with muscarinic receptors (Hall and Ogren, 1981), and the antagonistic action at these receptors is largely responsible for the dose-limiting side effects (Baldessarini, 1995). However, since cholinergic analgesia is thought to be mediated through nicotinic receptors, the anti-muscarinic actions of TCAs are unlikely to contribute to the analgesic mechanisms. ADs may also effect nicotinic receptors through open channel blocking properties (Eldefrawi et al., 1981; Scholfield et al., 1981; Arita et al., 1987; Rana et al., 1993; Fryer and Lukes, 1999). This action may contribute to the analgesic effect of ADs since nicotinic receptors have been localized to, and exert an excitatory effect on, cutaneous nociceptors (Morita and Katayama, 1984; Steen and Reeh, 1993; Roberts et al., 1995).

1.2.1.7 Antidepressants, adenosine and neuropathic pain

A final interaction of ADs (in particular amitriptyline) that has the potential to play a significant role in the antinociceptive actions of ADs is manipulation of endogenous adenosine levels. Earlier studies have suggested that amitriptyline may interact with endogenous adenosine systems by inhibiting cellular reuptake (Phillis and Wu, 1982; Phillis, 1984). This possibility is intriguing since adenosine has been shown to have anti-nociceptive properties in animal studies of acute nociception, inflammation and neuropathic pain (reviewed Sawynok, 1998, 1999). In humans, adenosine has been shown to be efficacious in the alleviation of neuropathic pain after intravenous infusions (Belfrage et al., 1995; Sollevi et al., 1995). Furthermore, a recent clinical study has demonstrated that intrathecal adenosine prevents the development of allodynia in an experimental model of sensitization (Rane et al., 1998). The importance of adenosine in neuropathic pain is also suggested by the reduced level of adenosine in the plasma and cerebral spinal fluid of patients with neuropathic pain (Guieu et al., 1996). Such observations suggest that there may be a beneficial endogenous tone of adenosine that is diminished as part of the pathophysiology of neuropathic pain.

Adenosine is an endogenously produced substance that has wide ranging and varying physiological effects (Collis and Hourani, 1993). The level of adenosine inside and outside of cells (approximately 10^{-8} to 10^{-7} M) is regulated by a balance of production, release, reuptake and metabolism (reviewed Geiger et al., 1997). Production of adenosine inside the cell involves breakdown of adenosine 5'-triphosphate (ATP), adenosine 5' diphosphate (ADP), cyclic adenosine 5' monophosphate (cAMP), and AMP specific 5'-nucleotidases, while

outside the cell adenosine production is a result of ATP breakdown by ecto-ATP dephosphorylase, ecto-ATPase, and ecto-5'-nucleotidase (reviewed Brundridge and Dunwiddie, 1997). Intracellular breakdown of adenosine occurs through deamination to inosine, by adenosine deaminase (ADA), phosphorylation to 5'-AMP by adenosine kinase (AK), and transformation to S-adenosyl homocysteine (SAH) by SAH-hydrolase (reviewed Brundridge and Dunwiddie, 1997). Intra- and extracellular adenosine levels are regulated by reuptake transporters. The transporters have been divided into two classes, the equilibrative and concentrative transporters, based on structure and action (reviewed Geiger et al., 1997). Basically, the equilibrative transporters are Na⁺-independent and act to diminish cross membrane differences in adenosine concentration. The equilibrative transporters have been further subdivided into equilibrium sensitive (*es*) and equilibrium insensitive (*ei*) based primarily on their affinity for the transport inhibitor nitrobenzylthioinosine (NBI) (Geiger et al., 1997). The concentrative transporters are Na⁺-dependant and act to couple adenosine transport to the movement of Na⁺, and will therefore act to change adenosine concentration as a function of membrane depolarization (reviewed Geiger, 1997).

The actions of extracellular adenosine are quite diverse, with effects on various biological systems including cardiovascular, respiratory, immunological, renal, gastro-intestinal and metabolic (Collis and Hourani, 1993). These effects are mediated through one or more of its four cloned receptors (A_1 , A_{2a} , A_{2b} , and A_3) coupled to intracellular G proteins (reviewed Fredholm et al., 1994). Adenosine A_1 receptors are coupled to pertussis sensitive $G_{i1,2,3}$ and G_o proteins (Freissmuth et al., 1991; Munshi et al., 1991) and as such, act to decrease

cAMP, decrease Ca^{2+} conductance, and increase K^+ conductance (Fredholm et al., 1994). Furthermore, A_1 receptor activation has been linked to stimulation of PLC and PKC (Gerwins and Fredholm, 1992). The adenosine A_{2a} receptors on the other hand are coupled to G_s proteins that mediate the activity of adenylyl cyclase (reviewed Fredholm, 1994). The A_{2a} receptors may also activate L-type Ca^{2+} channels (Birnbaumer, 1992) and thereby facilitate cell membrane depolarization as well as activation of intracellular protein kinases. The adenosine A_{2b} receptor interacts with G_s proteins (Stiles, 1997) to increase the activity of adenylyl cyclase as well as open Ca^{2+} channels. The final adenosine receptor, A_3 , is coupled to $G_{\alpha 2}$ and $G_{\alpha 3}$ proteins and results in a decrease in adenylyl cyclase activity as well as an activation of PLC (Palmer et al., 1995).

In acute pain models in animals, spinal administration of adenosine has been shown to be anti-nociceptive, an effect thought to be mediated by A_1 receptor activation (Karlsten et al., 1992; Doak and Sawynok., 1995, reviewed Sawynok, 1999). Intracerebroventricular injections of adenosine analogues produce antinociception (Holmgren et al., 1986), and the wider cerebral distribution of adenosine A_1 receptors as compared to that of the A_2 receptors suggests them as the principal contributors to adenosine supraspinal anti-nociception (Reppert et al., 1991). In various models of neuropathic pain, spinal adenosine analogues have been shown to alleviate neuropathic pain symptoms (Yamamoto and Yaksh, 1991; Lee and Yaksh, 1996; Sjölund et al., 1996; Cui et al., 1997). Similarly, as in acute pain tests, the spinal effect of adenosine in neuropathic pain is thought to largely occur through A_1 receptor activation in the substantia gelatinosa of the spinal cord (Sawynok and Sweeney, 1989; reviewed

Sawynok, 1999). Autoradiographic and binding studies have identified the substantia gelatinosa as containing the highest density of A_1 and A_2 receptors in the spinal cord (Goodman and Snyder 1982; Geiger et al., 1984; Choca et al., 1988a). The A_1 receptors in the spinal cord have been identified on both interneurons and projection neurons within the spinal dorsal horn (Geiger et al., 1984; Choca et al., 1988).

The inhibitory action of A_1 receptors on intrinsic dorsal horn neurons is thought to result from hyperpolarization of the cell membrane via enhanced K^+ conductance (Salter and Henry, 1985; De Konnick and Henry, 1992; Li and Perl, 1994; Reeve and Dickenson, 1995), as well as inhibition of membrane depolarization through inhibition of Ca^{2+} conductance (Sah, 1990). The post-synaptic action of adenosine A_1 receptor activation is illustrated by the adenosine mediated inhibition of nociceptive behaviors following intrathecal SP and NMDA administration (Doi et al., 1987; Delander and Wahl, 1988). Adenosine A_1 presynaptic inhibition of neurotransmitter release is also widely accepted as a mechanism for the anti-nociceptive effect of adenosine (Dunwiddie and Fredholm, 1997). In this context, A_1 receptor activation inhibits Ca^{2+} conductance and thereby inhibits the presynaptic terminal membrane depolarization necessary for synaptic release (Dolphin et al., 1986; MacDonald et al., 1986). Presynaptic A_1 receptor activation may also have an effect on the dynamics of synaptic vesicle docking and release (Dunwiddie and Fredholm, 1997). This action is validated by studies demonstrating A_1 receptor mediated inhibition of electrically evoked SP and CGRP release from capsaicin sensitive afferents (Vasko and

Ono, 1990; Sanctioli et al., 1992), and regulation of glutamate or aspartate release in the spinal dorsal horn (Conway and Yaksh, 1998).

While spinal adenosine is principally anti-nociceptive or anti-neuropathic, peripheral administration of adenosine is pronociceptive after intradermal administration in rats (Taiwo and Levine, 1990) and humans (Pappagallo et al., 1993), and augments the inflammatory pain induced by formalin injections (Karlsten et al., 1992; Doak and Sawynok, 1995). One problem in deciphering the pronociceptive effects of adenosine in the periphery is an apparent species difference (Sawynok, 1996). While A₁ receptors appear to be involved in the pronociceptive effect in humans (Pappagallo et al., 1993; Sawynok, 1996), the same effect is apparently mediated through the A₂ receptors in rodents (Taiwo and Levine, 1990, Karlsten et al., 1992; Doak and Sawynok, 1995).

In clinical studies, intravenous adenosine infusions of less than 70 µg/kg/ml have demonstrated analgesic effects of both the allodynic threshold (Segerdahl et al., 1994) and in the area of secondary hyperalgesia (Segerdahl et al., 1995). In both of these studies however the neuropathic conditions were experimentally induced and not ongoing as is the case with chronic neuropathic pain patients. The significance of the results may not be totally applicable, especially if neuropathic pain is a result of central sensitization and neuroplasticity-induced permanent changes. Nonetheless, a series of recent studies have indicated the potential benefit of adenosine in the clinical treatment of neuropathic pain. In a study of two patients with neuropathic pain, Sollevi et al. (1995) reported alleviation of spontaneous pain, tactile allodynia and warmth allodynia, as well as attenuation of pinprick hyperalgesia. The pain relief effect

lasted 4 to 6 hours after infusion, which is surprising in light of the reported adenosine half life of about 10 seconds (Sollevi, 1991). In a follow up adenosine infusion study, Belfrage et al (1995) reported a reduction in both spontaneous pain and pinprick hyperalgesia, and an increase in the allodynic threshold for seven patients with neuropathic pain. This study also reported a prolonged effect of the adenosine infusion lasting from 6 hours to 4 days. A recent clinical case of a post-surgical lesion revealed the effectiveness of intrathecal administration of the adenosine analogue R-N⁶-phenylisopropyladenosine (R-PIA), in alleviating both static (touch) and dynamic (brush) allodynia (Karlsten and Gordh, 1995).

The potential significance of endogenous adenosine levels to the analgesic action of ADs makes it important to consider interactions with compounds that affect adenosine actions. Acute administration of caffeine is understood to exert its pharmacological effects through antagonism of adenosine receptors at low doses, but exhibits undesirable effects at higher doses through inhibition of phosphodiesterase enzymes (Fredholm, 1995). Caffeine is a non-selective adenosine receptor antagonist being equally effective at both A₁ and A₂ receptors (Fredholm, 1995). Consumption of caffeine from various sources may therefore affect drugs acting either directly or indirectly on endogenous adenosine systems.

As described above, the analgesic potential of antidepressants may be exerted through multiple mechanisms following acute administration. Irrespective of the specificity of the acute mechanisms of each AD, there are common features that occur with chronic administration that are thought to account for the mood altering effects of this diverse class of drugs. As such,

studies of the chronic AD-induced changes are focussed on understanding the significance of the changes as they apply to depression. Nonetheless, an argument can be made for the beneficial aspects of these changes in terms of chronic pain treatment. It is important to remember however, that the desired mood altering therapeutic effect of ADs occurs after a longer lag time and requires a significantly higher dose than the analgesic effect (Sindrup, 1997). Furthermore, the analgesic effect is independent of the mood altering effects. For the purpose of this dissertation, the neuroplastic changes after chronic ADs will be briefly identified with a focus on changes implicated in the analgesic effect of chronic ADs.

1.2.2 Neuroplastic changes following chronic antidepressant treatment

While each of the individual ADs may have different potencies in their acute effects as described above, they all induce similar changes in receptor systems following chronic administration. In general these changes can be grouped into aminergic and non-aminergic (Leonard, 1996).

1.2.2.1 Chronic antidepressant-induced changes in aminergic systems

Probably the most well known change following chronic AD treatment is the desensitization and down regulation of cortical β -adrenoceptors (reviewed Leonard, 1996). As cortical β -adrenoceptors are thought to play an inhibitory role on cortical serotonin (5-HT) systems, this action would potentiate serotonergic transmission. Chronic ADs also decrease the functional activity of α_2 -autoreceptors and thereby decrease the inhibition of NA/5-HT release. On the

other hand, the up-regulation of α_1 -adrenoceptors by chronic AD administration (in Leonard et al., 1993, 1996) acts to potentiate the effects of noradrenaline release. There is also a decreased functional activity of dopamine autoreceptors, the functional significance of which remains to be determined.

The dominant mood altering effect of ADs seems to be exerted through manipulation of the serotonergic system. In general, all chronic ADs enhance serotonergic transmission by sensitizing forebrain neuronal receptors (5-HT_{1A}, 5-HT₂) to the effects of 5-HT, increasing the quantity of the 5-HT, and/or desensitizing autoreceptors (5-HT_{1A}, 1B) and transporters (reviewed Blier and de Montigny, 1995; Leonard, 1993; 1996). In a recent study, Mjellum et al. (1993) demonstrated a decrease in intrathecal NMDA-evoked behaviors with chronic desipramine, an effect that was reversed by a 5-HT_{1A} antagonist (NAN-190). Interestingly, the administration of a 5-HT_{1A} antagonist in controls potentiated the NMDA-induced response suggesting an endogenous serotonergic tone (Mjellum et al., 1993). A recent study also found that forebrain 5-HT_{1A} receptors became tonically active following chronic ADs (Haddjeri et al., 1998).

1.2.2.2 Chronic antidepressants induced changes in neurotransmitter systems

Possibly one of the most significant analgesia-linked alterations following chronic AD administration occurs with the NMDA receptor. The changes in the NMDA receptor are thought to involve both the receptor and second messenger systems. Chronic, but not acute imipramine has been shown to alter the ligand binding properties of glycine (both number and affinity) and glutamate recognition sites on the NMDA receptor (Nowak et al., 1993; Paul et al., 1993, 1994). As the

glycine and glutamate binding sites on the NMDA receptor are allosterically coupled (Williams et al., 1991; Carter, 1992), alterations in one or both of these sites will affect the actions of the NMDA receptor. Especially applicable to an analgesic role is the finding that the modifications in the NMDA receptor require approximately one week in order to become manifest. This time frame closely coincides with the time required to observe a therapeutic analgesic effect (reviewed Magni, 1991; Watson, 1994), but is significantly shorter than the 3 to 6 weeks required for the mood altering effect (reviewed Leonard, 1996; Sindrup, 1997).

It has been suggested that the effect of ADs is actually exerted through a common receptor coupled, or intracellular signaling pathway (Nestler et al., 1989; Duman, 1994; Mann et al., 1995). This rationale mainly comes from the repeated observation that the various treatment strategies for depression (pharmacotherapy and electroconvulsive therapy) fail to produce the same degree of changes in noradrenergic, dopaminergic, serotonergic, GABAergic or peptidergic transmission systems (Stone, 1983; Heninger and Charney, 1987; Vetulani, 1991). One important aspect common to chronic ADs, NMDA-R activation and central sensitization is altered Ca^{2+} conductance. A significant aspect of increases in intracellular Ca^{2+} is the activation of protein kinases and their subsequent effects. Two important kinases are PKC and Ca^{2+} calmodulin kinase (CaMK II). Chronic TCAs, cause an increase in expression and function of Ca^{2+} /cyclic AMP response element (CRE) DNA binding proteins (CREBS) in the rat hippocampus, suggesting a common adaptive mechanism regulated by 5-HT and NA (Duman et al., 1994; Nibuya et al., 1996).

Chronic administration of ADs are also thought to affect the expression of cortical GABA_B receptors, although the experimental results are equivocal showing both an increase and no change in density (reviewed Eschalier, 1999). Activation of GABA_B receptors inhibits voltage-sensitive Ca²⁺ currents in DRG neurons, inhibits K⁺-evoked release of 5-HT in cortical slices, and been shown to presynaptically inhibit neurotransmitter release (Desarmenien et al., 1982). As the GABA_B receptor is linked to activation of G_o proteins and a decrease in adenylate cyclase and PKC activity, the involvement of the GABA_B receptor may also be related to common second messenger modulation.

Another interesting action of chronic AD administration is the interaction with central glucocorticoid receptors (GR). Typical antidepressants increase the density of GRs (Pepin et al., 1989). Since GRs are known to function as DNA binding proteins that can modify gene transcription (Burnstein and Cidlowski, 1989), and GR have been localized to central catecholamine and 5-HT cell bodies (Harstrand et al., 1986), it is appealing to infer a relationship between GR receptor and subsequent neurotransmitter systems. However this postulate remains to be proven. The effect of chronic ADs on the glucocorticoid system may also involve a desensitization of corticotrophin releasing factor (CRF) receptors, and thereby decrease the assumed GR desensitization that occurs following the hypercortisolemia of depression (reviewed Leonard, 1996). Thus, chronic AD administration may effect gene transcription through regulation of GR, and this action may have long term consequences that contribute to the analgesic and antidepressant effect.

Yet another potentially beneficial effect of chronic ADs is the reduction in brain cyclo-oxygenase activity (Leonard, 1986; 1996). Reduction in cyclo-oxygenase would subsequently result in a decrease in the production of prostaglandins (most importantly prostaglandin E₂ [PGE₂]) that has been demonstrated following chronic ADs (Song and Leonard, 1995). Further, reduction in the activity of central macrophages by chronic ADs would act to reduce the release of interleukins and prostaglandins (Leonard, 1996), and thereby reduce their influence on neurotransmission.

A final action of chronic ADs that may contribute to the analgesic efficacy is highlighted by the *in vivo* inhibition of neurite outgrowth by amitriptyline (Wong et al., 1991). This study and others reviewed in Leonard (1993) suggests that chronic ADs may work, in part, by altering the cellular framework of the cell and ultimately reducing or eliminating in appropriate synaptic interactions. A reduction in the rate of cAMP synthesis has been implicated in this action, but further studies are required to ascertain the exact nature of this putative interaction.

As is evident from the two preceding sections the actions of ADs are quite diverse both in terms of their actions and in terms of the differences between acute and chronic paradigms. One goal of the basic science of the pharmacotherapy of neuropathic pain is to gain a better understanding of the mechanisms through which 'dirty' drugs like amitriptyline exert their effect. This may be accomplished pharmacologically using selective agonists and antagonists. Information may also be obtained by analyzing the changes induced by chronic AD administration on neurochemical markers.

1.3 Indicators of underlying pathophysiology following nerve-injury

That ADs provide relief in nociceptive and neuropathic pain paradigms is widely accepted. The question remains however as to whether the relief is primarily symptomatic, or whether ADs modify the underlying pathophysiology of the condition. To determine this first requires an appreciation of the changes following nerve injury. The mechanisms underlying the development and maintenance of neuropathic pain are thought to include anatomical changes such as altered chemical phenotype of primary afferents, and reorganization within the dorsal horn (section 1.1). Multi-level physiological changes also occur that include central sensitization, disinhibition, ectopic discharge of DRG cells, peripheral nociceptor sensitization, and sympathetic nervous system involvement (see Section 1.1). Changes in neuroactive substances have been studied in an effort to form a causal link with behavioral manifestations of neuropathic pain. For example, depending on the model, alterations in the expression of SP (Bennett et al., 1989; Munglani et al., 1995; Abbadie et al., 1996; Cameron et al., 1997), CGRP (Bennett et al., 1989; Cameron et al., 1991, 1997), GABA and GAP-43 (Woolf et al., 1990; Cameron et al., 1991; Somerville et al., 1991) have been observed. A detailed analysis of all the changes in these markers is beyond the scope of this study and therefore attention will be paid to certain markers because of their potential significance. Specifically, attention will be directed at the small 27kDA heat shock protein (Hsp27) because of its novelty in terms of response to nerve injury, neuropeptide Y (NPY) due to its putative role as a marker of reorganization, and SP because of its role in nociceptive processing by primary afferents.

1.3.1 Hsp27 and neuropathic pain

Recently, induction of the 27-kDa heat shock protein (Hsp27) was reported following nerve transection of the vagus (Hopkins et al., 1998) and sciatic nerves (Costigan et al., 1998). However, the significance of this increased Hsp27 expression following peripheral nerve transection still remains to be determined. Hsp27 has been shown to be constitutively expressed in sub-populations of sensory and motor neurons (Plumier et al., 1997c), and to be induced in astrocytes following kainic acid-induced status epilepticus (Plumier et al., 1996) and ischemic injury (Kato et al., 1994; Plumier et al., 1997a, 1997b). While the functional roles of Hsp27 are not completely understood, in non-neuronal cells Hsp27 has been shown to afford resistance to thermal (Landry et al., 1989) and oxidative (Mehlen et al., 1995) stress, to toxic agents (Huot et al., 1991) and to apoptosis induced by tumor necrosis factor- α (Mehlen et al., 1995, 1996). Other features of Hsp27 include chaperone functions (Jakob et al., 1993; Ehrnsperger et al., 1997) and actin-binding properties (Miron et al., 1991). Indeed, Hsp27 and its phosphorylation state may be involved in the regulation of actin filament dynamics (reviewed Landry and Huot, 1995) and are proposed to participate in trophic-induced cell migration (Rousseau et al., 1997; Piactrowicz et al., 1998).

1.3.2 Neuropeptide Y and neuropathic pain

Up-regulation of NPY in DRG, spinal dorsal horn and the gracile nucleus has been reported following loose ligation (Wakisaka et al., 1992; Munglani et al., 1995, 1996), crush (Wakisaka et al., 1992) or transection (Wakisaka et al., 1991

1992, Nahin et al., 1994; Zhang et al., 1995) of the sciatic nerve. While NPY is thought to have primarily anti-nociceptive effects in the spinal dorsal horn (Hua et al., 1991), the functional significance of the *de novo* synthesis of NPY in primary afferent neurons, and concomitant changes in the distribution of NPY-IR in the spinal dorsal horn and gracile nucleus (Zhang et al., 1995), has not been fully determined. Both up-regulation of NPY in myelinated cutaneous A β fibers located in laminae III-IV and sprouting of these fibers into lamina II has been reported following peripheral nerve injury (Koerber et al., 1994; Woolf et al., 1992; Lekan et al., 1996). These findings suggest that NPY plays a significant role in the central reorganization of pain pathways and concomitant behavioral responses.

Although the exact action of NPY in the spinal cord and DRG is not fully known, there is mounting evidence that it may play a role as an endogenous analgesic mediator in acute nociceptive paradigms (Hua et al., 1991; Xu et al., 1994). In more complex types of pain syndromes however NPY has been shown to be hyperalgesic when administered both locally (Tracey et al., 1995), and spinally (White, 1997; Xu et al., 1994; Xu et al., 1998).

Low level constitutive expression of NPY-immunoreactivity (NPY-IR) in the superficial spinal dorsal horn is thought to largely result from local interneurons, some bulbospinal projections (Gibson, 1984) and a minimal contribution from primary afferent terminals (Zhang et al., 1993c). In local interneurons of the spinal dorsal horn, NPY has been colocalized with GABA (Rowan et al., 1993). Following nerve injury however, there is a dramatic increase in the level of NPY-IR expression in the spinal dorsal horn and in the DRG (see Hökfelt et al., 1997).

A strong correlation exists between NPY mRNA and Y1 and Y2 receptor expression. Thus, localization studies of NPY and the Y1 and Y2 receptors suggest a beneficial alignment for influencing neuronal activity within the spinal cord.

It was originally thought that all NPY receptor subtypes were coupled to inhibitory G proteins (G_i) proteins that inhibit adenylate cyclase and result in a decrease the second messenger cyclic AMP (reviewed Grundemar and Hakanson, 1994; Blomquist and Herzog, 1997). However, NPY receptor activation has also been linked to increases in intracellular Ca^{2+} through the IP_3 pathway. Thus, the net outcome of NPY may be a combination of effects at the various receptor subtypes and the resulting effects on the second messenger/effector systems. Comparison of the profile of Y1 and Y2 receptor mRNA in naive and nerve injured animals also suggests an endogenous regulatory role. More specifically in normal animals Y1 mRNA is expressed in soma of local dorsal horn interneurons and in small diameter DRG cells (Mantyh et al., 1994; Zhang et al., 1994). However following axotomy, Y1 mRNA expression is decreased in small diameter and increased in medium and large diameter DRG cells (Hökfelt et al., 1997). This finding has been disputed in studies that found no change in Y1 receptor distribution following nerve injury (Mantyh et al., 1994; Marchand et al. 1999). The different findings may be related to the type of nerve injury as differences have also been found in the level of galanin whether the injury was a transection or a constriction (Ramer et al., 1998). A consistent finding with respect to the Y1 receptor is the somatic localization both in DRG cells (Zhang et al., 1994a; Zhang et al., 1994b) and

local interneurons (Zhang et al., 1994a). In contrast, the Y2 receptor has been reported to be transported centrally and peripherally ultimately becoming integrated in the axonal membrane as presynaptic receptors (Zhang et al., 1994b). Also, in contrast to that observed with the Y1 receptor, Y2 receptors are typically found in approximately 15% of all DRG cells, but following axotomy the percentage is increased to greater than 50% of the total cell population (Zhang et al., 1997). Furthermore, there is a reported shift in the proportion of Y2 receptors towards increased expression in small diameter afferents (Zhang et al., 1997). The above studies suggest a distribution of NPY-IR terminals and receptors in the spinal cord that may contribute to neuronal processing in the spinal cord.

This hypothesis is further supported by functional studies on the actions of NPY. For example, NPY inhibits depolarization-induced release of SP both *in vitro* (Walker et al., 1988) and *in vivo* (Duggan et al., 1991). This action may be related to the inhibition of Ca²⁺ influx observed in cultured DRG neurons (Walker et al., 1988) through activation of Y2 receptor (Bleakman et al., 1991; Wiley et al., 1993). The actual actions of NPY may be more complex as intrathecal NPY inhibits nociceptive reflexes in the anaesthetized rat (Hua et al., 1991), but exhibits biphasic actions on spinal reflexes following axotomy (Xu et al., 1994). NPY has also been shown to exacerbate neuropathy-induced hyperalgesia following local administration (Tracey et al., 1995). The inhibitory effect of NPY is independent of spinal α_2 -adrenergic or opioid mechanisms (Xu et al., 1994). Thus, functional studies indicate that NPY has a diverse effect depending on which receptors are activated and in which area (i.e. spinal dorsal horn, DRG or gracile nucleus). Indeed, NPY has been shown to inhibit C-fiber evoked

neurotransmitter release in general (Grundemar et al., 1990, 1993) and to specifically inhibit glutamate release (Colmers et al., 1991). Since SP and glutamate are colocalized and coreleased from primary afferents in the spinal cord (Battaglia and Rustioni, 1988; De Biasi and Rustioni, 1988), this suggests a potential dampening of the events leading to windup and central sensitization.

In addition to acting as a neuromodulator, studies have also shown that NPY induces neurite elongation (White and Mansfield, 1996) and this may contribute to the reorganization of the large diameter afferents in laminae III and IV following peripheral nerve injury.

1.3.4 Substance P in neuropathic pain

One particularly important neurochemical implicated in the processing of nociceptive stimuli is SP. SP is an undecapeptide member of the tachykinin family that also includes NKA and neurokinin B (NKB). Each of these peptides preferentially binds to one of the neurokinin subreceptor types, NK1, NK2, and NK3, although each of the receptor subtypes binds all the neurokinins with varying affinity (Routh and Helke, 1995). Specifically, SP preferentially binds to NK1 while NKA and NKB preferentially bind to NK2 and NK3 receptors, respectively (Kiyama et al., 1993). SP has been implicated in various functions including vasodilation and vasoconstriction (Saria, 1984; Helme et al., 1986). Perhaps however, the most widely known function for SP is as a neuromodulator in nociceptive processing.

The pattern of positive immunohistochemical labeling for SP (SP-IR) in the spinal dorsal horn is distinct and generally restricted to the superficial layers

(layer I and II_o), with some patchy labeling in layer V (Hökfelt et al., 1975).

These areas of SP-IR are also regions where nociceptive neurons have been localized (Hökfelt et al., 1975; Besson and Chauch, 1987). The SP-IR labeling in these areas is thought to be derived from three principal sources: termination of primary afferents, intrinsic interneurons, and descending raphe projections (Todd and Spike, 1993). Dorsal rhizotomy experiments, which eliminate the primary afferent contribution of SP to the spinal dorsal horn, have confirmed the large contribution of primary afferents to the pattern of SP-IR (Matthews et al., 1992). Although descending raphe projections are thought to contribute to the profile, the actual contribution from these projections is minimal since studies that utilize catecholaminergic neurotoxins to selectively destroy the descending projections did not significantly reduce the SP-IR labeling (Arvidsson et al., 1990). The component of SP derived from intrinsic interneurons is more difficult to ascertain, but can be inferred by considering the existence of immunopositive terminals after dorsal rhizotomy. Since dorsal rhizotomy would eliminate the contribution of SP-IR labeling due to primary afferents, and the descending contributions are minimal to none, then the remaining labeling can be inferred to be in intrinsic interneurons (reviewed Levine et al., 1993; Todd and Spike, 1993).

The presence of SP-immunoreactive (SP-IR) terminals in the region of spinal dorsal horn nociceptive neurons is highly indicative of a functional connection. However, there are many examples of a mismatch between the regional distribution of immunopositive terminals and receptors for the respective neurochemicals (Herkenham, 1987). The significance of the mismatch between receptors and immunopositive terminals is perplexing and may reflect a

hormonal-like mechanism involving diffusion of the peptide (Levine et al., 1993). However, SP has a short diffusion rate and is quickly degraded into terminal fragments by the enzyme endopeptidase (Levine et al., 1993). Therefore, the long distance effect of SP may require other neurochemical substances to inhibit the breakdown of SP and facilitate its diffusion (LeGreves et al., 1985).

SP-receptors (SP-R) have been localized post-synaptically to nociceptive primary afferent terminals in the spinal dorsal horn (Brown et al., 1995; Budai and Larson, 1996). Moreover, the SP-receptors were found to be in close proximity to nociceptive neurons, which is suggestive of SP involvement in nociception (Brown et al., 1995). Additionally, the presence of SP-IR terminals in the spinal dorsal horn further implicates the involvement of SP in nociceptive processing because of the importance of the marginal layer and substantia gelatinosa in nociceptive processing. In determining the role of SP in nociception, Duggan et al. (1988, 1990) found that noxious mechanical and electrical peripheral stimulation caused an increase in SP release in the spinal dorsal horn. Other studies analyzing spinal cord perfusate have confirmed a release of SP in the spinal dorsal horn in response to a noxious peripheral stimulus (Yonehara et al., 1986; Duggan et al., 1988). However, the exact source of the SP cannot be determined from such studies as the increase in SP could also reflect an increase in the release of SP from the intrinsic interneurons and/or descending raphe projections, in addition to that derived from primary afferent release.

Evidence for the involvement of SP in nociceptive processing arises not only from immunohistochemical localization of the terminals and receptors within a nociceptive processing region, but also from studies analyzing the actions of

SP, and exogenously applied agonists and antagonists to SP. The actions of SP vary depending on whether their action is peripheral, spinal, or central. Centrally, SP is implicated in neurotransmission, where it is involved in regulation of ventral motor neurons and preganglionic sympathetic neurons, as well as the transmission of nociceptive messages (Routh and Helke, 1995). Iontophoretic injection of SP into the dorsal horn excites nociceptive neurons (Levine et al., 1993), while having a minimal effect on non-nociceptive neurons (DeKonnick and Hendry, 1991). Similarly, intrathecal administration of SP elicits nociceptive behaviors such as biting and scratching that are morphine sensitive (Papir-Kricheli et al., 1987; Rusin et al., 1992), an indication of its putative involvement in nociception. The importance of SP as a nociceptive neuromessenger is further implied by anatomical and physiological studies that reveal a direct positive proportionality between the responsiveness of spinal dorsal horn nociceptive neurons and the number of SP synaptic contacts on these neurons (Ribiero-da-Silva, 1995). That is to say, that the greater the number of SP synaptic connections on the second order nociceptive neuron the greater the response.

The exact molecular mechanisms of SP in nociceptive processing are still under investigation and the details are beyond the scope of this thesis. In general however, SP is thought to act by potentiating and enhancing the action of glutamate on NMDA receptors, through a delayed but prolonged depolarization of cell membranes (Randik et al., 1990; Smullin et al., 1990; Rusin et al., 1992; Budai and Larson, 1996). In fact 35-65% of SP-IR fibers also contain glutamate

(Battaglia and Rustioni, 1988), which suggests co-release of the two neurotransmitters in response to various stimuli.

In models of nerve injury-induced neuropathic pain, the levels of SP-IR in the ipsilateral spinal dorsal horn are reduced to varying degrees depending on the model (reviewed Hökfelt et al., 1997). A role for SP in the generation of the neuropathic pain behaviors has also been confirmed in a study that used neonatal capsaicin to destroy C-fibers (Meller et al., 1992). In that study capsaicin pretreatment prevented the development of thermal hyperalgesia. Interestingly, it has also been recently shown that following sciatic nerve transection, small SP-positive sensory neurons form rings around large diameter DRG neurons in much the same manner as observed with noradrenergic fibers (McLachlan and Hu, 1998). This abnormal structural association may contribute to aberrant sensory processing following nerve injury (McLachlin and Hu, 1998). These highlighted studies suggest a role for SP in the development and maintenance of neuropathic pain although the level of contribution may vary with the injury.

1.4 Animal models of neuropathic pain

Several animal models have been developed to study the mechanisms involved in, and drug treatment of neuropathic pain (reviewed Bennett, 1994a; Kim et al., 1997). Three of these models involve varying degrees, and locations, of traumatic nerve injury that lead to subtle yet important differences in the presentation of neuropathic pains. These include chronic constriction injury (CCI; Bennett and Xie, 1988), partial nerve ligation (PNL; Seltzer et al., 1990), and

spinal nerve ligation (SNL; Kim and Chung, 1992). In this study, the SNL model of neuropathic pain was chosen because of the reproducibility of the injury to examine the effect of amitriptyline on different pain symptoms putatively resulting from different pathophysiological mechanisms. This model involves a restricted segmental unilateral spinal nerve ligation, resulting in a sympathetically dependent neuropathic pain state manifested as behavioral signs of cold and mechanical allodynia, and thermal hyperalgesia (Kim and Chung, 1991,1992; Kim et al., 1993).

1.5 Rationale, hypothesis and objectives

As discussed above, neuropathic pain is a multifactorial clinical problem that is not currently amenable to treatment. AD drugs are a widely used treatment strategy in the medical community but they only provide partial relief (Onghena and Van Houdenhove, 1992; McQuay et al., 1996). Interestingly, the multiple mechanisms of action of TCAs that contribute to their dose-limiting side effects, may also be the reason for their therapeutic efficacy in neuropathic pain. One potentially significant action is the manipulation of endogenous adenosine levels, especially in light of clinical studies that demonstrate a profound effect of adenosine in the treatment of neuropathic pain. **Therefore, the underlying hypothesis of this dissertation is that acute (and possibly chronic) antidepressants work in part by manipulating endogenous adenosine levels, and this action can have a long lasting effect on neuropathic pain.**

To study this required the development of a series of objectives to ascertain the effectiveness of antidepressants in an animal model of neuropathic

pain, the potential interaction with endogenous adenosine, and finally the effect of chronic antidepressants (amitriptyline) on the behavioral manifestations and underlying pathophysiology of neuropathic pain. Specifically the objectives of this thesis were as follows:

- 1) To determine the validity, in my hands, of unilateral L5/L6 segmental SNL as a model of nerve-injury induced neuropathic pain. This objective was designed around the time course of the expression of two behavioral manifestations, thermal hyperalgesia and static tactile allodynia.
- 2) To assess the efficacy of acute administration of several antidepressants (amitriptyline, desipramine and fluoxetine) on the behavioral symptoms of thermal hyperalgesia and static tactile allodynia.
- 3) To examine which of the therapeutically beneficial effects ascertained in objective (2) were affected by the concomitant administration of caffeine, a nonselective adenosine receptor antagonist.
- 4) Based on the results of the first three objectives, to determine the effect of chronic amitriptyline administered in the drinking water on thermal hyperalgesia and static tactile allodynia, and to determine the effects of chronic caffeine on the actions of chronic amitriptyline.

- 5) **To determine the immunohistochemical time course of Hsp27, NPY and SP expression in the lumbosacral spinal cord and the gracile nucleus, following SNL.**

- 6) **To determine whether the effects of chronic amitriptyline alone, and in combination with chronic caffeine, modifies the immunohistochemical expression of Hsp27, NPY and SP in the spinal dorsal horn and gracile nucleus.**

METHODS

2.1 Animals

Experiments were conducted on male Sprague-Dawley rats (100-120 g) from Charles River, Quebec, Canada and approved by the Dalhousie University Committee on Laboratory Animals. Animals were housed in pairs, maintained on a 12/12 hour light/dark cycle at $22^{\circ}\pm 1^{\circ}\text{C}$ and given ad-libitum access to food and water.

2.2 Surgical Procedures

All surgery was performed using an aseptic technique, under a dissection microscope, and with adherence to the guidelines of the IASP on animal experimentation in pain research (Zimmermann et al., 1983). Efforts were made to design experiments so as to limit the number of animals used.

2.2.1 Spinal Nerve Ligation.

Animals were rendered neuropathic through unilateral tight ligation of the 5th and 6th lumbar spinal nerves (L5 and L6), in accordance with the technique of Kim and Chung (1992)(see Appendix A). Animals were anaesthetized with halothane via an induction box and subsequently maintained at the appropriate anesthetic plane using 1.5-2.0% halothane. Rats were surgically clipped, administered Ringers lactate solution (5 ml; subcutaneous; s.c.), atropine (0.6 ml/kg; intramuscular), penicillin (Penlong; 1.0 ml/kg; s.c.) and topical eye ointment (Lacri-Lube; Allergen, Inc.). The surgical area was aseptically scrubbed

with alcohol and iodine, and a 3cm dorsal incision made using the ischium as the midline. Using blunt dissection and partial removal of the articular facet and the L4 transverse process, the L5 and L6 spinal nerves were exposed. The nerves were then tightly ligated with sterilized 6-0 silk. After ensuring hemostasis, the wound was closed in layers with subcutaneous and cutaneous suturing (3.0 Novophil). Animals were then placed in a heated area for surgical recovery and monitoring. For a more detailed description of the surgical procedure see Appendix A. Following a 48 hour recovery period, animals were again housed in pairs, and environmental enrichment added to the cages. Sham animals received the exact procedure as described above except for the ligation of the nerve.

2.2.2 Intrathecal cannulation

For animals used to study the effects of spinal administration of amitriptyline, intrathecal cannulas were implanted 7 days following spinal nerve ligation surgery in accordance with the technique previously reported in Sawynok and Reid (1990). Under halothane anesthesia, the atlanto-occipital membrane was exposed by dermal incision and spreading of the nuchal muscles. The membrane was then cut, and a 7.5cm length of saline filled PE-10 tubing was gently introduced into the subarachnoid space. The cannula was then held in place by a knot tied loosely to the nuchal muscles. The rostral end of the cannula was externalized through a small incision in the skin overlying the skull and capped with a stainless steel wire plug. Any animals showing signs of paralysis or motor impairment were euthanized.

2.3 Behavioral Assessment

All behavioral tests were conducted between 08:00 and 14:00 daily. In the acute drug paradigms, testing was performed at 7, 12, 17, and 22 days following nerve injury. An exception to this was the cannulated animals, which were tested at 7 and 12 days after cannulation (14 and 19 days after nerve injury, respectively). Following a recovery period of 7 days, animals were moved from the vivarium and acclimatized to the testing room for 40 minutes. After this initial period the animals were placed in the respective testing apparatus for 30-40 minutes or until exploratory behavior ceased.

2.3.1 Thermal Hyperalgesia

To test for thermal hyperalgesia, a paw thermal stimulator (UARDG, Dept. of Anesthesiology) was used to direct a focused beam of light at the paw, as described initially by Hargreaves et al. (1988). Rats were placed in pairs in a clear plexiglass box (22cm X 19 cm X 25 cm) on top of the temperature maintained glass surface ($30 \pm 0.1^\circ\text{C}$) of the stimulator. At each testing period a group consisted of 6 animals (2 per box with three boxes total). After the initial acclimatization period, rats were tested for baseline withdrawal latencies (seconds) of both paws once every 20 minutes until five consistent responses were achieved. In acute drug experiments, the animals were then returned to their cages for 30-40 minutes prior to drug administration and given ad-libitum access to food and water. Following acute drug administration, the animals were returned to the testing boxes and tested at each time period as defined by the observation protocol.

2.3.2 Static Tactile Allodynia.

Following weighing and acclimatization to the testing room, rats were placed in pairs in a clear plexiglass box (30 cm X 22 cm X 26 cm) on an elevated wire mesh platform to allow for access to the ventral surface of the hind paws. Rats were further acclimated to the testing chambers and the experimental baseline determined (once every 30 minutes) using Semmes-Weinstein monofilaments (Stoelting Comp., Wood Dale, IL). The 50% withdrawal threshold was determined using the Dixons up-down method (Chaplan et al., 1994). Briefly, filaments were applied to the ventral surface of the paw, starting with the 4.31 filament (2.04 g) and the response noted. Following a positive response (paw withdrawal, with characteristic pain behavior), a lower filament was then applied. Conversely, no response would necessitate subsequent application of higher filament. This pattern of application was repeated until a series of 6 responses was obtained. The 50% withdrawal threshold was then determined from the tabular value for the pattern of 6 responses (k), the final monofilament value (X_f ; in log units), and interpolated using the formula:

$$50\% \text{ g threshold} = (10^{X_f + \delta}) / 10,000$$

where δ is the mean difference between stimuli. Following determination of baseline values, the rats were returned to their original cages and left undisturbed with ad-libitum access to food and water for 30-40 minutes. After acute drug administration, the animals were again placed in the testing chambers and monitored for the appropriate behavioral periods depending on the route of drug administration. In these experiments a modification was made to the testing procedure of Chaplan et al. (1994), in that the filament was only applied to the

ventral surface of the paw for 3 seconds instead of 6. This modification was made as it was determined that 3 seconds was a sufficient time period to elicit a response from a neuropathic paw.

2.4 Acute drug treatment paradigms

For all experiments, the tester was unaware of the particular treatment group of each rat.

2.4.1 Differential route and test effects of amitriptyline, desipramine and fluoxetine

In both of the behavioral tests, amitriptyline, desipramine, fluoxetine (Sigma Chemical Co., St. Louis, MO) or saline was administered systemically by intraperitoneal (i.p.) injection, spinally through a chronically implanted intrathecal (i.t.) cannulas, or locally (s.c.) in the dorsal surface of the paw. Drugs were injected in a volume of 5 ml/kg for systemic injections, and a total volume of 20 μ l (10 μ l drug + 10 μ l saline flush) for i.t. injections. For local injections, the animals were briefly anaesthetized with halothane, and the solution administered s.c. in the dorsal surface of the ipsilateral or contralateral paw in a volume of 50 μ l using a 30 gauge needle attached to a Hamilton syringe.

2.4.2 Caffeine antagonism effects

Amitriptyline, desipramine, caffeine (Sigma Chemical Co., St. Louis, MO) or saline were administered alone or in combination, systemically (i.p.), spinally (i.t.), or peripherally (s.c.) in the dorsal aspect of the paw. Drugs were dissolved

in saline and injected in a volume of 5 ml/kg for systemic injections, and a total volume of 20 μ l (10 μ l drug + 10 μ l saline flush) for intrathecal injections. For local peripheral injections, the animals were briefly anaesthetized with halothane, and the solution administered s.c. in the dorsal aspect of the ipsilateral or contralateral paw in a volume of 50 μ l. Caffeine was co-administered with amitriptyline and desipramine in the systemic and local paradigms, but was given 10 minutes prior to amitriptyline only in the spinal paradigm.

2.5 Immunohistochemical Time Course

For the immunohistochemical time course animals were divided into experimental (n=4), and sham (n=3) at all time points, with inclusion of a naïve (n=3) group at alternate time points. Following perfusion for immunohistochemical processing, the L5 and L6 spinal nerves were exposed to determine both the integrity, and the position of the ligation.

2.5.1 Immunohistochemistry

For a detailed description of immunohistochemical processing see Appendix B. Animals were killed with a bolus dose of sodium pentobarbital and perfused transcardially with chilled (4°C) 350 ml of 0.1% sodium nitrite in 0.05M phosphate buffered saline (PBS), followed by 350 ml of 4% paraformaldehyde (PFA) dissolved in 0.1M phosphate buffer (PB). Following perfusion, the brains and spinal cords were removed and post-fixed in 4% PFA for 24-36 hours at 4°C, then placed in 20% sucrose in PB for 12-18 hours. The brains and spinal cords were cut (40 μ m) into 4 series using a sliding sledge microtome, and placed in

PBS. Sections were then placed in 1% hydrogen peroxide for 30 min, followed by 3 washes (⊗ 10 min each) in 0.2% Triton-X in PBS, and subsequently placed in primary rabbit polyclonal antibody raised against mouse Hsp25 (1:6000; StressGen Biotechnologies Corp.) or rabbit polyclonal antibody raised against rat NPY (1:500; Incstar Corp.), SP (1:500; Incstar Corp) diluted in 2% goat serum in 0.1% Triton-X in PBS, for 36-48 hours at 4° C. Following three rinses in PBS, sections were incubated in biotinylated goat anti-rabbit antibody, raised against rabbit IgG, diluted in 2% goat serum in 0.1% Triton-X in PBS (1:500; Vector Laboratories Inc.) for 90 minutes. Sections were then rinsed three times in PBS and incubated in avidin-biotin-horseradish peroxidase complex (ABC-Elite) (1:500; Vector Laboratories Inc.) for 90 minutes. The sections were immersed in 0.05% diaminobenzidine-tetrachloride (DAB)(Sigma) in PB, and reacted with 0.001% hydrogen peroxide. Sections were free floated on gelatin subbed slides air dried for 24 hours, dehydrated in increasing alcohol concentration series, cleared with Xylene, and coverslipped with Entellen coverslip medium.

2.5.2 Image Analysis

Coverslipped sections were viewed with an Olympus BH-2 microscope, and a qualitative assessment made of the immunoreactive staining intensity. The laminar position of the staining was determined by comparison to the cytoarchitecture described in Paxinos and Watson (1986) and verified using dark-field microscopy. Representative sections were also captured using a JVC video camera attached to the Olympus microscope. Digitized images were compiled and labeled using a Macintosh computer and Adobe Photoshop

software (v4.0) and printed using a dye-sublimation printer (Kodak XLS 8600, Rochester, NY).

Quantitative analysis was also made of the changes in immunoreactive expression of Hsp27-IR and NPY-IR in the spinal dorsal horn in the L5/L6 region of the spinal cord, in spinal nerve ligated animals. Five sections per animal were captured using a JVC camera attached to a Olympus BH2-RFCA microscope. The images were then converted to gray scale and a densitometric analysis made using NIH software (NIH Image 1.44). Values were obtained for laminae I-IV of both the ipsilateral and contralateral spinal dorsal horn. A further sub analysis was made of laminae III-IV for NPY only. For each individual section, a normalized value was calculated that reflected the percent change of the ipsilateral side compared to the contralateral side ($\% \text{ difference} = [\text{ipsilateral} / \text{contralateral}] \times 100$). The values were then combined according to the time point, and a mean \pm standard error of the mean (SEM) calculated for the ipsilateral and contralateral paw, as well as the percent difference.

2.6 Chronic drug administration paradigm

To study the effects of chronic drug administration, nerve injured rats were divided into four treatment groups: control (no drug), caffeine alone, amitriptyline alone, and amitriptyline-caffeine combination. All drugs were dissolved in the same water normally provided to animals in the Dalhousie Carleton Animal Care Facility at a concentration of 0.11 mg/ml for amitriptyline, and 0.05 mg/ml for caffeine. At the beginning of the spinal nerve ligation surgery, animals received a preemptive drug administration (i.p.) based on their assigned treatment group of

caffeine (7.5 mg/kg), amitriptyline (10.0 mg/kg), and a combination of amitriptyline (10 mg/kg) and caffeine (7.5 mg/kg) respectively. Saline controls were given a systemic injection (i.p.) of saline in an equivalent volume to the drug treatment groups. The animals then received the spinal nerve ligation surgery as previously outlined (section 2.2.1). Following surgery, and an initial observation period, the animals were housed individually for 48 hours to allow for wound healing. The animals were then paired back with their original cagemate for the duration of the experiment, and environmental enrichment was provided in the cages of all animals. The experimenter was blinded to the treatment group assignment of the rats.

2.6.1 Measurement of weight, fluid consumption and drug concentration

Handling of animals was limited to the experimenters during the study. Every 48 hour period the animals were weighed, had their cages changed and the fluids levels measured and changed. Fresh drug solutions were made every 48 hours. For each individual rat the dose of drug over the 48 hour period was estimated by first dividing the amount of fluid consumed by 4 (2 rats per cage X 2 days of consumption). The approximate dose was then calculated by multiplying the concentration of the drug in the water by the individual volume of fluid, and dividing this value by the weight at the respective time point.

2.6.2 Behavioural Analysis

Once every 7 days the animals were measured for the degree of thermal hyperalgesia (section 2.3.1) and static tactile allodynia (section 2.3.2). The

thresholds of these two evoked responses were obtained over a 2 day period (1 X behavioral test per day) to minimize the stress of repetitive testing. Animals were initially acclimated to the testing room and subsequently the testing apparatus (30–40 minutes each). The animals were then tested over a 90 minute period to determine the level of expression of the defined behaviors. Following the testing period the animals were returned to clean cages, and taken back to the vivarium.

2.6.3 Immunohistochemical analysis

Twenty-four hours after the third behavioral test point (day 22), the animals were killed and perfused for immunohistochemical processing as previously outlined (section 2.5.1). Following the procedure as outlined, the animals were processed for detection of Hsp27, NPY, and SP.

2.6.4 Blood sample collection

To determine the plasma levels of the drugs in the experimental animals approximately 2 millilitres (ml) of blood was collected prior to initiation of perfusion via a cardiac puncture. Blood was then quickly transferred to sterilized, vacuum sealed, heparinized tubes, gently agitated, and stored at 4°C until collection was completed from all animals. The tubes were then centrifuged (3000 RPM for 10 minutes) and the plasma taken off in 300 µl volumes and stored in individual aliquots at –70°C. This portion of the analysis was not completed within the confines of this dissertation, but will be analyzed at a later date in combination with samples from a clinical study.

2.7 Data Analysis

2.7.1 Acute drug administration paradigm

For each of the acute drug administration experiments, raw data for response thresholds of both paws in each animal were recorded and entered into a spreadsheet (Microsoft Excel 5.0). The data was then normalized (to account for inter animal variability in response thresholds) for each animal as Maximum Possible Effect (MPE) in terms of reversal of the neuropathic symptom being tested [MPE (anti-hyperalgesia or anti-allodynia)] or in terms of the analgesic effect of the contralateral paw [MPE (analgesia)]. These values were calculated as follows: $MPE \text{ (anti-neuropathic)} = (PDR - IBR) / (CBR - IBR)$, where PDR is the post drug response of the ipsilateral paw, IBR is the ipsilateral paw baseline response, and CBR is the contralateral paw baseline response. Similarly, the $MPE \text{ (analgesia)} = (PDR - CBR) / (\text{cutoff} - CBR)$, where cutoff was 20 seconds from the thermal stimulus and 15 g for tactile allodynia, and PDR is the post drug withdrawal threshold of the contralateral paw. Accordingly, the individual values reported or depicted are the $MPE \pm$ the standard error of the mean (SEM). The time course of the drug effect is also depicted as the cumulative MPE (ΣMPE) for each treatment and route of administration. This value was calculated as the sum of the individual MPE values through the time course. Individual time point data were statistically analyzed using a one way ANOVA for repeated measures with a pairwise comparison (Bonferroni post hoc analysis), or Student's t-test (two tailed). To compare ΣMPE values of multiple treatment groups a one-way

ANOVA with a pairwise comparison (Student-Neuman-Keuls post hoc analysis) was used. When comparing the means at individual time points, or the Σ MPE values of only two groups, a two tailed Students t-test was used with a Student-Neuman-Keuls post hoc correction. For all tests, a P value less than 0.05 ($P < .05$) was considered significant unless otherwise indicated in the figure legends.

2.7.2 Comparison of immunoreactive changes in Hsp27 and NPY

The quantitative changes in immunoreactive expression of Hsp27 and NPY were compared using a Spearman Correlational Analysis. Analysis was made between Hsp27-IR and NPY-IR in laminae I-IV, as well as between Hsp27-IR in laminae I-IV and NPY-IR in laminae III/IV only.

2.7.3 Chronic drug administration paradigm

The raw values of withdrawal thresholds of the paws for thermal (seconds; sec) and tactile (grams; gm) stimuli were recorded and the difference calculated. The means \pm the standard error of the mean (SEM) for the respective measure was calculated after grouping all the animals by their treatment group. Comparisons between groups for the specific measure (ipsilateral paw threshold, contralateral paw threshold, or difference) were made using a Students t-test (Student-Neuman-Keuls post hoc correction) for thermal hyperalgesia and Mann Whitney Rank Sum for allodynia, and the level of significance designated by the appropriate symbol on the figures.

RESULTS

3.1 Establishment and maintenance of neuropathic pain behaviors

Peripheral mononeuropathy from ligation of L5 and L6 spinal nerves resulted in neuropathic pain in the rat with symptoms of static tactile allodynia and thermal hyperalgesia that were maintained throughout the testing periods (days 7, 12, 17, 22) (Fig.3). A positive response in the neuropathic paw commonly consisted of shaking, biting, licking and guarding following a quick withdrawal from the stimulus. In the non-injured (contralateral) paw, the response involved a quick withdrawal from the stimulus without any of the secondary behaviors. The thermal threshold for the ipsilateral paw was determined to be approximately 7.98 ± 0.48 sec compared to 10.35 ± 0.63 sec for the contralateral paw ($P < .01$, $n=10$). Allodynia was manifested as 50% withdrawal threshold of 2.20 ± 0.28 g for the ipsilateral paw and 13.37 ± 0.37 g for the contralateral paw ($P < .001$, $n=10$). Threshold values remained relatively stable with no significant change in baseline values for either the ipsilateral or contralateral paws at each of the subsequent testing days. We did however observe a trend towards a gradual increase in the response threshold of both the ipsilateral and contralateral paws as the time course progressed and the animals got older.

Figure 3.

Graphic representation of the difference between the ipsilateral and contralateral paws in terms of the static mechanical allodynia 50% withdrawal threshold (grams) and thermal hyperalgesia (seconds) on successive days following spinal nerve ligation. Values depict group means (n=10 per group).

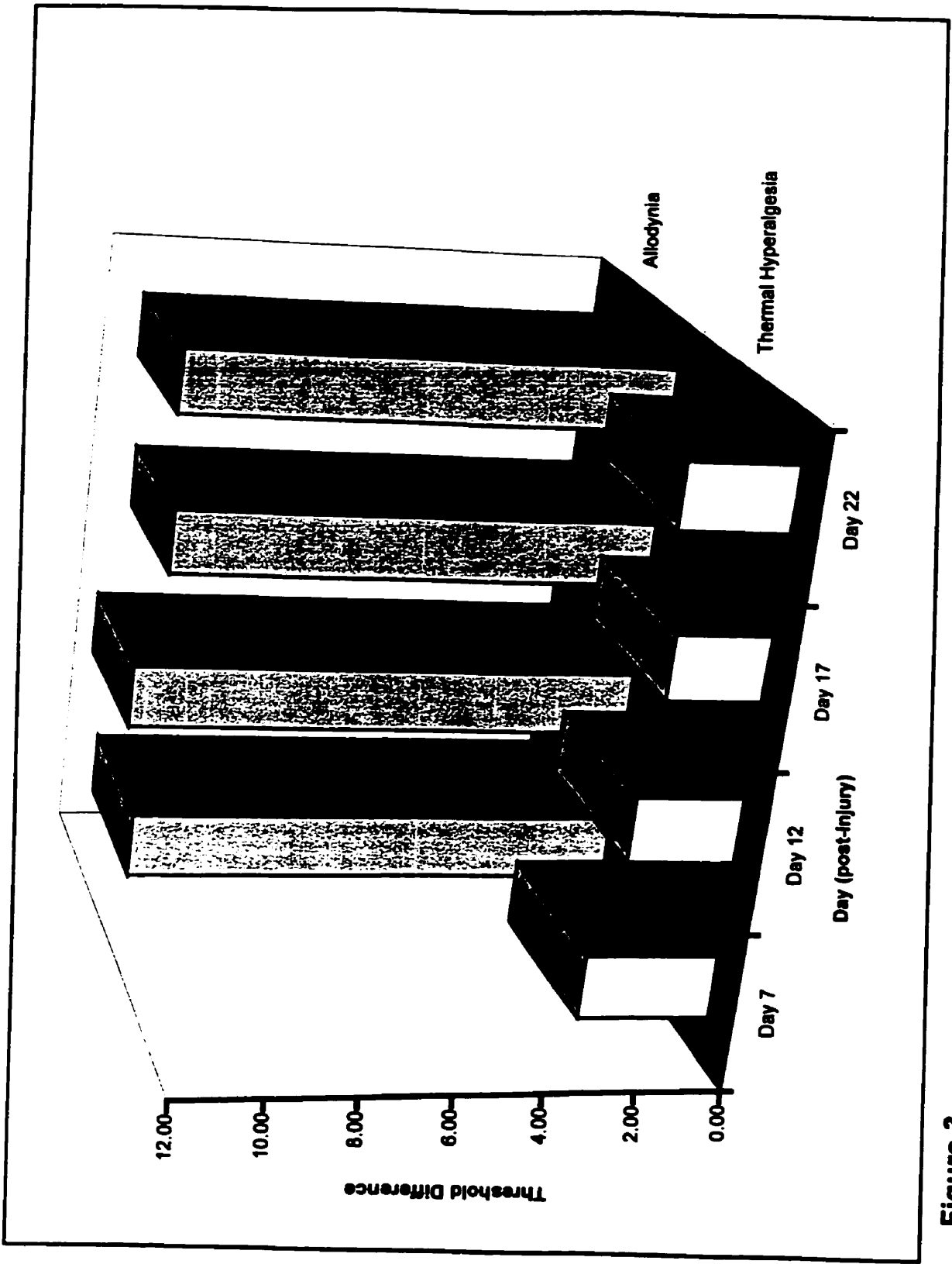


Figure 3

3.2 Effect of acute antidepressant administration on nerve injury-induced thermal hyperalgesia and static tactile allodynia

3.2.1 Effect of systemic amitriptyline, desipramine and fluoxetine on thermal hyperalgesia

Systemic administration of amitriptyline produced an anti-hyperalgesic effect on the thermal threshold of the nerve-injured paw (Fig. 4). This effect was greatest at 10 mg/kg, reaching an almost complete reversal of thermal hyperalgesia (MPE = 0.94±0.17) 60-80 minutes after injection (Fig. 4A). No statistically significant difference was detected between any dose in the contralateral paw (Fig. 4B). Comparison of the Σ MPE values for the ipsilateral paw (Fig. 4C) shows a statistically significant difference of 10 mg/kg compared to saline controls, as well as to 1 and 3 mg/kg responses. While both 1 mg/kg and 3 mg/kg were significantly different from the saline controls, they were not significantly different from each other. A slight sedative effect was observed in a few of the rats with the 10 mg/kg dose, but not at other doses. This effect did not hinder the ability of the animals to respond to the stimulus, as the nature of the response at threshold was similar to that observed during baseline determinations.

Systemic administration of desipramine (3 and 10 mg/kg) caused a significant thermal anti-hyperalgesic effect at individual time points (Fig. 5A), and in the cumulative effect (Fig. 5C). The magnitude of the effect was relatively consistent over the time period of observation with the maximal effect (40% reversal) occurring between 90 and 120 minutes (Fig. 5A). There was however

no significant difference in the cumulative values between the 3 and 10 mg/kg groups. A subsequent dose of 20 mg/kg was found to be highly sedative, which compromised accurate determination of the threshold and was therefore not included. Systemic fluoxetine (5 mg/kg) had no effect on the nerve injury-induced thermal hyperalgesia (data not shown).

3.2.2 Effect of spinal amitriptyline on thermal hyperalgesia

The intrathecal injection of amitriptyline (60 μ g) resulted in a partial reversal of the thermal hyperalgesia that was evident 20 minutes after injection, and persisted for the 60 minute testing period. As the 30 μ g dose was ineffective in the allodynia test, and 90 μ g was highly sedative, these doses were not used for testing the effect on thermal hyperalgesia. While values for the individual time points are not significantly different on their own (Fig. 6A), comparison of the Σ MPE values for saline and amitriptyline (60 μ g) were significantly different (Fig. 6C). No significant difference was observed in the contralateral paw withdrawal thresholds at either single time points or in comparison of the Σ MPE values (Fig. 6B and 6C).

Following intrathecal cannulation, the neuropathic animals showed signs of significant stress and general malaise that appeared to adversely effect their response ability. Because of this, and the fact that the effect of spinal amitriptyline was not robust, analysis of spinal desipramine or fluoxetine was not pursued.

Figure 4

Time course of the effect of systemic amitriptyline at various doses on thermal hyperalgesia in the ipsilateral paw (A), and thermal withdrawal threshold in the contralateral paw (B), and represented as the cumulative maximum possible effect (Σ MPE) over the time course (C). Values represent the MPE \pm SEM (n=5 for saline, 1 and 3 mg/kg, and n=15 for 10 mg/kg). *P<0.05 compared to saline controls, † P<0.05 compared to 1 and 3 mg/kg.

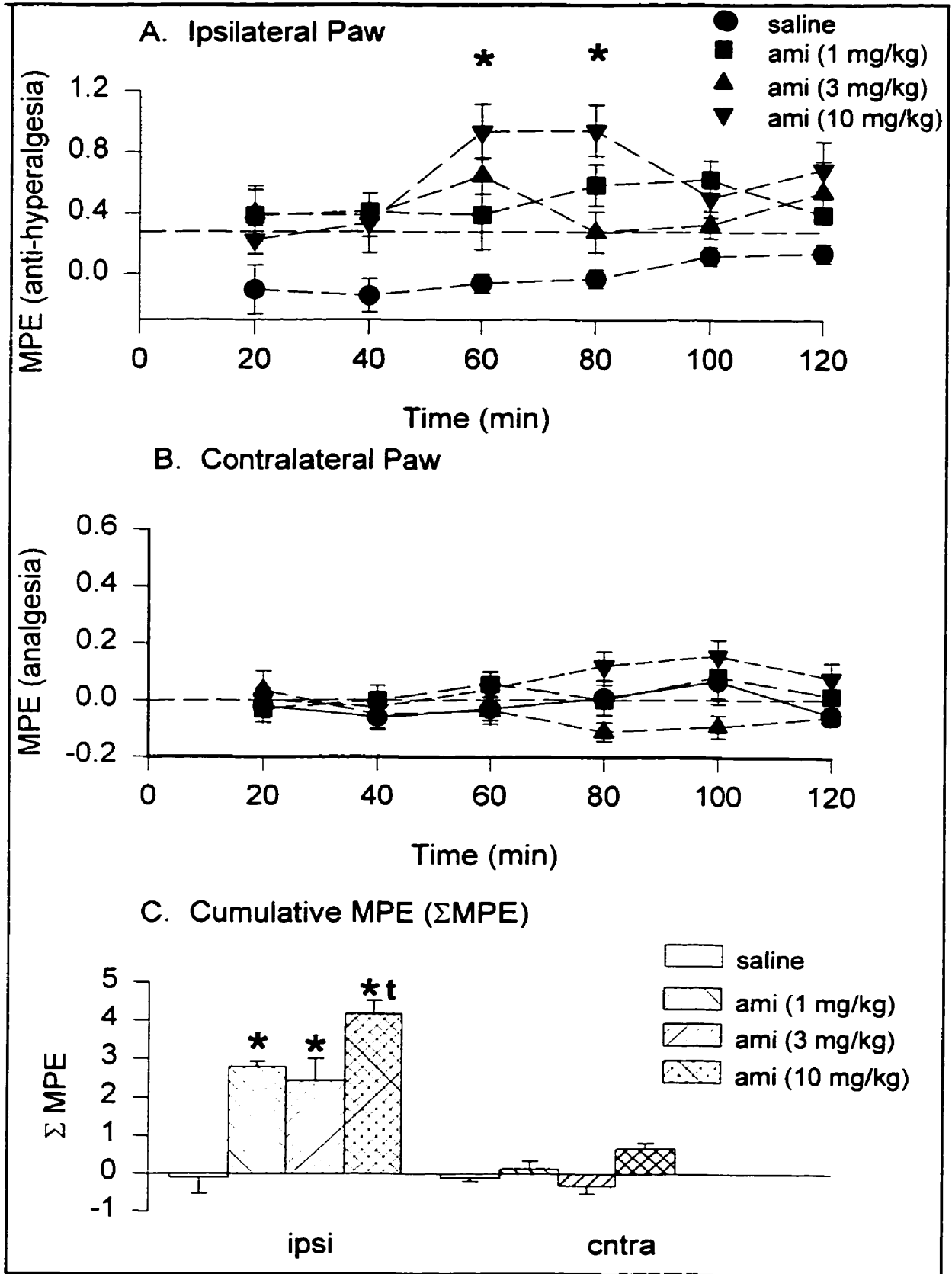


Figure 4

Figure 5

Time course of the effect of systemic desipramine at 3 and 10 mg/kg on thermal hyperalgesia in the ipsilateral paw (A), and thermal withdrawal threshold in the contralateral paw (B), and represented as the cumulative maximum possible effect (Σ MPE) over the time course (C). Values represent the MPE \pm SEM (n=5 for saline, 1, and 3 mg/kg, and n=15 for 10 mg/kg). *P<0.05 compared to saline controls.

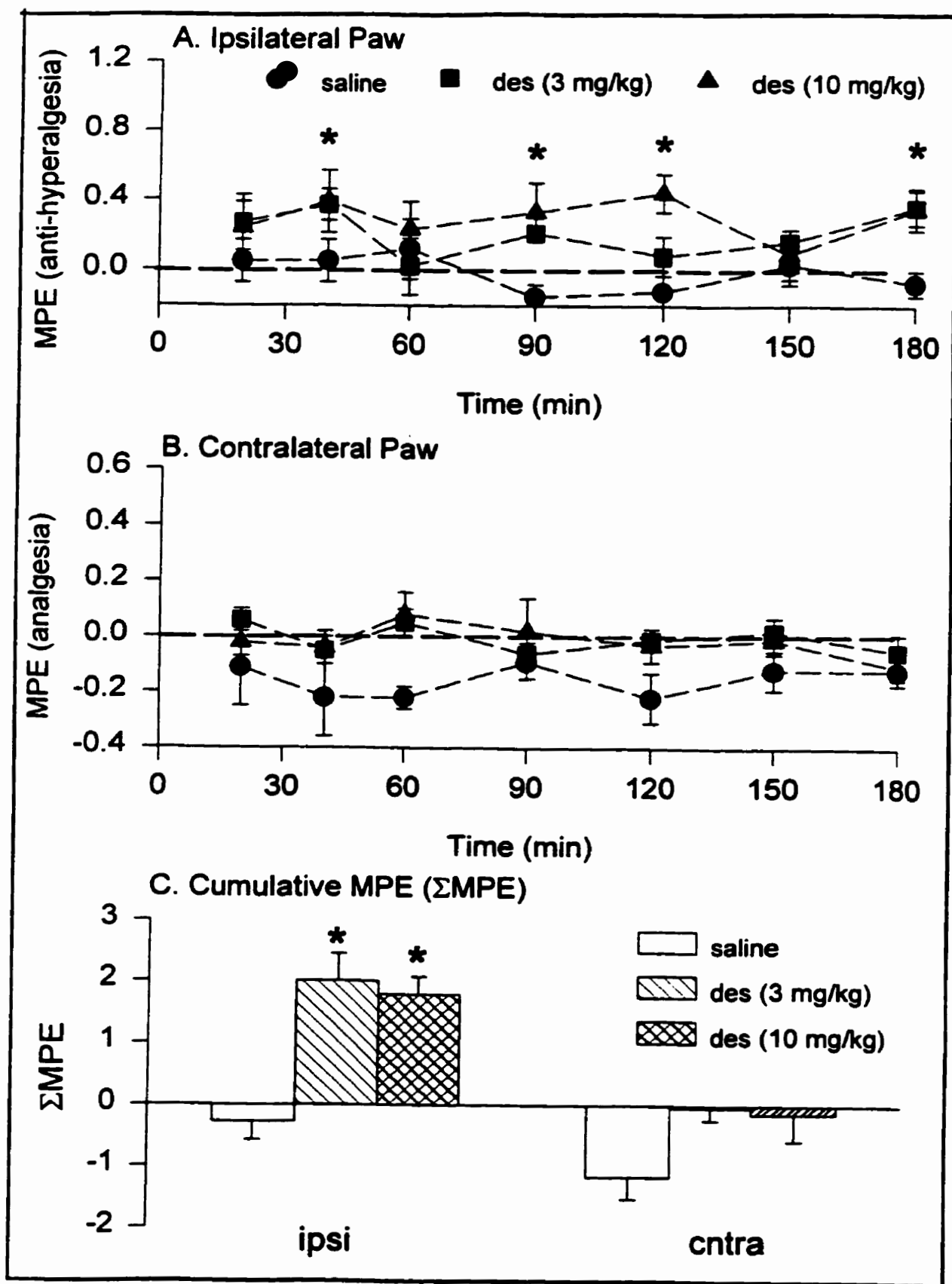


Figure 5

Figure 6

Time course of the effect of spinal amitriptyline (60 μ g) on thermal hyperalgesia in the ipsilateral paw (A), and the thermal withdrawal threshold in the contralateral paw (B), and represented as the cumulative maximum possible effect (Σ MPE) over the time course (C). Values represent the MPE \pm SEM (n=6 for both groups). *P<.05 compared to saline controls.

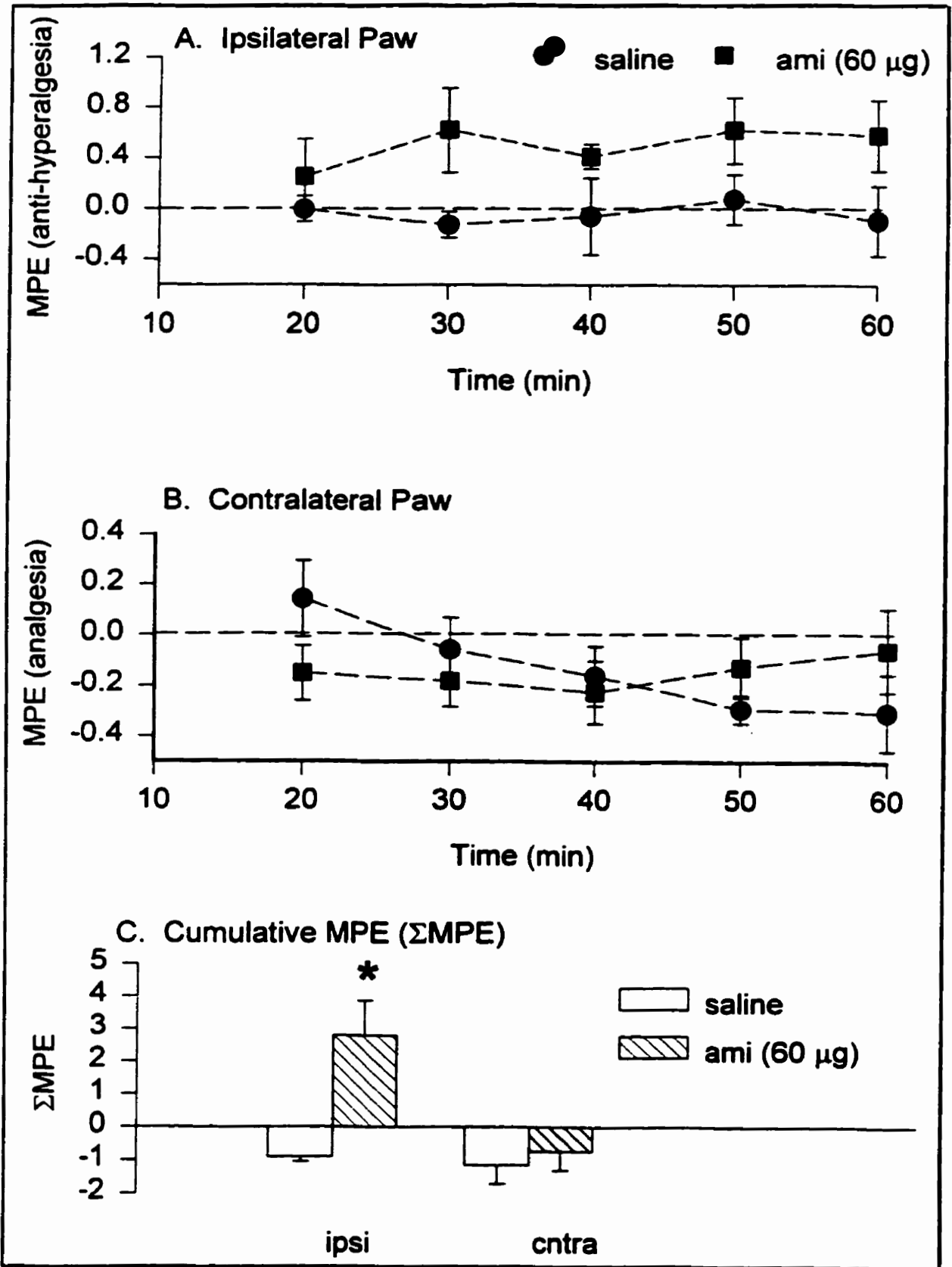


Figure 6

3.2.3 Effect of local peripheral injection of amitriptyline, desipramine and fluoxetine on thermal hyperalgesia

When injected locally into the neuropathic paw, amitriptyline at both 30 and 100 nmol, had an immediate statistically significant effect. At 100 nmol, amitriptyline almost completely reversed the thermal hyperalgesia (Fig. 7A), with the MPE values remaining significantly different from those of saline for the first 120 minutes. Local injection of amitriptyline (100 nmol) into the contralateral paw however, failed to show any significant effect on the withdrawal latency of the ipsilateral paw (Fig. 7A), arguing against a systemically mediated effect. Similarly, local injection of saline in the ipsilateral paw of nerve-ligated animals produced no effect. Furthermore, neither the ipsilateral nor contralateral local injections showed any significant effect on the contralateral paw withdrawal latency (Fig. 7B). Comparison of the Σ MPE values revealed a statistically significant difference of locally injected amitriptyline (30 and 100 nmol) on thermal hyperalgesia when administered in the injured paw (ipsi), as compared to values when saline was injected in the ipsilateral paw (saline), or when amitriptyline was injected in the contralateral paw (cntra)(Fig. 7C). There was also no alteration in the thermal withdrawal thresholds of naive animals after local injection of amitriptyline 100 nmol (n=4; data not shown).

Local administration of desipramine (100 nmol) caused an immediate and alleviation of nerve-injury induced thermal hyperalgesia that was significant as compared to controls (Fig. 8). This effect lasted for approximately 140 minutes as compared to the effect of saline. Increasing the dose of desipramine to 300

Figure 7

Time course of the effect of local injection of amitriptyline (30 and 100 nmol) on thermal hyperalgesia in the ipsilateral paw (A), when injected into the nerve-injured paw (ipsi) and non-injured contralateral (cntra) paw. Panel B represents the time course of the change in the thermal withdrawal latency in the contralateral paw, when amitriptyline is injected into the nerve-injured paw (ipsi) and non-injured paw (cntra). Panel C depicts the cumulative MPE value (Σ MPE). Values represent the MPE \pm SEM (n=9 for all groups). *P<0.05 compared to saline controls, tP<0.05 as compared to contralateral controls, \$P<0.05 100nmol ami compared to 30nmol ami.

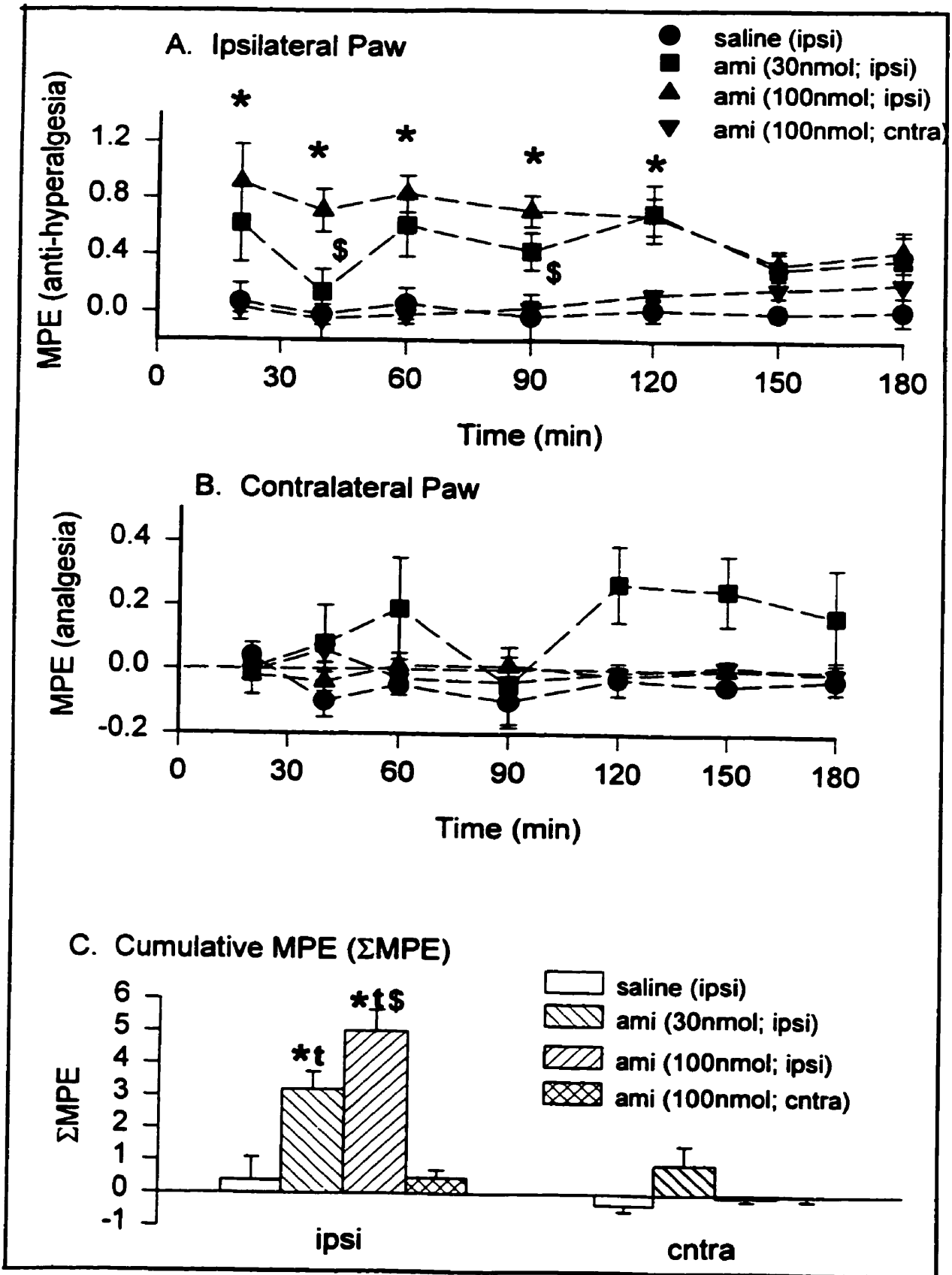


Figure 7

Figure 8

Time course of the local effect of ipsilateral desipramine (des; 100nmol) and fluoxetine (flx; 100nmol) administration on the thermal hyperalgesia of the nerve-injured paw (ipsi)(A) and withdrawal threshold of the contralateral paw (cntra)(B). Panel C depicts the cumulative MPE (Σ MPE) value. Values represent the mean \pm SEM (n=6 for all groups). *P<0.05 compared to saline controls.

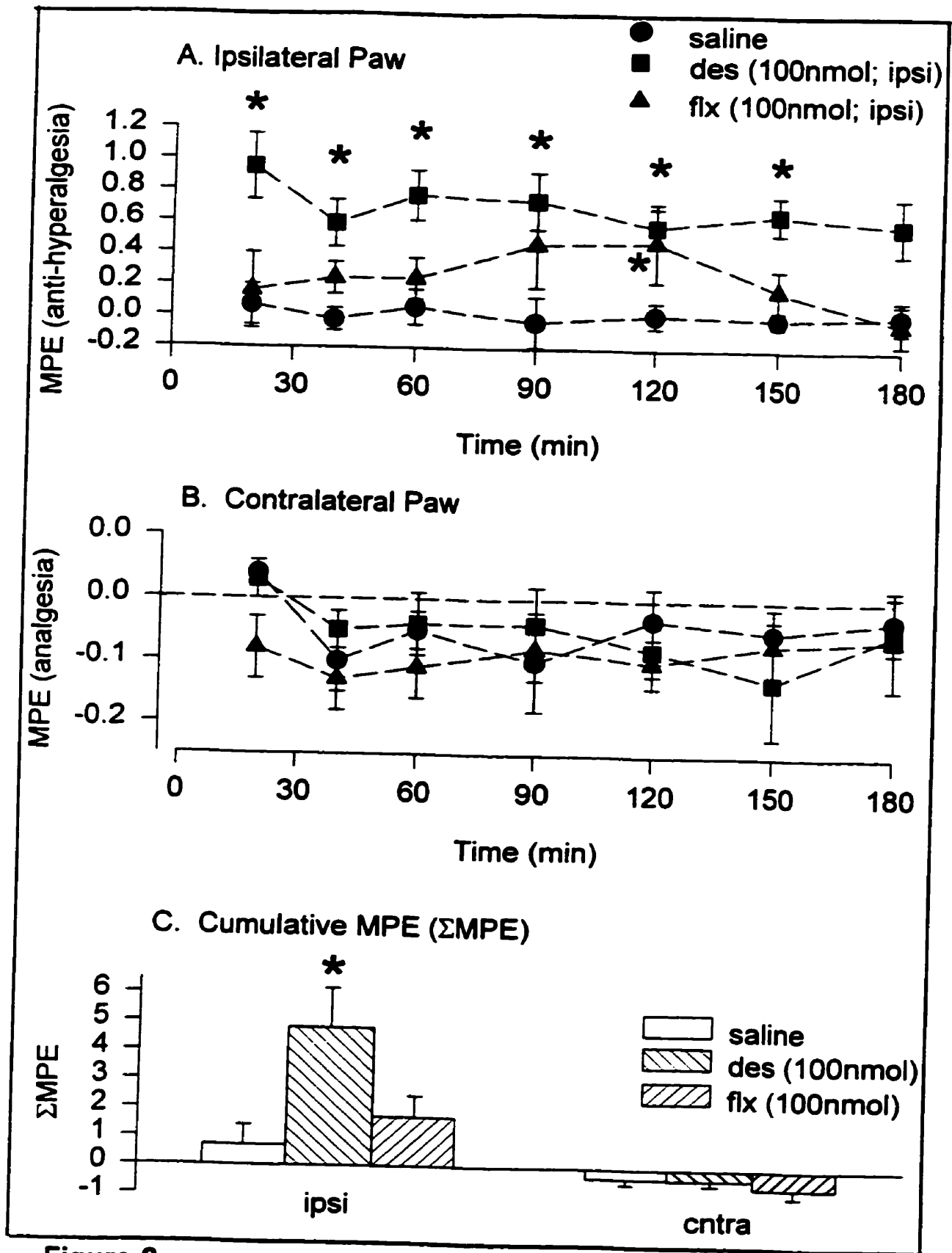


Figure 8

nmol did not result in any further increase in the thermal anti-hyperalgesic effect (data not shown). This effect was observed to be local since injection into the contralateral paw had no effect on the withdrawal threshold of the nerve injured (ipsilateral) paw (Fig. 8B). Similarly no effect was observed in the response threshold of the non-injured paw when amitriptyline was injected in the injured paw.

No significant anti-hyperalgesic effect was observed with fluoxetine (100nmol)(Fig. 8A and 8C). However, paw swelling was observed that was subsequently found to be statistically significant from the effect of a local injection of saline (Sawynok et al., 1999b).

3.2.4 Summary of systemic and local peripheral effects of amitriptyline, desipramine and fluoxetine on thermal hyperalgesia

Figure 9 is a summary/comparison of the systemic (Fig. 9A) and local peripheral (Fig. 9B) effects of each of the antidepressants on nerve injury-induced thermal hyperalgesia. As the figure depicts, the greatest effect was observed with amitriptyline for both routes although the overall effect of desipramine was very similar following local administration (Fig. 9B). Fluoxetine on the other hand had no effect on thermal hyperalgesia through either route and was found to cause caused significant paw swelling following local administration (Sawynok et al., 1999b).

Figure 9

Summary of various doses of systemic (A) and local peripheral (B) amitriptyline (ami), desipramine (des), and fluoxetine (flx) on thermal hyperalgesia of the nerve-injured paw (ipsilateral). Values represent the cumulative maximum possible effect (Σ MPE) of the respective time courses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared to saline controls. \$ $P < 0.05$ as compared to 10 mg/kg desipramine. Note: in (A) the cumulative MPE (Σ MPE) values for amitriptyline are from a 120 minute time course whereas those for desipramine are from a 180 minute time course. In (B) all Σ MPE values are from a 180 minute time course.

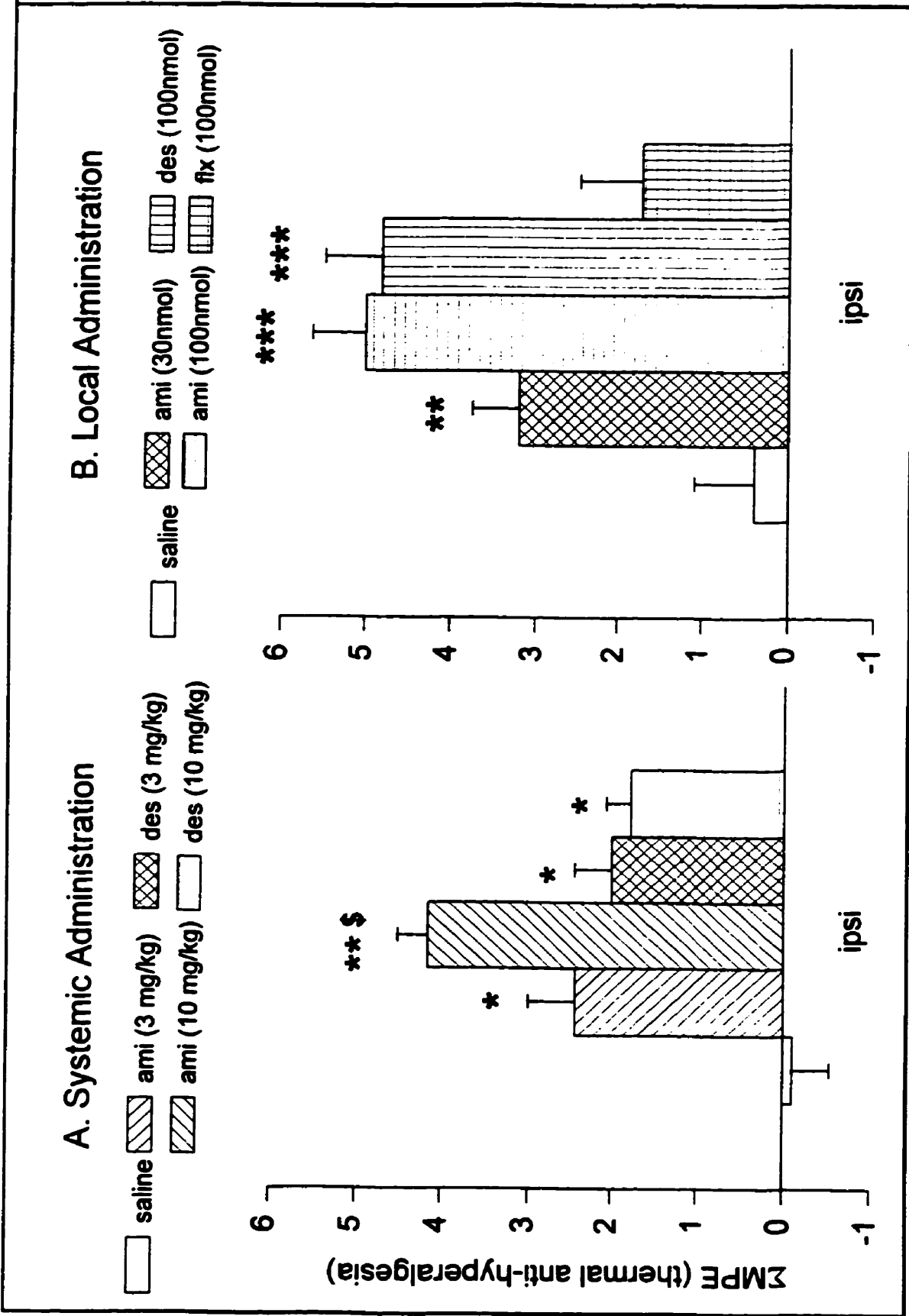


Figure 9

3.2.5 Effect of systemic amitriptyline, desipramine, and fluoxetine on static tactile allodynia

Systemically administered amitriptyline, at all doses tested (1, 5 and 10 mg/kg), had no significant effect on nerve injury-induced allodynia in the ipsilateral paw (Fig. 10A). Since 10 mg/kg was slightly sedative in some rats, a higher dose was not used, but this dose was used as a representative for the actions of systemic amitriptyline (Fig. 10). However, all doses of systemic amitriptyline caused a hyperaesthetic response in the contralateral paw, resulting in a reduced MPE (analgesia) value ranging from -0.4 to -0.8 (Fig. 10B). The Σ MPE value for 10 mg/kg differed significantly from saline (Fig. 10C). Qualitatively, the hyperaesthetic response was observed as a brisk withdrawal to the filament with no overt signs of pain such as biting, licking, vocalizations or guarding of the paw. As such, although the thresholds in the non-injured paws were similar to those in the injured paws, the responses were not categorized as 'neuropathic pain responses'. Threshold testing 24 hours after drug administration showed that both the ipsilateral and contralateral baselines returned to predrug values (data not shown), suggesting that the hyperaesthesia was drug related.

Neither desipramine (Fig. 10A) nor fluoxetine (data not shown) had any effect on the injury-induced static tactile allodynia. Systemic desipramine did however cause hyperaesthesia of the contralateral paw resulting in a decreased response threshold at individual time points (Fig. 10B) and in the cumulative MPE (Fig. 10C). Again, as in the hyperaesthetic effect observed with systemic amitriptyline, the response characteristically consisted of a brisk withdrawal from

Figure 10

Time course of the effect of systemic amitriptyline (ami; 10 mg/kg) and desipramine (des; 10 mg/kg) on static tactile allodynia in the ipsilateral paw (A), on the threshold of withdrawal to static tactile stimuli in the contralateral paw (B), and represented as the cumulative maximum possible effect (Σ MPE) over the time course (C). Values represent MPE \pm SEM (n=15 for ami and n=9 for des) *P<0.05, **P<0.01 compared to saline.

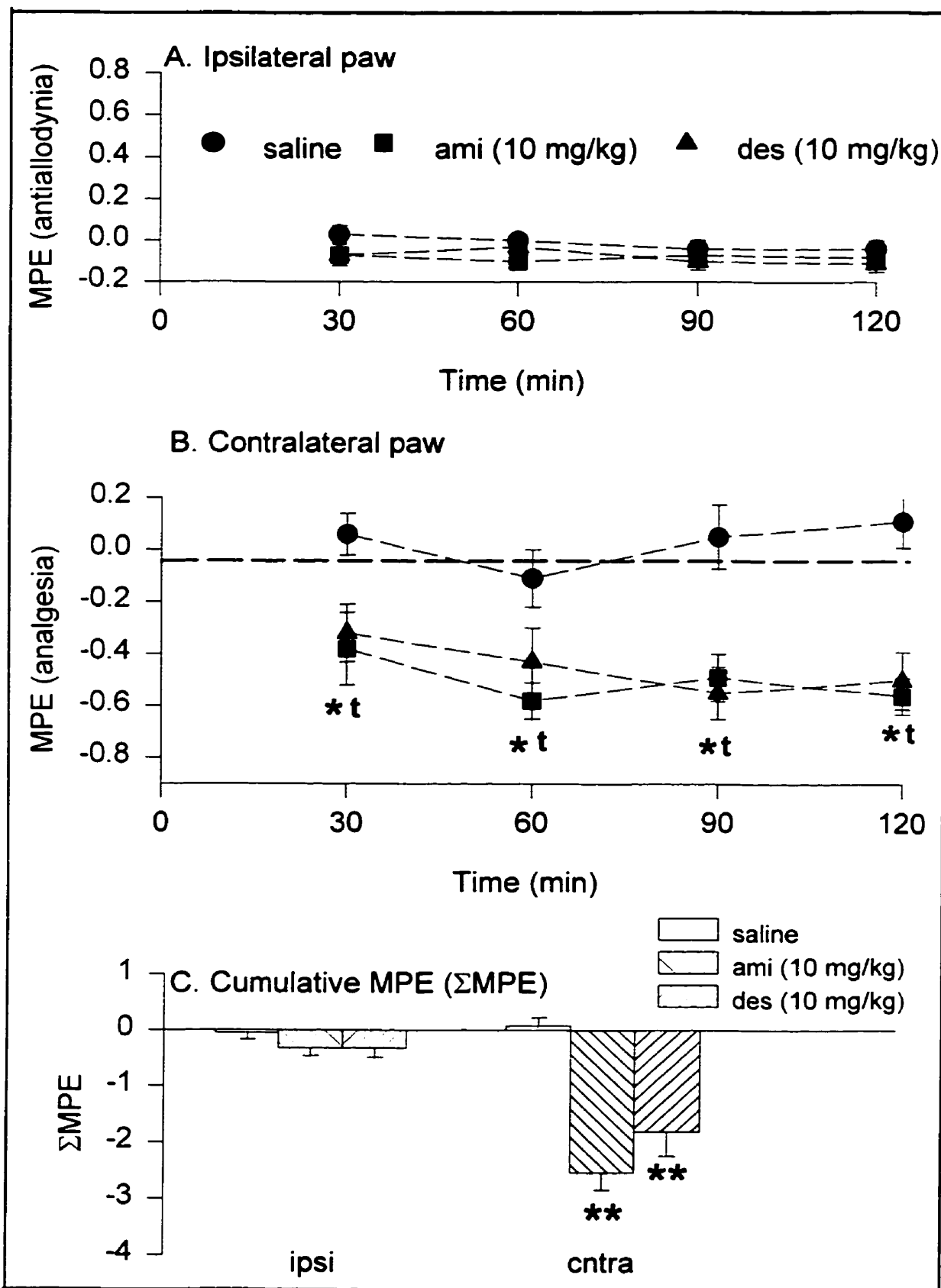


Figure 10

the stimulus without any of the secondary behaviors associated with the neuropathic pain response.

3.2.6 Effect of spinal amitriptyline on static tactile allodynia

Similar to systemic amitriptyline, spinal administration of amitriptyline failed to have a significant effect on allodynia in the ipsilateral paw (Fig. 11A). However, a comparison of the Σ MPE revealed the 60 μ g group to be significantly different from both the 30 μ g and the saline group (Fig. 11C). No significant effect of saline was observed although we did observe an anti-allodynic trend in the later time points (Fig. 11A). Spinal amitriptyline (60 μ g) also caused a hyperaesthetic response in the contralateral paw (Fig. 11B and 11C) that was similar in magnitude to that observed following systemic administration. A 90 μ g dose (n=3) produced a pronounced sedative effect that interfered with the accurate determination of the withdrawal threshold, and was therefore not included.

3.2.7 Effect of local peripheral injection of amitriptyline, desipramine and fluoxetine on static tactile allodynia

The local peripheral injection of amitriptyline (100nmol) failed to show any significant effect on the withdrawal response of the nerve-injured paw to tactile stimulation (Fig. 12A and 12C). However, this injection caused a reduction in the response threshold of the contralateral paw. A similar reduction in the threshold also occurred after direct local injection of amitriptyline (100nmol) into the non-injured contralateral paw (Fig. 12B). This effect was observed to be significantly

different from the response to saline in the same paw. The response following local injection of amitriptyline into the contralateral (non-injured) paw was similar to that following injection into the ipsilateral paw (Fig. 12B and 12C), suggesting a systemically mediated hyperaesthetic effect.

Neither desipramine nor fluoxetine had any effect on the tactile threshold of the ipsilateral paw when injected directly into the paw (Fig. 12A and 12C). In contrast, both caused contralateral paw hyperaesthesia following ipsilateral injection (Fig. 12B and 12C). This hyperaesthetic effect was significantly different from the effect of saline alone.

3.3 Effect of caffeine on the thermal anti-hyperalgesic action of amitriptyline and desipramine following nerve injury

Based on the differential symptom and route effects of amitriptyline and desipramine described in the first part of this thesis, the second part of the thesis sought to determine whether manipulation of endogenous adenosine contributed to the positive (thermal anti-hyperalgesia) and negative (contralateral hyperaesthesia) effects of amitriptyline or desipramine. This was achieved by using caffeine, a nonselective adenosine receptor antagonist. While an effect was observed against the action of amitriptyline on thermal anti-hyperalgesia, no effect was observed on the contralateral hyperaesthesia induced by either amitriptyline or desipramine (data not shown).

Figure 11.

Time course of the effect of spinal amitriptyline (30 and 60 μg) on static tactile allodynia in the ipsilateral paw (A), on the threshold of withdrawal to static tactile stimuli in the contralateral paw (B) and represented as the cumulative MPE (ΣMPE) over the time course (C). Values represent the MPE \pm SEM (n=5 for all groups). *P<0.05 compared to saline controls, tP<0.05 compared to ami (30 μg).

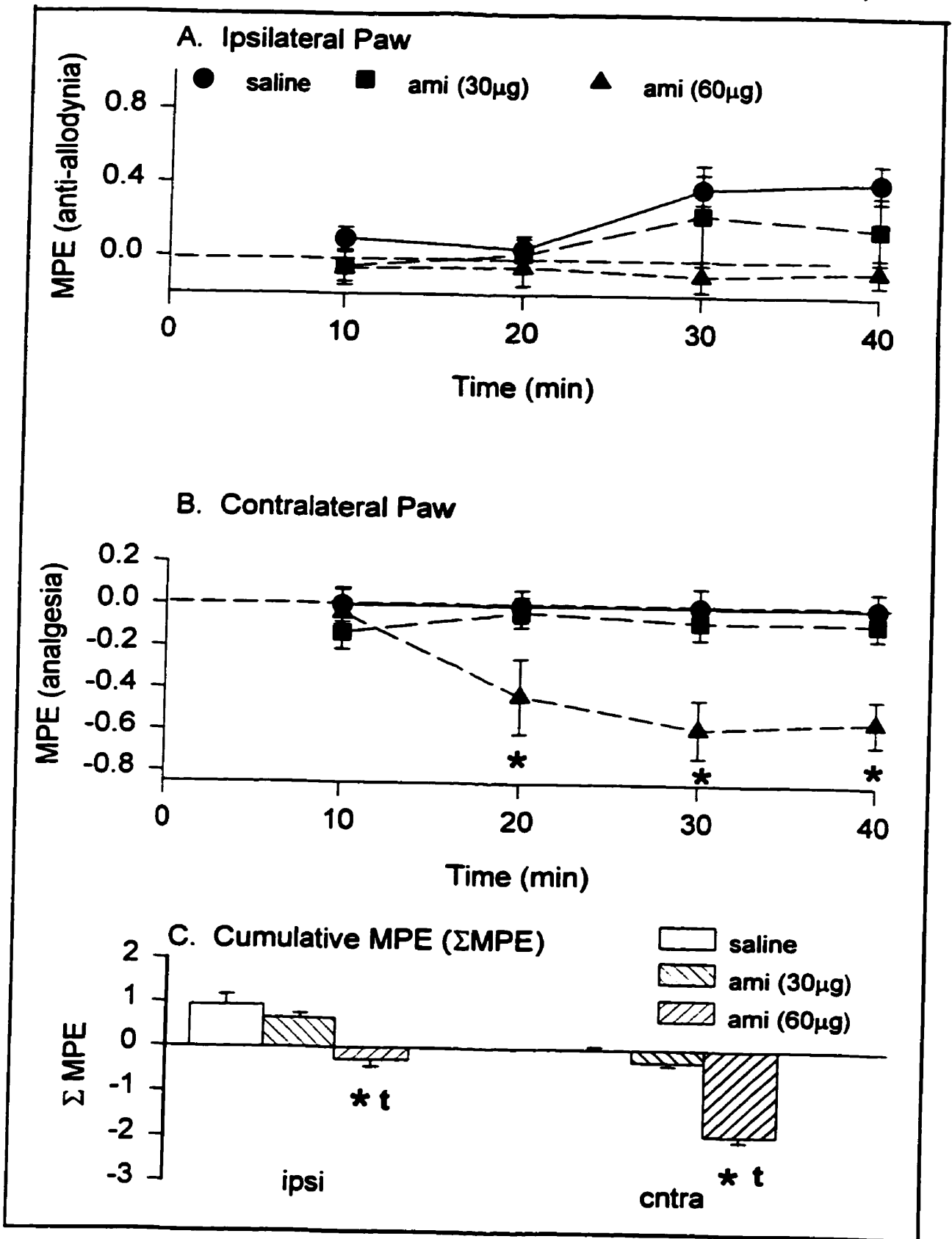


Figure 11

Figure 12

Time course of the effect of local peripheral injection of amitriptyline (100nmol) desipramine (100nmol), and fluoxetine (100nmol) on static tactile allodynia in the ipsilateral paw (A), when injected into the nerve-injured paw (ipsi) and non-injured contralateral (cntra) paw. Panel B represents the time course of the change in the tactile threshold of the contralateral paw (B), following injection into the nerve-injured paw (ipsi) and non-injured paw (cntra). Panel C depicts the cumulative maximum possible effect (Σ MPE) over the entire time course. Values represent the MPE \pm SEM (n=9 for saline and Ami groups and n=6 for desipramine and fluoxetine groups). *P<0.05 compared to saline controls, tP<0.05 ami (ipsi) compared to ami (cntra).

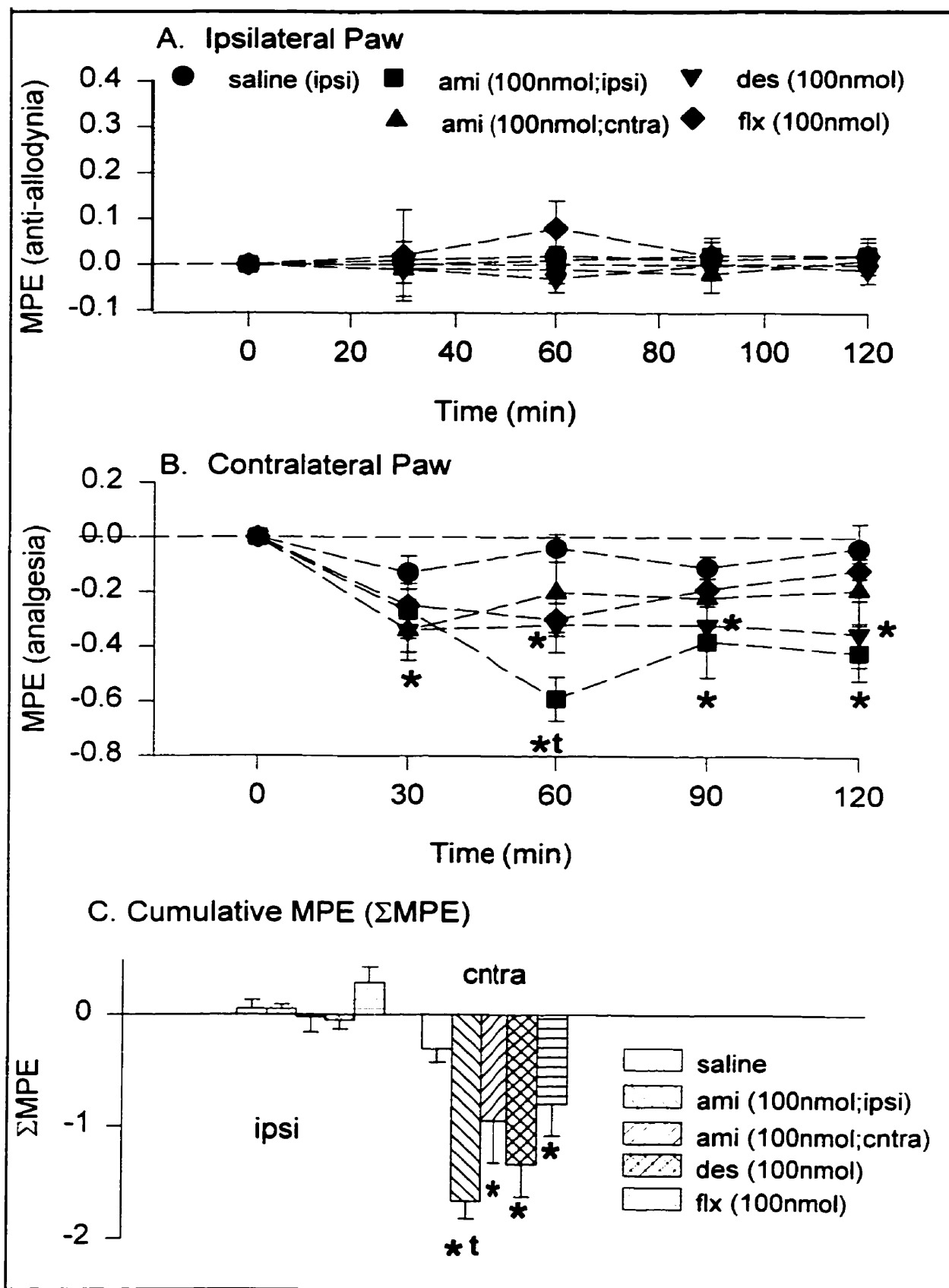


Figure 12

3.3.1 Intrinsic effect of systemic caffeine

Analysis of the intrinsic effect of caffeine revealed an apparent anti-hyperalgesic effect of 1.5 and 7.5, but not 3.75 mg/kg (Figs. 13A and 13B), as compared to saline. The cumulative MPE (Σ MPE) values for 1.5 and 7.5 mg/kg were not however different from that observed with 3.75 mg/kg (Fig. 13B). A further increase in the dose of caffeine to 15 mg/kg produced an increase in the activity level of the rat, an effect that was not observed for the 1.5, 3.75, or 7.5 mg/kg doses (data not shown). This behavioral response had an effect on the withdrawal threshold of the ipsilateral paw as accurate determination of the threshold requires the rat to remain still during presentation of the stimulus. No significant difference was found between the thermal withdrawal thresholds of the contralateral paw at any of the doses (Fig. 13B).

3.3.2 Effect of systemic caffeine on the thermal anti-hyperalgesic effect of systemic amitriptyline

Systemic amitriptyline (10 mg/kg) produced a significant reversal of nerve injury-induced thermal hyperalgesia from 60-180 minutes following administration (Fig. 14A), and with respect to the overall cumulative effect (Σ MPE) (Fig. 14D). Coadministration of 1.5 mg/kg of caffeine with amitriptyline had a statistically significant effect on the thermal anti-hyperalgesic effect of amitriptyline both at individual time points (Fig. 14A) and in the cumulative effect (Fig. 14D), where a 60% block of the thermal anti-hyperalgesic effect of amitriptyline was observed. Caffeine at 3.75 mg/kg completely antagonized (100% block) the thermal anti-

Figure 13

Time course of the systemic effect of various doses of caffeine (caff) on thermal hyperalgesia in the ipsilateral paw following nerve injury (A). The figure also depicts the cumulative maximum possible effect (Σ MPE) over the time course for both the ipsilateral (ipsi) and contralateral (cntra) paws (B). Values depict group means (n=6-9 per group) \pm SEM of the maximum possible effect. * P<0.05 compared to saline.

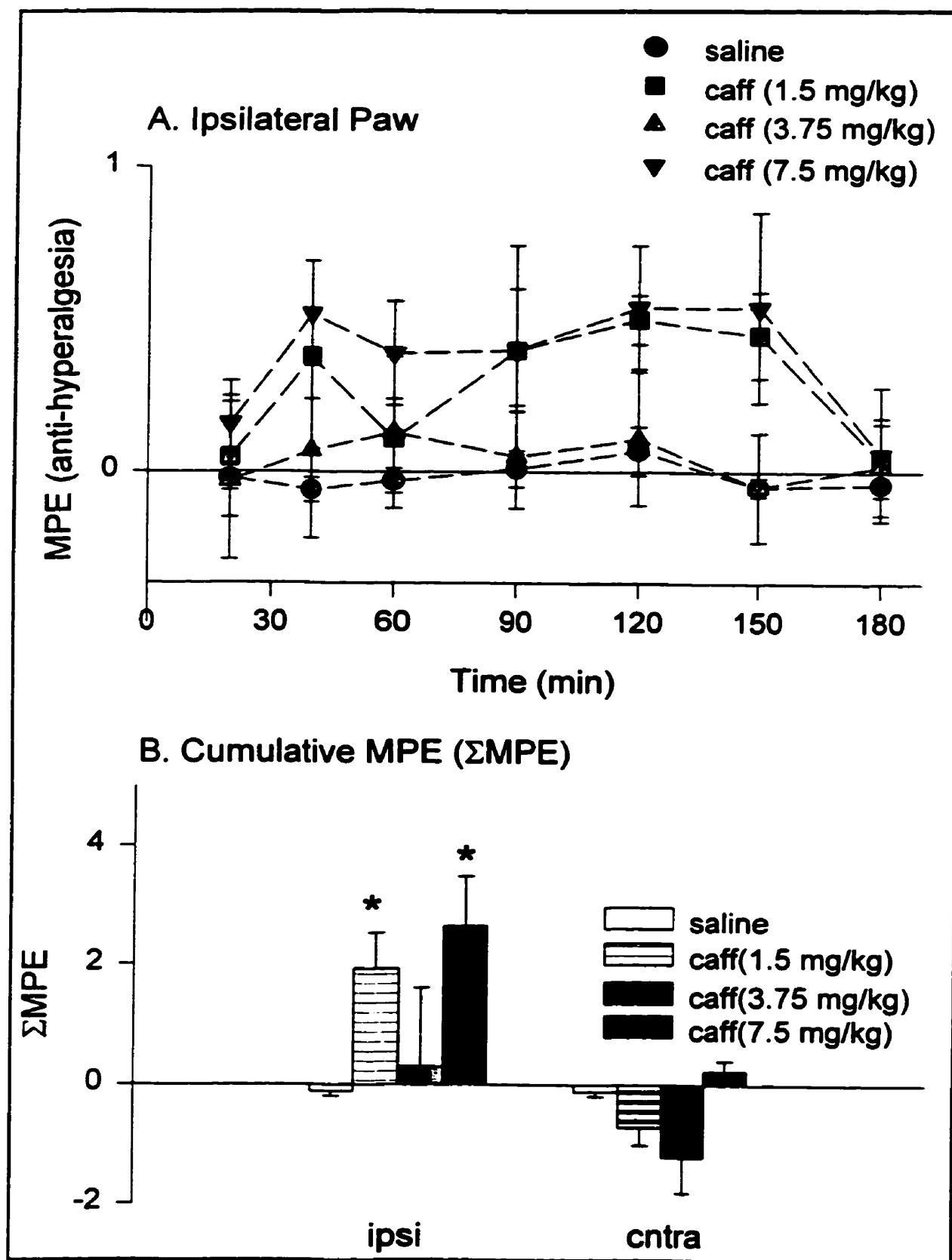
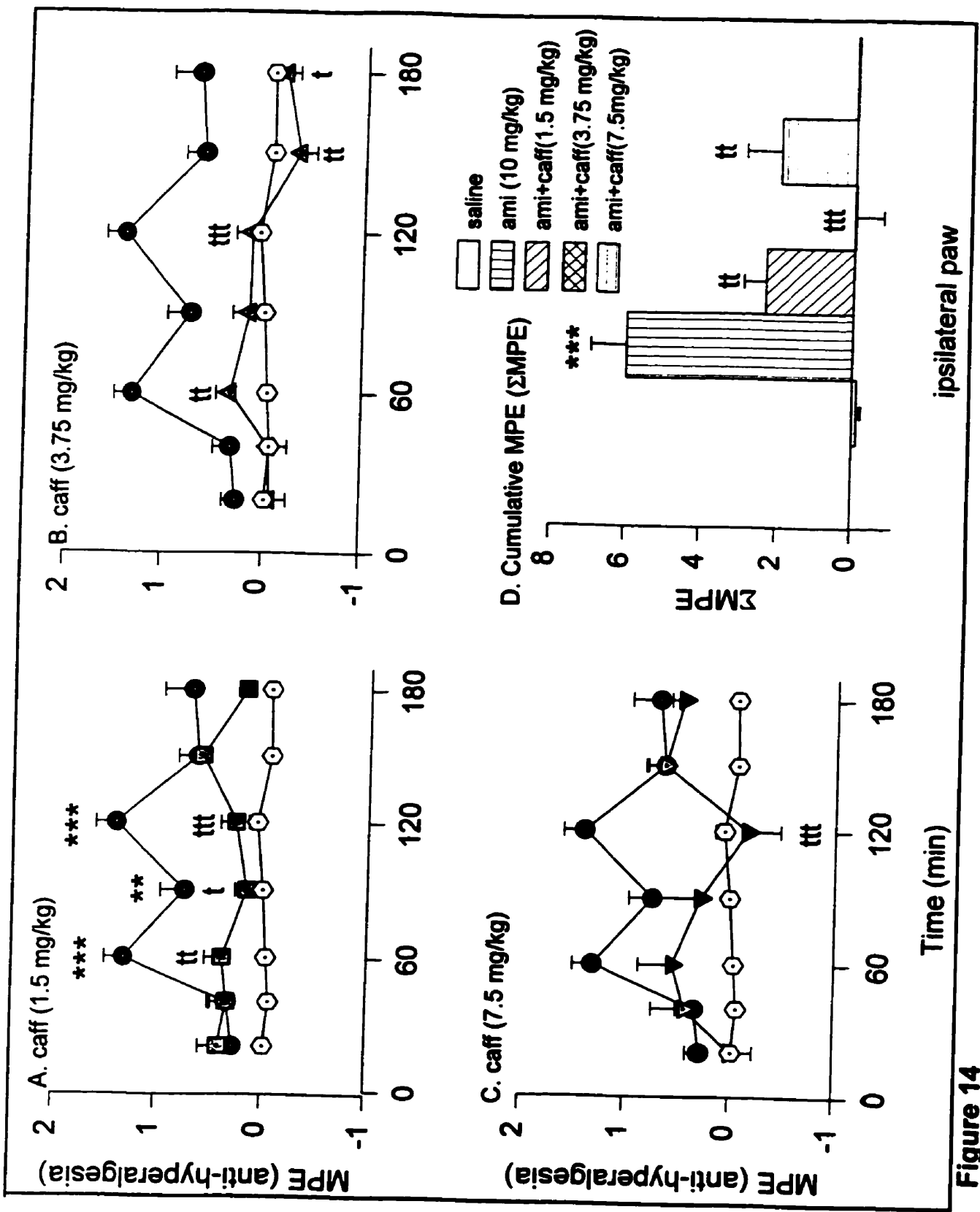


Figure 14

Time course of the effect of systemic co-administration of increasing doses of caffeine (gray symbols) on the systemic thermal anti-hyperalgesic effect of 10 mg/kg amitriptyline (ami) (black symbols) as compared to saline (hollow symbols) (A, B, and C). The overall effect is shown as the cumulative maximum possible effect (Σ MPE) (D). Values depict the mean (n=9 per group) \pm SEM. *P<0.05, **P<0.01, ***P<0.001 compared to saline. tP<0.05, ttP<0.01, tttP<0.001 compared to amitriptyline (10 mg/kg).



ipsilateral paw

Time (min)

Figure 14

hyperalgesic effect of amitriptyline (10 mg/kg) (Figs. 14B and 14D) without exerting any observable psychomotor stimulatory effect, as mentioned above. Similarly, while 7.5 mg/kg did not have any significant motor effect, it did partially block the thermal anti-hyperalgesic action of amitriptyline, at one individual time point (Fig. 14C) and in the cumulative effect (67% block) (Fig. 14D).

3.3.3 Effect of spinal caffeine on the thermal anti-hyperalgesic action of spinal amitriptyline

The spinal administration of amitriptyline (60 μ g) only produced a partial effect on nerve injury induced thermal hyperalgesia, and this effect was only significantly different from saline in the cumulative score (Fig. 15). Spinal pretreatment (10 min) with caffeine (100 μ g) slightly reduced the cumulative thermal anti-hyperalgesic action of spinal amitriptyline (Fig. 15B) but this effect was not statistically significant. This dose of intrathecal caffeine was without intrinsic motor or anti-hyperalgesic activity (Fig. 15A). Higher spinal doses of amitriptyline were not investigated, as they produced pronounced sedative and/or motor effects severely compromising the ability to determine the withdrawal thresholds as mentioned previously (section 3.2.2).

3.3.4 Effect of local peripheral caffeine on the thermal anti-hyperalgesic action of local peripheral amitriptyline

The local peripheral administration of amitriptyline (30 and 100 nmol) produced a significant thermal anti-hyperalgesic effect at individual time points

Figure 15

Time course of the effect of spinal amitriptyline (ami) and caffeine (caff) alone, and in combination, on nerve-injury induced thermal hyperalgesia of the ipsilateral paw (A) for individual time points. The data is also depicted in terms of the maximum possible effect (Σ MPE) (B). Values depict the mean (n=6 per group) \pm SEM. *P<0.05 compared to saline.

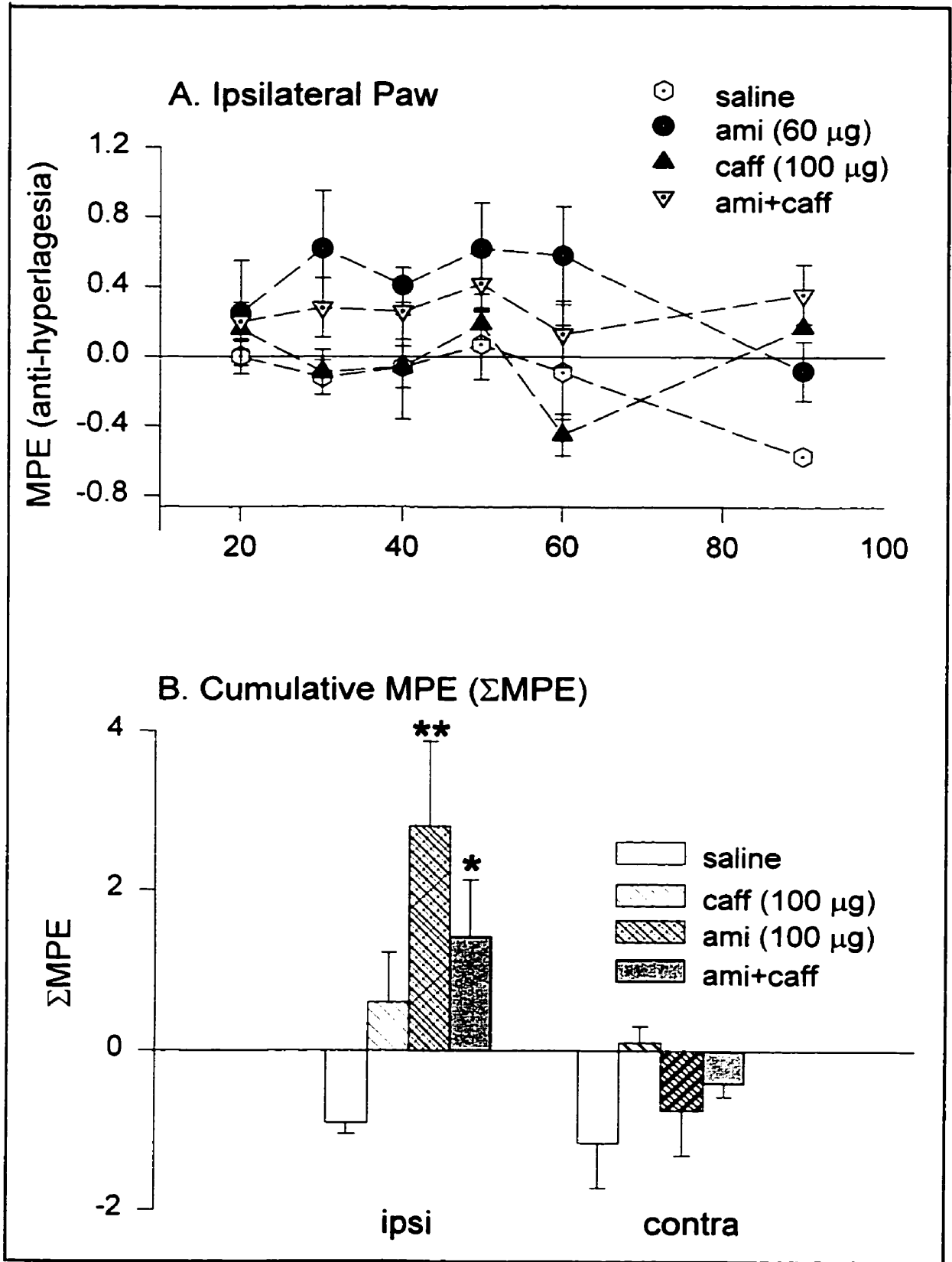


Figure 15

(Figs. 16B and 14C) and in the cumulative data (Fig. 16D). This effect was determined to be due to a local action as it was not observed following administration of amitriptyline into the contralateral paw (section 3.2.3). When co-administered peripherally with amitriptyline, caffeine (1500nmol) significantly reduced the local thermal anti-hyperalgesic effect of 30nmol (66% block) (Figs. 16B and 16D) and 100nmol (60% block) amitriptyline (Figs. 16C and 16D). Peripheral administration of caffeine alone however, failed to produce any significant effect (Fig. 16A). While there still remained a slight thermal anti-hyperalgesic effect following administration of the amitriptyline-caffeine combination, this effect was not significantly different from that of saline alone (Figs. 16B, 16C and 16D).

3.3.5 Effect of systemic and local caffeine on the thermal anti-hyperalgesic action of systemic and local peripheral desipramine

As identified in a previous section both systemic (10 mg/kg) and local (100nmol) desipramine had a significant effect on nerve injury induced thermal hyperalgesia. Neither systemic nor local co-administration of caffeine had an effect on the thermal anti-hyperalgesic action of desipramine (Fig. 17).

3.4 Time Course of immunoreactive expression of Hsp27, NPY, SP following unilateral spinal nerve ligation

The results from the previous two sections of this thesis identified a route and test specific effect of antidepressants in nerve injury induced neuropathic

Figure 16

Time course of the local peripheral effect of 1500nmol of caffeine (caff) alone (A) and on the thermal anti-hyperalgesic effect of 30nmol (B) and 100nmol (C) amitriptyline (ami). Amitriptyline alone is depicted by black symbols while combination of the respective doses with caffeine is depicted by gray symbols. The cumulative effect of the drugs alone, and in combination, on the maximum possible effect (MPE) on the ipsilateral paw, is shown in (D). Values depict the mean (n=9 per group) \pm SEM. *P<0.05, **P<0.01, ***P<0.001 compared to saline, tP<0.05, ttP<0.01, tttP<0.001 compared to the respective dose of amitriptyline alone.

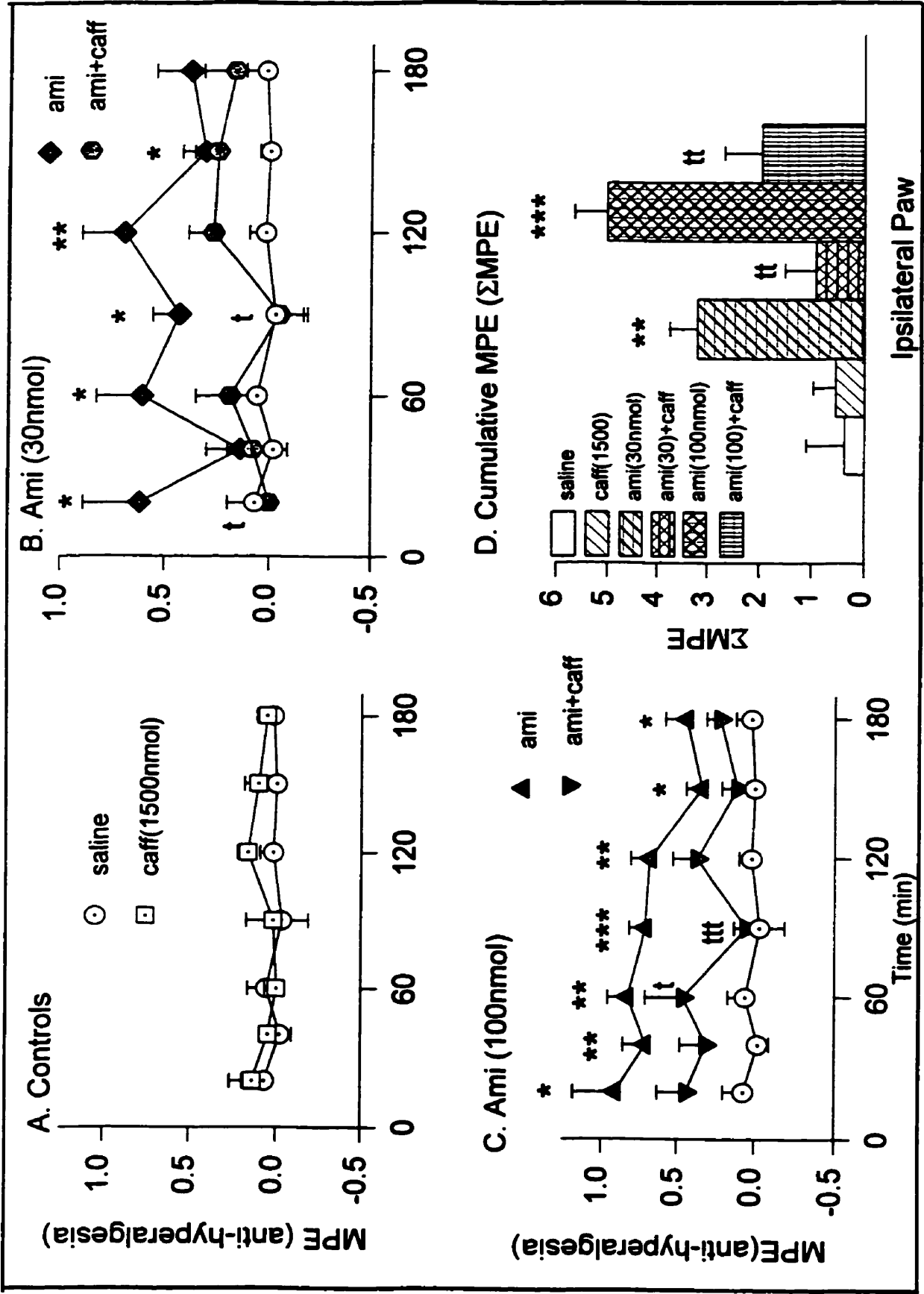


Figure 16

Figure 17

Time course of the systemic (A and B) and local peripheral (C and D) effect of desipramine alone and in combination with caffeine. Panel (A) depicts the time course of systemic desipramine (10 mg/kg) and caffeine (7.5 mg/kg) alone and in combination, while panel (B) represents the cumulative MPE (Σ MPE) values following systemic administration. Shown in panel (C) is the time course of the anti-hyperalgesic effect of desipramine (100nmol) and caffeine (1500nmol) alone and in combination, and shown in (D) is the cumulative MPE (Σ MPE) values over the time course. * $P < .05$, ** $P < .01$ compared to saline controls.

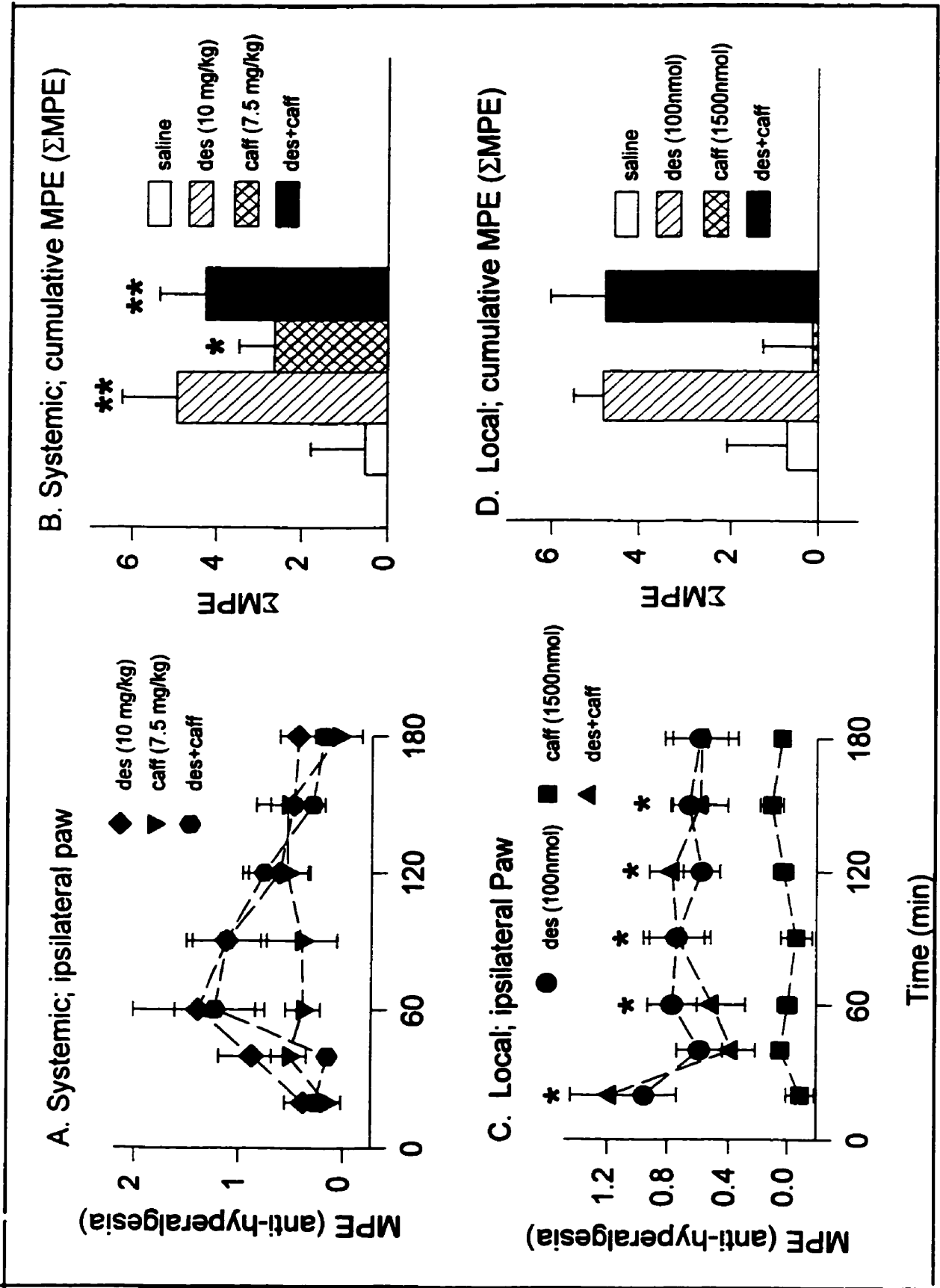


Figure 17

pain. In order to study the neuroanatomical effects of chronic drug treatment, the time course of immunoreactive expression of selective markers was performed.

3.4.1 Behavioral manifestations of peripheral nerve ligation pathophysiology

At each of the time points corresponding to the immunohistochemical analysis, a determination of the allodynic threshold was made. Figure 18 depicts the mean 50% withdrawal threshold for the ipsilateral and contralateral paws, as well as the difference between the two paws, in experimental animals at 4, 7, 12, and 17 days. The magnitude of the difference between the thresholds for the paws increased from day 4 to day 7, but then remained relatively stable until day 17. The figure also depicts a lower response threshold of the contralateral paw at 4 days that is significantly different from that of the contralateral paw at the later time points. There was also an occasional reduced threshold response in the ipsilateral paw of the sham animals that contributed to a higher variation in the response threshold for the group (data not shown). By 5 months however, we did not detect any difference in the response thresholds between the ipsilateral and contralateral paws, and both were above the cutoff (15 gram) threshold.

3.4.2 Changes in Hsp27-IR expression in the spinal dorsal horn and gracile nucleus

Hsp27-IR was examined in the spinal cord and brain stem of naive rats from the same group as the experimental animals coinciding with the 1, 4 and 12 day time points. For this study, Hsp27-IR is described only in the L4-S2 region of

Figure 18

Histogram showing the expression of tactile allodynia in the nerve ligated paw (ipsilateral) and the tactile response threshold of the non-injured (contralateral paw). Also shown is the difference in the response thresholds between the two paws at the successive time points. Values represent the mean \pm SEM for experimental animals ($n=4$ at each time point). $\phi P < 0.05$ as compared to the ipsilateral paw 4 day value, $*P < 0.05$ compared to the 7 day contralateral paw value, $\$P < 0.05$ compared to the 7 day difference value and $\xi P < 0.05$ compared to the 4 day ipsilateral paw value.

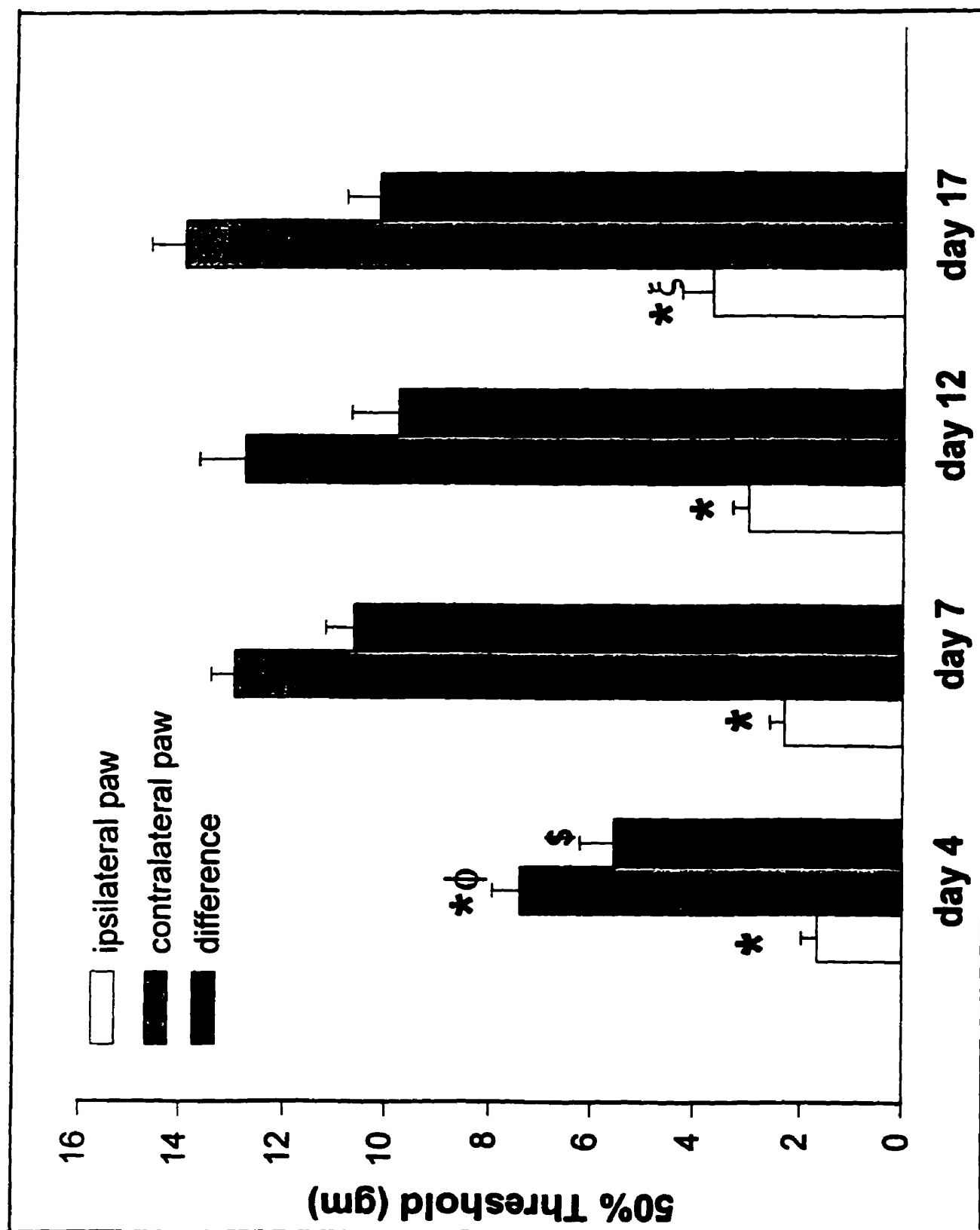


Figure 18

the spinal cord, as this was the spinal segments in which most of the changes in Hsp27-IR expression occurred following SNL. Hsp27-IR in the spinal cord of the naive animals was similar at all time points (Fig. 19A-E). Light labeling of Hsp27-IR was observed in LI and LIII-IV, but was noticeably absent in LII except for a few labeled fibers and axon terminal-like structures. At the L5 level, the Hsp27-IR was more dense in the medial one-half to two-thirds of the dorsal horn, and at L6, a small band of lightly stained fibers extended along the medial border of the dorsal horn towards the midline and crossed up towards LI on the opposite side.

Hsp27-IR was examined in the spinal cord and brain stem of SNL and sham rats at 6 and 24 hours, 4, 7, 12, 17 and 180 days. Relative changes in Hsp27-IR, as compared to naïve and sham ligated animals, did not become apparent in the dorsal horn until 4 days after SNL (see Fig. 19). By 4 days the SNL-induced Hsp27-IR was most apparent in the dorsal horn from L4-S2 (Figs. 19K-O). The Hsp27-IR was most dense in L6 and S1 and patchy labeling was observed in the superficial laminae of the L4, L5 and S2 segments. The Hsp27-IR in L6-S1 was most dense in LI, LII_o and the dorsal margin of LIII (LIII_d). Moderate Hsp27-IR was present in LII_i. In the sham ligated animals at 4 days, a dense patch of Hsp27-IR was located only on the lateral margins of LI, LII_o and LIII_d in spinal segments L5 to S1 (Fig. 19F-J).

Seven days after SNL, the intensity of Hsp27-IR in the ipsilateral dorsal horn from L5-S2 was not changed from that seen at 4 days (Figs. 19K-O). However, on the contralateral side of the spinal cord, we observed a reduction of Hsp27-IR in lamina III_d from L6-S1.

By 12 days after SNL, Hsp27-IR in the ipsilateral dorsal horn was most intense and appeared as a wide band of labeling that filled the medial to lateral extent of the dorsal horn from LI-LIII in L5-S1 (Figs. 20 and 21C). Less densely stained Hsp27-IR fibers appeared to spread further into the deeper laminae (LIV-LV) from the more densely stained superficial laminae. The Hsp27-IR labeling in L4 and S2 appeared as dense patches in LI-LIII with little or no SNL-induced change in Hsp27-IR in spinal segments above L4, or below S2. Moderate to dense Hsp27-IR extended along the medial border of the dorsal horn from L1-L6, crossed the midline above the central canal and extended up along the medial border of the contralateral dorsal horn to L1 (Fig. 21C). In addition, a few Hsp27-IR fibers in the neck region of the dorsal horn appeared to cross the midline above the central canal and extend towards the lateral-most border of the contralateral dorsal horn (Fig. 21C). Many of the Hsp27-IR fibers in the neck region of the ipsilateral dorsal horn appeared to overlap with branching dendritic processes that extended dorsally from neurons located in the ventral horn. A small patch of Hsp27-IR was observed in the region of the intermediomedial nucleus and a dense patch of Hsp27-IR fibers extended from the ventrolateral border of the dorsal horn into the region of the parasympathetic nucleus ipsilaterally (Fig. 22A). Dense Hsp27-IR fibers were observed in the dorsolateral region of the ipsilateral dorsal column in spinal segments L5-L6 (Figs. 20G, 20H, 20C, and 22B). The Hsp27-IR was traced rostrally in the dorsal column to the nucleus gracilis where Hsp27-IR fibers were dense in the medial region of the nucleus (Fig. 22D).

Figure 19

Hsp27-IR in the spinal dorsal horn (L4-S2) in naïve (A-E), sham-operated (F-J) and spinal nerve ligated animals (K-O) 4 days after surgery when changes first became apparent. The Hsp27-IR in the ligated animals is most dense in LI and the outer margin of LIII in the lower lumbar (L5-L6) and upper sacral (S1) spinal cord (L-N). Note that the labeling in LII is lighter than that seen in adjacent laminae of the ligated animals but darker than that seen in the sham-operated and naïve animals. Surgical exposure of the spinal nerves also induced some expression of Hsp27-IR in the lateral-most region of the superficial laminae including LI-LII and the outer region of LIII (G-I). The roman numerals in (A) indicate the different laminae of the spinal dorsal horn. Scale bar: 250µm.

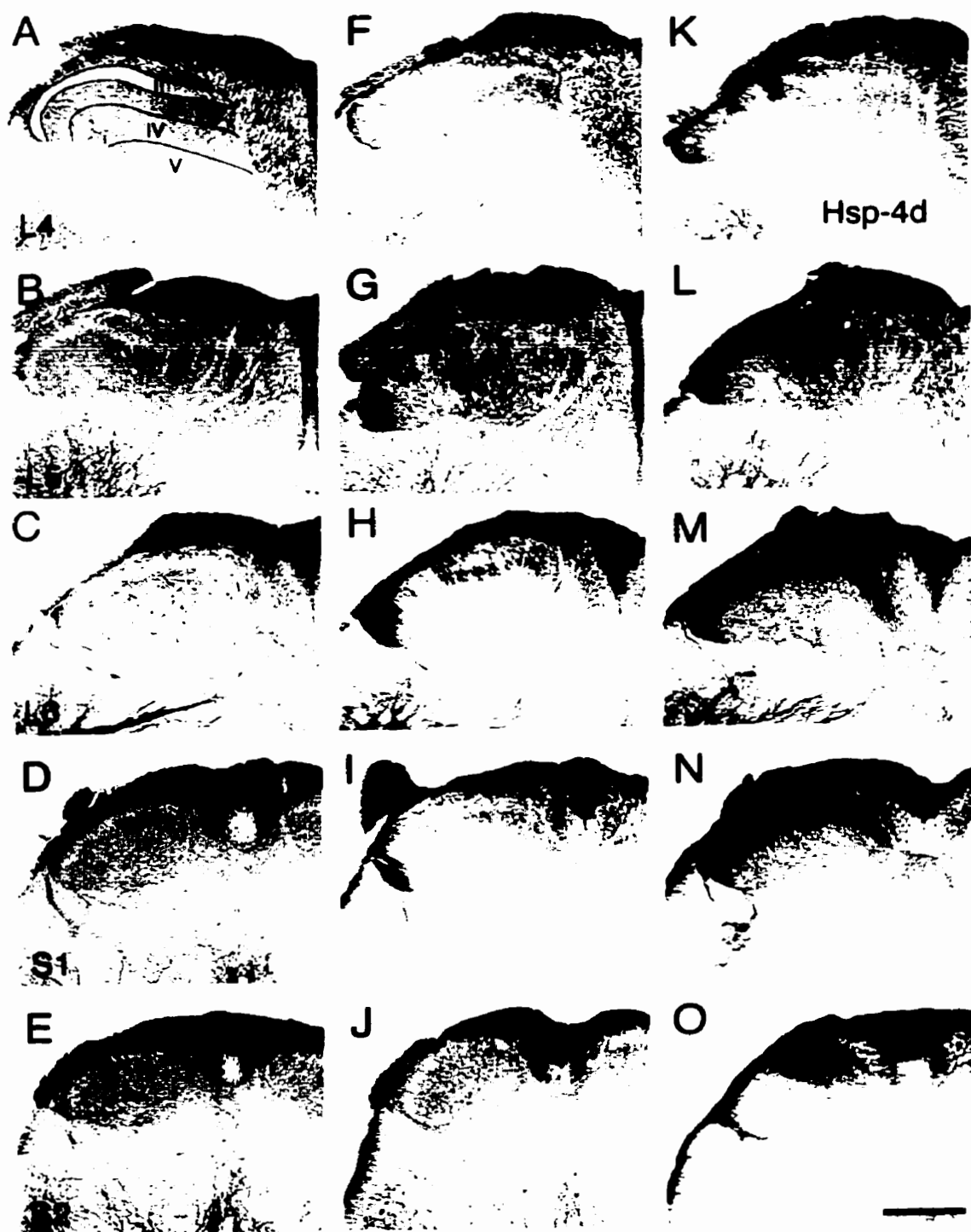


Figure 19

Figure 20

Hsp27-IR in the spinal dorsal horn (L4-S2) in sham-operated (A-E) and spinal nerve ligated animals (F-J) 12 days after surgery, when Hsp27-IR expression is at a peak. The Hsp27-IR expression in the ligated animals has intensified and spread into deeper laminae of the lumbo-sacral spinal cord. Hsp27-IR is now dense in LI-LIII and moderate to light in LIV and LV of the lower lumbar (L5-L6) and upper sacral (S1) spinal cord. Dense Hsp27-IR in the sham operated animals has also spread into intermediate and medial portions of LI and LII with moderate to light patchy labeling in LIII and LIV. The small lines in (A) indicate the approximate borders of the different laminae. Scale bar: 250 μ m.

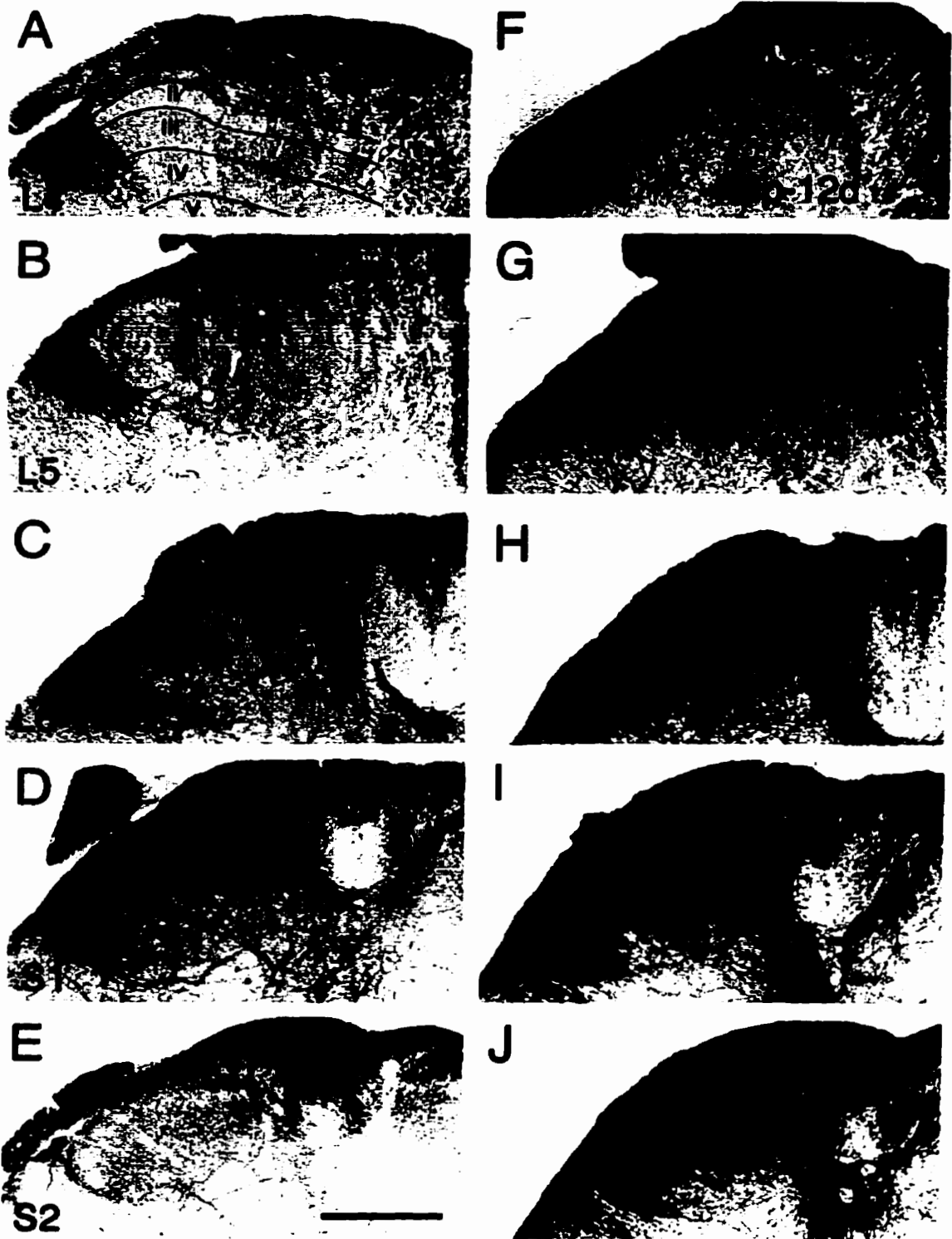


Figure 20

Figure 21

HSP27-IR in the L6 spinal segment 24 hr (A), 4 days (B), 12 days (C) and 180 days (D) after L5-L6 spinal nerve ligation. The Hsp27-IR is dense in the superficial laminae by 4 days, becomes most dense by 12 days and is greatly diminished by 180 days after spinal nerve ligation. By 12 days, the Hsp27-IR spreads into the midline and crossing fibers extend along the medial border of the dorsal horn, deep to the dorsal horn, to reach the superficial laminae on the contralateral side (see arrows). The small lines in (A) indicate the approximate borders of the different laminae. Scale bar: 250 μ m.

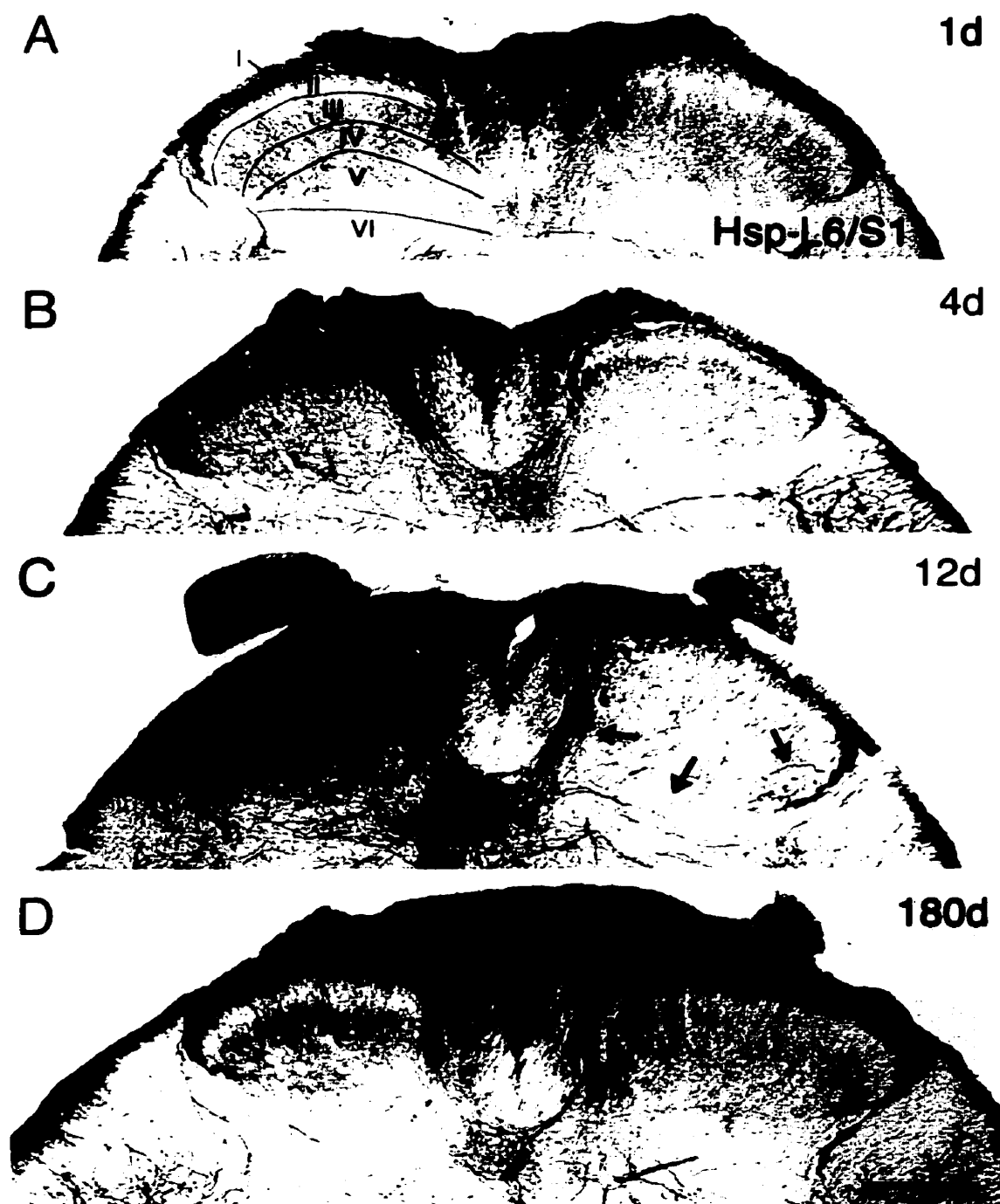


Figure 21

In the sham ligated animals at day 12, a dense band of Hsp27-IR was observed only in LI and on the lateral border of LI-LIII from L4-S1 (Figs. 20A to 20D). Moderate to light labeling was observed in LII-LIII from spinal segments L4-S1. At L6-S1, moderate to light Hsp27-IR extended along the medial border of the dorsal horn from LI-VI, crossed the midline above the central canal and extended up along the medial border of the contralateral dorsal horn to LI. Hsp27-IR fibers were sparse in the neck region of the dorsal horn and only the occasional Hsp27-IR fiber extended towards the midline. Moderate to dense Hsp27-IR was observed extending from the ventro-lateral border of the dorsal horn into the parasympathetic nucleus ipsilaterally. In the sham animals, little or no Hsp27-IR was observed in S2 at 12 days, and Hsp27-IR labeling in the dorsal columns and nucleus gracilis was light to moderate on both sides.

By day 17, Hsp27-IR in the SNL animals appeared to be less dense in LII-LV compared to that seen in the SNL animals at day 12. The differences in Hsp27-IR were most apparent in these laminae from L6-S1. Hsp27-IR in the other regions of L5-S2 were similar to that seen at day 12. By day 180, Hsp27-IR in the dorsal horn had returned almost to the same intensity as that seen in the naive animals (Fig. 21D). However, Hsp27-IR in the lateral half of LI and LIII, at spinal segments L6-S1 was more dense than that seen in the sham ligated or naive animals. In addition, Hsp27-IR was more dense in the dorsal and lateral part of the ipsilateral dorsal column as compared to the contralateral dorsal column (Fig. 21D).

Figure 22

Hsp27-IR in the spinal cord and brain stem 12 days after spinal nerve ligation (A, B and D) or sham-surgery (C). Panel (A) shows the typical pattern of Hsp27-IR in S1 and the presence of dense labeling in the lateral sacral parasympathetic nucleus (arrow). Panel (B) shows dense Hsp27-IR in the dorsolateral portion (arrows) of the ipsilateral fasciculus gracilis (gr) after SNL. Note the presence of dense HSP27-IR in the medial portion (arrows) of the ipsilateral nucleus gracilis (Gr) in the SNL animal (D) that is not as evident in the sham-operated animal (C). Scale bar: 250 μ m.

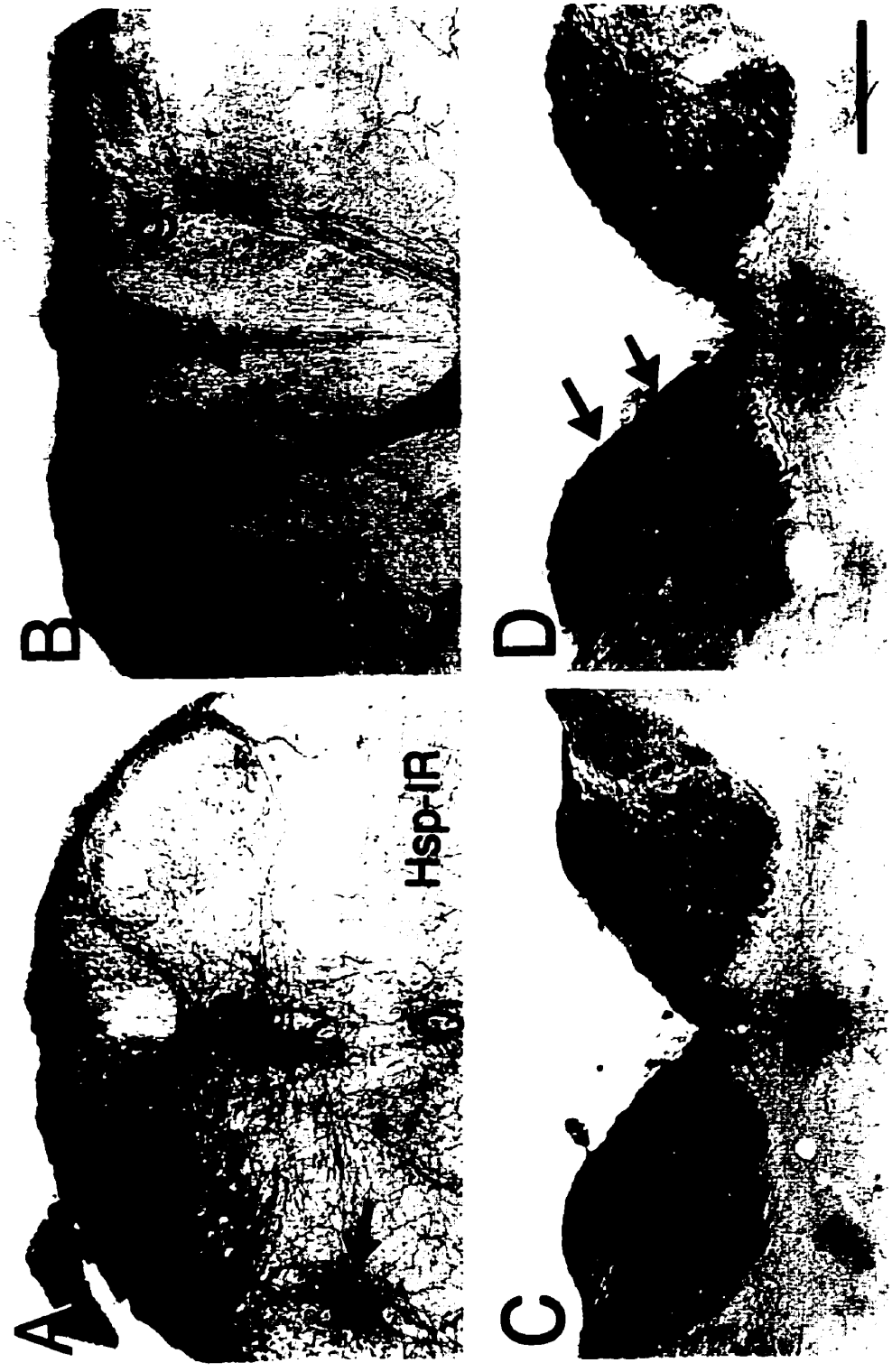


Figure 22

3.4.3 Changes in NPY-IR expression in the spinal dorsal horn and gracile nucleus

Changes in NPY-IR in the spinal dorsal horn and gracile nucleus were similar to the changes in Hsp27-IR observed following SNL. There was a marked increase in NPY-IR in laminae III and IV and the medial portion of lamina II of spinal segments L5-S1, predominately ipsilateral (Fig. 23 and 24). In contrast to the Hsp27-IR however, the NPY-IR was apparent by day 7 and was most intense by day 17 (Figs. 23K-O and 24). The increased NPY-IR was most dense in the medial to lateral extent of Laminae III and moderate in lamina IV by day 17. NPY-IR increased in the medial one-third of lamina II so a light to moderate patch appeared by day 7 that increased to a dense patch by day 17. By day 17, NPY-IR appeared to be increased in laminae I and II_c of the contralateral dorsal horn. Little or no change in NPY-IR was observed in spinal segment L4 and in the lateral one-third of S2.

In sham operated animals, an increase in NPY-IR was observed primarily in the lateral most region of lamina III of spinal segments L5-S1 that became evident by day 7 (Figs. 23F to 23J), was most prominent by day 17 and diminished after 180 days.

By day 17, NPY-IR was increased in the lateral border of the dorsal column (Fig. 24C) and dense NPY-IR filled the gracile nucleus caudal to the obex and was observed in the rostral most extent of the nucleus rostral to the area postrema (Fig. 24). In the sham animals there was a small patch of NPY-IR that was observed only in the medial part of the gracile nucleus caudal to the obex. The increase in NPY-IR in the spinal dorsal horn diminished by 180 days after

Figure 23

NPY-IR in the spinal dorsal horn (L4-S2) in naive (A-E), sham-operated (F-J) and SNL animals (K-O) 7 days after surgery when changes first became apparent. Changes in the density and distribution of NPY-IR in the SNL animals is most apparent in LI and LIII in the lower lumbosacral cord (L5- S1) spinal cord (see L-N). NPY-IR in LII appears to be increased primarily along the medial border of the lamina at L6-S1. At L4, there is little or no difference between groups and at S2, an increase in NPY-IR is only present in the medial two-thirds of LIII. Surgical exposure of the spinal nerves (F-J) increased NPY-IR in the lateral-most region of LIII at L6 (see H) and produced patchy labeling throughout LIII at S1 (see J). Scale bar: 250 μ m.

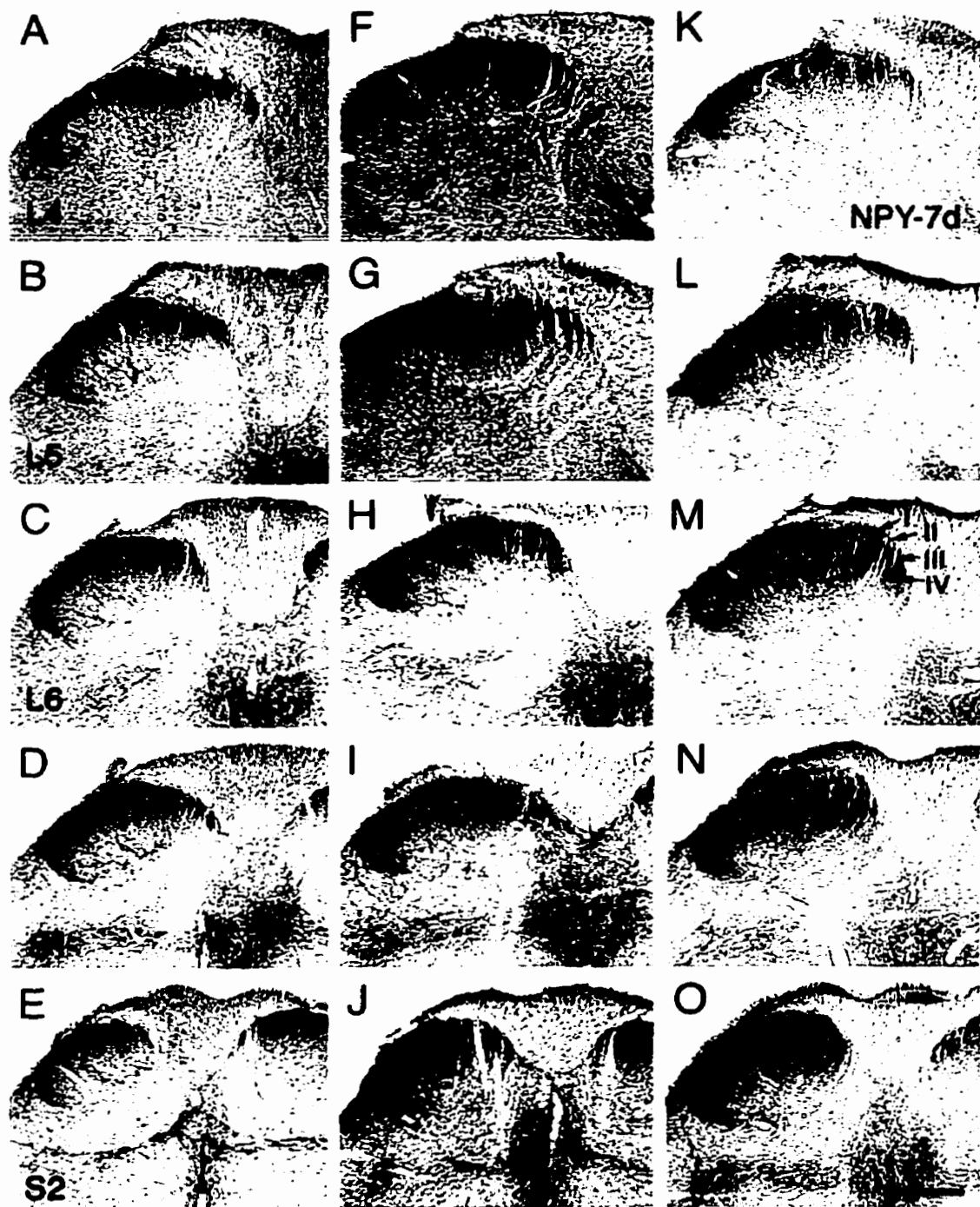


Figure 23

Figure 24

Representative spinal cord sections at L6/S1 showing the time course of the increase in NPY-IR above normal levels after SNL. There is little change in NPY-IR after 1 day but a marked increase in NPY-IR occurs in the spinal dorsal horn by 12 days (B). The intensity of NPY-IR peaks at 17 days (C) and diminishes to near normal levels by 180 days (D). At 17 days, the change in NPY-IR is most evident in LI, LIII-LIV and in the medial one-third of LII of the ipsilateral dorsal horn. Crossing NPY-IR fibers (arrows) were observed and NPY-IR appears increased in the contralateral LI and the medial border of LII 17 days after SNL. Scale bar: 250 μ m.



Figure 24

Figure 25

NPY-IR in the gracile nucleus (Gr) 17 days after SNL (A-B), 17 days after sham-surgery (C) and 180 days after SNL (D). Note that the NPY-IR is markedly increased in the ipsilateral gracile nucleus (A) compared to that seen on the contralateral side and in sham-operated animals (C). The increase in NPY-IR in the gracile nucleus extends from the caudal to its rostral-most extend (B). In the sham-operated animals, NPY-IR is increased mainly in the medial one-third of the nucleus (C). By 180 days after SNL, the increase in NPY-IR is still evident but is noticeably diminished especially in the medial one-third of the nucleus (D). Scale bar: 250 μ m.



Figure 25

Figure 26

Densitometric comparison of the percent change in immunoreactive intensity of Hsp27 and NPY in the spinal dorsal horn of the L6/S1 region of the spinal cord in experimental nerve-injured rats. The percent change in intensity (percent change = (ipsi-contra)/contra) is represented as the mean +/- SEM of 5 sections per animal at each of the time points.

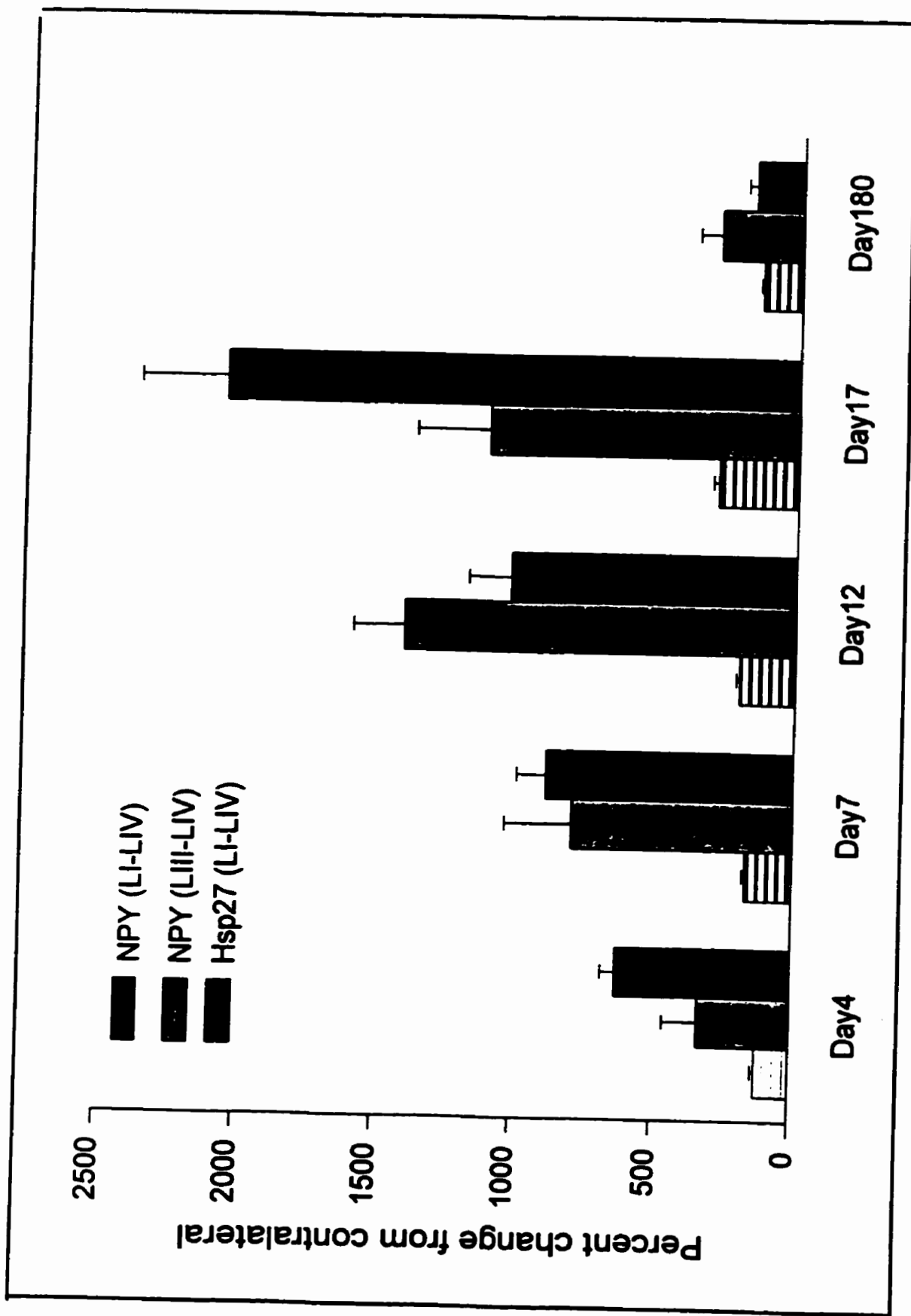


Figure 26

SNL so that it was similar to that seen in the control animals (Fig. 24D).

However, in the gracile nucleus light to moderate NPY-IR was still present 180 days after SNL (Fig. 25D).

Qualitative analysis of the immunoreactive changes in Hsp27 and NPY in both the spinal dorsal horn and gracile nucleus suggested a positive correlation. However, densitometric analysis of the change in immunoreactive labeling in the ipsilateral spinal dorsal horn as compared to that of the contralateral side using a Spearman Correlational analysis failed to confirm a positive correlation in the changes in Hsp27 and NPY (coefficient; $r_s = .91$, $P = .06$).

3.4.4 Changes in SP-IR expression in the spinal dorsal horn and gracile nucleus

In naïve and sham operated animals, dense immunoreactive expression of substance P (SP-IR) was observed in the superficial laminae of the dorsal horn. The labeling consisted of terminal plexus with some staining of the surrounding neuropil. There also appeared to be a lighter region of moderate SP-IR fibers in the area of the lamina II/III interface. The same pattern of expression was observed in the contralateral side in experimental animals (Fig. 27D), and on the ipsilateral side until day 7 when a reduction in the SP-IR was observed in the superficial laminae of the ipsilateral SDH (Fig. 27A). The depletion in SP-IR was most drastic at day 12 (Figs. 27B and 27E) and occurred in both the superficial lamina and in the deeper region of lamina II/III interface. A depletion in SP-IR

Figure 27

Comparison of the changes in immunoreactive expression of substance P (SP-IR) in the spinal dorsal horn of the L6/S1 segmental region. Depletion in the ipsilateral superficial laminae (I and II_o) was first evident at 7 days (A and D), greatest at 12 days (B and E), and still evident at 5 months (C and F). Staining intensity and distribution of naives is similar to that of the contralateral side at day 7 (D). Scale bar: 250 μ m.

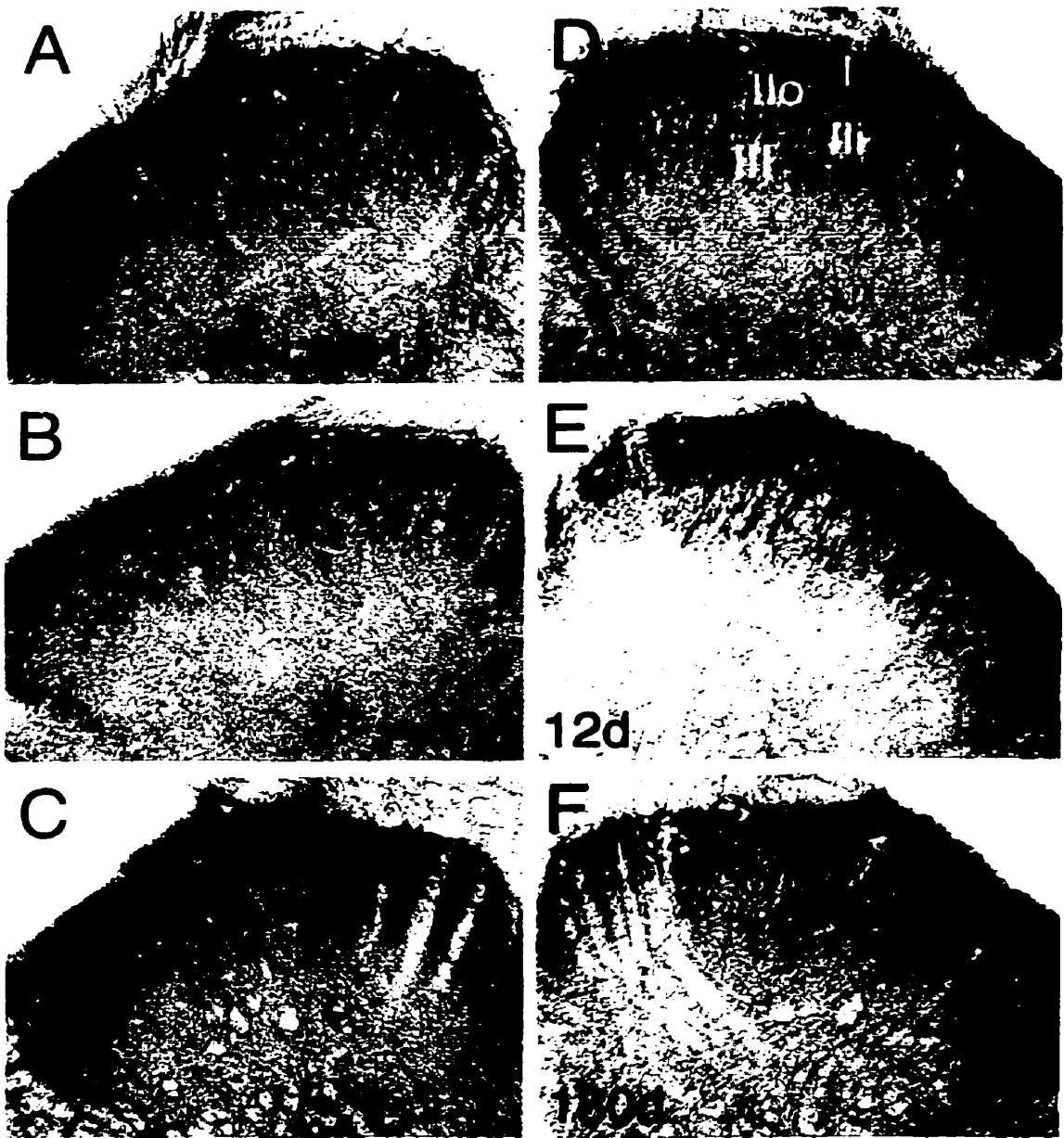


Figure 27

was still observed in the superficial lamina 180 days following injury (Figs. 27C and 27F). Very light SP-IR terminal labeling was observed in the gracile nucleus in all animals. No changes in gracile nucleus SP-IR were evident following nerve injury (not shown).

3.5 Effects of chronic drug administration on behavior and immunohistochemistry following unilateral spinal nerve injury

To determine whether chronic administration of amitriptyline has an effect on nerve injury induced neuropathic pain behaviors, amitriptyline was administered in the drinking water. Caffeine was also administered alone and in combination with amitriptyline to determine if the blocking effect observed in the acute experiments also occurred following chronic administration. Due to limitations imposed by immunohistochemical processing, the groups were divided into two equal series of 4 animals from each treatment group. The data was then pooled after consistency was confirmed between the series.

Amitriptyline hydrochloride is subject to degradation when in aqueous solution and exposed to light for long periods (3-4 days)(Buckles and Walters, 1976). To minimize this effect, drugs were administered in opaque bottles and fresh solutions were given every 48 hours, which falls within the window of the acceptable limit for stability.

3.5.1 *Weight gain, fluid consumption, and drug levels.*

Figure 28 shows the mean +/- SEM weight of all the animals in the first and second series of experiments. No statistical significance was observed

between any of these values for any time point. As shown, all groups gained weight at approximately the same rate. These results suggest that the drug treatment did not adversely effect the continued development of the animals.

Figure 29 depicts the volume of fluid consumed over successive 48 hour periods expressed as the mean \pm SEM of the group. As indicated in the figure, all groups appeared to consume roughly the same volume of fluid over the time periods. These results further suggest that the drug treatment did not adversely effect 'normal' drinking behavior.

Figure 30 depicts the approximate dose of drug received by the respective groups based on the weight, fluid consumption, and initial concentrations of 0.11 mg/ml for amitriptyline, and 0.05 mg/ml for caffeine. Comparison of the drug concentration between groups revealed that animals receiving amitriptyline alone (ami) or in combination with caffeine (ami (combo)) received approximately the same dose (15-18 mg/kg)(Fig 30). Similarly, there was no difference in the dose of caffeine (6-8 mg/kg) obtained by animals receiving caffeine alone (caff) or in combination with amitriptyline (caff (combo)). The initial difference between the groups receiving the drug alone compared to those receiving the combination reflects an initial methodological error, and a dose titration in the first week of the first series. Although the initial differences in the drug doses were significantly different, there were no significant variations in the behavioral effect (see below).

Figure 28

Average weight (kg) of rats, measured every 48 hours, in each treatment group over the course of the chronic drug administration period (21 days). Values represent the mean \pm SEM of n=8 for each of amitriptyline (ami), caffeine (caff), combination (ami+caff) and control groups.

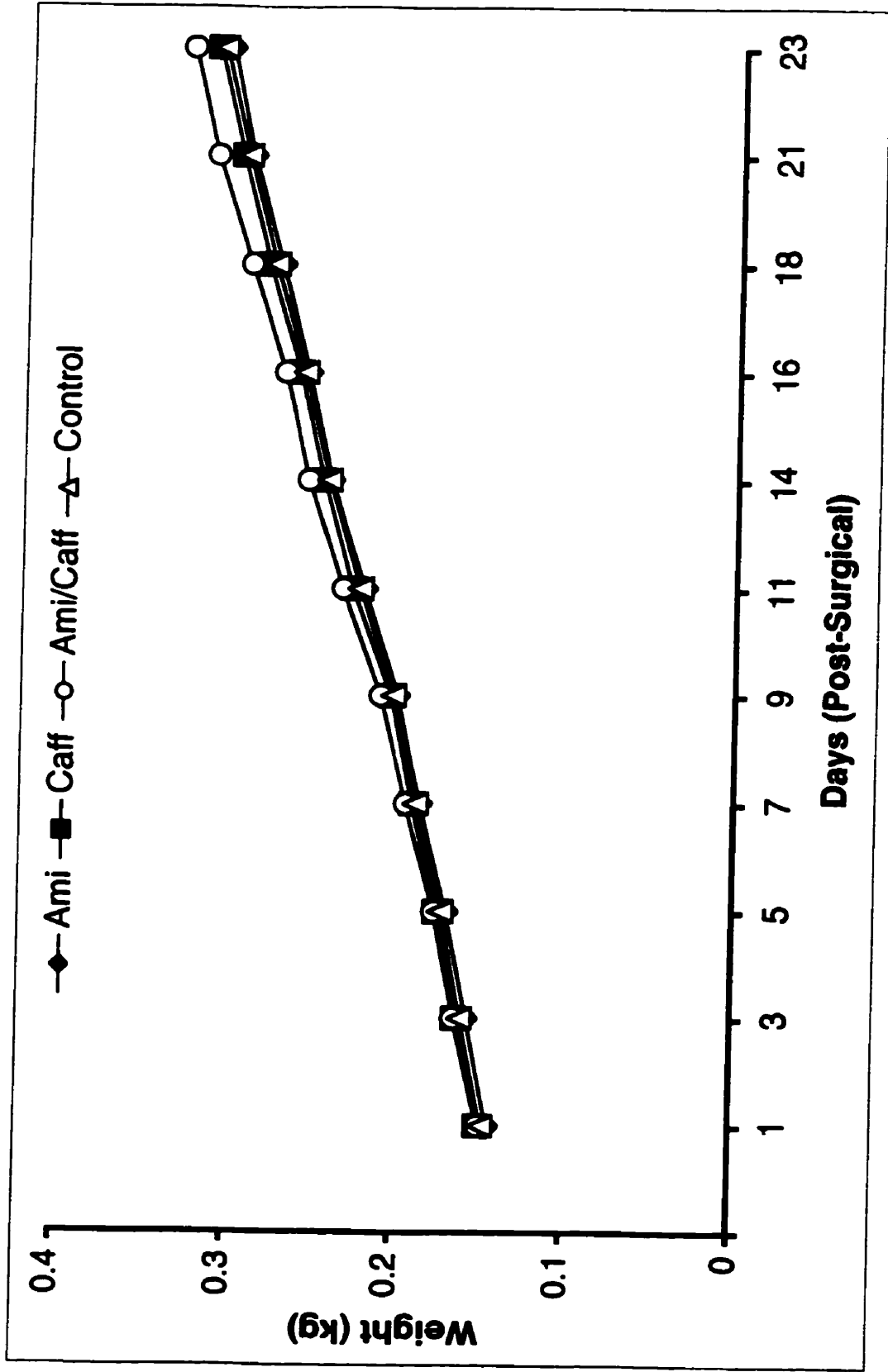


Figure 28

Figure 29

Average fluid consumption (ml/day), measured at 48 hour intervals, in each treatment group over the course of the chronic drug administration period (21 days). Values represent the mean \pm SEM of n=8 for each of amitriptyline (ami), caffeine (caff), combination (ami+caff) and control groups.

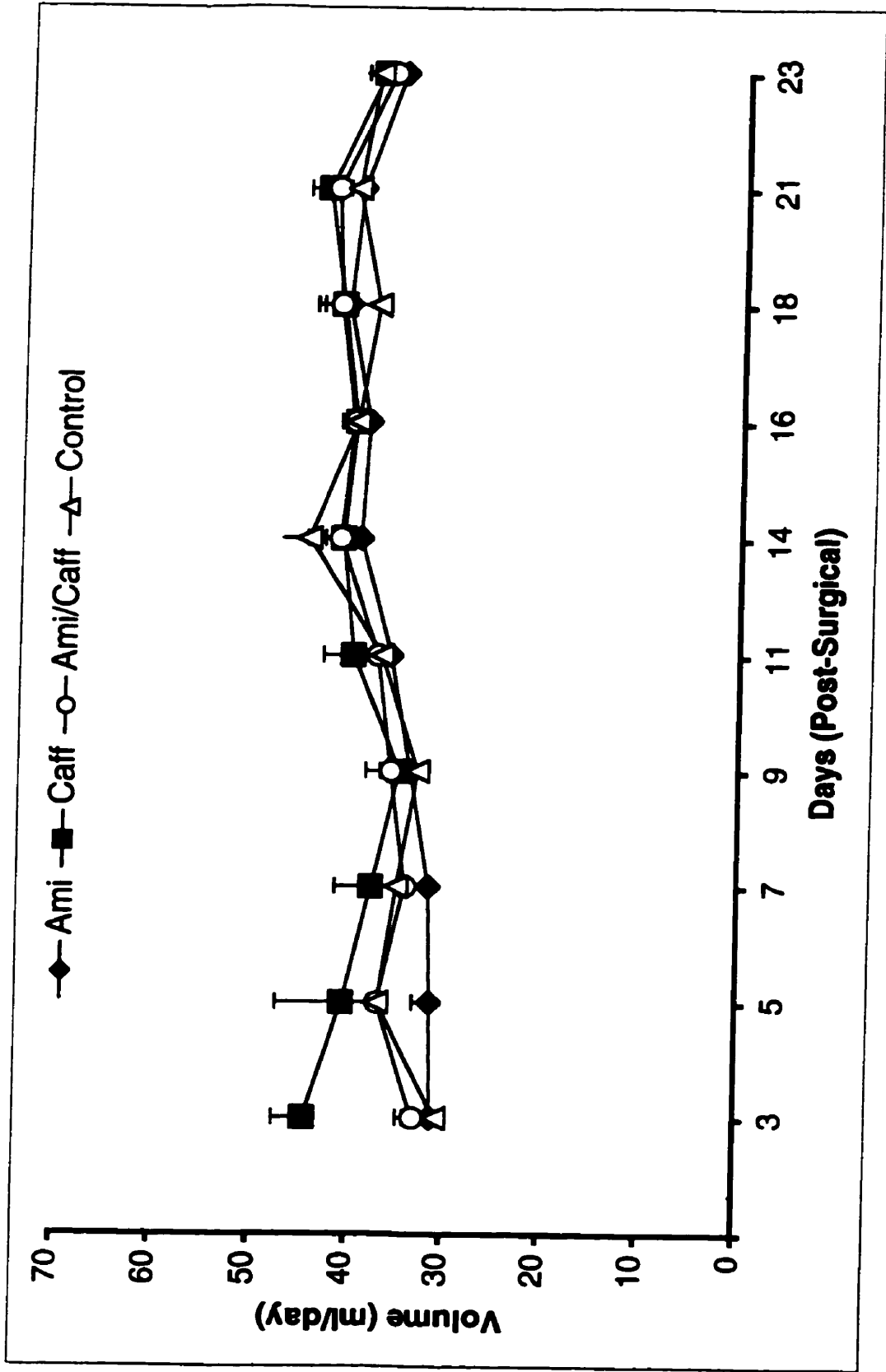


Figure 29

Figure 30

Graph of drug concentration of amitriptyline (11 mg/ml) and caffeine (0.05 mg/ml) administered in the drinking water over the 21 day evaluation period. Values depict the mean \pm SEM of n=8 animals, for each group at each time point. Lines represent the concentration of amitriptyline when administered alone (ami) and in combination with caffeine (ami (combo)), and caffeine when administered alone (caff) and in combination with amitriptyline (caff (combo)).

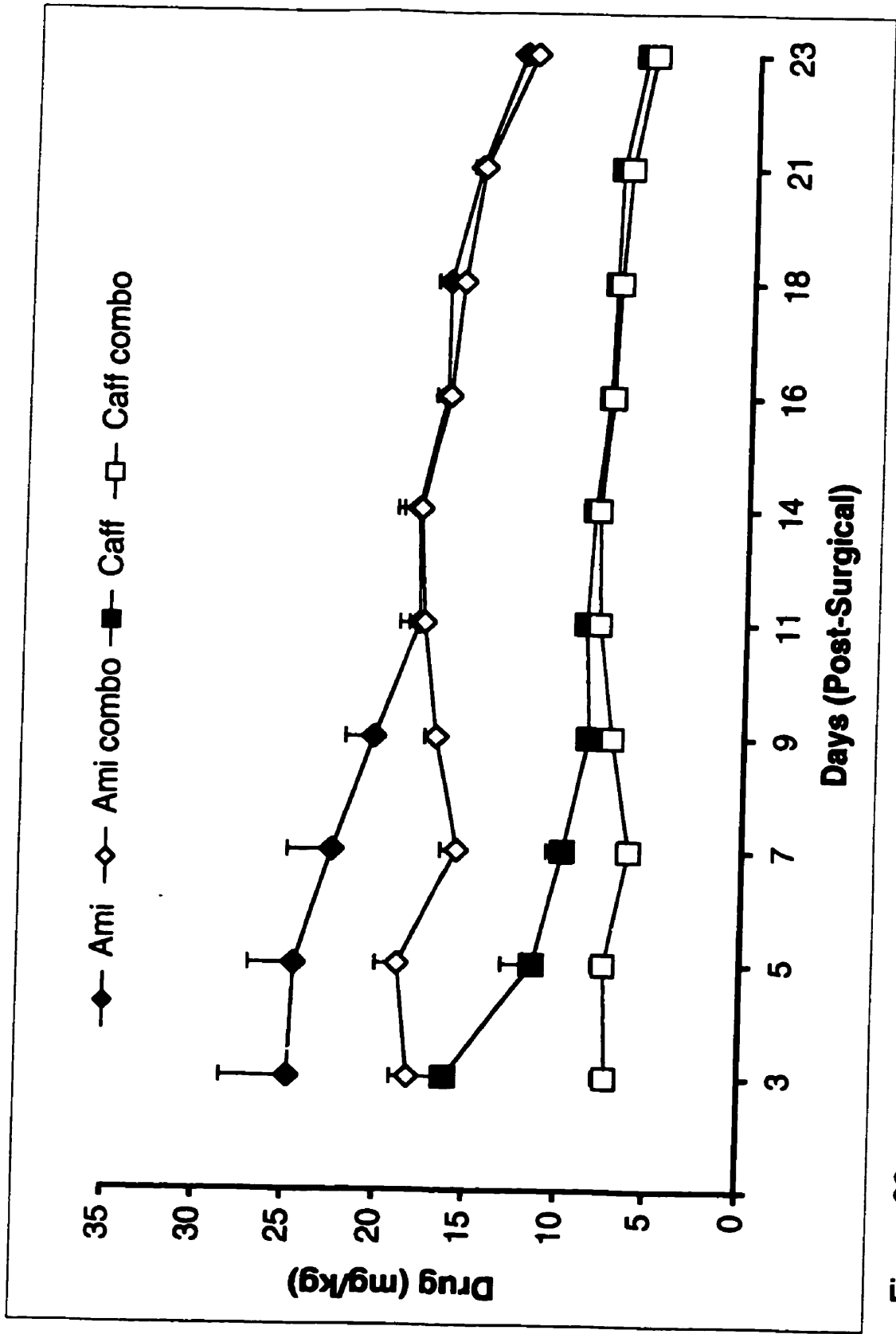


Figure 30

3.5.2 Behavioral effects of chronic drug administration

Assessment of the behavioral effects of chronic drug treatment involves an appreciation of the actions of the drug on each paw as well as changes in the difference value between the paws. The results from the pooled groups for the chronic study are shown in Figures 31 and 32. The control groups (neuropathic, no drug) were first compared to the naïve groups to ensure a significant difference in the response thresholds and therefore expression of the neuropathic pain behavior. At each time point for both thermal hyperalgesia and static tactile allodynia a statistical comparison was made for each of the paws as well as the difference score against the respective values in the control group (indicated by * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). A comparison was also made between respective values in the amitriptyline-caffeine combination group and those on the amitriptyline only group (indicated by t $P < 0.05$, tt $P < 0.01$, ttt $P < 0.001$).

3.5.2.1 Thermal hyperalgesia

Seven days following surgery and chronic drug administration rats in the drug treatment groups were showing signs of differences in thresholds as compared to the controls (Fig. 31A). In control animals, the value of the ipsilateral paw was significantly reduced from either the contralateral paw or naïve animals, indicating a robust neuropathic condition. Caffeine treated animals exhibited a reduced ipsilateral paw threshold and greater paw difference value, as compared to the controls (Fig. 31A). Of all groups, the caffeine animals demonstrated the strongest evidence of neuropathic pain. On the other hand, compared to controls, chronic amitriptyline had a greater ipsilateral paw

threshold, a lower contralateral paw threshold, and less of a difference between paws (Figure 31A). Combination of caffeine with amitriptyline partially inhibited the anti-hyperalgesic effect of amitriptyline (Fig. 31A). The difference between the paws in the combination group was not significantly different from that of the amitriptyline group, but this may have been a result of the significant decrease in the response threshold of contralateral paw in the combination group.

After two weeks of chronic drug administration, the same general trends were observed as seen at day 7, with some important additions (Fig. 31B). Thus, the caffeine group again demonstrated the most robust thermal hyperalgesia as evidenced by the lowest ipsilateral paw threshold, and the greatest paw difference score (Fig. 31). Additionally, in the caffeine group, the contralateral paw threshold was greater than that observed in naïve rats, suggesting that the animals avoided bearing weight on the injured paw. Compared to controls, the amitriptyline group had a greater ipsilateral paw threshold, a lower contralateral paw threshold, and a lower paw difference score (Fig 31B). Combination of caffeine with amitriptyline again caused a block of the thermal anti-hyperalgesic effect of amitriptyline, an effect that was greatest at this time point.

At the third week (Day21) of the study the results were still comparable to those observed at the first two time points (Fig. 31C). The most robust thermal hyperalgesia was observed in the control and caffeine treated animals (Fig. 31C). Chronic amitriptyline again blocked expression, or development, of thermal anti-hyperalgesia. The effect of amitriptyline was blocked by co-administration of caffeine (Fig. 31C) but not to the extent of that observed at 14 days.

Figures 31A, 32B, 33C

Histograms showing the effect of chronic drug administration of amitriptyline (ami :15-18 mg/kg/day) and caffeine (caff: 6-8 mg/kg/day) alone and in combination (ami+caff) on nerve injury-induced thermal hyperalgesia of the ipsilateral paw (ipsi), the thermal withdrawal threshold of the contralateral paw (cntra), and the difference between the two responses (diff; where diff=ipsi-cntra). Analysis was made at 7 (A), 14 (B), and 21 (C) days after surgery. Naïve animals had no surgical or drug intervention, while control animals received spinal nerve ligation surgery but no drug (normal drinking water). The treatment groups are identified below the respective group of three histograms. Histogram values represent the mean \pm SEM of n=4 for the naïve group and n=7-8 for control and treatment groups. #P<0.05, ##P<0.01, ###P<0.001 compared to naïve, *P<0.05, **P<0.01, ***P<0.001 compared to control, tP<0.05, ttP<0.01, tttP<0.001 for combination group (ami+caff) compared to the appropriate amitriptyline value (ami).

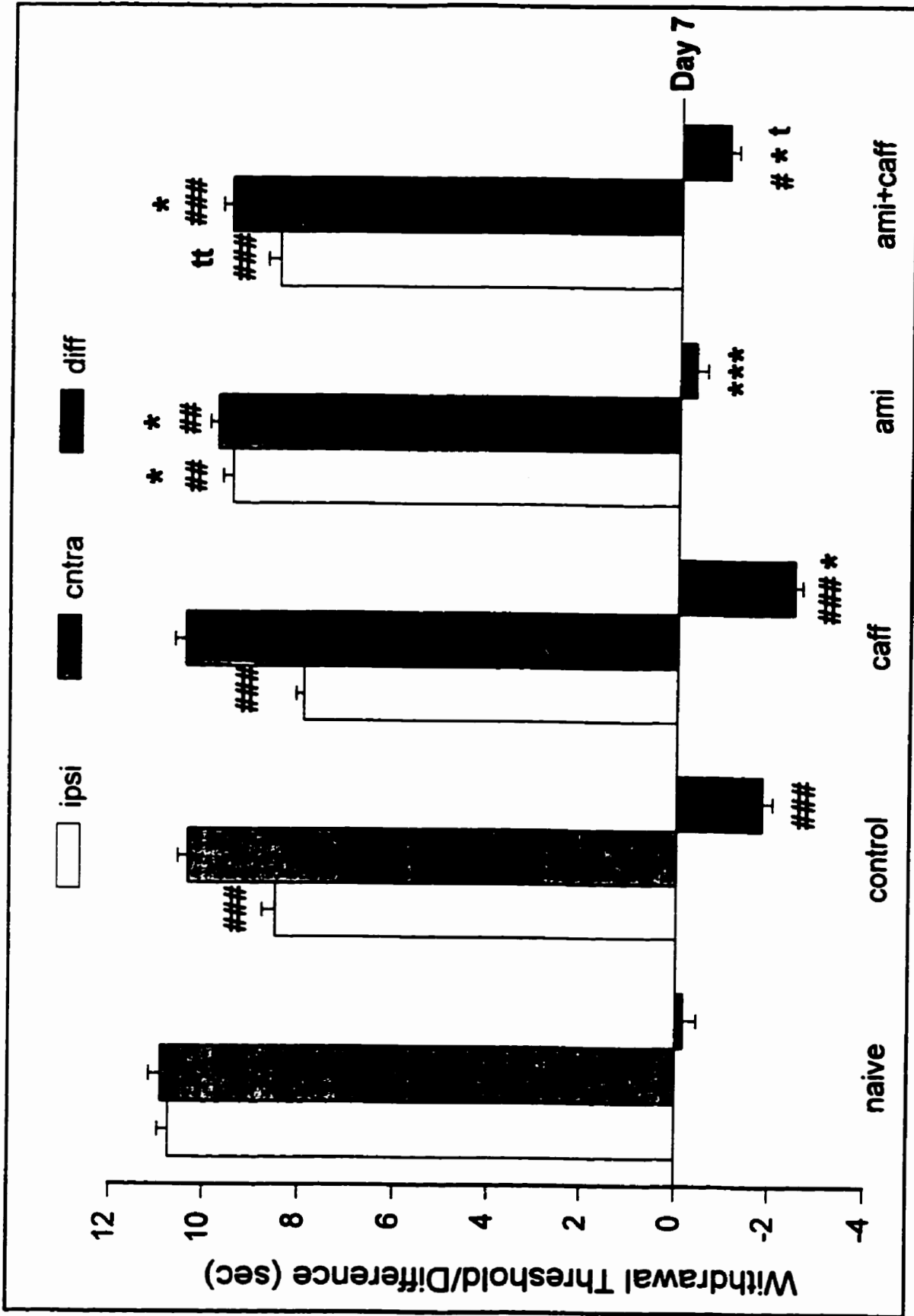


Figure 31A

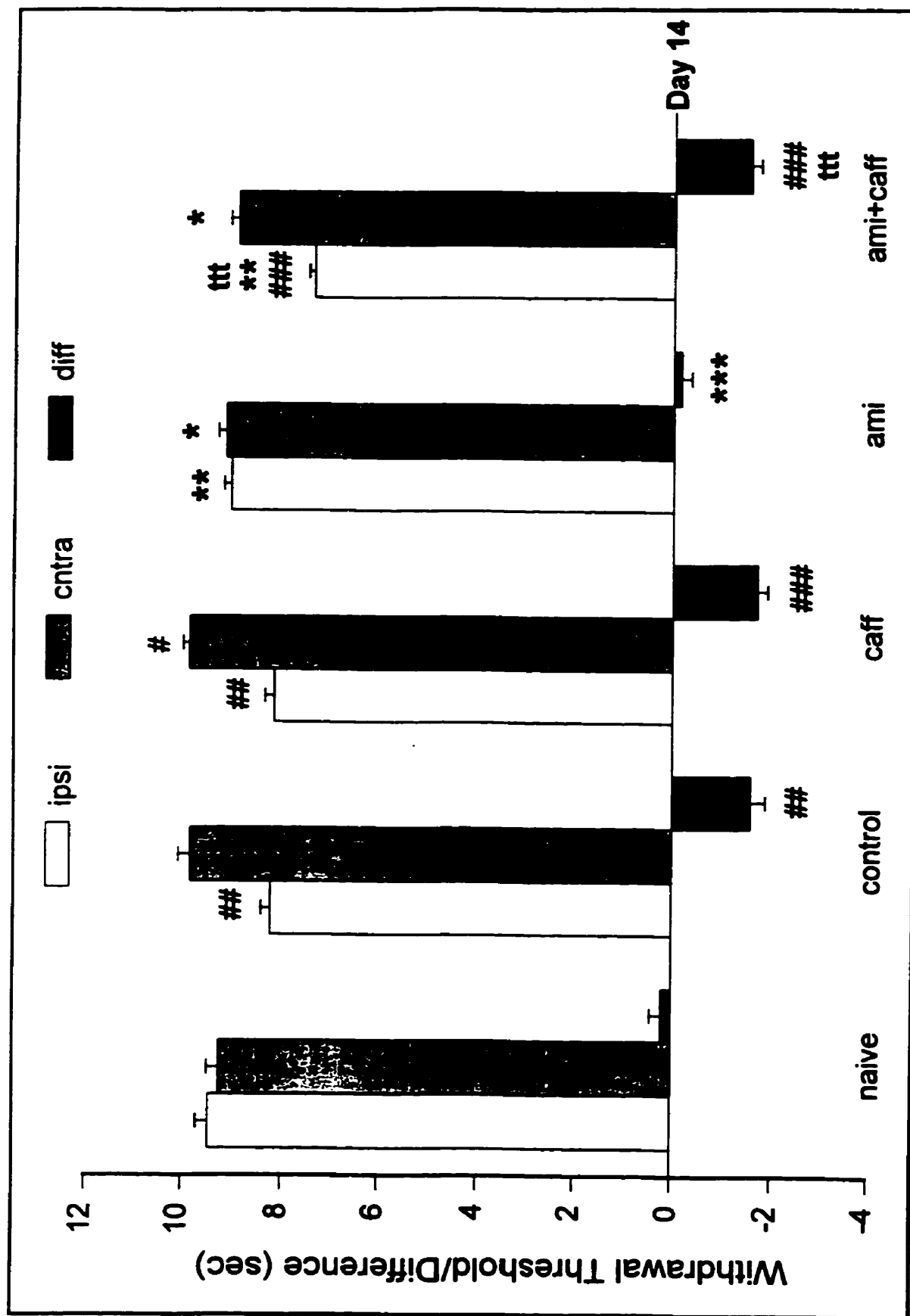


Figure 31B

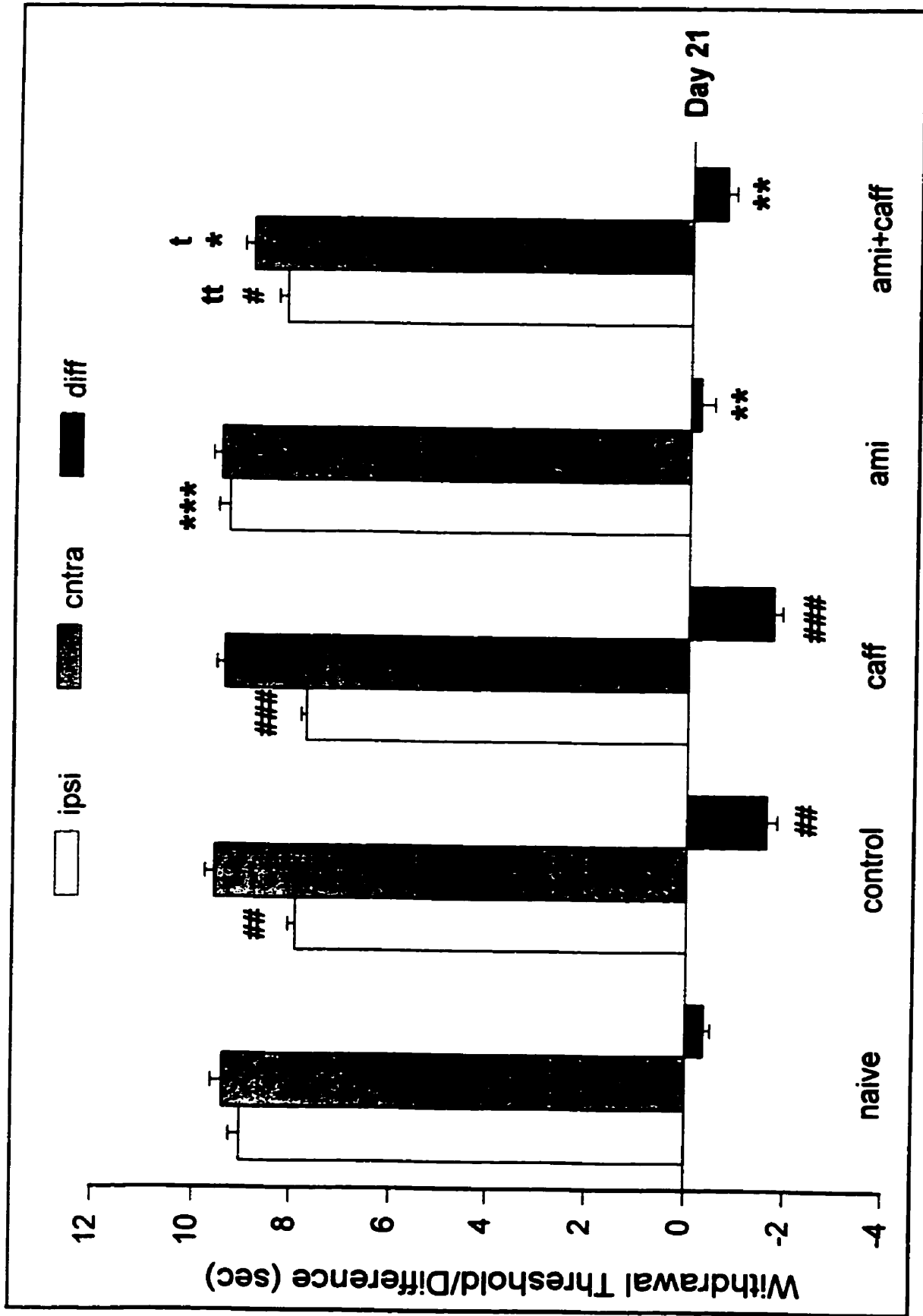


Figure 31C

3.5.2.2 Static tactile allodynia.

At day 7 there was no difference in the ipsilateral paw threshold for any of the treatment groups compared to the controls. However a hyperaesthetic effect on the contralateral paw in both the amitriptyline and amitriptyline-caffeine combination groups was observed at all time points (Fig.32A). As in the acute amitriptyline experiments (section 3.2.5), the contralateral response threshold was significantly lower than that of control animals. Qualitative assessment of the response however differentiated it from that observed in the neuropathic paw. The hyperaesthetic response typically involved a rapid withdrawal from the stimulus but with none of the secondary behaviors observed in the neuropathic paws (i.e. shaking, biting, licking and guarding). Animals in the amitriptyline had a significantly lower contralateral paw withdrawal threshold from that observed in the control (Fig. 32A). A further decrease in the contralateral paw threshold was observed in the combination group so that the value was significantly less than that of any other treatment groups including amitriptyline alone (Fig. 32A). The net result of these lower contralateral paw thresholds is a reduction in the paw difference scores (Fig. 32A), as compared to controls, which taken alone could be interpreted as an anti-allodynic effect.

At day 14, the same general trends were observed as in day 7. As such, there was no effect on the ipsilateral paw threshold, yet there was a significant hyperaesthetic effect observed in the contralateral paw of both the amitriptyline, and amitriptyline-caffeine combination groups (Fig. 32B). Once again caffeine coadministration with amitriptyline resulted in a significantly lower contralateral paw threshold as compared to the amitriptyline alone group (Fig. 32B).

Figures 32A, 32B, 32C

Histograms showing the effect of chronic drug administration of amitriptyline (ami: 15-18 mg/kg/day) and caffeine (caff: 6-8 mg/kg/day) alone and in combination (ami+caff), on nerve injury-induced static tactile allodynia of the ipsilateral paw (ipsi), tactile withdrawal threshold of the contralateral paw (cntra), and the difference between the two responses (diff; where diff=ipsi-cntra). Analysis was made at 7 (A), 14 (B), and 21 (C) days after surgery. Naïve animals had no surgical or drug intervention, while control animals received spinal nerve ligation surgery but no drug (normal drinking water). The treatment groups are identified below the respective group of three histograms. Histogram values represent the mean \pm SEM of n=4 for the naïve group and n=7-8 for control and treatment groups. #P<.05, ##P<.01, ###P<.001 compared to naïve, *P<.05, **P<.01, ***P<.001 compared to control, tP<.05, ttP<.01, tttP<.001 for combination group (ami+caff) compared to the appropriate amitriptyline histogram (ami).

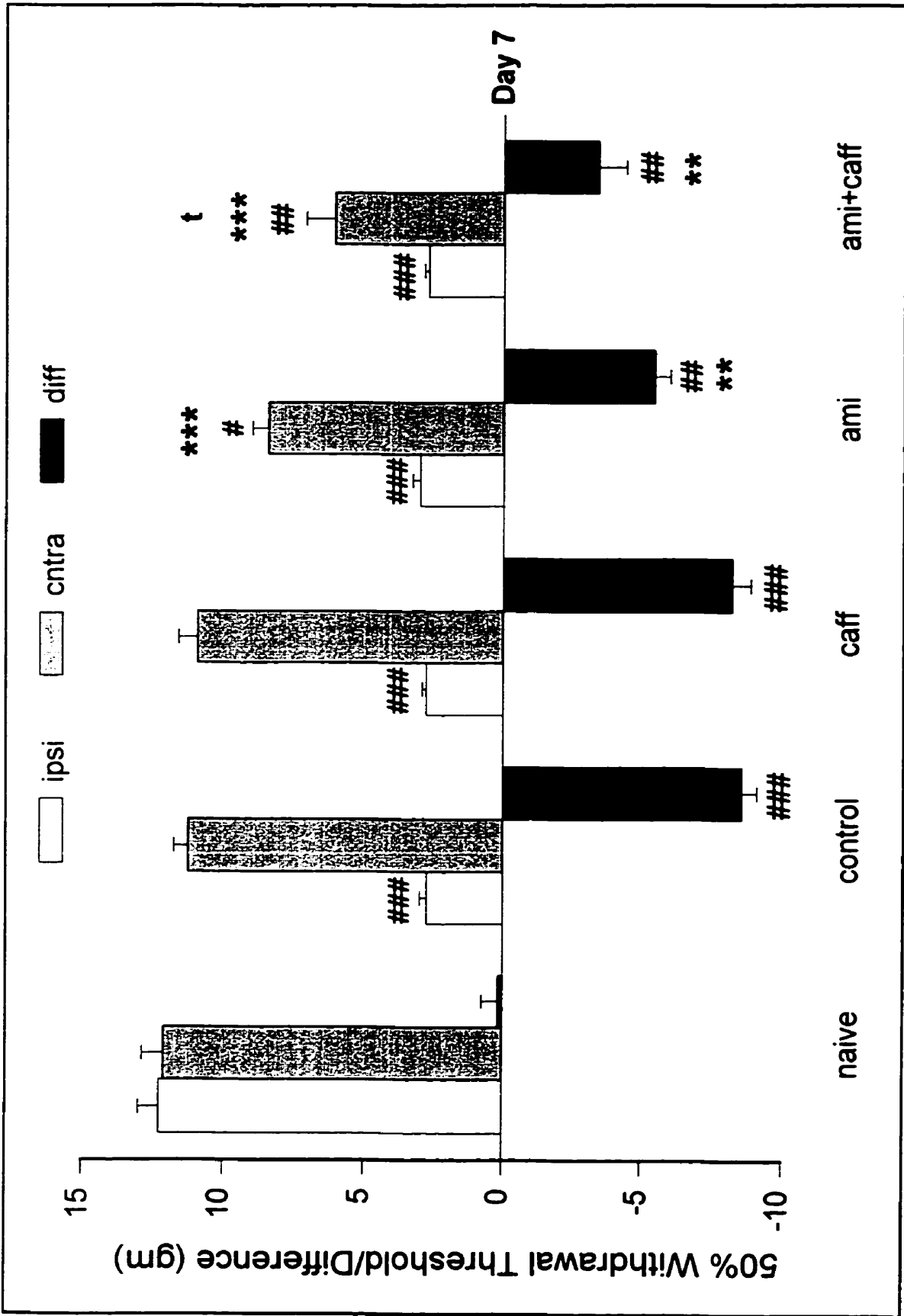


Figure 32A

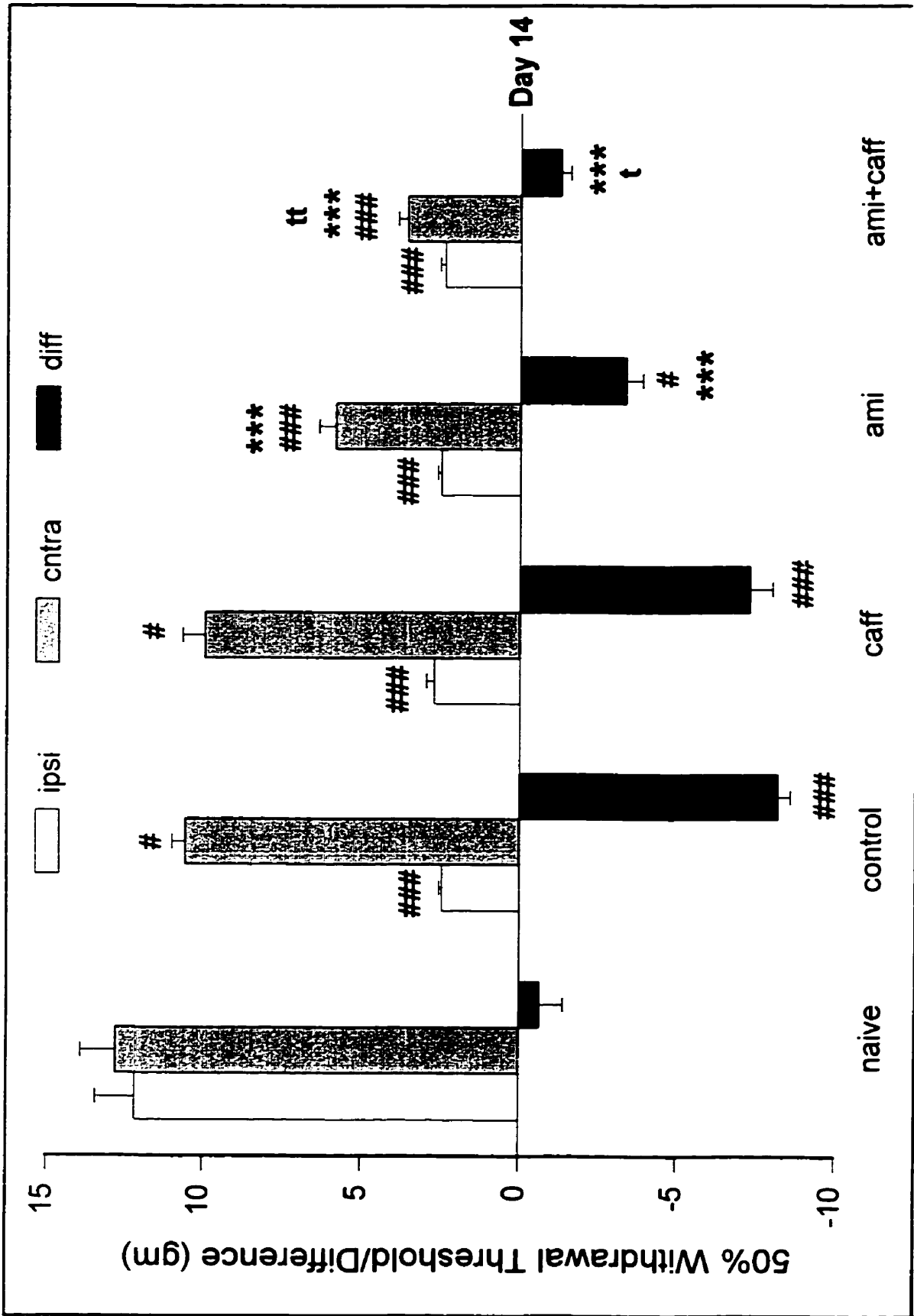


Figure 32B

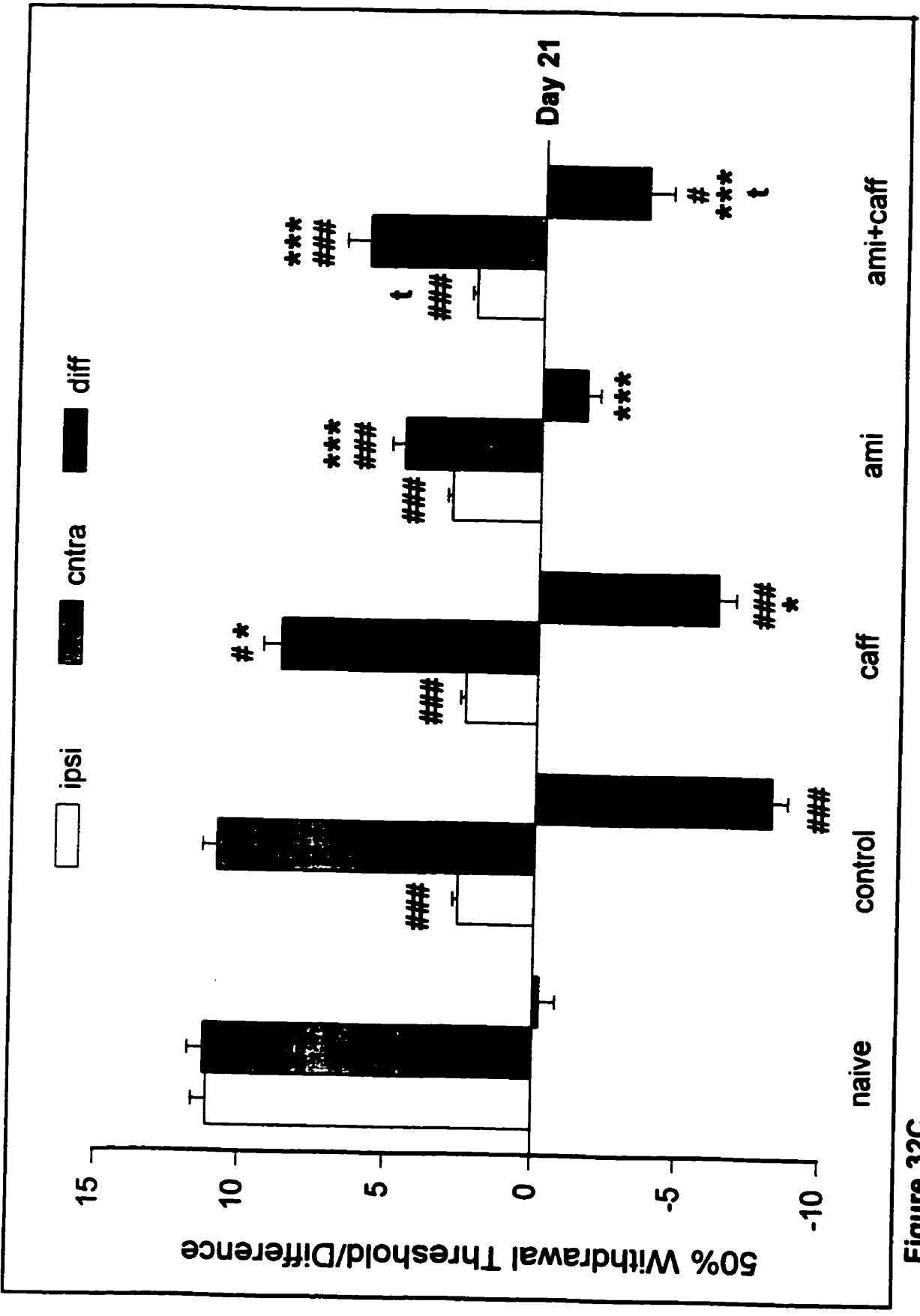


Figure 32C

By day 21 (Fig. 32C), the amitriptyline-caffeine group no longer had an exacerbating effect on the contralateral paw hyperaesthesia as compared to amitriptyline alone. Thus, while both groups were significantly hyperaesthetic as compared to controls, they were not different from each other (Fig. 34C).

3.6 Effect of chronic drug administration on Hsp27-IR, NPY-IR, and SP-IR in the spinal dorsal horn and gracile nucleus

To determine if the anti-hyperalgesic and hyperaesthetic effect of the chronic drug treatments were symptomatic, or whether they were manifestations of changes in the underlying pathophysiology, we examined changes in immunoreactive expression of several immunohistochemical markers. This analysis was made 24 hours after the final behavioral test to minimize manipulation-induced effects on the expression (i.e. at day 22 after surgery). For the purpose of this thesis, analysis was restricted to the lower lumbar and upper sacral sections of the spinal cord, but included the full extent of the gracile nucleus.

3.6.1 Hsp27 immunoreactivity in the spinal dorsal horn and gracile nucleus.

In the ipsilateral spinal dorsal horn of control animals Hsp27-IR in the lower lumbar and upper sacral segments consisted of dense expression in the superficial lamina and in the lamina II/III interface. In lamina II, however, there appeared to be a reduction in the intensity of the Hsp27-IR expression (Fig. 33A). This same pattern of Hsp27-IR expression was observed in ipsilateral SDH of the caffeine group (Fig. 33B). In the amitriptyline group (Fig.33C), there was dense

Figure 33

Comparison of the effect of chronic drug administration on Hsp27-IR expression in the spinal dorsal horn in the L6/S1 region following unilateral spinal nerve ligation. Panel (A) is a representation of the Hsp27-IR expression in nerve-injured animals receiving only drinking water. Subsequent panels depict the effect of chronic caffeine (caff: 6-8 mg/kg/day)(B), amitriptyline (ami: 15-18 mg/kg/day)(C) alone, and in combination (ami+caff)(C) administered in the drinking water. Special attention is directed at the lamina III/IV region of the ipsilateral dorsal horn (arrows). Scale bar: 100 μ m.

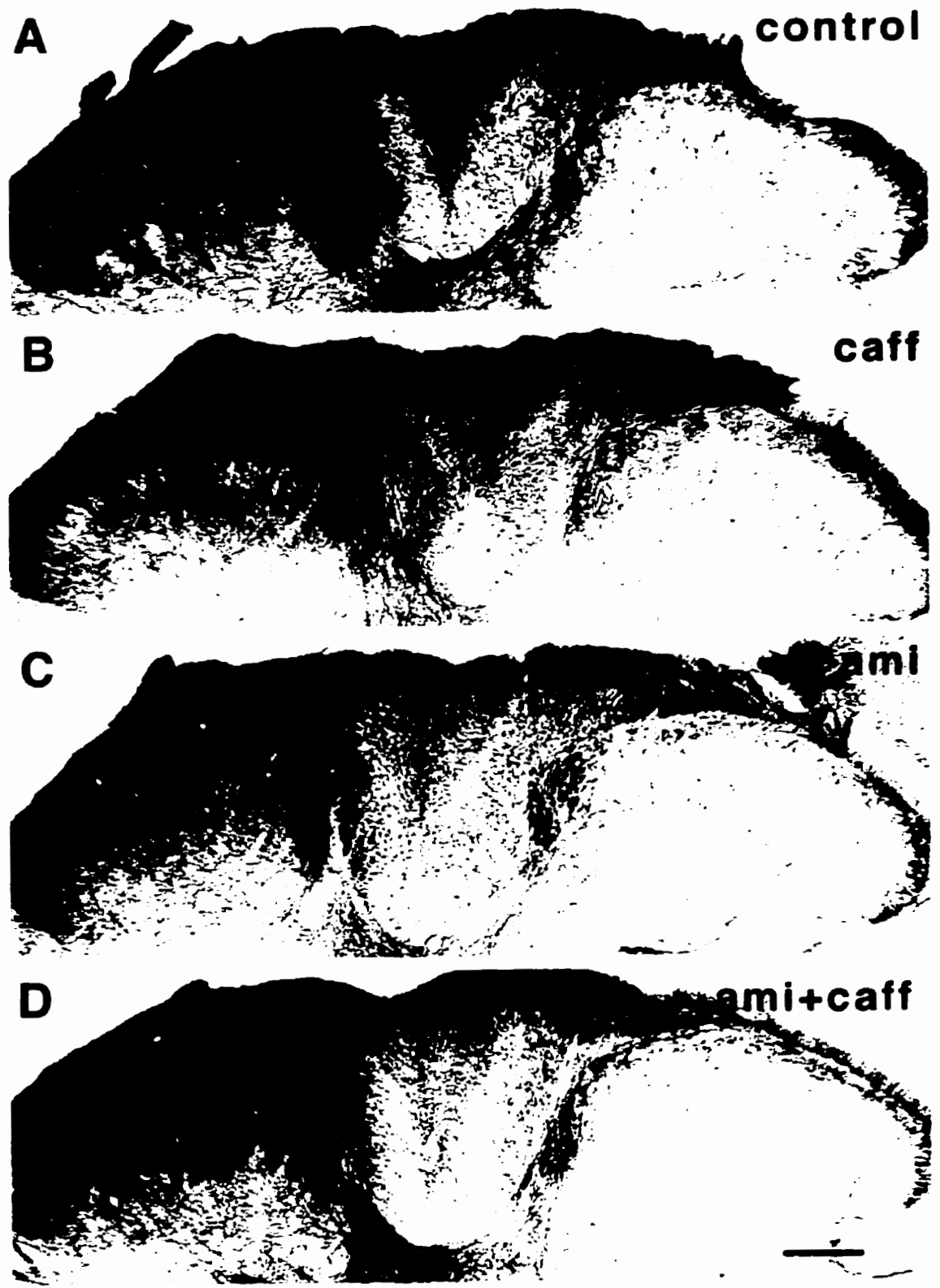


Figure 33

Figure 34

Comparison of the effect of chronic drug administration on Hsp27-IR expression in the gracile nucleus at the caudal level of the area postrema, following unilateral spinal nerve ligation. Panel (A) is a representation of the Hsp27-IR expression in nerve-injured animals receiving only drinking water. Subsequent panels depict the effect of chronic caffeine (caff: 8-10 mg/kg/day)(B), amitriptyline (ami: 15-20 mg/kg/day)(C) alone, and in combination (ami+caff)(C) administered in the drinking water. Special emphasis is given to the increased expression in the contralateral gracile nucleus (arrows). Scale bar: 80 μ m.

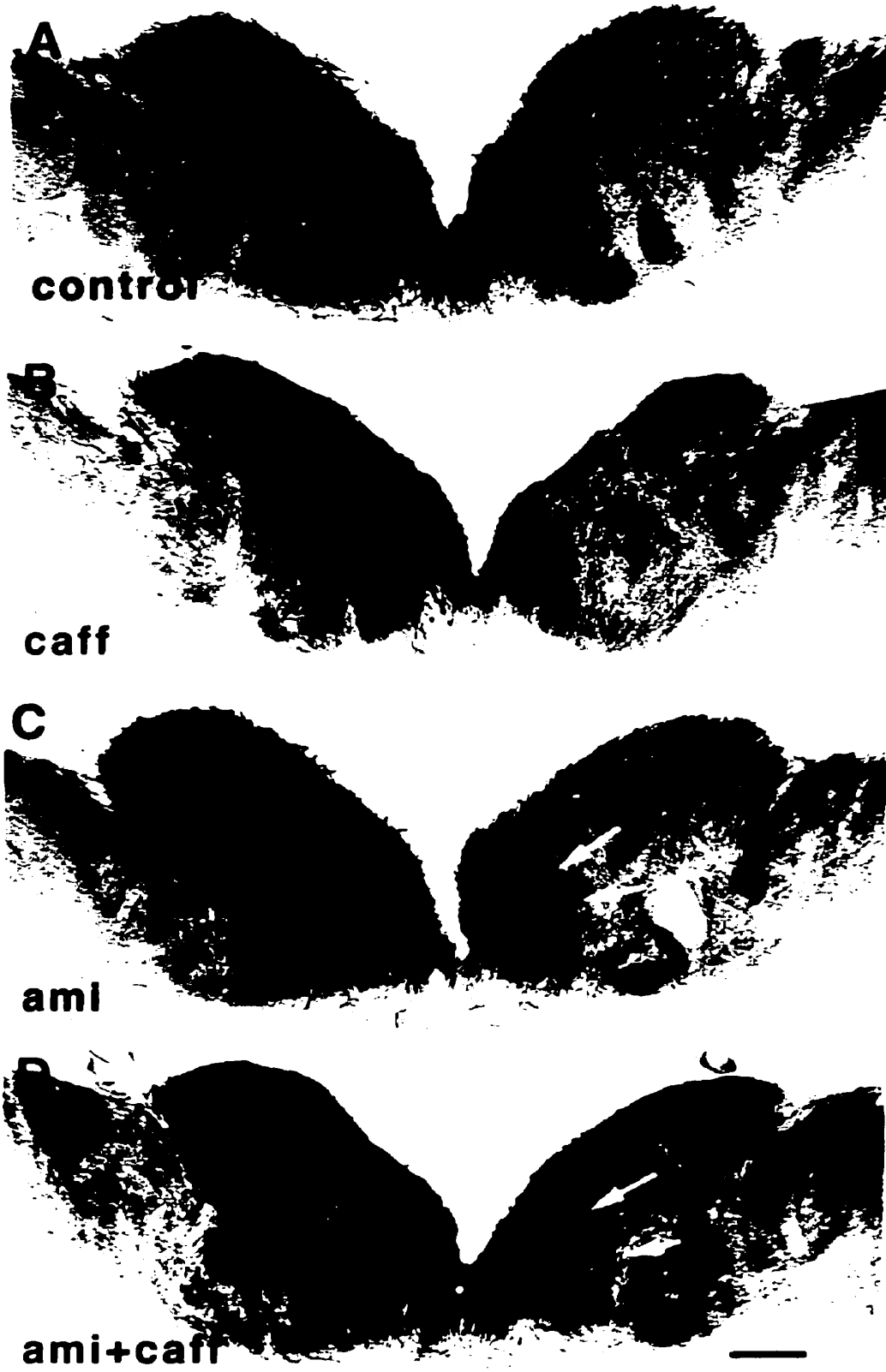


Figure 34

expression in the superficial lamina, as observed in the control and caffeine groups, but the labeling in laminae II, III and IV was homogeneous so that the dense band of labeling at the lamina II/III interface was no longer apparent. In the combination group (Fig. 33D), Hsp27-IR in the ipsilateral SDH was similar to that observed in the control and caffeine groups.

In the contralateral SDH, there appeared to be an increase in Hsp27-IR labelling in the medial 1/3rd region of the lamina II/III interface in both the amitriptyline (Fig. 33C) and combination groups (Fig. 33D) as compared to either the control or caffeine groups. In these same groups there also appeared to be an increase in the Hsp27-IR in the superficial laminae especially the lateralmost region.

In the ipsilateral gracile nucleus, Hsp27-IR was increased in the amitriptyline and combination groups compared to the control and caffeine groups (Fig. 34). This difference was most evident at the caudal level of the area postrema, but was observed through the rostral extent of the nucleus. In this region there also appeared to be an increase in the Hsp27-IR labeling in the medial region of the contralateral gracile nucleus.

3.6.2 NPY immunoreactivity (NPY-IR) in the spinal dorsal horn and gracile nucleus

Following drug treatment there appeared to be a reduction in NPY-IR in the lamina II/III interface in the ipsilateral SDH of the amitriptyline animals (Fig. 35C). Furthermore, whereas in the control, caffeine, and combination groups dense immunoreactive expression was observed in the superficial laminae and

the lamina II/III interface, in the amitriptyline group, dense NPY-IR expression was only observed in the superficial laminae (Fig. 35). The labeling consisted of punctate terminal-like endings and fibers.

Contralateral SDH immunoreactive expression of NPY consisted mainly of a thin region of terminal like endings in the superficial laminae. In the amitriptyline and control groups however, there appeared to be an increase in the extent of the labeling in the medial 1/3rd of the lamina II/III interface and at the lateralmost region of the superficial laminae (Fig. 35C and 35D).

In the ipsilateral gracile nucleus, increased NPY-IR was observed through the extent of the nucleus in the amitriptyline and combination groups as compared to that observed in the control and caffeine groups (Fig. 36C and 36D).

3.6.3 SP immunoreactivity (SP-IR) in the SDH and gm

Changes in the SP-IR expression following nerve injury (data not shown) were less obvious than those observed with either Hsp27-IR or NPY-IR. Nonetheless there appeared to be an increase in SP-IR in the lamina II/III interface of the ipsilateral dorsal dorsal horn although there was considerable variation between animals.

Figure 35

Comparison of the effect of chronic drug administration on NPY-IR expression in the spinal dorsal horn in the L6/S1 region following unilateral spinal nerve ligation. Panel (A) is a representation of the Hsp27-IR expression in nerve-injured animals receiving only drinking water. Subsequent panels depict the effect of chronic caffeine (caff: 8-10 mg/kg/day)(B), amitriptyline (ami: 15-20 mg/kg/day)(C) alone, and in combination (ami+caff)(C) administered in the drinking water. Arrows indicate special emphasis on the lamina III/IV region of the ipsilateral dorsal horn (black arrows), and lamina II/III of the contralateral dorsal horn (white arrows). Scale bar: 100 μ m.

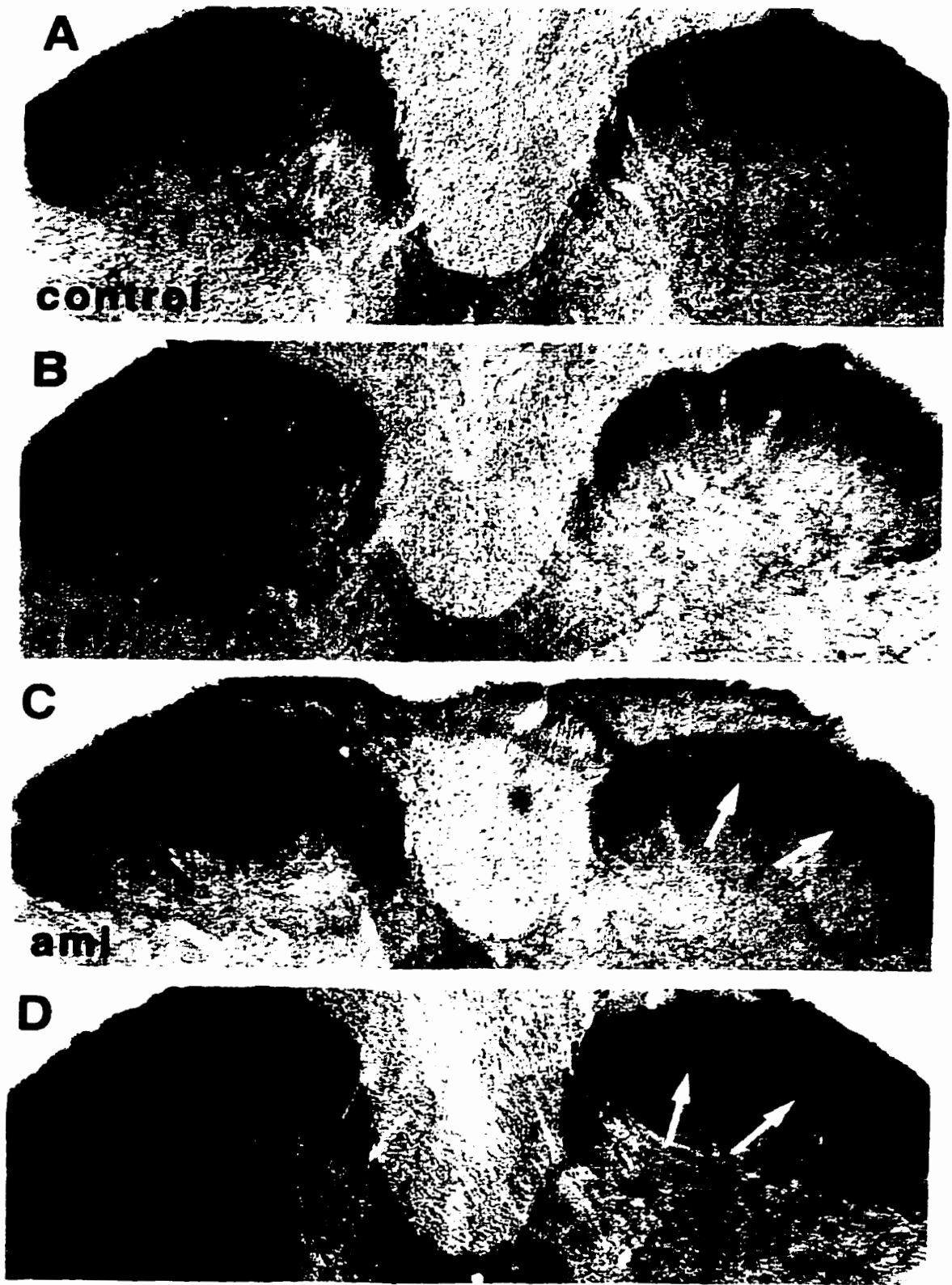


Figure 35

Figure 36

Comparison of the effect of chronic drug administration on NPY-IR expression in the gracile nucleus, at the caudal level of the area postrema, following unilateral spinal nerve ligation. Panel (A) is a representation of the Hsp27-IR expression in nerve-injured animals receiving only drinking water. Subsequent panels depict the effect of chronic caffeine (caff: 8-10 mg/kg/day) (B), amitriptyline (ami: 15-20 mg/kg/day) (C) alone, and in combination (ami+caff) (C) administered in the drinking water. Special emphasis is given to the increased expression in the ipsilateral gracile nucleus in the amitriptyline treated animals (arrows). Scale bar: 80 μ m.



Figure 36

DISCUSSION

The underlying purpose of this dissertation was to determine whether ADs were effective in alleviating symptoms of neuropathic pain in a rat model of nerve injury. Additionally, the study was designed to determine whether the relief afforded by ADs was primarily symptomatic, or a result of effects on the underlying pathophysiology. Also of interest was a determination of whether manipulation of endogenous adenosine contributes to the action of various ADs.

The principal findings of this thesis are as follows: (1) Acute administration of amitriptyline and desipramine is effective in alleviating thermal hyperalgesia through multiple routes of administration. (2) The thermal anti-hyperalgesic effect of amitriptyline, but not desipramine, is partially blocked by caffeine administration at the same sites. (3) Hsp27-IR and NPY-IR show a positive correlation in their increased expression in the spinal dorsal horn and gracile nucleus. (4) Chronic administration of amitriptyline alleviates thermal hyperalgesia, and this effect is blocked by chronic administration of caffeine. (5) Chronic administration of amitriptyline also causes hyperaesthesia in the contralateral paw, an effect that is exacerbated by combination with chronic caffeine administration. (6) The behavioral effects of chronic amitriptyline administration are mirrored by changes in immunoreactivity of Hsp27 and NPY in the spinal dorsal horn.

Collectively, the results of this thesis suggest that ADs (in particular amitriptyline) may be effective in treating neuropathic pain symptoms of thermal hyperalgesia but not those of static tactile allodynia. With respect to the latter symptom, amitriptyline may actually cause an untoward effect (contralateral

tactile hyperaesthesia). The results also suggest that part of the efficacy of amitriptyline involves a manipulation of endogenous adenosine levels, an action that may be amenable to enhancement through combination with other agents such as adenosine kinase or deaminase inhibitors. In contrast, the ability of caffeine to block the beneficial anti-hyperalgesic action of amitriptyline suggests that caution may need to be exercised in patients using ADs for the treatment of neuropathic pain and who regularly consume caffeine from various sources. This possibility needs to be verified in controlled clinical trials.

4.1 Symptom and route effect of acute ADs

The results of the behavioral studies indicate that amitriptyline produces heterogeneous effects on different manifestations of neuropathic pain and may therefore be useful for the treatment of some, but not all, neuropathic pain symptoms. Specifically, in the rat model of unilateral spinal nerve ligation, amitriptyline produced an almost complete reversal of thermal hyperalgesia, but had no effect on static mechanical allodynia. Site and test specific actions of amitriptyline have been described previously in studies using models of acute nociception (tail flick, hot plate, vocalization) (Dirksen et al., 1994; Korzeniewska-Rybicka and Plaznik, 1998), but few studies have examined the profile of amitriptyline in this model of neuropathic pain. While other studies of neuropathic pain have shown amitriptyline to be active against hyperalgesia (Ardid and Guilbaud, 1992; Courteix et al., 1994), they did not report any effect against allodynia. Recently, two studies have reported an anti-allodynic effect of

systemic amitriptyline (Abdi et al., 1998; Field et al., 1999), but the magnitude of the effect was minimal in terms of the extent of the condition, and dose related effects were not shown. Additionally, the methods for determining the allodynic threshold were different from that used in this study and this may have also contributed to the discrepancy in the anti-allodynic effects of amitriptyline between those studies and this thesis. The findings of these studies, in conjunction with the present results, may partially explain the equivocal clinical results seen with amitriptyline and other ADs in the treatment of neuropathic pains (Ollat and Cesaro, 1995; McQuay et al., 1996), since it may act preferentially against some symptoms and not others.

The results in this thesis also indicate that the thermal anti-hyperalgesic effect of amitriptyline occurs following systemic, spinal, or local administration while the effect of desipramine occurs following systemic and local administration (spinal route not tested). In all cases, the anti-hyperalgesic effects of amitriptyline and desipramine were observed without a concomitant analgesic effect in the contralateral paw. When administered systemically, amitriptyline almost completely reversed the nerve injury-induced thermal hyperalgesia while desipramine had less of an effect. While this effect may be due to dose limitations, further testing was precluded by the occurrence of sedative effects. The spinal site of action of amitriptyline against thermal hyperalgesia shown in this study has already been demonstrated in the carrageenan model of persistent inflammation (Eisenach and Gebhart, 1995a), and in acute nociceptive tests (Hwang and Wilcox, 1987). The present study also demonstrates a local peripheral thermal anti-hyperalgesic effect of both amitriptyline and desipramine,

the duration of which was similar to that observed following systemic injection, but with a longer duration. The local nature of this effect was verified when injections into the contralateral paw did not have any effect on ipsilateral response latencies. Furthermore this local effect did not appear to be a result of the potential local anesthetic-like actions of tricyclic ADs (Deffois et al., 1996), since no change was seen in the thermal threshold of (a) the contralateral paw following local administration into that paw, or (b) the paw of naive animals following local administration. This finding is in agreement with the local actions of amitriptyline and desipramine in the rat formalin model (Sawynok et al., 1999a, 1999b) and of clomipramine, imipramine and desipramine in a model of noxious colorectal distention (Su and Gebhart, 1998). However, the results are in contrast to the lack of local effect observed with clomipramine in the rat carrageenan pressure threshold model (Ardid et al., 1991). The disparity between the two inflammatory models may be due either to a difference in the intensity of the inflammatory injury, or to a potential difference in the profile of different drugs in the same class. The present observations represent the first report of a local, peripheral site of action of both amitriptyline and desipramine in a neuropathic pain model.

In contrast to the pain alleviating actions against thermal hyperalgesia, both amitriptyline and desipramine were ineffective in the treatment of nerve injury-induced static tactile allodynia. There was even a slight untoward effect following spinal administration of amitriptyline (60 μ g) that became statistically significant when the cumulative score was analyzed. Increasing the spinal concentration of amitriptyline to 90 μ g caused a significant sedative effect in that

the animals were flaccid in the testing apparatus and this hindered their ability to respond to the stimuli. An interesting observation with the use of both agents in the tactile threshold test was the decrease in response threshold observed in the contralateral paw, which was termed a hyperaesthetic tactile response. While the hyperaesthetic response threshold was similar to that of the nerve injured paw, qualitatively the observed behavioral response (a brisk withdrawal) differed from that exhibited by a normal neuropathic pain response (i.e. biting, licking, and guarding). A similar magnitude of hyperaesthetic effect was observed following all routes of administration for both agents. While no other studies using neuropathic pain models have reported a pain facilitating or pronociceptive effect, amitriptyline has been shown to have pronociceptive actions in the rat tail flick test when administered spinally (Dirksen et al., 1994). In most other models, amitriptyline produced antinociception when administered systemically (rat hot plate, tail flick, withdrawal reflex, and acetic acid writhing tests) (Dirksen et al., 1994; Casas et al., 1995; Korzeniewska-Rybicka and Plaznik, 1998). Collectively, the findings of this and the other studies indicate a distinct test and route specific effect of amitriptyline and desipramine.

In contrast to amitriptyline and desipramine, systemic and local peripheral administration of fluoxetine was ineffective against either thermal hyperalgesia or static tactile allodynia. Further, the local peripheral administration of fluoxetine was found to cause paw oedema (Sawynok et al., 1999b). The lack of effect of fluoxetine in this study is in agreement with others showing a minimal effect of SSRIs in the treatment of experimental and clinical neuropathic pain (reviewed Sindrup, 1997).

While the focus of this study was not a complete mechanistic analysis of the action of ADs against these two symptoms of neuropathic pain, it is useful to consider the general pathophysiological mechanisms of neuropathic pain that may account for differential actions. The development of neuropathic pain is thought to involve reorganization of A β afferents into lamina I and II, central sensitization, loss of inhibitory interneurons (reviewed Coderre et al., 1993; Woolf and Doubell, 1994), sensitization of peripheral nociceptors (reviewed Simone, 1992), and possibly a phenotypic switch in the response properties of the peripheral receptors following inflammation (Schaible and Schmidt, 1988; Meyer, 1995; Neuman et al., 1996). Another important factor in neuropathic pain is a sympathetic maintenance and dependence (reviewed Kim et al., 1997) as a result of reorganization of noradrenergic fibers infiltrating the dorsal root ganglia (McLachlan et al., 1993; Ramer and Bisby, 1997; Rubin et al., 1997). While central sensitization, loss of inhibitory interneurons, and sympathetic dependence may be involved in both allodynia and hyperalgesia, reorganization of A β afferents and sensitization of respective nociceptors appears to be of particular relevance to allodynia. Furthermore, it has been shown that allodynia may be spinally mediated through (RS)- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on wide dynamic range neurons, while hyperalgesia may be spinally mediated through NMDA-R on the same neurons (Leem et al., 1996). This finding correlates with the increased expression of the GluR1 subunit of the AMPA receptor in LIII, LIV and LV following dorsal rhizotomy (Carlton et al., 1998), regions which are thought to undergo reorganization following peripheral nerve injury. These mechanisms may explain

the amitriptyline-induced hyperaesthetic effect in the contralateral paw, and the lack of effect of amitriptyline against allodynia in the ipsilateral paw. However, it has also been shown that spinal NMDA antagonists are effective in alleviating nerve injury-induced allodynia (Chaplan et al., 1997) which argues against a simple receptor differentiation between the two symptoms.

ADs are known to exert a number of pharmacological actions (reviewed Richelson, 1990; Leonard, 1993; Eschalier et al., 1994; Eschalier et al., 1999). The most prominent of these actions is the prevention of both noradrenaline and serotonin reuptake (Richelson and Pfenning, 1984), which in the long term leads to receptor up-regulation or downregulation and this is thought to partly underlie their efficacy in treating depression (Blier and de Montigny, 1995). With regard to pain mechanisms, enhancement of central noradrenergic tone may contribute to alleviating neuropathic pain from chronic nerve constriction injury through activation of descending adrenergic inhibitory circuits as suggested by Ardid and Guilbaud (1992). However, if this mechanism was prominent in the present model, one might expect an anti-allodynic action of amitriptyline and desipramine as spinal administration of α_2 -adrenergic receptor agonists produce pronounced anti-allodynic actions (Yaksh et al., 1995). Peripherally, noradrenaline can produce hyperalgesia (Levine et al., 1986; Tracey et al., 1995a) or antinociception (Davis et al., 1991; Khasar et al., 1995) via activation of different α_2 -adrenergic receptor subtypes (Khasar et al., 1995). While blockade of the pronociceptive peripheral α_2 receptors might potentially be involved in the local action of amitriptyline and desipramine, this was not deemed to underlie the efficacy in an inflammatory model (Sawynok et al., 1999a, 1999b). The role of

serotonin in pain modulation is also complex, since activation of serotonergic receptors produces pro- or anti-nociceptive actions in the spinal cord, depending on the receptor subtype being activated (reviewed Cesselin et al., 1994; Sawynok, 1996, 1998), and pronociceptive actions at peripheral sites (Abbott et al., 1996; Doak and Sawynok, 1997). Interestingly, it has been shown recently that 5-HT released from immortalized cells transplanted into the lumbar subarachnoid space was able to reverse the development of chronic neuropathic pain (Eaton et al., 1997). However, there is not much literature on the role of 5-HT itself in neuropathic pain tests (but see Cesselin et al., 1994). Nonetheless, the duality of actions observed with both amines may have relevance to the heterogeneity of results seen with amitriptyline and to a lesser extent desipramine.

Part of the pathophysiology of the nerve injury-induced neuropathic pain syndrome is a peripherally expressed, sympathetically mediated, pain (Kim and Chung, 1991; Kim et al., 1993; Ramer and Bisby, 1997). Thus, surgical (Shir and Seltzer, 1991; Kim et al., 1993; Kinnman and Levine, 1995), and chemical (Neil et al., 1991; Desmeules et al., 1995) sympathectomy relieve neuropathic pain symptoms. The benefit of sympathectomy may also be both model- and technique-dependent (Kim et al., 1997; Ramer and Bisby, 1997). Prevention of the reuptake of noradrenaline could augment neuropathic pain by stimulating a sympathetically-dependant discharge from the ganglia, and this could manifest as symptoms of spontaneous and ongoing pain. This might explain why amitriptyline causes a tactile hyperaesthetic response, especially in light of reports indicating that an increased sympathetic drive augments mechano-

hypersensitivity after spinal nerve ligation (Kim et al., 1993; Kinnman and Levine, 1995).

Tricyclic ADs also have antagonistic actions at the NMDA receptor (Reynolds and Miller, 1988; Semagor et al., 1989; Cai and McCaslin, 1992; McCaslin et al., 1992), and this action could explain the anti-hyperalgesic action of amitriptyline in the spinal cord (cf. Leem et al., 1996; Chaplan et al., 1997). Block of NMDA-R in the spinal cord has been shown to alleviate pain in animal models of acute nociception (Gordh et al., 1995; Nichols et al., 1997), inflammation (Eisenach and Gebhart, 1995a; Chaplan et al., 1997) and neuropathic pain (Davar et al., 1991; Kawamata and Omote, 1996; Chaplan et al., 1997; Nichols et al., 1997). Spinal amitriptyline has specifically been shown to antagonize the hyperalgesic effects of spinal NMDA administration (Eisenach and Gebhart, 1995a). Blockade of peripheral NMDA-R (cf. Zhou et al., 1996) might contribute to the local anti-hyperalgesic effect of amitriptyline in this study, but such an action was not implicated in the local action of amitriptyline in the formalin model (Sawynok et al., 1999a). Whether peripheral EAA receptors are involved in pain signaling following nerve injury remains to be determined.

Another possible mechanism for the action of ADs is through interactions with endogenous opioid systems (Hall and Ögren, 1981; Isenberg and Cicero, 1984), as naloxone blocks the systemic effects of amitriptyline (Ardid and Guilbaud, 1992; Gray et al., 1998) and clomipramine (Eschalier et al., 1981; Antesuategui et al., 1989). However, in neuropathic pain, a spinal mode of action for opioids seems unlikely since in neuropathic pain, such an effect is greatly reduced (Bian et al., 1995). However, an effect of opioids on neuropathic

pain has been observed following supraspinal (Bian et al., 1995; Martin et al., 1998; Suzuki et al., 1999) and systemic (Bian et al., 1995; Suzuki et al., 1999) administration.

Finally, a local anesthetic like action of TCAs has been suggested (Deffois et al., 1996), and studies have shown local anesthetics to be effective in animal models of neuropathic pain (Jett et al., 1997; Abdi et al., 1998) as well as in the treatment of clinical neuropathic pain (Rowbotham et al., 1995; Galer et al., 1999). However, in the present study no effect was observed on the thermal threshold in the contralateral paw, or in the paws of naïve animals, when the ADs were administered systemically or locally. Thus, these results do not support a straightforward local anesthetic effect of amitriptyline and desipramine in this study. The results do not however, preclude the possibility that the local anaesthetic effect is confined to the neuropathic paw because of the pathophysiological changes in Na⁺ channel expression and dynamics following peripheral nerve injury. This remains a distinct possibility especially in light of the fact that local anesthetics are particularly effective when Na⁺ channels are in an active state (Tanner et al., 1997; Kral et al., 1999).

The findings of this study confirm those of other animal studies showing a profile of amitriptyline that is dependent on the dose, the route of administration, and the pain test (Dirksen et al., 1994; Casas et al., 1995; Mestre et al., 1997; Korzeniewska-Rybicka and Plaznick, 1998). These findings suggest that the pathophysiology of thermal hyperalgesia and mechanical allodynia are maintained through distinct mechanisms, as has been suggested by the differential profile of other drugs like dextromethorphan on these two endpoints

(Tal and Bennett, 1992). The clinical implications of this study, and others showing a specificity of action are evident, since the efficacy of amitriptyline in a patient may be determined by which of the neuropathic symptoms is the most strongly expressed. It will therefore be interesting to see if other pharmacotherapies also show a preferential efficacy for subtypes of pain symptoms, which may be indicative of an effect against specific pathophysiological mechanisms underlying neuropathic pain.

Another important implication of the present study is the demonstration of a peripheral anti-hyperalgesic effect of amitriptyline and desipramine when administered locally. This is the first time a local effect of tricyclic ADs has been demonstrated in a neuropathic pain model, although it has been reported in a model of noxious colorectal distention (Su and Gebhart, 1998), and in inflammatory pain (Sawynok et al., 1999a, 1999b). This particular finding suggests that an alternative mode of administration (e.g. cream or patch) may be effective in alleviating certain manifestations of neuropathic pain. The possibility of developing topical treatments for neuropathic pains is an emerging issue in the development of new therapeutic strategies for this condition (Rowbotham et al., 1995; Galer et al., 1999).

4.2 Caffeine interaction with ADs in neuropathic pain

In the first part of this study, a thermal anti-hyperalgesic effect was detected with both amitriptyline and desipramine. Both of these ADs act through multiple mechanisms as noted above, but amitriptyline has the additional putative mechanism of manipulating endogenous adenosine levels. This mechanism

could potentially be very significant since systemic and spinal adenosine and its analogues have been shown to alleviate human (Belfrage et al., 1995; Karlsten and Gordh, 1995) and rat neuropathic pain (Yamamoto and Yaksh, 1991; Lee and Yaksh, 1996). To investigate this potential mechanism, caffeine was administered via the same route as the AD (amitriptyline and desipramine). Caffeine is a non-selective adenosine receptor antagonist and provides an opportunity to look at this interaction with a clinically relevant antagonist.

Caffeine blocked the thermal anti-hyperalgesic effect of amitriptyline but not desipramine, and this was observed following both systemic and peripheral administration. As blockade of adenosine receptors is thought to underlie many of the pharmacological effects of caffeine, especially at moderate doses (reviewed Fredholm, 1995), these observations suggest involvement of endogenous adenosine in the thermal anti-hyperalgesic effect of amitriptyline. Other studies have previously reported involvement of adenosine receptors in the antinociceptive effect of systemically administered ADs in the tail flick (Pareek et al., 1994) and writhing tests (Sierralta et al., 1995), and of peripheral amitriptyline in the formalin test (Sawynok et al., 1999a). Collectively, these observations suggest that adenosine is an important endogenous mediator of the analgesic properties of a number of (but not all) tricyclic ADs and of amitriptyline in particular.

The mechanism by which amitriptyline interacts with endogenous adenosine systems is likely through inhibition of adenosine uptake, as nortriptyline (the active metabolite of amitriptyline) was shown to be the most potent of a number of tricyclic ADs in inhibiting the neuronal uptake of adenosine

(Phillis and Wu, 1982)(no actual uptake data was presented for amitriptyline). Consistent with this interpretation is the observation that ADs enhance the inhibitory effects of adenosine on neuronal firing, but do not affect the actions of an adenosine analog which is not subject to uptake (Phillis, 1984). While neither an amitriptyline-facilitated release of adenosine nor a direct binding of amitriptyline to adenosine receptors have been reported, these actions remain mechanistic possibilities for the adenosine-linked actions of amitriptyline. In this manner, amitriptyline is well known for its ability to bind to a diverse number of different receptor sites (reviewed Eschalier et al., 1994). However, with the exception of opioid receptors, these actions are primarily inhibitory, which would tend to argue against a direct adenosine receptor agonist-mediated action of amitriptyline.

Adenosine analogs have been shown to produce pain relieving effects in a variety of neuropathic pain models following both spinal (Sosnowski and Yaksh, 1989a, 1989b; Sjölund et al., 1996; Lee and Yaksh, 1996; Cui et al., 1997; Khandwala et al., 1998) and peripheral administration (Liu and Sawynok, 1998). Considering the proposed adenosine-linked action of amitriptyline and the prominent efficacy of adenosine analogs in neuropathic pain models, it is not clear why we only observed a modest thermal anti-hyperalgesic effect of spinally administered amitriptyline. The spinal dose of amitriptyline used in the present study (60 μ g) has been reported to produce complete reversal of thermal hyperalgesia following intraplantar carrageenan (Eisenach and Gebhart, 1995a), while having no effect on the response to radiant heat in a non-inflamed situation (Eisenach and Gebhart, 1995b). The apparent lack of block of the spinal action

of amitriptyline by caffeine may reflect a low level of intrinsic adenosine activity. Interestingly, the greatest effect of spinal amitriptyline (Eisenach and Gebhart, 1995b) and other tricyclic ADs (Hwang and Wilcox, 1987a) was observed in combination with other agents. Therefore it may be possible that a more robust effect of spinal amitriptyline in neuropathic pain would occur as a result of combinations with other agents.

The manipulation of endogenous adenosine by amitriptyline, while important, is unlikely to be the sole anti-hyperalgesic mechanism involved since amitriptyline is known to produce a diverse number of biological actions. As discussed previously, these include inhibition of monoamine uptake, blockade of muscarinic, histamine, α -adrenergic, NMDA and SP receptors, and quinidine-like actions on sodium channels (reviewed Eschalier et al., 1994, 1999), and a number of these actions may contribute to the efficacy of amitriptyline (and desipramine). Additional lines of reasoning also support the conclusion that the pharmacology of acute amitriptyline is not solely a result of an interaction with endogenous adenosine systems, or perhaps with any other single mechanism for that matter. Thus, while spinal administration of adenosine analogs exerts a prominent anti-allodynic effect in the spinal nerve ligation model (Lee and Yaksh, 1996), amitriptyline did not exhibit any anti-allodynic effect in this study. A similar argument can be made with respect to potential NMDA receptor antagonism by amitriptyline. In this study, amitriptyline and desipramine were effective against thermal hyperalgesia, but not static tactile allodynia. However, high potency NMDA antagonists have been shown to produce both anti-allodynic (Chaplan et al., 1997) as well as anti-hyperalgesic (Mao et al., 1992a, 1992b; Yamamoto and

Yaksh, 1991; Mao et al., 1993) effects following nerve injury. It appears therefore, that blockade of NMDA-R can at best only partially account for the actions of amitriptyline and desipramine. While such a mechanism for spinal amitriptyline was proposed recently in an inflammatory pain model (Eisenach and Gebhart, 1995a), the pharmacological profile of NMDA antagonists at blocking inflammatory versus neuropathic pain can differ (Chaplan et al., 1997).

An important point to note regarding these observations is that the doses of caffeine (1.5, 3.75 and 7.5 mg/kg) that blocked the systemic effect of amitriptyline are modest ones. These doses did not produce overt behavioral stimulation in this study, and are at the low end of the minimal range for producing psychomotor stimulant effects in rodents (Nehlig et al., 1992). Acute ingestion of two cups of strong coffee (2x100 mg; Barone and Roberts, 1996) would generate a human dose of approximately 2.8-3.6 mg/kg (70 kg male or 55 kg female). On the other hand, it has been estimated that the average daily consumption rate of caffeine, in the United States and Canada, is 2.4 to 4.0 mg/kg/day (170 to 300 mg/day for 60-70 kg individual) (reviewed Fredholm et al., 1999). While direct extrapolation between rats and humans may not be entirely appropriate due to pharmacokinetic and other variables, the doses of caffeine which block the action of amitriptyline are clearly low enough to be relevant to human dietary intake levels. Therefore the issue of whether caffeine consumption in a dietary context might interfere with the efficacy of amitriptyline in neuropathic pain needs to be addressed directly in human studies. This potential interaction could perhaps contribute to the limited efficacy of

amitriptyline observed in a recent meta-analysis of ADs used for the treatment of clinical neuropathic pain (McQuay et al., 1996).

In summary, the results of this portion of the study suggest that the thermal anti-hyperalgesic effect of acute amitriptyline, but not of acute desipramine, is mediated in part through manipulation of endogenous adenosine. This interaction occurs following both systemic and peripheral administration of amitriptyline, but is less apparent following spinal administration. This study raises the possibility that caffeine consumption might influence the efficacy of amitriptyline in alleviating neuropathic pain in humans.

4.3 Immunohistochemical changes in Hsp27-IR, NPY-IR and SP-IR following nerve injury

While the findings of the first two sections of this thesis yielded some interesting results, the acute administration paradigm may not be fully representative of the mechanisms following ADs use in the treatment of neuropathic pain. Thus, in the acute drug administration paradigm, the duration of the thermal anti-hyperalgesic effect was short lived as the thermal hyperalgesia typically returned by the end of the observation period. In this context, the acute AD administration appears to provide symptomatic relief. To analyze the effectiveness of ADs in a more clinically relevant paradigm, a chronic administration regime was developed. Furthermore, to determine if the relief was primarily symptomatic or reflected an alteration in the underlying pathophysiology, immunohistochemical analysis was performed on selective markers in the spinal cord, dorsal columns and gracile nucleus. Before this could

be accomplished however it was necessary to determine the time course of the changes in expression of selected markers following nerve injury.

The third component of this study was designed to achieve this end and consisted of an analysis of the time course of the behavioral expression of mechanical allodynia in conjunction with the segmental and temporal expression of Hsp27-IR, NPY-IR, and SP-IR expression. The results indicate that the temporal expression of Hsp27-IR and NPY-IR in the dorsal horn closely coincides with the expression of mechanical allodynia following SNL nerve injury. Following resolution of the allodynia, the altered long term expression of Hsp27-IR and NPY-IR could be indicative of ongoing plasticity, suggesting an involvement of both Hsp27 and NPY in the degeneration and regeneration phases following nerve injury.

4.3.1 Tactile Allodynia following SNL injury

The pathophysiological events following SNL result in symptoms of mechanical and thermal hyperalgesia (exaggerated responses to noxious stimuli) as well as mechanical and thermal allodynia (exaggerated response to normally innocuous stimuli) (reviewed Bennett, 1994a, 1994b; Kim et al., 1997). These behaviors are maximal approximately 14 days following injury, but wane to undetectable levels by 4 months (Kim and Chung, 1992). Only expression of static tactile allodynia was monitored as an indication of the neuropathic pain level in this series of experiments, as this is a more robust manifestation of neuropathic pain.

Significant expression of mechanical allodynia was observed from 4 through to 17 days following SNL. There was also a reduced response threshold in the contralateral paw of the SNL animals at 4 days that was not apparent at 7 days. However, allodynia was not detectable 180 days following SNL, as has been previously characterized in this (Kim and Chung, 1992) and other rat nerve injury models (Bennett and Xie, 1988; Seltzer et al., 1990). The fact that tactile allodynia could not be detected may simply reflect a technical limitation in measuring sensory differences between the two paws in older animals. Alternatively, post mortem analysis of the ligated (L5 and L6) spinal nerves at this final time point revealed a compromise in the integrity of the ligation from around the nerve, as the suture material had dissolved while the knot from the ligation remained. Furthermore, there appeared to be a partial decrease in the constriction of the ligated spinal nerves from that observed with intact ligations at day 17. This finding suggests that alleviation of the neuropathic syndrome in this study could be due in part to a regeneration of previously injured fibers, although this remains to be proven.

4.3.2 Temporal expression of Hsp27-IR following SNL

Low levels of constitutive expression of Hsp27-IR have been previously described in lamina I, III, and IV of the spinal cord (Plumier et al., 1997c). In addition, axotomy has recently been reported to induce Hsp27 expression in motor neurons of the vagus nerve (Hopkins et al., 1998), and in DRG neurons following peripheral, but not central, nerve transection (Costigan et al., 1998). Moreover, medium to large dorsal root ganglion cells showed up-regulation of

Hsp27 mRNA while *de novo* synthesis of Hsp27 mRNA occurred in small DRG cells (Costigan et al., 1998). After SNL, expression of Hsp27-IR, in terms of both intensity and extent, peaked at 12 days. Five months following SNL, Hsp27-IR was still increased in laminae I and III of the ipsilateral dorsal horn. Laminae I, II, and V are commonly associated with nociceptive transmission from primary afferents. While laminae I and V have been shown to receive A δ - and C-fibers, lamina II is thought to principally receive C- fibers (reviewed Willis and Coggeshall, 1991). Hsp27-IR increased expression in laminae I and II could therefore represent an up-regulation of Hsp27 in medium size DRG cell bodies of A β - fibers and *de novo* synthesis in small diameter DRG cell bodies of C- fibers, as observed after nerve transection (Costigan et al., 1998). In addition, it is possible that Hsp27 is upregulated in local interneurons or glial cells of the dorsal horn, but this remains to be verified with ultrastructural studies of the spinal dorsal horn of mononeuropathic animals.

In the dorsal most aspect of lamina III (III_d), there was a robust up-regulation of Hsp27-IR. Previous studies have suggested the possibility of reorganization of large diameter afferents at the lamina II/III interface (Woolf et al., 1992; Shortland and Woolf, 1993; Woolf and Doubell, 1993; Lekan et al., 1996) that eventually infiltrate into lamina II and potentially contribute to painful mechanosensitivity inherent to neuropathic pain (Campbell et al., 1998; Ochs et al., 1989). Therefore, the results in this study could be indicative of synaptic reorganization in this region as they correspond to the time frame of large diameter fiber reorganization, and manifestation of large diameter fiber mediated neuropathic pain (allodynia). The potential significance of a correlation between

increased Hsp27-IR and a reduced response threshold is also suggested by the immunohistochemical and behavioral findings in the contralateral paw of SNL animals at 4 days. At 4 days, we observed a reduced response threshold and an increase in Hsp27-IR in lamina III_c of the contralateral paw in SNL animals.

In addition to up-regulation of Hsp27-IR in the spinal dorsal horn, there was an increase in Hsp27-IR in the ipsilateral dorsal column and gracile nucleus from 12 days through to 5 months post-SNL. These findings suggest that Hsp27 up-regulation coincides with increased afferent traffic of myelinated fibers to the gracile nucleus, which in turn projects to the thalamus. A study by Miki et al. (1998) recently suggested that the increased activity within the dorsal column medial lemniscus (DCML) pathway, following sciatic nerve chronic constriction injury, contributes to the pathophysiology of neuropathic pain by changing the endogenous tone of gracile second order neurons. In turn, these neurons project to the ventroposterolateral nucleus of the thalamus, a region that also serves as an integrative site for nociceptive information transmitted through the spinothalamic pathway. The findings in this study compliment those of others that allude to the potential contribution of the DCML pathway and the ventroposterolateral nucleus of the thalamus in neuropathic pain (Guilbaud et al., 1986a, 1986b; Kupers and Gybel, 1993; Berkley and Hubscher, 1995). Conversely, the increased Hsp27-IR in the dorsal column could also represent transmission of nociceptive information through a dorsal column polysynaptic pathway (reviewed Willis and Coggeshall, 1991) that has recently been implicated in other pain models (Al-Chaer et al., 1998). It therefore seems

plausible that plasticity of primary afferent pathways through the dorsal columns contributes to the maintenance of the neuropathic state.

An increase in Hsp27-IR was also noted in the ipsilateral parasympathetic nuclei in the intermediolateral gray. This labeling could reflect ectopic afferent drive from injured DRG neurons of the 5th and 6th lumbar segments, since visceral afferent terminals have been shown to project to these nuclei through the L6 spinal nerve (de Groat et al., 1996). The up-regulation of Hsp27-IR in the parasympathetic nuclei could also be an unmasking of input to these nuclei as a result of dying back of the injured afferents. This remains speculative however and the functional implications of this altered input still needs to be determined.

4.3.3 Role of Hsp27 following nerve injury

The diverse roles for Hsp27, such as protection against cell death and regulation of actin filament dynamics (see Introduction), suggests that Hsp27 may have distinct time- and event-dependent roles following nerve injury. Indeed, peripheral nerve injury involves phases of both degeneration and regeneration (Coderre et al., 1993; Woolf and Doubell, 1994). The results of this study and those of others (Costigan et al., 1998; Hopkins et al., 1998) suggest that the short-term onset and long-term duration in the alteration of Hsp27 expression may contribute to both events, albeit through different actions. The up-regulation and *de novo* synthesis of Hsp27 within 24 hours of nerve injury may reduce putative apoptotic mechanisms, as up-regulation of Hsp27 has been linked to increased cell survival from cytotoxic challenge (Mehlen et al., 1996; Samali and Cotter, 1996) On the other hand, the more persistent Hsp27

expression may reflect a role for Hsp27 in regeneration through involvement in actin filament dynamics (Lavoie et al., 1995). The temporal expression and neuroanatomical distribution of Hsp27-IR corresponds to the time course of A β -fiber reorganization, maximal expression of allodynia, and expression of GAP-43 which is thought to be indicative of synaptic reorganization in the dorsal horn (Woolf et al., 1989; Coggeshall et al., 1991; Nahin et al., 1994). Whether functional reorganization occurs, and the exact factors that facilitate this process is not fully known. Only 50% of the small DRG cells die following peripheral nerve lesion by 32 weeks (Coggeshall et al., 1997). It has also been recently demonstrated that C-fiber atrophy and the ensuing vacancy in the dorsal horn is insufficient in and of itself, and that there are intrinsic factors present in damaged C-fibers that are necessary to facilitate the reorganization (Chong et al., 1996; Mannion et al., 1998). Therefore it is possible that Hsp27 expression in small diameter DRG cells contributes to the survival of C-fibers and this survival is important to the reorganization that occurs in lamina II.

4.3.4 Temporal expression of NPY-IR following SNL

The present study also describes changes in NPY-IR following SNL that permits a more precise correlation of the spinal nerve and spinal segmental level where neuroplastic and neurochemical changes occur 6 hours to 180 days after nerve injury. Neuropeptide Y expression is increased in similar regions of the lumbo-sacral spinal cord in the SNL model as in the sciatic nerve loose ligation or transection models (reviewed Hökfelt et al., 1998). Following SNL, the increase in NPY first becomes evident at 4 days, is most intense by 17 days and is almost

completely diminished by 180 days. Furthermore, there was an increase in NPY-IR in laminae I and II_o of the contralateral spinal dorsal horn 17 days after SNL. Projections to the contralateral dorsal horn most likely originated from the ipsilateral side of the cord since NPY immunostained fibers were observed extending across the midline dorsal to the central canal towards the contralateral side. Following SNL, NPY in the ipsilateral gracile nucleus was most dense at 17 days and still evident 180 days after SNL. It is interesting to note that both Hsp27-IR and NPY-IR were increased in similar regions of the lumbosacral dorsal horn, dorsal column white matter and gracile nucleus. Moreover, maximal expression of the NPY-IR was delayed by about 3-5 days compared to that of the Hsp27-IR, suggesting that changes in the two were positively correlated.

The results described in this portion of the thesis confirm and extend those of previous studies that have described changes in NPY-IR levels in the spinal dorsal horn following loose ligation (Wakisaka et al., 1992; Munglani et al., 1995, 1996), crush (Wakisaka et al., 1992) or transection (Wakisaka et al., 1991, 1992; Nahin et al., 1994; Zhang et al., 1995) of the sciatic nerve. These studies have consistently demonstrated an increase in expression or *de novo* synthesis of NPY in either dorsal root ganglia or laminae III and IV of the lower lumbar spinal cord at 14, 28 or 100-120 days after nerve injury. In the dorsal root ganglia, expression of NPY is mainly increased in large and medium-sized neurons after sciatic nerve transection (Wakisaka et al., 1991, 1992; Zhang et al., 1994b). The large and medium-sized dorsal root ganglion neurons may account for much of the increased NPY seen in the spinal cord as well as the dorsal columns and gracile nucleus since they have large myelinated fibers that terminate in laminae

III and IV of the spinal dorsal horn (Rivero and Grant, 1990) and ascend in the spinal dorsal columns (Giuffrida and Rustioni, 1992). In contrast, however, changes in NPY-IR in laminae I-II may result from increased synthesis in local NPY-containing neurons in this region or from SNL induced sprouting of afferent fibers located in the deeper laminae (i.e. laminae III or IV). Up-regulation of NPY in myelinated cutaneous A β fibers located in laminae III-IV and sprouting of these fibers into lamina II has been reported following peripheral nerve injury (Koerber et al., 1994; Woolf et al., 1992; Lekan et al., 1996).

4.3.5 Role of NPY following nerve injury

The exact actions of NPY following nerve injury are complex and depend on the type and location of its receptor subtypes (i.e. supraspinal, spinal, or peripheral). Low level constitutive expression of NPY-IR in the superficial spinal dorsal horn is thought to largely result from local interneurons, some bulbospinal projections (Gibson et al., 1984) and a minimal contribution from primary afferent terminals (Zhang et al., 1993). In local interneurons of the spinal dorsal horn, NPY has been colocalized with GABA (Rowan et al., 1993). In the spinal dorsal horn and DRG, intrathecal NPY inhibits nociceptive reflexes in the anaesthetized normal rat (Hua et al., 1991), but has biphasic actions on spinal reflexes following axotomy (Xu et al., 1994). It is also worth mentioning that peripherally administered NPY augments hyperalgesia, an effect thought to be the result of an interaction with the sympathetic nervous system and which may contribute to peripheral sensitization (Tracey et al., 1995a, 1995b).

This analgesic potential of NPY in the spinal cord is also supported by mechanistic studies on the actions of NPY. For example, NPY inhibits depolarization-induced release of substance P both *in vitro* (Walker et al., 1988) and *in vivo* (Duggan et al., 1991). This action may be related to the inhibition of Ca²⁺ influx observed in cultured DRG neurons (Walker et al., 1988) through activation of Y2 receptors (Bleakman et al., 1991; Wiley et al., 1993). As substance P is known to be pain facilitating, decreasing its release could in effect suppress pain. In addition to acting as a neuromodulator, other studies have shown that NPY induces neurite elongation (White and Mansfield, 1996) and this may contribute to the reorganization of the large A β afferents in laminae III and IV following peripheral nerve injury.

4.3.6 Changes in SP-IR following nerve injury

A decrease in the intensity SP-IR expression was identified in the superficial laminae of the ipsilateral dorsal horn in the L4 through S2 spinal segments. Light to no SP-IR terminal expression was observed in the gracile nucleus in any treatment group at any of the time points observed. These results are consistent with the substantial body of literature that has demonstrated a decrease in SP in the spinal dorsal horn and DRG following nerve injury (reviewed Hökfelt, 1994). The decrease in synthesis and release of SP from primary afferents has the potential to attenuate transmission of peptide-mediated information from primary afferents. Conversely, expression of SP-IR has been shown to be increased in spared or adjacent DRG neurons following partial sciatic nerve injury (Ma and Bisby, 1998), suggesting that SP may contribute to

the development and maintenance of neuropathic pain. That no increase in SP-IR was observed in this study could be related to the extent of the injury as the model used by Ma and Bisby (1998) does not damage all the fibers in the nerve, while the model used in the present study is for all intents and purposes, a transection.

4.3.7 Summary of immunohistochemical time course

In summary, the immunohistochemical time course suggests a close positive association between the expression of Hsp27-IR and NPY-IR in the spinal cord, dorsal columns and gracile nucleus. Furthermore, the changes in expression of these two markers closely coincides with the development of static tactile allodynia. On the other hand, SP-IR was reduced in the ipsilateral dorsal horn, which is suggestive of a loss of primary afferent fibers in this region. Although the exact roles of Hsp27 and NPY have yet to be determined, several important possibilities exist.

The first possibility is based on the observation that the expression of Hsp27 in the dorsal horn coincides with two temporally separated yet related events following nerve injury. In the initial stages, Hsp27-IR expression may be indicative of a neuroprotective response mechanism within the spinal cord and DRG as an attempt to limit the amount of injury induced damage. At later time points, the expression of Hsp27 may be indicative of regenerative or adaptive processes that allow the organism to survive despite the injury. The second possibility is based on the enhanced expression of NPY in the superficial and deeper laminae that could be part of an endogenous modulatory mechanism

acting through the presynaptic Y2 receptor to limit the release of excitatory neurotransmitters glutamate, SP and CGRP. This postulate can be extended to the gracile nucleus where a number of studies have revealed the importance of this nucleus in the expression of tactile hypersensitivity. Increased expression of NPY could therefore be a means of dampening the increased afferent input through this system as a result of the injury - a type of relief valve for effects of the excitatory barrage. This effect could also be exerted through Y1 receptors although this action may be facilitative if the receptors were located on local inhibitory interneurons. However, studies on the pathophysiology of nerve injury suggest that part of the underlying pathology that leads to persistent pain is a loss of inhibitory interneurons (Sugimoto et al., 1990; Bennett, 1991). If Y1 receptors are located on the interneurons that are lost, then the facilitative aspect of NPY in the spinal cord may be lost. Perhaps this is why the action of spinal NPY appears to be inhibitory in nerve injured animals and biphasic in inflammatory models where there is no loss of the interneurons. While this remains speculative it could be evaluated directly using selective Y1 and Y2 agonists in the various pain models. Studies have also proposed that the up-regulation of NPY is maladaptive (Munzlani et al., 1995, 1996), and in this sense NPY up-regulation could indicate a pathophysiological mechanism involved in sustaining the neuropathic condition.

The third interesting aspect of the immunohistochemical time course is the close correlation between the change in expression of NPY-IR and Hsp27-IR in the ipsilateral dorsal horn and gracile nucleus. This finding suggests that the actions of Hsp27 may play a role in the subsequent up-regulation of NPY either

directly or indirectly. This relationship seems plausible especially in light of the temporal offset between the expression of these two markers.

4.4 Effect of chronic amitriptyline on neuropathic pain behaviors and expression of Hsp27, and NPY in the spinal cord, dorsal columns and gracile nucleus

In previous sections of this dissertation, it was determined that acute administration of amitriptyline and desipramine was effective in treating thermal hyperalgesia but not static tactile allodynia. This effect was observed following acute administration, and this is interesting but it may not accurately represent the clinical treatment of neuropathic pain where drugs are administered over longer periods. In order to study AD pharmacotherapy of neuropathic pain more precisely, a chronic drug paradigm was developed to examine the effect of chronic amitriptyline both alone and in combination with chronic caffeine. The end points of this part of the study were the changes in the neuropathic behaviors resulting from the nerve-injury as well as changes in the immunohistochemical expression and localization of Hsp27, NPY and SP.

Drugs were administered in the drinking water to reduce the stress of repeated intraperitoneal injections, and because oral administration is the most clinically applicable route. One concern with a drinking water paradigm is spillage of the fluid that may give an inaccurate dose calculation. Since all groups consumed approximately the same amount of fluid, it can be assumed that the amount of spillage was comparable between the groups. Allowing for a spillage of 20% of the volume would yield dose concentrations of approximately

10-12 mg/kg/day for amitriptyline and 4-6 mg/kg/day for caffeine (regardless if the drug was received alone or in combination). The presence of alterations in the neuropathic responses also suggests that an effective dose was obtained. Also of concern in using this method of administration is the potential degradation of amitriptyline that occurs over time when the drug is in aqueous solutions (Buckles and Walters, 1976; Enever et al., 1977). This effect was minimized by using opaque water bottles to reduce the effect of light, and by changing the drinking solution every 48 hours. According to the pharmacokinetic studies, the level of degraded product (ketone bodies) becomes unacceptable after 3-4 days (Buckles and Walters, 1976), which falls outside the time frame that the rats were exposed to the solution in this study. Furthermore, the greatest catalyst in the degradation of aqueous amitriptyline is the ion content of the water (Enever et al., 1977). To correct for this problem Enever et al. (1977), used chelating agents and antioxidants, but these were not used in the present paradigm as they may create an unpleasant taste. In spite of these methodological concerns, the fact that amitriptyline caused an ipsilateral anti-hyperalgesia and a contralateral hyperaesthesia which were similar to the effects observed following acute administration suggests that the drug was active during the study.

Many studies have attempted to determine the mechanisms of action of chronic ADs but this has typically been done in the context of determining how they relieve depression rather than their actions as analgesics. While studies on the effects of chronic ADs do not always involve the same agent, the profile for the TCAs in general is very similar with differences being more a matter of degree. The changes that occur following chronic AD treatment have primarily

focussed on changes in neurotransmitter receptors as well as non-aminergic mechanisms in the brain (reviewed Richelson, 1990; Leonard, 1996). Changes to neurotransmitter receptors include down regulation of cortical β -adrenoceptors, GABA_B and 5-HT_{2A} receptors, a decreased functional activity of 5-HT_{1A} and 5-HT_{2A} receptors as well as α_2 -adrenergic and dopamine autoreceptors, and an up-regulation of cortical α_1 -adrenoceptors (Leonard, 1996). Changes in non-aminergic mechanisms include modulation of NMDA and/or sigma-1 receptors, sensitization of glucocorticoid receptors, desensitization of corticotrophin releasing factor (CRF) receptors in the limbic system, and inhibition of the brain cyclo-oxygenase pathway (Leonard, 1996). As is evident from the list of affected receptor systems, chronic ADs modify systems that may in turn explain their efficacy in the treatment of neuropathic pain.

Just as the acute effects of ADs may not necessarily account for their actions following chronic administration, the same may be true for caffeine. While acute administration of caffeine at the doses used in this study probably produces an antagonism of adenosine receptors, the action of chronic caffeine even at the low dose achieved in this study may not be as straight forward. Thus at clinically relevant doses chronic caffeine has been shown to upregulate the expression of adenosine A₁ and to a lesser extent A₂ receptors, decrease the density of cortical β -adrenoceptors, cause alterations in the GABA receptor channel function, and reduce the excitatory effect of acetylcholine release on rat cerebral cortical neurons (reviewed Daly, 1993).

4.4.1 Thermal anti-hyperalgesic effect of chronic amitriptyline and changes in Hsp27-IR and NPY-IR in the spinal cord and gracile nucleus

In this study, chronic amitriptyline (15-18 mg/kg/day) administration was found to be effective in alleviating thermal hyperalgesia of the ipsilateral paw for the three week observation period. This effect was reduced by concomitant chronic caffeine (6-8 mg/kg/day) at each of the evaluation time points. The behavioral effects of chronic amitriptyline and caffeine appeared to coincide with changes in Hsp27-IR and NPY-IR expression in the ipsilateral spinal dorsal horn, especially in the region of laminae III and IV. It is not possible from these results to determine the exact mechanism(s) for the anti-hyperalgesic effect of chronic amitriptyline, although the blocking effect of caffeine strongly suggests an adenosine link. Similarly, it is difficult to infer a direct causal relationship between the behavioral effects of the drug treatment and the difference in expression of Hsp27-IR and NPY-IR between the groups. Nonetheless, several hypotheses can be made based on the putative mechanisms of chronic amitriptyline and on the functions of Hsp27 and NPY.

4.4.2 Manipulation of endogenous adenosine levels

The caffeine block of the thermal anti-hyperalgesic effect of amitriptyline suggests an involvement of endogenous adenosine in the action of chronic amitriptyline, a potential that has been suggested by several lines of evidence. Thus, ADs inhibit uptake of adenosine in neuronal populations (Phillis and Wu, 1982), and enhance the inhibitory effects of adenosine on neuronal firing but not of an adenosine analog which is not subject to uptake (Phillis, 1984). More

recently, amitriptyline was shown to inhibit the veratridine-evoked release of glutamate in the rat prefrontal cortex (Golembiowska and Zylewska, 1999), and this effect was blocked by 10 mg/kg (IP) caffeine (Krystyna Golembiowska, personal communication). Interestingly, the ability of desipramine to inhibit the veratridine-evoked glutamate release (Golembiowska and Zylewska, 1999) was not blocked by caffeine. It has also been demonstrated recently that intrathecal administration of N⁶-cyclohexyladenosine, an adenosine analog, blocks NMDA-evoked glutamate and adenosine release in the perfusate of the rat spinal cord (Conway and Yaksh, 1998). These results, in combination with the behavioral evidence for the anti-neuropathic effect of adenosine, highlight the potential importance of this mechanism in the analgesic action of AD. Thus, increased extracellular adenosine may act presynaptically to inhibit neurotransmitter release, or postsynaptically to inhibit the generation of post synaptic potentials.

Manipulation of the endogenous adenosine levels by amitriptyline may potentially afford relief from thermal hyperalgesia through interaction with opioid receptors. The use of opioids in neuropathic pain is controversial as systemic and supraspinal administration affords relief from neuropathic pain symptoms, yet higher spinal doses are required to elicit reduced effects in comparison to that observed in nociceptive pain (Portenoy 1990). With these higher doses, a risk of side effects occurs (Foley, 1991). It has been proposed that part of the efficacy of spinal opioids in nociceptive paradigms results from adenosine release (Sawynok et al., 1989). Thus, spinal administration of adenosine receptor antagonists reduces the antinociceptive effects of spinal morphine (Sweeney et al., 1987; Delander et al., 1992; Cahill et al., 1995). Recently, a synergistic effect

interaction between spinal adenosine and morphine was demonstrated in this model of neuropathic pain (Lavand'homme and Eisenach, 1999). Of particular interest was the dramatic effect of spinal administration of dipyridamole, an adenosine uptake inhibitor, on the anti-allodynic effect of spinal morphine (Lavand'homme and Eisenach, 1999). Dipyridamole by itself however did not have a significant anti-allodynic effect. This result may help explain why no significant effect of spinal amitriptyline was observed in our study, as it appears that manipulation of endogenous spinal adenosine is insufficient by itself to produce a direct effect in this neuropathic model.

4.4.3 Chronic amitriptyline-induced changes in aminergic neurotransmitter receptor systems

Alterations in the density and functional activity of various aminergic receptors, is one possible mechanism for the analgesic effect of chronic amitriptyline. In general, effects appear mostly to involve down regulation and decreased functional activity (reviewed Leonard, 1996). These actions may be applicable to the analgesic effect if there is a decrease in the autoreceptors controlling NA and 5-HT release in bulbospinal pathways, whereby such an effect could enhance the actions of normal descending modulation. This may be particularly important in chronic neuropathic pain, as one of the factors in the maintenance of the condition is a loss of inhibitory tone in the spinal dorsal horn as a result of a loss of local inhibitory interneurons (Woolf and Doubell, 1994). Thus, a potential increase in the gain of descending modulation by chronic amitriptyline could help compensate for the loss of the inhibitory interneurons.

On the other hand, increases in peripheral NA and 5-HT may have pain potentiating effects on neuropathic pain, which would argue against the benefit of increasing NA and 5-HT.

A complex association between NMDA-R and 5-HT_{1A} receptors was suggested in a study by Mjellum et al. (1993). In the Mjellum study, chronic desipramine reduced the behavioral effect of intrathecal NMDA, and this effect was blocked by intrathecal administration of a 5-HT_{1A} antagonist (NAN-190). Also of interest is the finding that in the control animals (no desipramine) intrathecal NAN-190 augmented the NMDA induced behavioral response while a 5-HT_{1A} agonist (8-OH-DPAT) inhibited it. These results suggest a tonic inhibition of NMDA-R through 5-HT_{1A} receptors following chronic ADs, which may also be applicable in neuropathic pain.

4.4.4 Chronic amitriptyline-induced non-aminergic changes

The effects of chronic amitriptyline on non-aminergic mechanisms are complex and variable. For the sake of brevity, the discussion of these mechanisms will be confined to those with the greatest potential of contribution to amitriptyline-induced thermal anti-hyperalgesia observed in this thesis. As well, the mechanisms will be discussed in the context of the differential expression of Hsp27-IR and NPY-IR in the spinal dorsal horn and gracile nucleus.

4.4.4.1 NMDA receptor antagonism

From a mechanistic point of view, block of the effects of NMDA-R activation is an appealing mechanism to account for the thermal anti-hyperalgesic effect of chronic amitriptyline. The chronic AD-induced changes in the NMDA-R complex indicate that it is a slowly developing and adaptive phenomenon (Paul et al., 1994). This mechanism could therefore explain the lag in therapeutic effect observed following initiation of AD treatment. Furthermore, the downstream consequences of NMDA-R adaptation are potentially very important in the long term effect of chronic amitriptyline. The ability of ADs from all classes to affect adaptive changes in the NMDA-R has also been suggested as a final common pathway of AD pharmacotherapy (Paul et al., 1994). The method by which ADs interact with the NMDA-R is not fully known. However, a recent study by McCaslin et al. (1992) determined that while amitriptyline blocked NMDA induced toxicity, it did not block NMDA-evoked glutamate release. Further, the antagonistic effect of ADs on the NMDA receptor appear to require an open channel (Semagor et al., 1989; Tohda et al., 1995). This finding may be especially important in neuropathic pain because of the enhanced state of NMDA-R in central sensitization (i.e. relief of the Mg^{2+} block). Another important point of note is that the AD-induced alterations in ligand binding properties at both glycine and glutamate recognition sites of the NMDA-R requires a chronic AD administration of 7 to 14 days to be expressed (Nowak et al., 1993; Paul et al., 1993, 1994). Thus, ADs have NMDA-R antagonistic and receptor modifying properties that have significant potential for reducing central sensitization.

4.4.4.2 Neurotoxicity, TNF α and NGF

The reduction in Hsp27-IR could correlate to an amitriptyline-facilitated reduction in the neurotoxic state within the spinal dorsal horn or DRG as a result of the nerve-injury. This could be a result of a combination of antagonistic actions at NMDA and SP receptors, manipulation of endogenous adenosine with subsequent activation of antinociceptive adenosine A1 receptors, a decrease in ectopic activity of spinal dorsal horn or DRG cells and a decrease in intracellular Ca²⁺ conductance. Additionally, TCAs have been shown to reduce astrocytic and microglial secretion of interleukins and thereby prevent the initiation of the cytokine cascade that may act to sensitize peripheral nociceptors or spinal cord neurones (reviewed Kobierski, 1997). The combination of these actions of amitriptyline could reduce the requirement for the molecular chaperone and neuroprotective actions of Hsp27, which would be manifested as less intense Hsp27-IR in spinal dorsal horn. This seems plausible since one function of Hsp27 is to afford protection from TNF- α (Mehlen et al., 1997), a cytokine that is up-regulated in cells of the macrophage-lineage following nerve injury and purported to be involved in the maintenance of the chronic pain state (DeLeo et al., 1997). Curiously, the pattern of immunoreactive labeling in the dorsal horn also appears to have been one of an up-regulation of Hsp27-IR in lamina II, which could correspond to a survival of C fibers projecting to this region if one accepts that the source of Hsp27-IR is the DRG as suggested by Costigan et al. (1998) and that a certain percentage of small diameter afferents die following nerve injury (Coggeshall et al., 1997). Therefore, the maintained expression in

the amitriptyline animals could indicate some degree of spared fibers although this seems unlikely since the traumatic injury is still maintained.

In inflammatory pain, endogenous adenosine has also been shown to reduce levels of TNF- α in the circulation as well as at the lesion site (Firestein et al., 1994; Cronstein et al., 1995). Adenosine also inhibits the secretion of TNF- α in stimulated monocytes (Parmely et al., 1993) and adenosine A₂ receptor activation inhibits endotoxin stimulated production of TNF- α (Thiel and Chouker, 1995). Although the results of these studies are more applicable to inflammatory pain, they nonetheless highlight the protective potential of endogenous adenosine against TNF- α . The manipulation of endogenous adenosine by chronic amitriptyline may aid in reducing the effects of TNF- α . Furthermore, by potentially inhibiting expression of TNF- α , this could reduce the subsequent expression of NGF in the spinal cord and DRG. This reduced NGF may in turn reduce the degree of sympathetic sprouting in the DRG and decrease the level of NPY gene induction.

With respect to the decrease in NPY-IR induced by chronic amitriptyline, the difficulty in interpretation arises initially from determining whether the up-regulation in the nerve-injury (no drug) animals is compensatory or maladaptive. If up-regulation of NPY is a compensatory means of presynaptic autoregulation through the Y2 receptor as described previously, it is not surprising that there is increased expression in spinal dorsal horn in nerve injured animals. In this respect, amitriptyline could be acting to reduce or prevent the development of central sensitization within the spinal dorsal horn, in which case there would be

less of a requirement for the increased synthesis of NPY, and this could result in decreased expression. Conversely, if NPY is pronociceptive as suggested by others (Munzlani et al., 1995; White 1997), then the reduction in the pain state may be due in part to a decrease in the synthesis and release of NPY. For this to occur, there would have to be a reduction in the signaling molecules/mechanisms that are responsible for the nerve-injury induced up-regulation. There are several factors involved in the expression of NPY. These include activators of cAMP, calcium or phospholipid dependent protein kinases, glucocorticoids and NGF (reviewed Heilig and Widerlöv, 1995). Chronic ADs have been shown to regulate the activity (Perez et al., 1989) and nuclear translocation (Nestler, 1989) of cAMP-dependant protein kinases. Effects of chronic ADs have also been noted on the phosphoinositol-PKC pathway, and on expression of neurotrophins in the brain (reviewed Duman, 1994). Thus, chronic ADs have the potential to affect the expression of NPY in neuronal and glial cells. The question still remains as to whether the decrease in NPY-IR in the amitriptyline group is a result of the attenuated development of thermal hyperalgesia, or the cause.

The potential manipulation of NGF expression by amitriptyline is important for reasons other than altering the expression of NPY. NGF has been shown to be important in up-regulation of other neuroactive peptides, and levels of NGF are increased in the spinal dorsal horn following nerve injury (reviewed Anand, 1995). It may therefore be possible that part of the action of amitriptyline is to reduce the level of NGF in the spinal dorsal horn or DRG. Increases in NGF have been shown to be important in the sympathetic sprouting and the formation

of perineural baskets of sympathetic fibers around the DRG cells that contribute to the sympathetic dependency of some neuropathic pains (McLachlan et al., 1993; Chung et al., 1996; Ramer and Bisby, 1997). Interestingly, it has also been shown recently that NGF facilitates the formation of SP-IR perineural baskets around DRG neurons of large diameter afferents, that may also contribute to the generation and maintenance of the neuropathic pain state (McLachlan and Hu, 1998). The potential benefit of blocking the bioactivity of NGF is also exemplified in a recent study that demonstrated an antiallodynic effect of a NGF receptor antagonist (ALE-0540)(Owolabi et al., 1999). This effect was observed following both systemic and intrathecal administration.

Many of the above considerations have the underlying assumption that the NPY-IR expression occurs in neurons. It is also possible that the expression of NPY-IR in this study may be a result of astrocytic expression as NPY has been shown to be induced during *in situ* reactive gliosis (Bamea et al., 1998). Thus the decrease in NPY-IR observed in the chronic amitriptyline group may reflect a reduction in reactive gliosis in the spinal cord. The consequences of reactive gliosis in terms of the release of pronociceptive mediators is well known (reviewed Kcbierski, 1997), and a reduction in this effect by chronic amitriptyline could contribute to the anti-hyperalgesic effect.

4.4.4.3 Cation channels and intracellular messengers

Amitriptyline may also act to reduce the spontaneous activity within the DRG through inhibition of Na⁺ or Ca²⁺ channels. The manipulation of Na⁺ channels will affect the ability of the cell membrane to depolarize and thereby

prevent post-synaptic action potentials, ectopic discharges and presynaptic neurotransmitter release. In addition to these effects, alteration in the dynamics of Ca^{2+} influx through inhibition of voltage dependent calcium channels (VDCCs), or via NMDA-R facilitated influx, may affect subsequent gene expression (Finkbeiner and Greenberg, 1998) and in turn, peptide synthesis. Amitriptyline-facilitated block of Na^+ and Ca^{2+} conductance could therefore be effective in reducing central sensitization both presynaptically and postsynaptically. Additionally, amitriptyline block of facilitated increases in intracellular Ca^{2+} that occurs in the spinal dorsal horn during central sensitization, or in the DRG following nerve injury, has the potential to effect neuronal gene expression. Thus, the effect on gene expression of DRG neurons could explain the less intense Hsp27-IR and NPY-IR since the increased expressions of both of these in the spinal dorsal horn are thought to be a result of increase synthesis and expression in primary afferent terminals (as discussed previously).

Amitriptyline has also been suggested to interact with second messenger systems either directly or indirectly. With respect to the latter, this most likely occurs as a result of manipulation of intracellular Ca^{2+} . In terms of direct manipulation of second messengers, penetration of the neuronal cell membrane and direct influence on phosphatidyl inositol hydrolysis has been suggested (Leonard, 1993). As alluded to above, the NMDA-R plays an important role in the generation and maintenance of neuropathic pain, as well as in the effects of chronic ADs. An important aspect of the maladaptive changes to the NMDA-R in neuropathic pain is the phosphorylation of the receptor by intracellular kinases

(reviewed Coderre et al., 1993). Therefore manipulation of these kinases has the potential to alter the adaptive changes in the NMDA-R.

4.4.4.4 Other actions of chronic amitriptyline

Amitriptyline has also been shown to inhibit neurite outgrowth (Wong et al., 1991), and in so doing potentially prevent the development of inappropriate synaptic contacts (Leonard, 1993). This possibility is intriguing in light of the importance of structural reorganization to the development and maintenance of neuropathic pain. As suggested above, the difference in the expression of Hsp27-IR and NPY-IR between treatment groups could reflect an effect on the development of the neuropathic condition rather than a decrease in an already established neuropathic state. Based on a comparison of the Hsp27-IR and NPY-IR, the treatment-dependent effect of chronic amitriptyline appears to be greatest in the laminae II_o, III, and IV regions of the ipsilateral spinal dorsal horn. It may be possible therefore that chronic AD treatment is reducing the maladaptive reorganization in this region of the ipsilateral spinal dorsal horn and this facilitates the thermal anti-hyperalgesic effect of amitriptyline in this study.

4.5 Lack of anti-allodynic effect and tactile hyperaesthetic effect of chronic amitriptyline and caffeine

Chronic amitriptyline showed no effect on nerve injury-induced static tactile allodynia. An increase in expression of NPY-IR and Hsp27-IR in the ipsilateral gracile nucleus was observed that coincides with the lack of effect of amitriptyline on the static tactile allodynia in the ipsilateral paw. Thus, the

increased expression of Hsp27-IR and NPY-IR in the gracile nucleus and the decreased expression in the spinal dorsal horn, suggests involvement of two different fiber types in the mediation of allodynia and thermal hyperalgesia. The concept that hyperalgesia is mediated by C and A δ fibers and allodynia by A β fibers is widely accepted (reviewed Woolf and Doubell, 1994). The differential behavioral and immunohistochemical effects of chronic amitriptyline further suggest symptom-related specificity of amitriptyline as was observed following acute administration.

Curiously, chronic amitriptyline also resulted in an increased sensitivity of the contralateral paw, manifested as a significantly reduced response threshold to tactile stimulation that did not include any of the secondary neuropathic pain behaviors. There also was an increase in Hsp27 and NPY-IR in the medial portion of lamina II-IV in the contralateral spinal dorsal horn. This is the region of primary afferent termination of tactile afferents from the ventral surface of the paw (Swett and Woolf, 1985). In this case, either the ipsilateral afferents are projecting bilaterally or for some reason the drug groups have induced up-regulation of NPY in the contralateral DRG. The first of these outlined possibilities seems more likely.

The hyperaesthetic effect of amitriptyline may also be related to the finding that while amitriptyline inhibits NMDA-induced toxicity, it does not inhibit the NMDA-induced release of glutamate (McCaslin et al., 1992). As a result of this, the excess glutamate would be available to interact with the AMPA or kainate receptors that have been implicated in allodynia.

4.6 Changes in SP-IR following chronic amitriptyline

No difference was observed in SP-IR expression in the spinal cord following chronic treatment with any of the drug regimens, as compared to the controls. Thus, in all groups there was a reduction in immunoreactive labeling for SP-IR. This was not wholly unexpected since a significant contribution to the SP-IR in the spinal cord is from primary afferents (Nahin et al., 1991 and references therein). As these fibers are severely damaged from the injury, it did not seem reasonable to expect the drug treatment to alter the expression. It may have been possible to discern a difference using quantitative methods but this requires an appropriate control. The best control from a methodological standpoint would be the contralateral paw since it would have the exact same immunohistochemical processing. However, since contralateral behavioral effects were observed in the drug treatment groups, it did not seem appropriate to use the contralateral side as a control.

CONCLUSIONS

The findings of this dissertation suggest several interesting and potentially important points regarding the AD pharmacotherapy of nerve injury-induced neuropathic pain.

First of all, the model of unilateral spinal nerve ligation appears to be a good model for studying the pharmacological intervention in neuropathic pain as it reliably produces strong manifestations of static tactile allodynia and thermal hyperalgesia that are analogous to clinically observed phenomena. The fact that there was a differential symptom and route effect of amitriptyline and desipramine suggests that (a) the model mimics what happens clinically since not all neuropathic pain patients respond to AD analgesic treatment which may be due to differential dominance of their symptoms, and (b) antidepressants may work better on symptoms of thermal hyperalgesia. The caffeine block of the thermal anti-hyperalgesic action of amitriptyline may be clinically relevant since consumption of caffeine could act to limit the effectiveness of amitriptyline. Conversely, this problem could possibly be avoided by using desipramine, which provided relief from thermal hyperalgesia, but this effect was not blocked by caffeine. However, it may also be advantageous to exploit the potential link between amitriptyline and manipulation of endogenous adenosine levels since adenosine and its analogues have been shown to have a profound effect on neuropathic pain. This may be accomplished through combination therapy with adenosine kinase or deaminase inhibitors. The local peripheral effect of amitriptyline and desipramine suggests the potential for development of cream or

patch formulations to increase the local concentration while limiting the systemic absorption and the ensuing side effects.

Part of the problem in developing effective treatments for neuropathic pain is an incomplete understanding of the underlying mechanisms in the development and maintenance of this condition, and the effects of the treatments on these parameters. In this study, acute amitriptyline and desipramine reversed thermal hyperalgesia but this effect was only temporary as the symptoms quickly returned. This finding suggests that in this context (acute administration) amitriptyline and desipramine are affording short term symptomatic relief. Chronic amitriptyline administration also provided relief from thermal hyperalgesia, but it is uncertain whether this is symptomatic relief or attenuation of the pathophysiological mechanisms of nerve injury-induced neuropathic pain. The alteration in Hsp27-IR and NPY-IR expression in the dorsal horn suggests that there is a chronic amitriptyline-induced manipulation of the underlying pathophysiology, a speculation that remains to be proven. However, if it is assumed that the preemptive and chronic amitriptyline paradigm used in this study was effective in treating the underlying pathophysiology, then it may be beneficial to investigate the use of a similar paradigm (preemptive plus chronic maintenance) in other situations that involve nerve damage.

FUTURE EXPERIMENTS

Throughout the discussion, an effort was made to suggest future experiments in the sections with the appropriate results. Nonetheless, there are several interesting areas that deserve reiteration.

(1) Differential route and symptom effects of ADs

The differential symptom and route effects of amitriptyline and desipramine raise the issue as to whether differential effects occur in other models of neuropathic pain. A number of earlier studies have looked at the analgesic action of antidepressants in neuropathic pain. Nonetheless, it would be useful for a single laboratory to do a comprehensive profile of ADs in models of neuropathic pain ranging from nerve injury (spinal nerve ligation, partial nerve ligation, and chronic constriction injury), metabolic disturbances (diabetic neuropathy), viral infection (HIV, Herpes Simplex II) and chemical agents (chemotherapeutic agents). The advantage of one laboratory performing such a comparison would be the built in control in terms of the behavioral tests (these do differ in detail and application between studies), and could therefore provide a useful profile of ADs in neuropathic pain of differing etiologies.

(2) Contralateral hyperaesthesia

The contralateral hyperaesthesia induced by both acute and chronic administration of amitriptyline in this study is somewhat perplexing. It would therefore be interesting to determine whether amitriptyline alters the electrophysiological properties of neurons in the spinal cord, gracile nucleus and possibly the thalamus. Similarly, it would also be interesting to determine whether the same hyperaesthetic effect is observed in other nerve injury models

and if so, is it also observed in other models of non-nerve injury models of neuropathic pain.

(3) Caffeine block of amitriptyline

The ability of caffeine to block the thermal anti-hyperalgesic action of amitriptyline was very intriguing. The question still remains however as to whether systemic caffeine is able to block the thermal anti-hyperalgesic actions of locally administered amitriptyline. This question is particularly applicable to the potential development of local patch or cream formulations, especially since caffeine is usually administered orally. The lack of effect of spinal amitriptyline in this study was unexpected, but could have been a result some degree of trauma induced by intrathecal cannulation. Therefore, for the purpose of using this model, it would be useful to do a study using an acute lumbar puncture procedure for administration of amitriptyline (and desipramine). Should this procedure yield a spinal effect, then it would be useful to determine if the effect is blocked by spinal and systemic caffeine.

The observation that chronic amitriptyline provided relief from nerve-injury induced thermal hyperalgesia was not wholly unexpected. However, the question remains as to whether the effect was primarily symptomatic or if there was a modification of the underlying pathophysiology. To determine this, it may be useful to stop the chronic drug administration after one week and see if, and when the thermal hyperalgesia becomes manifested (as well as whether the contralateral hyperaesthesia is resolved). It would also be useful to investigate the therapeutic window and determine if there is a critical period where, after which, the effects of amitriptyline may be lost. This is especially important since

in a practical sense preemptive administration may not always be possible. In the discussion a lot of speculation was made as to the mechanistic effects of chronic amitriptyline. Some of these possibilities could be explored using molecular biological techniques (e.g. changes in mRNA levels) to look for changes in the expression of various mediators (e.g. TNF- α , NGF) in the various neurotransmitter systems that can be influenced by chronic ADs.

(4) Relationship between the behavioral and immunohistochemical changes

Whether the observed increases in NPY are maladaptive or adaptive in neuropathic pain remains to be definitively answered. However, this could be addressed using spinally delivered NPY antagonists and observing the result on thermal hyperalgesia and allodynia. It may also be possible to use knockout and knockdown technology to analyze the role of NPY in development and maintenance of neuropathic pain. The exact role of Hsp27 in peripheral nerve injury also remains to be determined, but it does appear to be a sensitive marker of pathophysiological changes following nerve injury. Correlation of the effects of chronic ADs on nerve injury-induced Hsp27 expression (protein and mRNA) with the effects of other anti-neuropathic drugs, such as NMDA antagonists, may provide a better understanding of the significance of the changes in Hsp27 expression.

APPENDIX A

Methodology for Spinal Nerve Ligation surgery.

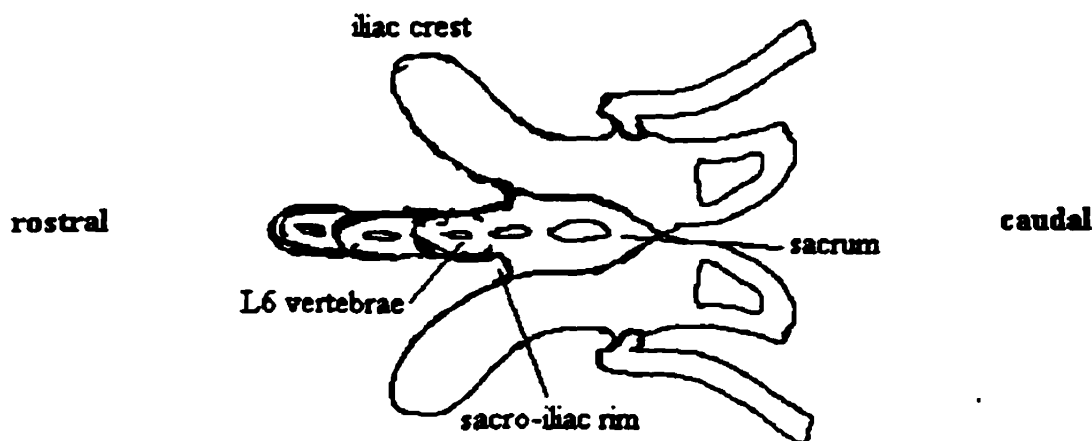
A. Pre-surgical Preparations.

1. Sterilize sutures: 6-0 silk in individual packages for the number of surgeries per day.
2. Ensure glass probes/hooks are suitable (plus spares).
3. Ensure all supplies; 3-0 vicryl swagged (or novofil) for closure, scalpel blades (#15), cotton swabs on wooden sticks, gauze, alcohol, iodine, eye ointment, LRS, sterile saline and penicillin.
4. Clean all instruments thoroughly, then sterilize with the microbead sterilizer.

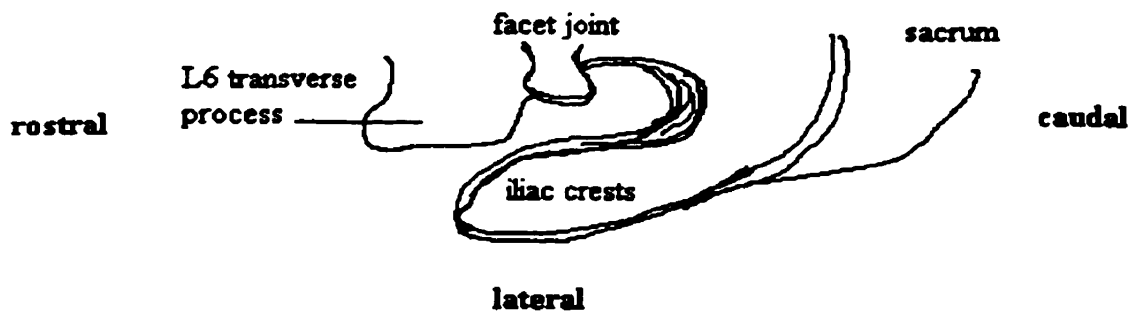
B. Neuropathic Surgery.

1. Anaesthetize the rat (100-150gm) using halothane and place on a heated blanket. Surgically clip the dorsal pelvic area (approximately 5 cm both sides of the midline), and apply eye ointment.
2. Administer 5 cc LRS (ip/sc), and 0.1 cc penicillin (im).
3. Adjust halothane as necessary (1.5-2.0 %) throughout, remembering that the procedure takes 40-60 minutes (prevent overdosing).
4. Surgically scrub/prep the incision area using EtOH and iodine.
5. Make a 3 cm skin incision along the midline using the iliac crests as a midpoint (don't cut too deep as the bone at this developmental stage is soft, and easily penetrated).

Make a stab incision (#15 scalpel blade) on the left side of the vertebral column, at the mid-sacral level. The blade should hit the sacrum at the depth of the cut, as below (see Figure).



6. Using the scalpel blade gently cut along the left side of the vertebral column for about 2 cm rostral to the stab incision.
7. Insert scissor tips in incision site and gently spread open the exposure.
8. Place retractor at the L 4 vertebrae (right side), and a second retractor to counter-retract the muscle away from the vertebral column.
9. Clear the sacrum and iliac crests using blunt dissection technique (by rotating the blunt end of the Q-tip).
10. Identify the sacro-iliac rim, the L6/S1 articular facet, and the L6 transverse process.



11. Use rongeurs to gently nip off the articular facet close to the vertebral column, removing any connective tissue as well.

12. With a third retractor, open the exposure site further by scraping and retracting the muscle from the transverse process. Clear off the transverse process, using blunt dissection as necessary.
13. Clear away area between the transverse process and the sacro-iliac rim, exposing the connective tissue underneath.
14. Detach the connective tissue from the sacro-iliac rim, to allow access to the L6 transverse process.
15. Slowly nip away the L6 transverse process, moving cranial to allow for exposure and access to the L4/L5 nerve bundle (nerves appear snow white). When complete, the bone edges should be smooth to prevent any damage to the nerves. **Caution:** the nerve bundle lies encapsulated in the muscle and surrounding fascia.
16. Using a glass hook 'fish' around the nerve bundle on the medial side, advancing the tip ventral and cranial to get underneath the bundle. Gently elevate the bundle and separate it from the surrounding tissue (be careful in stretching the nerves, as stretch of L4 will result in foot drop). Slowly move the glass hook medially under the bundle until L4 drops off (more lateral and ventral nerve). Allow the glass hook tip to break through the fascia connecting the nerves. Ensure that the hook is cranial to the region where the two nerves join.
17. Once L4 is isolated/free, wrap a piece of sterilized 6-0 silk around the ball tip of the glass hook, and pass the hook tip underneath and back around the nerve.
18. Ligate the nerve ('finger-tight') using the deep tie method, and a square knot, so that the nerve bulges on both sides of the knot. Make sure that not doesn't also strangulate muscle, or any other tissue. Trim suture ends. **Note:** it is also possible to triple knot L4 to prevent loosing of the knot.
19. 'Fish' for L6 under the sacro-iliac rim, at a 45° angle (parallel to the sacro-iliac notch). Carefully work the ball tip cranial along the underside of the sacrum, as L6 lies tightly against the sacrum. Once the ball tip is clear of the sacro-iliac notch, gently pull it through the fascia. Ligate L6 as done for L5, ensuring that the nerve ligature doesn't also include surrounding tissue. **Caution:** Since there is minimal mobility to L6, it can easily snap or become pulled from the peripheral roots.

20. Swab out the surgery area, debride any necrotic muscle, remove retractors and gently 'roll' the muscle back into place (ensuring that no air pockets remain).
21. Close the wound first with subcutaneous sutures (continuous) using 3-0 novofil, followed by repeated cutaneous sutures.

C. Post-Operative Care.

1. Place animal in recovery box, with heated lamp, for observation.
2. Once ambulatory, return the rat to cage and include mash dish.
3. House each rat individually for 3-4 days, then return to double housing with environmental enrichment.
4. During initial recovery period, check for ambulation, eating, coat condition (groomed), and hydration levels.
5. Administer additional LRS and/or penicillin as required.

APPENDIX B

DAB Immunohistochemistry Protocol for detection of SP, NPY, CGRP, Hsp25/27

1. Place fixed brain in sucrose buffer (20% in 0.1 M PB) overnight.
2. Section using freezing microtome (4 series, 40 μ m thick) and collect sections in 50 ml beakers containing 0.05 M phosphate buffer saline (PBS).
3. Incubate sections in 1% H₂O₂ in PBS for 30 minutes
4. Wash 3 times in 0.2% triton-X in PBS (15 minute each wash).
5. Make 2% rabbit serum (dilute 200 μ l aliquot of goat serum into 10 ml of 1% triton-X in PBS and wash pipette tip 3 times in the serum).
6. Pellet sections and add 2 ml of 2% goat serum plus primary antibody 1:500 (SP and NPY), and 1:6000 (Hsp25/27). If there are many sections (i.e., more than half a brain) then use more than 1 ml of solution. Incubate sections for 48 hours at 4°C on a shaker.
7. Wash 3 times in PBS, then pellet.
8. Incubate sections in 1 ml of BIOGAR 1:500 (BIOTinylated Goat Anti-Rabbit; add 40 μ l aliquot of BIOGARS to 2 ml of 2% goat serum) 1–2 hours.
9. Make up ABC solution in preparation for step 10 (see notes below).
10. Wash 3 times in PBS, then pellet.
11. Incubate in 1 ml of ABCelite 1:500 (Avidin-Biotin-peroxidase Complex) 1–2 hours (for 1 ml of ABC*, pipette 2 μ l from solution "A" and add to 1 ml of 1% triton-X in PBS, then with a clean tip pipette 2 μ l from solution "B" and add to the 1% triton-X solution, allow to mix for 30 minutes).
12. Wash 3 times in PBS, then pellet.
13. Weigh out a small piece of DAB (diaminobenzidine; stored in freezer; you will need at least 0.0025 g for each series). Wear gloves.
14. Dissolve the DAB in a small amount of distilled water.
15. Add ? ml of 0.1 M Phosphate buffer (PB) such that the DAB is at a 0.0005 g/ml concentration.

16. **Make up hydrogen peroxide (H₂O₂) fresh each time (dilute 1 ml of 30% H₂O₂ into 100 ml of distilled water).**
17. **React sections in DAB, and monitor reaction rate by removing representative sections and analysing staining intensity (add 5 ml of the 0.5 mg/ml DAB solution and 150 μ l (30 μ l/ml of DAB) of the dilute H₂O₂ solution to the sections).**
18. **Stop reaction by rinsing sections 4 times in succession in PBS.**
19. **Pour the DAB solution into the waste DAB bottle and wash the sections 3 times in PBS. Store sections in the fridge.**

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