A STUDY OF THE ANTINOCICEPTIVE INTERACTION BETWEEN INTRATHECAL OPIOIDS AND a-ADRENERGIC AGONISTS IN THE SPINAL CORD OF THE RAT

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A STUDY OF THE ANTINOCICEPTIVE INTERACTION BETWEEN INTRATHECAL OPIOIDS AND α-ADRENERGIC AGONISTS IN THE SPINAL CORD OF THE RAT

by

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ABSTRACT

The role and mechanism of action of α_1-adrenoceptors in adrenergic spinal antinociception is uncertain. α_1-Adrenoceptors are normally excitatory in the CNS, suggesting that any inhibitory effect on nociception must be indirect. Recently, we showed that intrathecal (i.t.) α_1-agonists potentiate α_2-induced spinal antinociception in the rat by a δ-receptor mediated, enkephalin-dependent process. If enkephalins, released from spinal interneurons, mediate the antinociceptive effect of α_1-adrenoceptors, then non-selective α-agonists (i.e. norepinephrine; NE) should exhibit cross-tolerance to δ-agonists (i.e. DADLE), but not to μ-agonists (i.e. morphine), in the spinal cord. Conversely, the antinociceptive effect of α_2-selective agonists (i.e. dexmedetomidine; DX) should be unaffected by spinal opioid tolerance. To test these hypotheses, male, Sprague-Dawley rats (300-400g) were continuously infused with i.t. saline (1 μl/h), morphine (MOR; 5, 10, or 20 μg/h) or DADLE (10 μg/h) for 6 days using ALZET osmotic mini-pumps. Antinociception was assessed using the tail flick (TF) test. In an initial time course study, significant recovery from DADLE and MOR tolerance did not occur until day 3 and 4 post-infusion (PI), respectively. In subsequent cross-tolerance experiments, NE and DX dose-response curves were determined on days 1 and 2 PI for DADLE-pretreated rats, and on days 1-3 PI in MOR-pretreated rats. The table of ED_{50} ratios for i.t. NE and DX in opioid- and saline-infused animals demonstrates that NE exhibits significant (✳) cross-tolerance to DADLE, but not morphine. No cross-tolerance was observed between DX and DADLE.

INFUSION	TEST DRUG	ED_{50}(opioid)/ED_{50}(saline)
MOR 5	NE	1.12
MOR 10	NE	1.51
MOR 20	NE	1.35
DADLE 10	NE	2.54✳
DADLE 10	DX	0.66

In separate groups of rats, i.p. naloxone significantly attenuated the antinociceptive effect of MOR, but not DADLE, on day 1 of infusion (time of peak antinociception); i.p. naltrindole significantly antagonized DADLE, but not MOR. These data indicate that μ- and δ-receptor selectivity was retained during infusion, consistent with the different cross-tolerance results to NE. The marked antagonism of i.t. DX by Wyeth 27127, but not by prazosin, confirmed the α_2-selectivity of DX at the doses used. The results of this study are consistent with the hypothesis that α_1-adrenoceptors facilitate the release of enkephalins in the spinal cord which, in turn, effect antinociception by a δ-receptor mechanism.

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07:00h	7 o'clock in the morning
08:00h	8 o'clock in the morning
10:00h	10 o'clock in the morning
14:00h	2 o'clock in the afternoon
±	plus and minus, in addition to, subtracted from (about the mean)
%	percent
>	greater than
<	less than
≈	approximately equal to
5-HT	serotonin
α	alpha, a Greek letter, a subtype of adrenergic receptor
α_1	alpha-1, a subtype of α-adrenergic receptor
α_2	alpha-2, a subtype of α-adrenergic receptor
β	beta, a Greek letter, a subtype of adrenergic receptor
°C	degrees Celsius
δ	delta, a Greek letter, a subtype of opioid receptor
μ	mu, a Greek letter, used to indicate micro in metric units, also a subtype of opioid receptor
μg	microgram, 10^{-6} grams, unit of mass
μl	microliter, 10^{-6} liters, unit of volume
μm	micrometer, 10^{-6} meters, unit of length
Aß	A-beta, a class of primary afferent neurons
Aδ	A-delta, a class of primary afferent neurons
ANOVA	analysis of variance
AUC	area under the curve
C	a class of primary afferent neurons
Ca^{++}	calcium ion
CCK	cholecystokinin
CGRP	calcitonin gene-related peptide
CI	confidence interval
Cl^-	chloride ion
cm	centimeter(s), unit of distance
CNS	central nervous system
Co.	company
Corp.	corporation
CSF	cerebrospinal fluid
D	dextrorotatory
D_1	dopamine-1, a subtype of the dopamine receptor
D_2	dopamine-2, a subtype of the dopamine receptor
DADLE	D-Ala^2, D-Leu^5-Enkephalin, a δ-opioid agonist

DAMGO	[D-ala^2, MePhe^4, Gly-ol^5]enkephalin, a μ-opioid agonist
DPDPE	[D-Pen^2, D-Pen^5]enkephalin, a δ-opioid agonist
de novo	new
DMSO	dimethyl sulfoxide, an organic solvent
DSTBULET	Tyr-D-Ser(OtBu)-Gly-Phe-leu-Thr, a δ-opioid agonist
DX	dexmedetomidine, an α_2-receptor agonist
ED_{50}	effective dose for 50 percent response (ED50 used in figures)
e.g.	exempli gratia (for example)
ENK	enkephalin
et al.	*et alia* (and others)
g	gram(s)
GABA	γ-aminobutyric acid, γ-aminobutyrate
h	hour(s)
HCl	hydrogen chloride
ICI 174,864	N,N-diallyl-Tyr-Aib-Aib-Phe-Leu, a δ-opioid antagonist
ID_{50}	inhibitory dose decreasing a response by 50 percent
i.e.	id est (that is)
in vitro	in glassware
in vivo	in the living body
i.p.	intraperitoneal(ly)
i.t.	intrathecal(ly)
i.v.	intravenous(ly)
K^+	potassium ion
kg	kilogram(s), 10^3 grams
L	levorotatory
LC	locus coeruleus
log	logarithm (base 10)
Ltd.	limited
mg	milligram(s), 10^{-3} grams, unit of mass
min	minute(s)
ml	milliliter(s), 10^{-3} liters, unit of volume
mm	millimeter(s), unit of distance
MOR	morphine, a μ-opioid agonist
MPE	maximum percent effect
mRNA	messenger ribonucleic acid
MX	methoxamine, an α_1-receptor agonist
n	number of determinations
NE	norepinephrine, a non-selective α-agonist
ng	nanogram(s), 10^{-9} grams
nmol	nanomole(s), 10^{-9} moles
NTI	naltrindole, a δ-opioid antagonist
NX	naloxone, a μ-opioid antagonist

P	probability of error
PAG	periaqueductal gray
PE-10	size 10 polyethylene tubing (diameter≈0.61 mm)
PE-60	size 60 polyethylene tubing (diameter≈1.22 mm)
PL 017	Tyr-Pro-NMePhe-D-Pro-NH_2, a selective μ-agonist
POMC	pro-opiomelanocortin
PZ	prazosin, an α_1-antagonist
s	second(s)
s.c.	subcutaneous(ly)
SAL	saline
SCH 32615	Schering 32615, a neutral endopeptidase inhibitor
SD	standard deviation
SEM	standard error of the mean
SP	substance P, a neurotransmitter
ST-91	2-[2,6-diethylphenylaminol]-2-imidazoline, an α_2-agonist
TF	tail flick
VIP	vasoactive intestinal peptide
WB-401	2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane hydrochloride, an α_1-antagonist
WY	Wyeth 27127 (disulphonamino-benzoquinoline), an α_2-antagonist

1.0 INTRODUCTION

1.1 Statement of the Research Problem

The perispinal administration of α-adrenoceptor agonists produces robust antinociception in a variety of experimental animals, without affecting muscle strength, motor reflexes and locomotor activity (see review by Yaksh, 1985). Extensive pharmacological studies using receptor-selective agonists and antagonists have yielded structure activity profiles indicative of an antinociceptive effect mediated by spinal α_2-adrenoceptors. These results are consistent with receptor binding and autoradiographic data demonstrating the dense localization of α_2-binding sites in the substantia gelatinosa (Unnerstall *et al.*, 1984; Sullivan *et al.*, 1987; Simmons and Jones, 1988), and the inhibitory role played by α_2-adrenoceptors in the CNS. Thus, the activation of spinal α_2-adrenoceptors has been shown to inhibit neurotransmitter release from the central terminals of primary afferent pain fibers (Howe and Zieglgänsberger, 1987; Ueda *et al.*, 1995); and to depress high threshold stimulus-evoked activity in wide dynamic range neurons (Fleetwood-Walker *et al.*, 1985; Omote *et al.*, 1991). Importantly, the functional and behavioral inhibition mediated by spinal α_2-adrenoceptors appears to be independent of endogenous opioid systems (Figure 1).

The contribution of α_1-adrenoceptors in spinal adrenergic antinociception is less clear. Radioligand binding studies and quantitative autoradiography indicate a homogeneous distribution of α_1-adrenoceptors throughout the grey matter of the

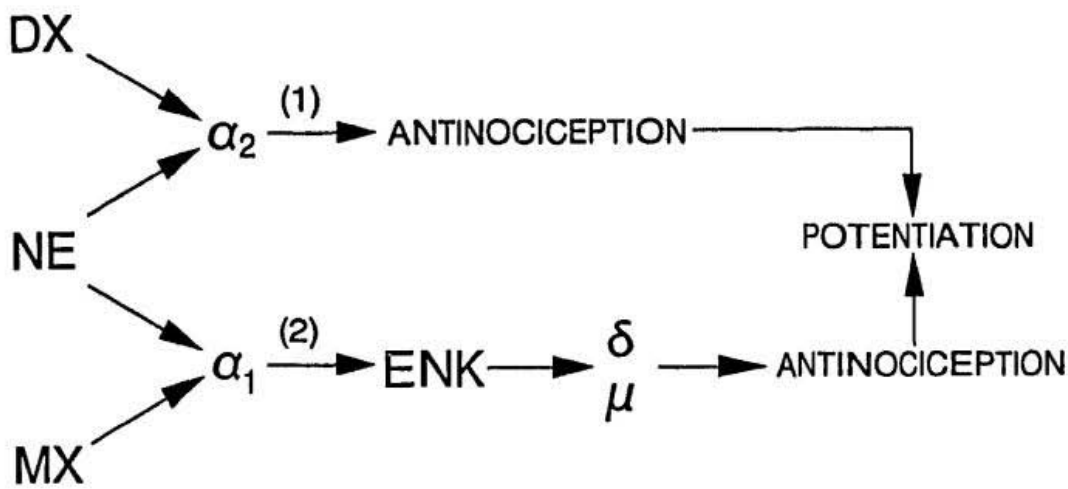

Figure 1. Proposed mechanisms underlying spinal adrenergic antinociception, and the hypothesized opioid link to α_1-adrenoceptors. Activation of the α_1-receptor by drugs such as methoxamine (MX) or norepinephrine (NE) may facilitate the release of enkephalins which act preferentially on δ-opioid receptors to effect antinociception. Co-activation of α_1- and α_2-receptors could lead to the potentiation of spinal adrenergic analgesia via an opioid-α_2 interaction.

rat spinal cord (Giron *et al.*, 1985; Simmons and Jones, 1988; Roudet *et al.*, 1993). That these binding sites are functionally coupled is demonstrated by the pharmacological effects of α_1-selective agonists and antagonists on motor, autonomic and sensory function in the spinal cord of experimental animals (Davis and Astrachan, 1981; Astrachan *et al.*, 1983; Howe *et al.*, 1983; Yaksh, 1985; Loomis and Arunachalam, 1992). In the latter case, intrathecal (i.t.) α_1-agonists like methoxamine and phenylephrine significantly increased the thermal nociceptive threshold of conscious rats (tail-flick and hot-plate tests) (Howe *et al.*, 1983; Yaksh, 1985). Unlike α_2-agonists however, antinociceptive doses of methoxamine and phenylephrine facilitated motor reflexes, raising questions about the selectivity of action and thus the potential of α_1-agonists as spinal analgesic drugs. Nevertheless, the contribution of α_1-adrenoceptors in spinal adrenergic antinociception/analgesia has never been disproved by experimental data.

α_1-Adrenoceptors are excitatory in the CNS (Aghajanian and Rogawski, 1983), enhancing neuronal depolarization and Ca^{++} influx. Consequently, the ability of α_1-agonists to effect spinal antinociception suggests that they activate, or at least facilitate, an inhibitory neural input on primary afferent and/or projection neurons in nociceptive pathways. Enkephalin, one of three major endogenous opioid peptides, is known to selectively modulate nociceptive transmission in the spinal cord (see review by Yaksh, 1993). Enkephalin-containing neurons, and the δ-opioid receptors that are believed to mediate the biological activity of enkephalin, are densely

localized in the dorsal horn (Dado *et al.*, 1993; Todd and Spike, 1992; Ruda *et al.*, 1986). There is an extensive literature documenting the antinociceptive synergy between opioids, including enkephalin analogues, and α-agonists, following co-injection into laboratory animals (Ossipov *et al.*, 1990; Omote *et al.*, 1991; Roerig *et al.*, 1992). A similar interaction has also been reported in humans (Motsch *et al.*, *1990;* Gordon *et al.*, 1992; Siddall *et al.*, 1994). Recent studies in our laboratory have shown that, in the rat, a threshold dose of i.t. methoxamine significantly potentiates the antinociceptive effect of the highly selective α_2-agonist, dexmedetomidine, via a δ-receptor-mediated, enkephalin-dependent process (Loomis *et al.*, 1992a, 1992b, 1993). Although a pharmacokinetic interaction between the two drugs could not be totally excluded, the data suggest that methoxamine, and presumably other α_1-agonists, augment the antinociceptive activity of α_2-agonists in the rat spinal cord by facilitating the local release of enkephalin (see Figure 1). Extracellular enkephalin would then be free to inhibit nociceptive transmission through its normal opioid receptor-coupled mechanisms in the spinal dorsal horn. In this manner, the co-activation of α_1- and α_2-adrenoceptors with appropriate receptor agonists could induce, albeit indirectly, an α_2-opioid interaction that is known to be both supra-additive and antinociceptive. Considering the fact that norepinephrine (NE), the neurotransmitter released from bulbospinal neurons of the endogenous pain control system, is a *non-selective* α-agonist, and the proximity of enkephalin-containing neurons to the terminals of these

noradrenergic fibers, it is possible that such an interaction also underlies the modulatory effect of NE, at least in the rat.

If the hypothesis illustrated in Figure 1 is correct, then non-selective α-agonists like NE, and α_1-selective agonists like methoxamine, should exhibit cross-tolerance to δ-agonists in the rat spinal cord. Conversely, highly selective α_2-agonists such as dexmedetomidine (DX) should be unaffected by tolerance to i.t. δ-agonists. Using dose-response analysis, we determined the degree of cross-tolerance between opioid- and α-receptor subtype selective agonists in the rat tail-flick test. Opioids were delivered by continuous i.t. infusion using ALZET osmotic mini-pumps. The antinociceptive effect of the i.t. α-agonist was determined in tolerant and non-tolerant animals by means of dose response analysis. Agonist selectivity was verified using receptor subtype-selective antagonists.

1.2 Spinal Opioid Analgesia

Although opioids unquestionably produce analgesia by a supraspinal action, basic and clinical studies have shown that they also act directly in the spinal cord to inhibit the transmission of pain (see reviews by Yaksh and Noueihed, 1985; Yaksh, 1993). Electrophysiological studies in animals first demonstrated the potent depressant actions of morphine on the electrical responses of spinal neurons evoked by noxious stimuli; an effect antagonized by specific opioid antagonists (Belcher and Ryall, 1978; Duggan *et al.*, 1977; Sastry, 1978). The conclusion from

these studies was that, at appropriate doses, opioids could selectively depress the activity of small afferent fibres involved in pain transmission without affecting large diameter fibers.

The development of the technique of chronic catheterization of the subarachnoid space in several animal species (Yaksh and Rudy, 1976a) made it feasible to apply opioids to the spinal cord of conscious animals. Using this procedure, spinal opioids were shown to produce segmental analgesia that was dose-dependent, stereospecific and antagonized by naloxone (Yaksh and Rudy, 1976b). At analgesic doses, the acute i.t. injection of opioids in animals has no major effect on respiration, muscle activity or autonomic function (Yaksh and Rudy, 1976b), although large dose can produce hind limb weakness and convulsions (Frenk *et al.*, 1984; Watkins *et al.*, 1984).

Within three years of the first report of spinal opioid analgesia in rats, spinal morphine was tested in humans (Wang *et al.*, 1979). Wang and coworkers reported that the i.t injection of 0.5-1.0 mg of morphine relieved severe back and leg pain in patients with inoperable malignancies of the genitourinary tract for up to 24 h. Since that first clinical report, spinal opioids have been used successfully in the treatment of postoperative pain, obstetric pain, pain associated with trauma, deafferentation pain and pain arising from chronic illness, including cancer. In the clinical management of pain, both i.t. and epidural routes of administration are used.

The selective and localized effect of spinal opioids offers a number of

advantages over other forms of pain control. First, the duration of analgesia is longer with spinal injection than with systemic administration of the same agent. In humans, systemically administered morphine (10 mg, s.c.) produces analgesia lasting for 4-5 h (Jaffe and Martin, 1990), while the equivalent dose administered epidurally is effective for up to 18 h (Cousins and Mather, 1984). The duration of spinal analgesia permits a longer dosing interval during repeated administration, thereby providing more freedom to the patient and the attendant medical personnel. A second advantage is that the i.t. or epidural injection of opioids concentrates the drug near its site of action in the dorsal horn. Consequently, effective analgesia can be produced with lower doses of opioids than those required for systemic administration. For example, in the treatment of cancer pain, doses of i.t. morphine ranging from 0.5-16 mg, and doses of epidural morphine ranging from 2-30 mg have been recommended (Payne, 1987). This is in contrast to oral doses of 30-60 mg (Foley and Inturrisi, 1987) which must also be given more frequently than i.t. morphine. As a result, the concentration of drug eventually reaching the systemic circulation after spinal injection is low and the incidence of adverse effects mediated at supraspinal sites, such as mental clouding and drowsiness, is reduced (Payne, 1987). A third advantage is that the selective action of spinal opioids avoids the complication of sympathetic blockade associated with spinal anesthesia. Overall, the absence of autonomic and motor effects with spinal opioids provides patients with adequate pain control over long periods of time, while remaining ambulatory

and having an otherwise functional sensorium.

The advantages afforded by spinal opioids have been tempered however by the development of adverse effects, including delayed respiratory depression, pruritus, urinary retention, tolerance and withdrawal. Respiratory depression is a life-threatening consequence of spinal opioid use that generally occurs 6-12 h after injection. The use of more lipid soluble opioids such as fentanyl has partially overcome this problem but patients do require continuous and intensive monitoring. In the management of chronic pain, tolerance is a factor limiting the use of spinal opioids. For example, of 62 patients receiving i.t. morphine for cancer pain, 74% of the patients experienced effective analgesia without severe respiratory depression or loss of motor or sensory functions (Wang, 1985). Many of the patients were effectively managed as outpatients. However, 43% of patients given repeated i.t. morphine injections, and 50% of patients receiving continuous i.t. infusion via drug pumps developed tolerance and severe complications (pruritus, sphincter disorder, and somnolence). In addition, withdrawal in patients receiving epidural opiates has been reported following discontinuation of treatment or when treatment is antagonized by substitution of a pure agonist with a partial agonist or agonist-antagonist (Cousins and Mather, 1984). While i.t. opioids are useful in the management of chronic pain, it is clear that more effective drugs or combinations of drugs are needed to reduce the problems of tolerance and the incidence of adverse effects. Moreover, some types of pain, particularly those of a non-

malignant nature, do not respond to opioids administered either spinally or systemically (i.e. neural injury pain) (Arnèr and Meyerson, 1988; Siddal *et al.*, 1994).

Synaptic transmission between primary afferent fibres and second order neurons that comprise the spinothalamic tract is subject to modulation by a large number of neurotransmitters/neuromodulators within the spinal cord (substance P, VIP, CCK, somatostatin, neurotensin, angiotensin, met-enkephalin, bombesin, excitatory amino acids, GABA, norepinephrine, and serotonin)(Hökfelt *et al.*, 1977; Barber *et al.*, 1978; Salt and Hill, 1983; Basbaum and Fields, 1984; Besson and Chaouch, 1987; Yaksh, 1993). Drugs that mimic the actions of spinal neurotransmitters or neuromodulators that inhibit pain transmission could be useful as spinal analgesics. To the extent that these drugs exert their effect through a receptor subtype and an intracellular mechanism that is distinct from those of the opioid peptides, they could be used as adjuncts with spinal opioids or as an alternative therapy to optimize analgesia.

1.3 Endogenous Pain Control System - Descending Inhibitory Pathways

Anatomical and pharmacological studies have identified descending and intrinsic neuronal systems in the spinal cord that modulate nociceptive transmission in the dorsal horn (Dahlstrom and Fuxe, 1965; Basbaum and Fields, 1984; Fields and Basbaum, 1989). Neurons utilizing serotonin (5-HT) as a neurotransmitter descend from the nucleus raphe magnus in the medulla through the dorsolateral

funiculus of the spinal cord to terminate in the outer laminae of the dorsal horn. Similarly, noradrenergic neurons descend from nuclei in the pons, including the locus coeruleus (LC), the medial and lateral parabrachial nuclei, nucleus subcoeruleus, the A5 nucleus, and the A7 nucleus (see reviews by Proudfit, 1988; Jones, 1991) to terminate in the outer dorsal horn. Following their release in the spinal cord, NE and 5-HT inhibit the evoked discharge of spinothalamic neurons.

These descending (bulbospinal) inhibitory pathways are thought to provide a regulatory feedback loop, whereby nociceptive transmission through the dorsal horn is modulated by afferent inputs reaching the thalamus, periaqueductal grey (PAG) and brainstem. For example, afferent input in the spinomesencephalic tract (and probably other tracts ascending through the anterolateral quadrant of the spinal cord), as well as input from the hypothalamus, thalamus and cortex, activate the PAG. By mechanisms that remain poorly understood, the PAG appears to coordinate the response of these inhibitory bulbospinal pathways to noxious stimulation (Fields and Basbaum, 1989).

The discovery of these monoaminergic (and other) systems, the identification of the neurotransmitters underlying their modulatory effect, and subsequent studies of their release and spinal pharmacology, provided important evidence for *non-opioid* modulation of noxious sensory processing in the spinal cord of experimental animals.

1.4 NE and 5-HT Effect Spinal Antinociception

Consistent with their inhibitory role in the spinal cord, and like the opioids, NE and 5-HT were shown to depress the discharge of dorsal horn neurons driven by noxious stimulation (Headley *et al.*, 1978). In subsequent rodent behavioral studies, i.t. NE and 5-HT significantly inhibited the escape responses evoked by noxious thermal stimuli, at doses that did not affect muscle strength, normal reflexes or locomotor activity (Yaksh and Wilson, 1979; Yaksh and Reddy, 1981; Milne *et al.*, 1985). However, NE was approximately 30 times more potent than 5-HT. The spinal antinociceptive effect of NE could be blocked by α-, but not β-adrenoceptor antagonists, suggesting the former to be the relevant receptor subtype. Antinociception was also potentiated by monoamine oxidase inhibitors and monoamine reuptake blockers (Kuraishi *et al.*, 1979; Reddy *et al.*, 1980; Reddy and Yaksh, 1980; Yaksh and Wilson, 1979). In view of the fact that vasoconstrictors, such as angiotensin II, and vasodilators, such as bradykinin, given i.t., were without effect in these behavioral tests, it was concluded that i.t. monoamine-induced antinociception is not secondary to changes in spinal cord blood flow. Rather, it is a direct effect on sensory neurons in the spinal cord, consistent with the results of electrophysiological studies described above. While their short time course of action (<30 min) makes i.t. NE or 5-HT impractical for clinical use, these studies provided direct experimental evidence that monoamines could selectively inhibit the behavioral responses to noxious stimuli in conscious behaving animals.

Importantly, the antinociceptive effect of i.t. NE is shared by other α-agonists. For example, low dose i.t. clonidine produced significant antinociception without disturbing motor function (Yaksh and Wilson, 1979; Yaksh and Reddy, 1981; Milne *et al.*, 1985b). In general, the rank order of potency of α-agonists in behavioral tests of nociception parallels that observed in other pharmacological preparations utilizing α-adrenoceptors. A fundamental question arising from these results, and one critical for the rational selection of an adrenergic spinal analgesic, is the α-adrenoceptor subtype(s) mediating antinociception in the spinal cord.

1.5 Spinal Adrenergic Receptors and Antinociception

1.5.1 Binding Studies

Early binding studies with radiolabelled ligands that could discriminate between α_1-, α_2- and ß-adrenoceptors confirmed the presence of all three binding sites in the spinal cord (Jones *et al.*, 1982). Autoradiography with [^{3}H]WB-4101, an α_1-selective antagonist, indicated a moderate density of α_1-binding sites in the substantia gelatinosa and the spinal trigeminal nucleus of the rat (Young and Kuhar, 1980). Similar studies with [^{3}H]para-aminoclonidine showed the highest density of α_2-binding sites in rat and human spinal cords to be in the substantia gelatinosa and the intermediolateral cell column (Young and Kuhar, 1980; Unnerstall *et al.*, 1984). In contrast, α_2-binding sites were very low in the ventral horn. Using the fluorescent probe, 9-amino-acridine propranolol, a high density of ß-receptors was located in the

region of the ventral horn containing α-motoneurons (Melamed *et al.*, 1976). ß-Receptor binding was sparse in the dorsal horn except in the substantia gelatinosa where moderate ß-receptor binding was observed.

In a subsequent study, the lesioning of descending adrenergic fibers with 6-hydroxydopamine was shown to have no detectable effect on the density of α_2-binding sites in the gray matter of the cat lumbar spinal cord (Howe *et al.*, 1987a). These data indicate that the majority of α_2-binding sites are present on cells in the lumbar spinal gray matter, and not on the spinal terminals of descending adrenergic neurons. This is further supported by autoradiographic data indicating that α_2-adrenoceptors are located on spinal dorsal horn neurons in the rat (Sullivan *et al.*, 1987). Howe *et al.* (1987b) also showed that a unilateral ganglioectomy of the dorsal roots in the cat yielded only a 20% reduction in the total number of α_2-binding sites in the ipsilateral lumbar dorsal horn. It was inferred that the remaining 80% of α_2-sites are post-synaptic to the primary afferents neurons (i.e. on projection neurons and/or interneurons).

1.5.2 Pharmacological Studies

The observation of α_1-, α_2- and ß-binding sites in the substantia gelatinosa, an area of the dorsal horn known to modulate nociceptive input, raised the possibility that all three types of adrenergic sites could be "analgetically coupled". However, the i.t. injection of the ß-receptor agonist, isopropylnorepinephrine, had no effect on

the behavioral responses of rats to thermal nociceptive stimuli, Likewise, i.t. NE-induced antinociception was not reversed by the ß-antagonist, propranolol (Reddy *et al.*, 1980). In contrast, a variety of α-agonists, with differing selectivities for α_1- and α_2-adrenoceptors, produced significant, dose-dependent antinociception following i.t. administration to laboratory animals (Reddy *et al.*, 1980; Yaksh and Reddy, 1981; Milne *et al.*, 1985b; Loomis *et al.*, 1985; Sherman *et al.*, 1987); an effect that was dose-dependently inhibited by phentolamine, but not by propranolol (Reddy *et al.*, 1980; Yaksh and Reddy, 1981; Milne *et al.*, 1985b). The resulting rank order of potency in the rat tail flick, hot plate and acetic acid writhing tests was: ST-91 (α_2-selective agonist) = NE > methoxamine (α_1-selective agonist) >> isopropylnorepinephrine = 0. Similar results were reported in the primate shock titration test (Yaksh and Reddy, 1981). Thus, the ability of i.t. NE and other adrenergic agonists to elevate nociceptive threshold appears to be uniformly mediated by α-adrenoceptors.

To assess the relative role of spinal α_1- and α_2-adrenoceptors in noradrenergic antinociception, dose-response studies using α_1- and α_2-selective antagonists were undertaken. Intrathecal yohimbine significantly inhibited i.t. ST-91 in the tail flick and hot plate test. Consistent with its selective blockade of α_2-adrenoceptors in this experiment, the ID_{50} of yohimbine was 1/10th that of i.t. prazosin (Howe *et al.*, 1983). In contrast, i.t. prazosin was approximately 10 times more potent yohimbine in antagonizing the antinociceptive effect of i.t. methoxamine

and NE. The relative order of activity in reversing thermal antinociception with i.t. NE was: prazosin, phentolamine, rauwolscine, yohimbine, coryanthine, propranolol (=0) (Yaksh, 1985; unpublished observations). The relative order of activity in reversing ST-91 was: yohimbine, rauwolscine, prazosin, phentolamine, coryanthine, propranolol (=0) (Yaksh, 1985). Thus, the dominant population of adrenergic receptors mediating spinal antinociception in the rat exhibit a pharmacological profile characteristic of α_2-adrenoceptors. Nevertheless, i.t. NE (a non-selective α-agonist) was more potently antagonized by i.t. prazosin than i.t. yohimbine (Howe *et al.*, 1983; unpublished observations), suggesting that spinal α_1-adrenoceptors may contribute to the overall inhibitory effect.

Indeed, α_1-selective agonists, including methoxamine, cirazoline and phenylephrine, were shown to inhibit the behavioral responses to thermal and mechanical nociceptive stimuli (Reddy *et al.*, 1980; Howe *et al.*, 1983; Yaksh, 1985; unpublished observations). A non-selective effect at α_2-adrenoceptors, particularly at high doses, could not account for these results as methoxamine was more potently antagonized by prazosin than yohimbine (Howe *et al.*, 1983). The α_1-agonists had a lower maximum antinociceptive effect compared to NE or to the α_2-selective agonists. In agreement with this observation, phenylephrine and cirazoline were shown to be less efficacious than NE in stimulating the maximum accumulation of inositol phosphate (Chiu *et al.*, 1987), the second messenger coupled to α_1-adrenoceptors (Minneman and Johnson, 1984). However,

antinociceptive doses of i.t. methoxamine and phenylephrine also produced clear motor effects. In the primate, these include hind limb tremor and exaggerated reflex withdrawal responses to non-nociceptive stimuli. In the rat, dose-dependent, cutaneous hyperreflexia, clonic flexion of the hindlimbs, rigidity and serpentine movements of the tail have been observed (Yaksh, 1985; unpublished observations). These exaggerated reflexes, consistent with the facilitatory role of spinal α_1-adrenoceptors on motor neurons (Tanabe *et al.*, 1990), could explain the apparent lower efficacy of α_1- as compared to α_2-agonists given spinally. At the very least, the measurement of the behavioral responses to nociceptive stimuli following i.t. α_1-agonists, involving both reflex and supra-spinally co-ordinated motor responses, are confounded by such an effect. Thus, the role of spinal α_1-adrenoceptors in adrenergic antinociception has not been completely or accurately investigated.

1.6 Cellular Mechanisms Underlying Spinal α_1- and α_2-Adrenoceptor-Mediated Antinociception

The activation of neuronal α_2-adrenoceptors results in the opening of outwardly directed K^+ channels. This causes a hyperpolarization of the cell, which results in the suppression of neuronal firing (see figure 3) (Nakamura *et al.*, 1981; Egan *et al.*, 1983). As well, α_2-adrenoceptor activation can inhibit voltage sensitive calcium channels (Williams and North, 1985), thereby suppressing Ca^{++} influx and

the fusion of neurotransmitter-filled vesicles with the synaptic membrane. These actions on ion channels are believed to be mediated through a G-protein (Dunlap *et al.*, 1987). Consistent with these observations, it has been shown that α-agonists, acting primarily through the α_2-adrenoceptor (Kuraishi *et al.*, 1985; Go and Yaksh, 1987) can inhibit the release of substance P in mammalian spinal cords, using both *in vivo* (Kuraishi *et al.*, 1985; Go and Yaksh, 1987) and *in vitro* (Pang and Vasko, 1986) preparations. Similarly, α_2-receptor activation has been shown to inhibit the release of calcitonin gene-related peptide (CGRP) (Holz *et al.*, 1989). α_2-Adrenoceptors also hyperpolarize receiving neurons in the spinal cord and thus attenuate their response to neurotransmitters released from adjacent nerve terminals.

α_1-Adrenoceptors have been shown to regulate the level of excitability of cells, rather than participating in the transmission of rapid signals (Aghajanian and Rogawski, 1983). Using iontophoretic drug delivery and intracellular recording from motoneurons in the facial nucleus, NE produced a long-lasting depolarization that was associated with a decrease in the membrane conductance to K^+. Thus, NE brought these cells closer to the threshold for action potential generation, explaining the ability of α_1-receptor activation to facilitate neuronal transmission (Aghajanian and Rogawski, 1983). Electrophysiological studies using *in vitro* spinal cord preparations have also shown that NE can excite unidentified dorsal horn neurons; an effect mediated by α_1-receptors (North and Yoshimura, 1984).

Hence, it appears that α_2- and α_1-adrenoceptors affect K^+ conductance in opposing ways. α_2-Adrenoceptor activation increases K^+ conductance leading to cell hyperpolarization and the inhibition of neuronal transmission. In contrast, α_1-adrenoceptor activation decreases K^+ conductance leading to depolarization and an increase in neuronal excitability. Unlike the electrophysiological results of the α_2-agonists, these data appear inconsistent with an antinociceptive effect. Indeed, electrophysiological recordings have confirmed the depolarization of many dorsal horn neurons following the local application of NE or α_1-agonists (North and Yoshimura, 1984; Todd and Millar, 1983; Howe and Zieglgänsberger, 1987).

1.7 α_1-Facilitation of Enkephalinergic Inhibition in the Rat Spinal Cord

In view of the depolarizing effect of α_1-adrenoceptors in the CNS (Aghajanian and Rogawski, 1983), the ability of α_1-agonists to effect spinal antinociception suggests that they activate, or at least facilitate, an inhibitory neural input on primary afferent and/or projection neurons in nociceptive pathways. Enkephalin, one of three major endogenous opioid peptides, is known to selectively modulate nociceptive transmission in the spinal cord (see review by Yaksh, 1993). Enkephalin-containing neurons, and the δ-opioid receptors that are believed to mediate the biological activity of enkephalin, are densely localized in the dorsal horn (Dado *et al.*, 1993; Todd and Spike, 1992; Ruda *et al.*, 1986). Enkephalin is known to be released in the spinal cord following a noxious stimulus in a number of

species. Noxious mechanical stimulation has been shown to increase the release of met-enkephalin-like material in the spinal cord of anesthetized rats (LeBars *et al.*, 1987), and high intensity stimulation of peripheral nerves and the intra-arterial injection of bradykinin produces an enhanced release of met-enkephalin-like material in the spinal perfusates of anesthetized cats (Yaksh and Elde, 1981). Given the excitatory nature of α_1-adrenoceptors in the CNS, and the location of enkephalin-containing neurons in the dorsal horn, activation of these receptors could facilitate the local release of enkephalin in the spinal cord.

Intracellular recordings indicate that opioids induce a hyperpolarization of neurons, secondary to an increase in K^+ conductance (Williams *et al.*, 1982; Yoshimura and North, 1983). The relevance of these data to the antinociceptive effect of opioids is indicated by the observation that: a) the elevated mouse tail-flick latency induced by μ-, δ-, or κ-agonists is attenuated by K^+ channel blockers; and b) antinociception induced by K^+ channel openers is blocked differentially by i.t. opioid antagonists (norbinaltorphimine, ICI 174,864, and naloxone) (Welch and Dunlow, 1993). The results also suggest that these two classes of drugs probably do not interact at a common receptor, but rather with a common second messenger system (Welch and Dunlow, 1993).

Opioid-induced hyperpolarization of dorsal horn neurons inhibits the release of nociceptive neurotransmitters (see Figure 1). Thus, μ-agonists have been shown to inhibit the release of substance P *in vivo* (Yaksh *et al.*, 1980; Kuraishi *et al.*, 1983;

Go and Yaksh, 1987) and *in vitro* (Jessell and Iversen, 1977; Pang and Vasko, 1986). Substance P is a neurotransmitter found in small primary afferent fibers and released by Aδ/C-fiber activity (Yaksh *et al.*, 1980; Kuraishi *et al.*, 1983; see review by Yaksh and Noueihed, 1985). Similarly, enkephalin-analogues (i.e. δ-agonists) attenuate the evoked release of substance P *in vitro* (Jessell and Iversen, 1977; Mudge *et al.*, 1979) and *in vivo* (Go and Yaksh, 1987); an effect that is antagonized by naloxone. Anatomical and electrophysiological data also suggest that opioids can act at receptors located post-synaptically to primary afferent terminals to suppress nociceptive processing (see Figure 2) (see review by Yaksh and Noueihed, 1985). D-Ala^2-D-Leu^5-enkephalin (DADLE) attenuated glutamate-induced excitation of attached rootlets *in vitro* (Zieglgänsberger and Sutor, 1983), and intracellular recordings *in vitro* showed that application of morphine, DADLE, and met-enkephalin results in hyperpolarization. These effects were antagonized by naloxone. Additional evidence for a post-synaptic action of opioids has been provided by recordings of cell bodies in the nucleus proprius following the application of opioids near the terminals in the substantia gelatinosa. In particular, morphine applied to the dorsal lamina has been shown to depress responses evoked by noxious thermal stimuli, but has no effect when administered near the cell body (see review by Yaksh, 1985).

These data indicate that enkephalin-containing interneurons, and the opioid receptors mediating their effect, are strategically located in the dorsal horn where

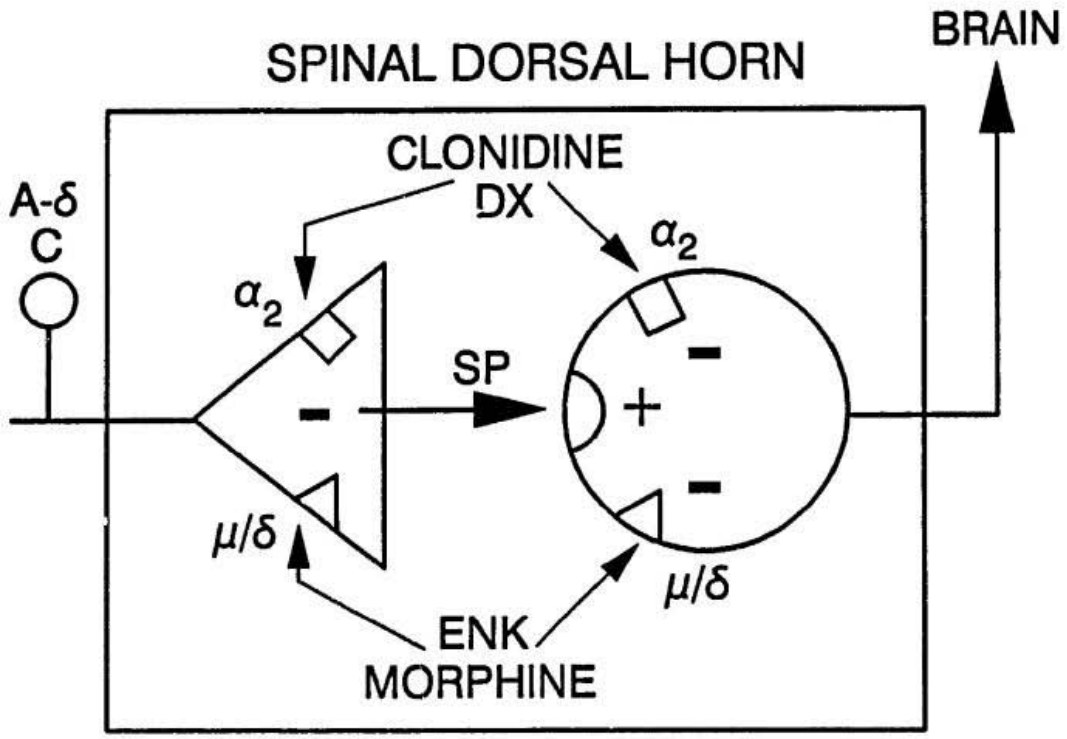

Figure 2. Pre- and post-synaptic sites of antinociceptive action of opioid- and α_2-adrenergic agonists in the dorsal horn of the spinal cord. The large triangle represents a central nerve terminal of a small diameter, myelinated (Aδ) or unmyelinated (C) primary afferent fiber from which substance P (SP) is released. The large circle represents the soma of an adjacent second-order neuron in the pain pathway which is activated (+) by substance P. The small triangles represent μ- and δ-opioid receptors mediating the inhibitory (-) effects of morphine and enkephalin (ENK), respectively. α_2-Adrenoceptors, mediating the inhibitory effects of clonidine or dexmedetomidine (DX), are indicated by the small squares.

they normally modulate nociceptive transmission across the first synapse in the pain pathway. By decreasing K^+ conductance, α_1-adrenoceptor activation would bring these neurons closer to the threshold for action potential generation thereby facilitating the endogenous release of enkephalin under conditions of noxious stimulation.

1.8 Antinociceptive Interaction Between Spinal α_1- and α_2-Agonists in the Rat - Opioid and Adrenergic Synergy

Previous studies in our laboratory have shown that a threshold dose of i.t. methoxamine (10 µg) potentiates the effect of i.t. dexmedetomidine (α_2-selective agonist) in the rat tail-flick and paw pressure tests (Loomis *et al.*, 1992a, 1992b, 1993). Dexmedetomidine (0.01-1 µg i.t.) alone produced dose-dependent antinociception (ED_{50} = 45 ng in the tail-flick test and 252 ng in the paw pressure test). The addition of a fixed dose of methoxamine (10 µg i.t.), yielding <5% MPE in the tail-flick test and 0% MPE in the paw pressure test when injected alone, significantly shifted the dexmedetomidine dose response curve to the left (ED_{50} = 8.1 ng; tail-flick test and 10 ng; paw pressure test). Methoxamine did not prolong dexmedetomidine's duration of action suggesting that a pharmacokinetic interaction was unlikely. Moreover, pretreatment with SCH 32615 (75 µg i.t.; a neutral endopeptidase inhibitor), but not vehicle, produced a further parallel leftward shift of the methoxamine + dexmedetomidine dose response curve (ED_{50} = 1.8 ng; tail-

flick test and 7.7 ng; paw pressure test). This potency shift exceeded the effect of SCH 32615 on dexmedetomidine alone. A fixed dose combination of methoxamine (10 µg) + dexmedetomidine (0.025 µg), producing near maximal activity in the tail-flick test and intermediate activity in the paw pressure test, was significantly blocked by each of the following pretreatments: prazosin (10 µg i.t.), WY 27127 (0.5 µg i.t.), naloxone (30 µg i.t.), ICI 174,864 (75 µg/kg i.p.), and antiserum to Met-enkephalin (10 µl i.t.). These data strongly suggest that the synergy exhibited between MX and DX involves a spinal enkephalin-dependent process, consistent with the known antinociceptive synergy between spinal opioid- and α_2-agonists in experimental animals (Ossipov *et al.*, 1990; Omote *et al.*, 1991; Roerig *et al.*, 1992), and in humans (Motsch *et al., 1990;* Gordon *et al.*, 1992; Siddall *et al.*, 1994). The interaction described between α-agonists is not specific to MX and DX. Intrathecal NE-induced antinociception in the rat was also potentiated by SCH 32615, and antagonized by i.t. naltrindole (δ-selective antagonist) or i.t. naloxone (Loomis *et al.*, 1993). Thus, α_1-adrenoceptors may be contribute to spinal adrenergic antinociception by facilitating the local enkephalinergic-modulation of nociceptive transmission (Figure 1).

1.9 Rationale, Research Hypothesis and Specific Objectives

There is an extensive and consistent body of evidence indicating that α_2-adrenoceptor mediated antinociception is a direct and selective inhibitory effect on

relevant sensory neurons in the spinal cord that is independent of endogenous opioid systems. The pharmacological activation of spinal α_1-adrenoceptors effects antinociception in experimental animals. However, these receptor subtypes are normally excitatory in the CNS suggesting that their ability to interrupt nociceptive transmission in the spinal cord must involve the activation or facilitation of an inhibitory neural input. Considering the dense localization of enkephalinergic interneurons in the dorsal horn, the selective modulatory effect of enkephalin on nociception in the spinal cord, and the well characterized antinociceptive interaction between opioid- and α_2-agonists in the spinal cord of experimental animals, we hypothesized that spinal α_1-adrenoceptors facilitate the release of enkephalin in the rat spinal cord. The observation that i.t. methoxamine significantly potentiated the inhibitory effect of i.t. DX in thermal and mechanical nociceptive tests via an enkephalin-dependent process provides further support for such a mechanism.

If this hypothesis is correct, then non-selective α-agonists (i.e. those with affinity for both α_1- and α_2-adrenoceptors), given i.t. to the rat, should exhibit antinociceptive cross-tolerance to i.t. δ-selective agonists. That is, down-regulation of spinal δ-opioid receptors following the continuous i.t. infusion of a δ-selective agonist (i.e. DADLE) should attenuate the *hypothesized* enkephalinergic mechanism underlying α_1-mediated antinociception in the spinal cord (see Figure 1). In contrast, α_2-selective agonists (e.g. DX), that do not utilize an enkephalin-dependent mechanism, should exhibit no such cross-tolerance (Figure 1).

To test this hypothesis, male Sprague-Dawley rats were continuously infused with either morphine (μ-agonist), D-Ala^2-D-Leu^5 enkephalin (DADLE; highly specific δ-agonist) or vehicle for 6 days using ALZET osmotic mini-pumps. Infusion doses producing robust antinociception via distinct spinal opioid sites (μ versus δ) were verified with μ- and δ-selective antagonists. Antinociception was determined during and after infusion using the tail-flick test. After the 6-day infusion was discontinued, dose-response curves for i.t. NE (non-selective α-agonist) or DX were determined in morphine-tolerant, DADLE-tolerant and vehicle-infused rats. The magnitude of cross-tolerance was determined from the shift in the dose-response curve (e.g. ED_{50} potency ratio). The specific objectives of the thesis research were:

1. To determine the time-course of recovery of antinociceptive activity following the continuous i.t infusion of morphine or DADLE.

2. To determine if rats, made tolerant to i.t. morphine, exhibit antinociceptive cross-tolerance to i.t. NE.

3. To determine if rats, made tolerant to i.t. DADLE, exhibit antinociceptive cross-tolerance to i.t. NE.

4. To determine if rats, made tolerant to i.t. DADLE, exhibit antinociceptive cross-tolerance to i.t. DX.

5. To verify the selectivity of i.t. DX for α_2-adrenoceptors by comparing its sensitivity to the α_2-antagonist, WY 27127, and the α_1-antagonist, prazosin.

6. To verify the selectivity of morphine and DADLE for μ- and δ-receptors, respectively, during continuous i.t. infusion by comparing their sensitivity to the δ-antagonist, naltrindole, and the μ-antagonist, naloxone.

2.0 METHODS AND MATERIALS

2.1 Animals

All procedures were approved by the Animal Care Committee of Memorial University in accordance with the Guidelines of the Canadian Council on Animal Care. Male, Sprague-Dawley rats (Charles River Canada, St. Constant, Canada) weighing 250-350 g were used for all experiments. Animals were housed in a climate controlled room with a 12 hour light-dark cycle (lights on at 07:00 h). Tap water and rodent laboratory chow were freely provided. Animals were housed individually and allowed a 2-6 days acclimatization period before use.

2.2 General Methods

The general experimental protocol, consisting of the surgical implantation of i.t. catheters, baseline testing, continuous i.t. infusion via osmotic mini-pumps, and termination of the infusion with removal of the mini-pumps, is shown in Figure 2.

2.2.1 Intrathecal Catheter Implantation

Rats were anesthetized with halothane (Halocarbon Laboratories, River Edge, N.J.) and implanted with i.t. catheters using the method of Yaksh and Rudy (1976a), as modified by Loomis *et al.* (1987b). All i.t. catheters were constructed from PE10 tubing. A permanent loop was made in the catheter for attachment to the

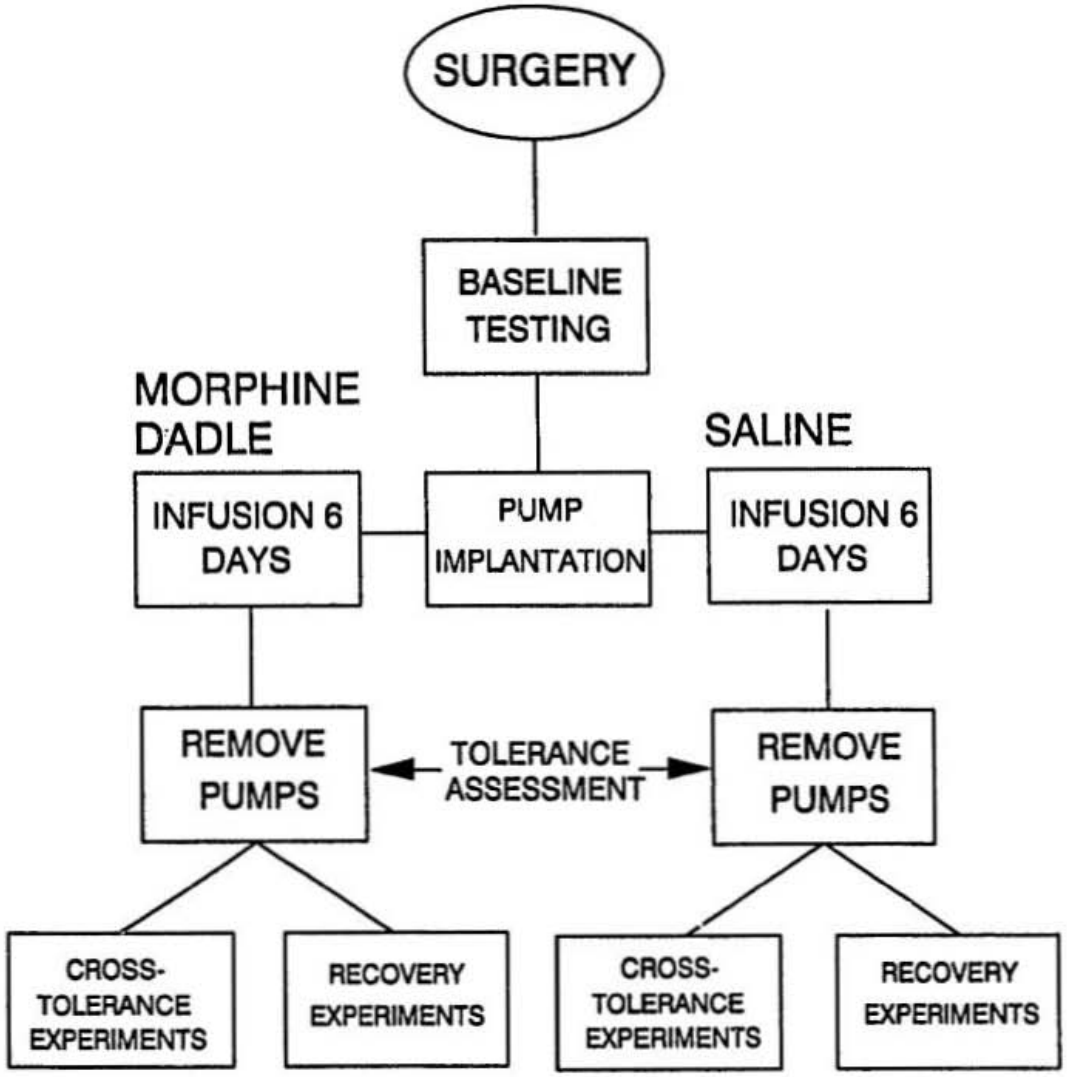

Figure 3. The general protocol for experiments using continuous i.t. infusion.

surrounding musculature and the tubing was cut 7.5 cm distal to the loop. The proximal end of the catheter was connected to a piece of PE 60 tubing (3 cm in length) and the junction was fused with heat. The larger tubing was used as an adaptor to connect the i.t. catheter to an osmotic mini-pump (ALZET 2001; ALZA Corporation, Palo Alto, USA) for continuous i.t. drug infusion. After filling the catheter-adaptor assembly with normal saline, the i.t. catheter was inserted through a small slit in the cisternal membrane, and carefully guided through the spinal subarachnoid space so that the tip was positioned near the lumbar enlargement. The catheter loop was sutured to the overlying muscle and the delivery system was flushed with normal saline. The tip of the PE 60 adaptor was heated with a soldering iron and pinched to seal the end. The adaptor was then inserted into a subcutaneous pocket on the back of the animal, the incision was closed with 3-4 sutures and the animal was allowed to recover for at least 4 days. Only animals exhibiting normal motor function and having normal baseline responses in the tail-flick test (D'Amour and Smith, 1941) were used for experimentation.

2.2.2 Osmotic Pump Preparation and Implantation

One day prior to pump implantation, each osmotic mini-pump was filled with freshly prepared drug solution or vehicle (see below) and incubated in normal saline at room temperature overnight. On the following day, the pumps were transferred to a water bath and heated to 37°C 2-5 h before surgery.

Animals, previously implanted with i.t. catheters, were anesthetized with halothane. An incision was made in the skin overlying the s.c. pocket that contained the PE 60 adaptor and the tip was externalized. The sealed tip was cut with scissors, the catheter-adaptor assembly was flushed with 20 µl of normal saline, and the adaptor was trimmed to a final length of about 7 mm. The obturator of the osmotic pump was fitted directly into the PE 60 adaptor and the pump was inserted into the s.c. pouch. The wound was then closed with sutures and the animals were allowed to recover overnight. The combined dead volume of the i.t. catheter and the trimmed adaptor was 10.6 ± 0.3 µl (mean ± SD; n = 28).

2.2.3 Continuous Intrathecal Infusion

Animals received either morphine sulfate (5, 10 or 20 $\mu g \cdot h^{-1}$), [D-Ala^2 D-Leu^5]-enkephalin (DADLE; 10 $\mu g \cdot h^{-1}$) or saline (1 $\mu l \cdot h^{-1}$) by continuous i.t. infusion for 6 days. Tail-flick latency was determined daily between 08:00 and 10:00 h to minimize the effects of diurnal variation on behavioral responses (Frederickson *et al.*, 1977). Body weight was also recorded daily and room temperature was maintained between 23-25°C. Immediately after testing on day 6, the animals were lightly anesthetized with halothane and an incision was made in the skin overlying the mini-pump. The pump-adaptor assembly was removed from the s.c. pocket and cut near the catheter-adaptor junction with scissors. The rostral end of the catheter was passed s.c. to the back of the head, externalized through the skin, and the

incision was closed with sutures. The catheter, having a dead volume of 7.9 ± 0.2µl (n=47), was flushed with 10 µl of sterile saline to clear the residual drug solution, except for rats infused with morphine (20 $\mu g \cdot h^{-1}$). In this case, the catheter was initially flushed with 5 µl of saline followed approximately 0.5 h later with another 10 µl of saline to clear the remaining drug solution. Two separate saline flushes, spaced 0.5 h apart, were necessary to minimize the development of high dose morphine hyperesthesia (Yaksh *et al.*, 1986; Stevens *et al.*, 1988; Stevens and Yaksh, 1992). The catheters was sealed with a stainless steel plug and used for all subsequent i.t. drug injections. The animals were allowed to recover overnight before being assigned to either: A) the opioid-recovery experiments; or B) the cross-tolerance experiments.

2.2.4 Recovery from Opioid Tolerance

To determine the time-course of recovery from morphine tolerance, animals were infused with i.t. saline (1 $\mu l \cdot h^{-1}$) or morphine (5 $\mu g \cdot h^{-1}$) for 6 days. The infusion was terminated (as described above) and a test dose of i.t. morphine (4 µg) was injected beginning on day 1 post-infusion. The test dose of morphine was chosen from previous dose-response experiments to yield a near maximal effect in the tail flick test. To minimize the possibility of prolonging opioid tolerance with repeated test doses of i.t. morphine, each rat received only one test dose during the recovery period (days 1-5 post-infusion). Thus, separate groups of rats were used for each

of the 5 post-infusion days. A similar protocol was used to determine the time-course of recovery following continuous i.t. DADLE infusion (10 $\mu g \cdot h^{-1}$), except that a test dose of 3 µg of i.t. DADLE was used. In another group of morphine (5 $\mu g \cdot h^{-1}$)- and saline (1 $\mu l \cdot h^{-1}$)-infused rats, dose-response curves of i.t. morphine were determined in the post-infusion period. The ED_{50} ratio was then calculated to quantitate the magnitude of opioid tolerance.

2.2.5 Cross-Tolerance Experiments

The magnitude of cross-tolerance between opioids and α-agonists was determined using dose-response analysis. In morphine-tolerant rats, test doses of NE (2.5, 5.0, or 10 µg i.t.) were injected on days 1-3 post-infusion. In DADLE-tolerant rats, test doses of NE or DX (0.1, 0.2, 0.3 and 0.5 µg i.t.) were injected on days 1-2 post-infusion. These time points were chosen from our study of the recovery from morphine and DADLE tolerance. All behavioral testing was conducted between 08:00 and 14:00 h. Rats received only one i.t. dose of NE or DX per day. Tail-flick latencies were measured immediately before and 30, 60, and 90 min after injection of the test dose of NE or DX. Body weight was recorded daily throughout the cross-tolerance experiments.

2.2.6 α-Antagonist Experiments

Separate groups of rats were surgically implanted with i.t catheters as

described above, except that no PE 60 adapter was used. Rather, the s.c. end of the catheter was externalized on the back of the head and sealed with a stainless steel plug. The dead volume of the catheter was 7.0 ± 0.8 µl (n=46). The selectivity of i.t. DX (0.5 µg) for α_2-adrenoceptors was assessed by challenging the agonist with i.t. Wyeth 27127 (WY; α_2-selective antagonist) or prazosin (PZ; α_1-antagonist). Tail flick latencies were initially measured before and 15 min after i.t. DX. Immediately following this 15-min determination, rats were given a second i.t. injection of either WY (10 µg), PZ (10 µg), or vehicle (DMSO or saline). Tail flick latencies were then determined 30, 45, and 60 minutes after DX administration. As these experiments required two i.t. injections spaced ≈15 min apart, WY and PZ were injected in a volume of 5 µl, followed by 10 µl of saline.

2.2.7 Opioid Antagonist Experiments

The selectivity of morphine and DADLE for µ- and δ-receptors during continuous i.t. infusion was assessed by challenging each agonist with naloxone (1 mg/kg i.p.) or naltrindole (1 mg/kg i.p.; determined from previous experiments). Rats were continuously infused with morphine (20 $\mu g \cdot h^{-1}$) or DADLE (10 $\mu g \cdot h^{-1}$) as described above. Naloxone or naltrindole was injected on day 1 of infusion (i.e. when antinociception was maximal) in a volume not exceeding 0.2 ml for naloxone and 0.5 ml for naltrindole. Tail flick latency was measured before, and 15, 30, 45, 60, and 90 min after i.p. administration. For graphical purposes, data from each rat

were calculated as the % of the peak tail flick latency to correct for the difference in maximum antinociception induced by morphine and DADLE. However, all statistical analysis was conducted on the raw data.

2.2.8 Verification of Catheter Position

At the completion of each experiment, animals were injected with 10 µl of 5% lidocaine followed by 10 µl of saline. Rats were subsequently observed for evidence of hind limb weakness or paralysis, indicative of the correct placement of the spinal catheter. A laminectomy was also performed on rats randomly selected from the experimental groups to visually confirm the position of the catheter.

2.3 Drugs

Morphine sulfate (BDH Chemicals, Toronto, Canada) or [D-Ala^2, D-Leu^5]-enkephalin (Acetate Salt, Sigma Chemical Company, St.Louis, USA) was dissolved in 0.9% saline. These solutions were filtered through a sterile Millex-GS filter (0.22 µm; Millipore Products, Bedford, USA) as the osmotic mini-pumps were filled. Norepinephrine bitartrate (Sigma Chemical Company, St. Louis, USA), dexmedetomidine HCl (Orion Corp. Farmos, Turku, Finland), WY 27127 HCl (Wyeth Ltd., Philadelphia, USA), naloxone HCl (Research Biochemicals Incorporated, Natick, MA), naltrindole HCl (Research Biochemicals Incorporated) and lidocaine HCl (Sigma Chemical Company) were dissolved in 0.9% sterile saline. Prazosin

HCl (Sigma Chemical Company) was dissolved in dimethyl sulphoxide (DMSO, 98%; BDH Chemicals, Toronto, Canada). Drug solutions were freshly prepared on the morning of the experiment, except lidocaine which was stored at 4°C for up to 2 weeks. Except where indicated, all drugs were injected i.t. in a volume of 10 µl followed by 10 µl of saline using a hand held µl-syringe. All doses are expressed as the salt.

2.4 Algesiometric Testing and Data Analysis

Tail flick latency was measured using a Tail Flick Analgesia Meter, model MK-330 (Muromachi Kikai Co. Ltd., Tokyo, Japan); a cut-off of 10 s was imposed to avoid tissue damage. All data are expressed as the mean ± the standard error of the mean (SEM). For the cross-tolerance experiments, data were analyzed as both absolute tail flick latency (sec) and as the maximum percent effect (MPE). MPE was calculated using the equation:

$$\text{MPE} = \frac{\text{post drug response - predrug response}}{\text{cutoff - predrug response}} \times 100$$

MPE values below baseline were assigned an MPE of zero (Russell *et al*, 1987). Area under the tail flick latency versus time curve (AUC) for each rat was calculated by trapezoidal approximation.

Dose-response analysis, including the ED_{50} and 95% CI, the least-squares regression line, and tests for parallel shifts of dose-response curves, were based on

the methods outlined in Tallarida and Murray (1987). ED_{50}'s and 95% CI's were calculated using both peak tail flick latencies (where the ED_{50} is the midpoint of the regression line extending from the mean control baseline to the 10 s cut-off), and MPE's (where ED50=50% MPE). ED_{50} ratios were defined as the ED_{50} of the test drug in opioid-infused animals divided by the corresponding ED_{50} in saline-infused rats [ED_{50} (opioid)·ED_{50} (saline)$^{-1}$]. A significant difference between two groups was determined using either the unpaired Student's t-test or Mann-Whitney two-sample test. Repeated measures, one-way ANOVA was used to analyze the within-group time-course data; completely randomized, one-way ANOVA was used for all other multiple group comparisons. Post-hoc analysis was performed using the Newman-Keuls test and differences with a probability of $P<0.05$ were considered statistically significant. A significant shift in the ED_{50} of NE or DX in opioid- versus saline-infused rats was indicated when the 95% CI's did not overlap. Data were recorded and analyzed using a commercial spread-sheet program (Quattro Pro) on a Wise Data System computer. Statistical analyses were conduced using a commercial program (Instat).

3.0 RESULTS

3.1 Morphine Experiments

3.1.1 Induction of Morphine Tolerance with Continuous Intrathecal Infusion

Spinal morphine (5, 10 and 20 $\mu g \cdot h^{-1}$) induced significant antinociception as compared to saline-infused rats on days 1 to 2 of infusion (Figure 4). Tail-flick latency peaked on day 1 and then progressively declined to baseline over the next 5 days. The area under the curve (AUC) for each morphine infusion dose was also significantly different from saline (data not shown), confirming the antinociceptive effect. Saline had no effect on tail-flick latency throughout the infusion period. A parallel, rightward shift in the morphine dose-response curve, representing a 6-fold increase in the ED_{50}, was observed on day 1 post-infusion in rats that had received morphine (5 $\mu g \cdot h^{-1}$) for 6 days. The ED_{50} and 95% CI of i.t. morphine was 1.02 µg (0.87-1.20) in the saline-infused group and 6.22 µg (4.76-7.99) in the morphine - infused group. These data are consistent with the induction of morphine tolerance following continuous i.t. morphine infusion.

3.1.2 Behavioral Effects with Morphine Infusion

The general behaviour of rats appeared to be unaffected during morphine or saline infusion. Morphine-treated rats, especially those receiving 20 $\mu g \cdot h^{-1}$, exhibited increased muscle tone in the hind limbs and stiff tails on the first day of infusion. Generally, these effects disappeared by the next morning and were not observed

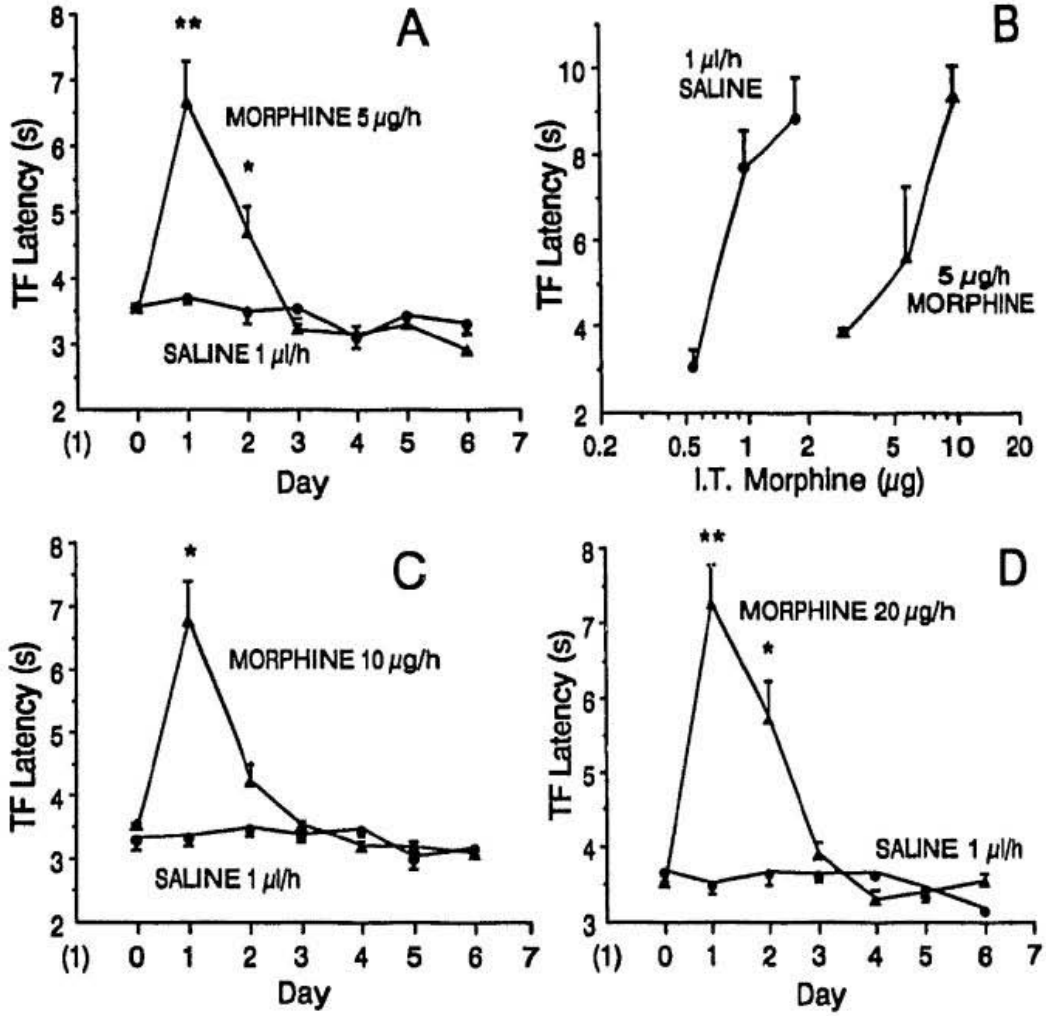

Figure 4. The time course of tail-flick latency during the continuous i.t. infusion of: A) morphine 5 μg·h^{-1}; C) morphine 10 μg·h^{-1}; or D) morphine 20 μg·h^{-1} for 6 days. Control animals received continuous i.t. saline (1 μl·h^{-1}). Each point represents the mean ± SEM of 6-16 morphine-treated and 4-10 saline-treated rats. All points were significantly different from saline control on days 1 and 2, except morphine (10 μg·h^{-1}) on day 2 (Student's t-test or Mann-Whitney two sample test; A: ✳✳P<0.005; ✳P<0.05; B: ✳✳P<0.005; D: ✳✳P<0.0005; ✳P<0.005). AUC's for opioid infused animals were significantly different from saline (Mann-Whitney two sample test;P<0.05). **B):** Morphine dose-response curves determined one-day after the saline or morphine (5 μg·h^{-1}) infusion had been discontinued. The ED_{50} and 95% CI of i.t. morphine was 1.02 μg (0.87-1.20) in saline-infused rats and 6.22 μg (4.76-7.99) in morphine-infused rats.

thereafter. The highest dose of morphine (20 $\mu g \cdot h^{-1}$) produced hematuria in two rats. All groups displayed a comparable increase in body weight during the infusion period (days 1-6; Figure 5), indicating that the i.t. catheter and s.c. osmotic pump were well tolerated.

After removal of the pumps on day 6, some morphine-infused rats exhibited signs of opioid withdrawal. Diarrhea, piloerection, sensitivity to touch and increased vocalization upon handling were observed on day 1 post-infusion. A significant decrease in body weight was also observed after the morphine-infusion had been discontinued (as compared to saline-infused rats; Figure 5). This decrease was: a) maximal on day 1 post-infusion and coincided with the appearance of opioid withdrawal behaviour; b) opioid dose-dependent; and c) temporary, with animals regaining their body weight 4 days after terminating the infusion (data not shown). Following removal of the pump and subsequent flushing of the i.t. catheter with saline, 52% of animals (7 of 15) receiving morphine (20 $\mu g \cdot h^{-1}$) displayed a transient writhing, biting and scratching of the dermatomes, suggestive of hyperesthesia.

3.1.3 Recovery from Morphine Tolerance

To determine the time course of recovery from tolerance following low dose (5 $\mu g \cdot h^{-1}$)-morphine infusion, a probe dose of morphine (4 µg i.t.) was tested once daily during the post-infusion period (Figure 6). After saline-infusion, morphine

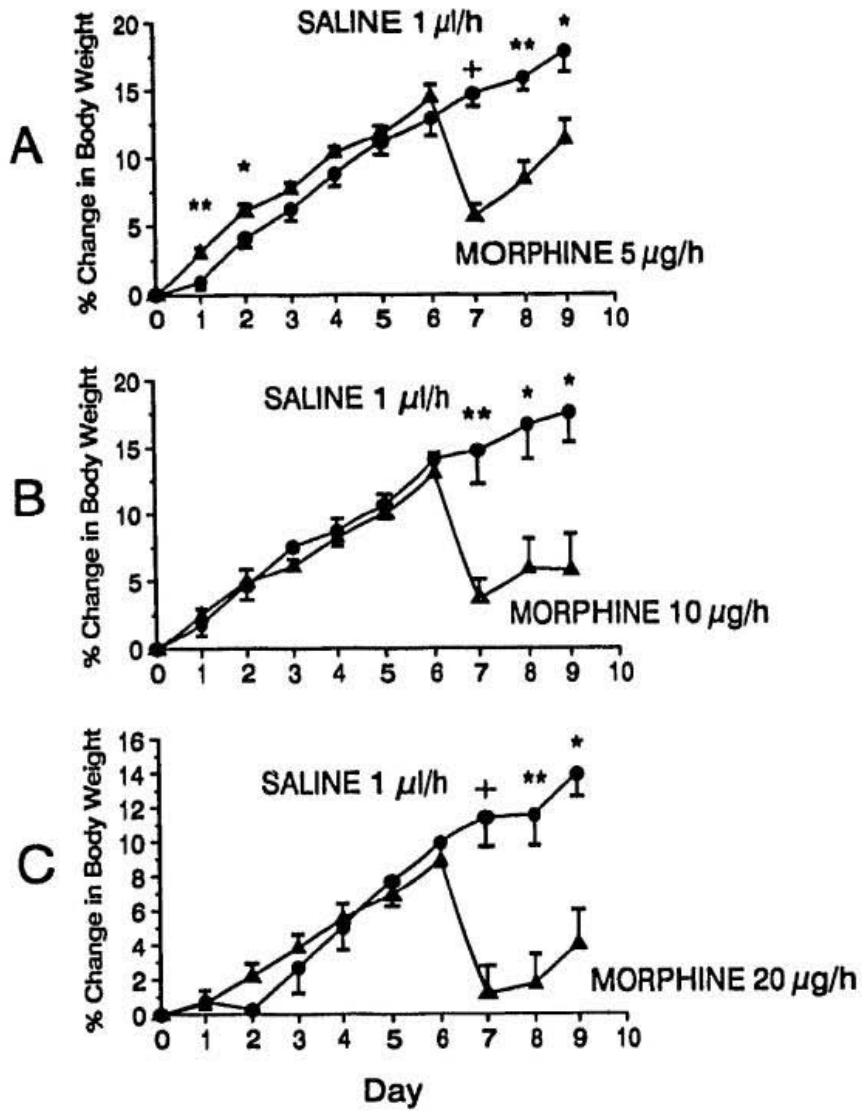

Figure 5. The change in body weight during (days 1-6) and after (days 7-9) the continuous i.t. infusion of: A) morphine (5 µg·h^{-1}) or saline (1 µl·h^{-1}); B) morphine (10 µg·h^{-1}) or saline; and C) morphine (20 µg·h^{-1}) or saline. Data are expressed as the percent of body weight before infusion and each point represents the mean ± SEM of 4-10 saline-infused and 6-16 morphine-infused rats. Symbols indicate a significant difference from saline-infused rats at the corresponding time point (unpaired Student's t-test or Mann-Whitney two-sample test, where applicable; +P<0.0001; ✳✳P<0.005; ✳P<0.05).

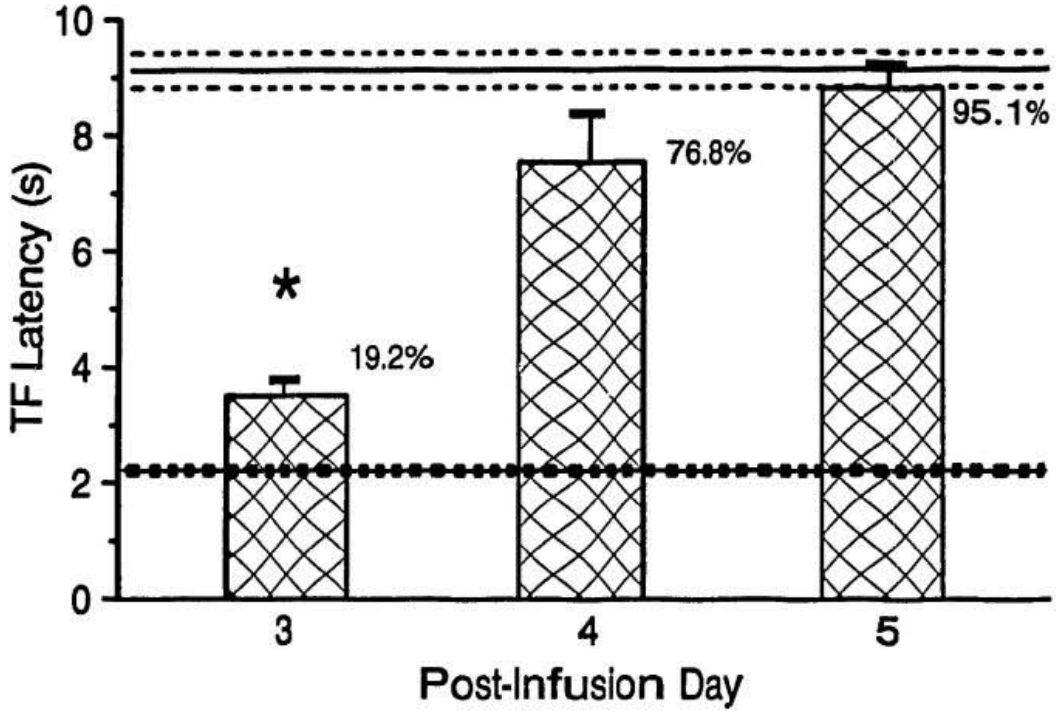

Figure 6. The time course of recovery from morphine tolerance following the continuous i.t. infusion of morphine (5 μg·h^{-1}) for 6 days. Histograms indicate the maximum tail-flick response to a test dose of morphine (4 μg i.t.), injected on consecutive days after the infusion was discontinued. Separate groups of rats were used on each post-infusion day and the results represent the mean ± SEM of 6-7 rats. The results are also expressed as a percent of the morphine effect in saline-infused rats. The upper solid horizontal line and adjacent dashed lines represent the maximum tail-flick latency ± SEM produced by the morphine test dose in saline-infused rats (n=23). The lower solid line and adjacent dashed lines indicate the baseline response ± SEM before injection of the test dose. An asterisk denotes a significant difference from saline-infused animals (one-way ANOVA followed by Newman-Keuls test; $P<0.05$). Significant recovery from tolerance was not observed until day 4 post-infusion.

produced an average tail-flick latency of 9.1 ± 0.3 s (n = 23), determined over a 14-day post-infusion period (see upper horizontal line and adjacent dashed lines in Figure 6). The same test dose had a minimal effect in morphine-infused rats up to day 3 post-infusion (n=6-7). Partial recovery from morphine tolerance was observed on day 4 post-infusion (76.8% of morphine activity in saline-infused rats), and by day 5, recovery was complete (95.1% of morphine activity in saline-infused rats). These results indicate that i.t. infused animals remain significantly tolerant to the antinociceptive effect of morphine three days after morphine is discontinued. Accordingly, cross-tolerance experiments with i.t. NE were conducted on days 1-3 post-infusion.

3.1.4 Cross-Tolerance Studies between Morphine and NE

The time course of tail-flick latency following the acute i.t. injection of NE (2.5, 5.0 and 10 μg) in rats that been infused with morphine (5 $\mu g \cdot h^{-1}$) or saline (1 $\mu l \cdot h^{-1}$) are shown in Figure 7. All doses of NE significantly increased tail-flick latency in both treatment groups. Peak antinociception occurred 30 min after injection, with tail-flick latency returning to baseline 60 min after the 2.5- and 5.0-μg doses, and 90 min after the 10-μg dose. AUC analysis of the NE time-course data indicated no significant differences between the morphine- and saline-infused groups (Table I), except for NE (2.5 and 10 μg) in morphine (10 $\mu g \cdot h^{-1}$)-infused rats. Both doses produced a marginally but significantly lower antinociceptive effect in this morphine-

TABLE I. Summary of the Differences in the Area Under the Tail-Flick Latency versus Time Curve (AUC) Following the Intrathecal Injection of Norepinephrine (NE) in Rats Previously Infused with Morphine or Saline for 6 Days

NE DOSE (μg)	TREATMENT (dose)	AUC (s·min)
2.5	SALINE (1 $\mu l \cdot h^{-1}$)	315 ± 54
	MORPHINE (5 $\mu g \cdot h^{-1}$)	274 ± 27
5.0	SALINE (1 $\mu l \cdot h^{-1}$)	399 ± 28
	MORPHINE (5 $\mu g \cdot h^{-1}$)	385 ± 24
10	SALINE (1 $\mu l \cdot h^{-1}$)	579 ± 35
	MORPHINE (5 $\mu g \cdot h^{-1}$)	504 ± 32
2.5	SALINE (1 $\mu l \cdot h^{-1}$)	329 ± 20
	MORPHINE (10 $\mu g \cdot h^{-1}$)	266 ± 13*
5.0	SALINE (1 $\mu l \cdot h^{-1}$)	406 ± 41
	MORPHINE (10 $\mu g \cdot h^{-1}$)	354 ± 22
10	SALINE (1 $\mu l \cdot h^{-1}$)	573 ± 42
	MORPHINE (10 $\mu g \cdot h^{-1}$)	448 ± 30*
2.5	SALINE (1 $\mu l \cdot h^{-1}$)	343 ± 26
	MORPHINE (20 $\mu g \cdot h^{-1}$)	314 ± 13
5.0	SALINE (1 $\mu l \cdot h^{-1}$)	402 ± 30
	MORPHINE (20 $\mu g \cdot h^{-1}$)	379 ± 24
10	SALINE (1 $\mu l \cdot h^{-1}$)	559 ± 40
	MORPHINE (20 $\mu g \cdot h^{-1}$)	492 ± 31

* $P<0.05$

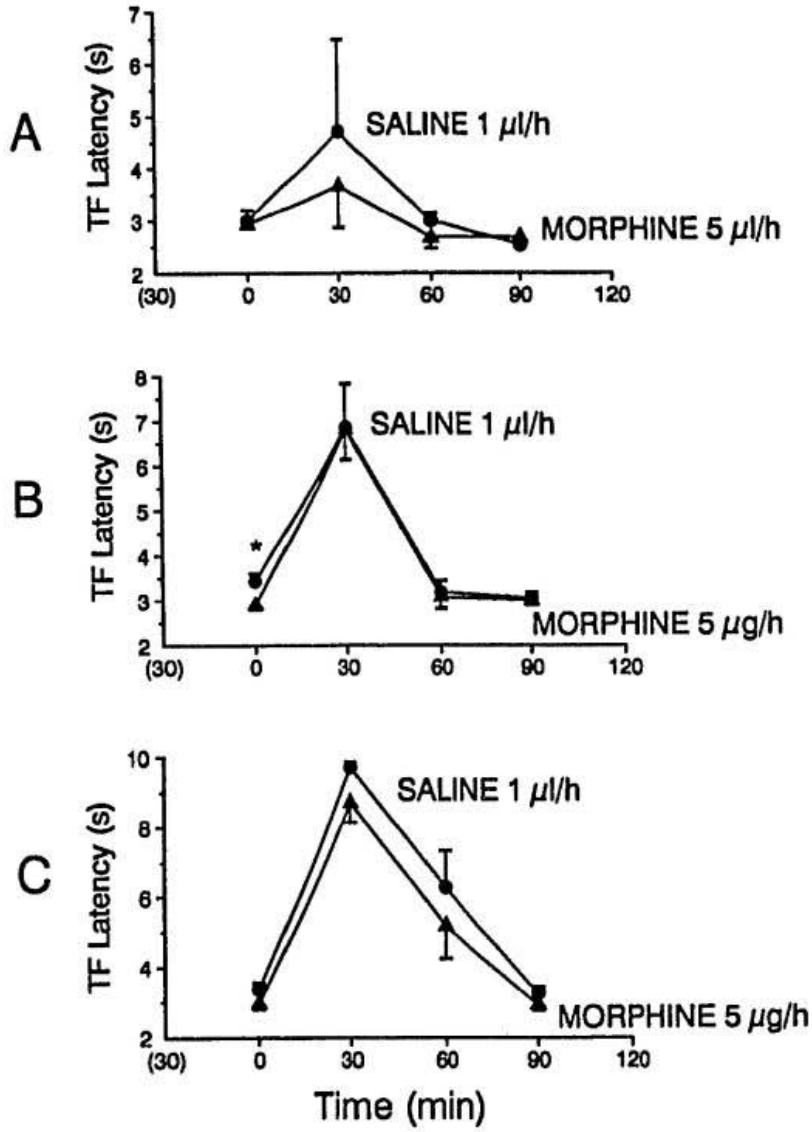

Figure 7. The time course of tail-flick latency following the acute i.t. injection of NE: A) 2.5 µg; B) 5.0 µg; and C) 10 µg to rats previously infused with morphine (5 µg·h^{-1}; ▲) or saline (1 µl·h^{-1}; ●). Data are expressed as the mean ± SEM of 10 saline- and 15 morphine-infused rats, except Figure 7A where n = 4 and 6, respectively. Doses of NE were injected on days 1-3 post-infusion. There were no significant differences between the two groups except at time 0 (baseline) in Figure 7B (unpaired Student's t-test or Mann-Whitney two-sample test, where applicable; * $P<0.01$).

infused group (Table I). Identical NE time course studies were conducted following the continuous 6-day infusion of i.t. morphine (10 and 20 $\mu g \cdot h^{-1}$) and saline (1 $\mu l \cdot h^{-1}$) (data not shown). Using the peak tail-flick response (30 min) from these data, NE dose-response curves were constructed (Figures 8-10). Regardless of the infusion rate, there was no significant shift in the NE dose-response curve in morphine-tolerant as compared to saline-infused animals. The dose-response curves did not differ from parallelism ($P>0.05$) and the ED_{50}'s of i.t. NE were comparable in both treatment groups (overlapping 95% CI's; Table II). The ED_{50} ratio was 1.12, 1.51, and 1.35 for the 5-, 10- and 20-$\mu g \cdot h^{-1}$ infusion dose, respectively (Table II). These data indicate that there was no significant cross-tolerance between i.t. NE and morphine in the rat tail-flick test at morphine infusion rates up to 20 $\mu g \cdot h^{-1}$ for 6 days.

3.2 DADLE Experiments

3.2.1 Induction of DADLE Tolerance with Continuous Intrathecal Infusion

The time-course of tail-flick latency during the continuous infusion of DADLE (10 $\mu g \cdot h^{-1}$) or saline (1 $\mu l \cdot h^{-1}$) is depicted in Figure 11. Like morphine, significant antinociception was observed on days 1 and 2 of infusion ($P<0.05$). The AUC for the DADLE-infused group was also statistically different from that of saline ($P<0.05$). Peak tail-flick latency occurred on day 1 and then gradually declined to baseline on day 3, reflecting the development of opioid tolerance in the spinal cord.

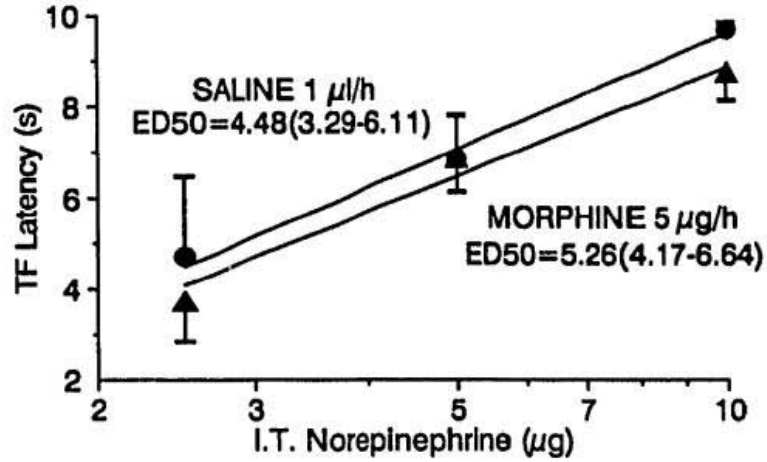

Figure 8A. NE dose-response curves (expressed as peak tail-flick latency in seconds) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or morphine (5 µg·h^{-1}; ▲) for 6 days. NE was tested on days 1-3 post-infusion with only one dose injected daily. Data are expressed as the mean ± SEM of 4-15 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

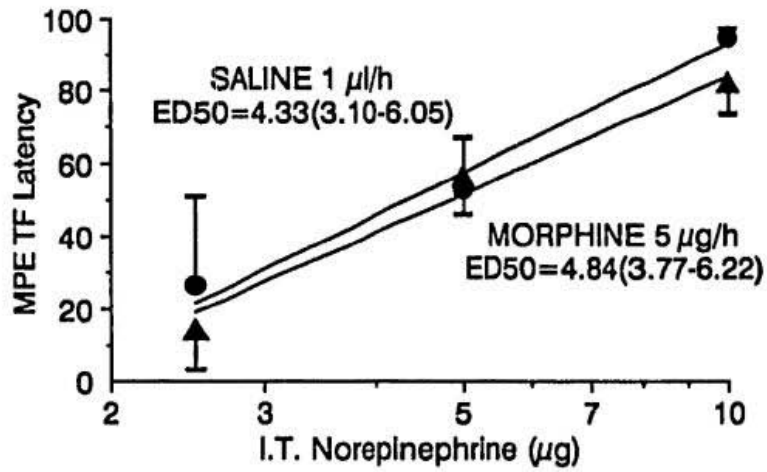

Figure 8B. NE dose-response curves (expressed as MPE) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or morphine (5 µg·h^{-1}; ▲) for 6 days. NE was tested on days 1-3 post-infusion with only one dose injected daily. Data are expressed as the mean ± SEM of 4-15 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

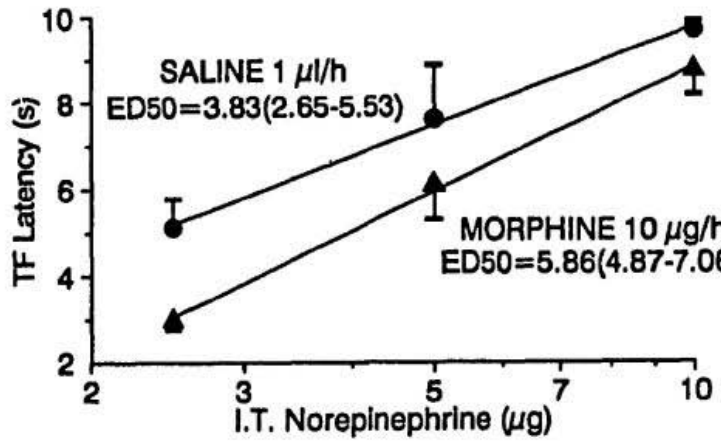

Figure 9A. NE dose-response curves (expressed as peak tail-flick latency in seconds) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or morphine (10 µg·h^{-1}; ▲) for 6 days. NE was tested on days 1-3 post-infusion with only one dose injected daily. Data are expressed as the mean ± SEM of 5-9 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

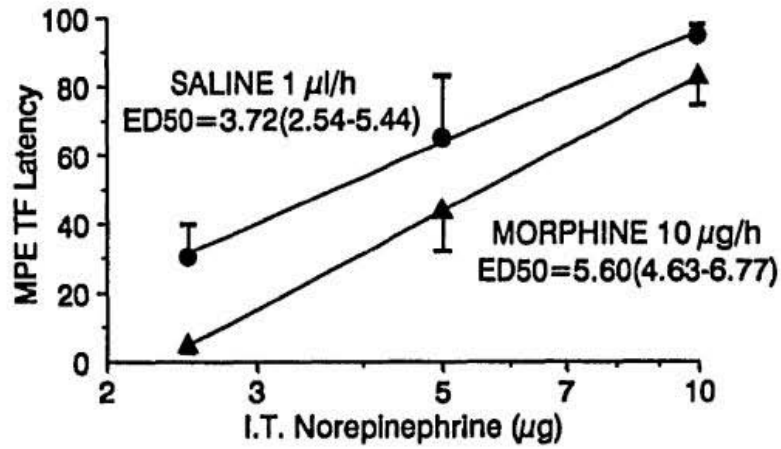

Figure 9B. NE dose-response curves (expressed as MPE) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or morphine (10 µg·h^{-1}; ▲) for 6 days. NE was tested on days 1-3 post-infusion with only one dose injected daily. Data are expressed as the mean ± SEM of 5-9 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

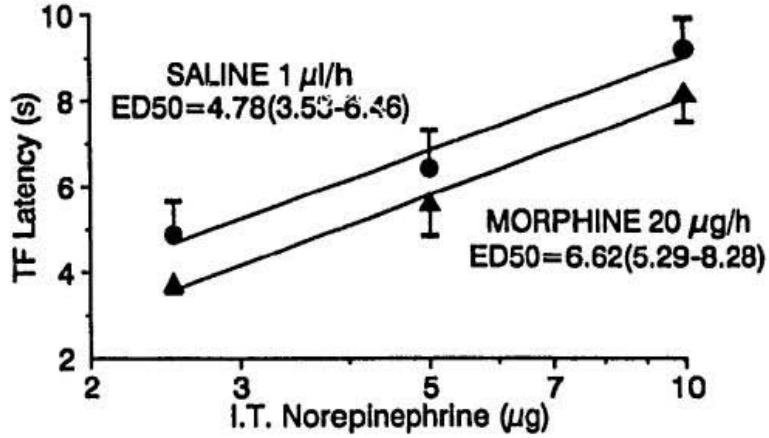

Figure 10A. NE dose-response curves (expressed as peak tail-flick latency in seconds) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or morphine (20 µg·h^{-1}; ▲) for 6 days. NE was tested on days 1-3 post-infusion with only one dose injected daily. Data are expressed as the mean ± SEM of 7-14 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

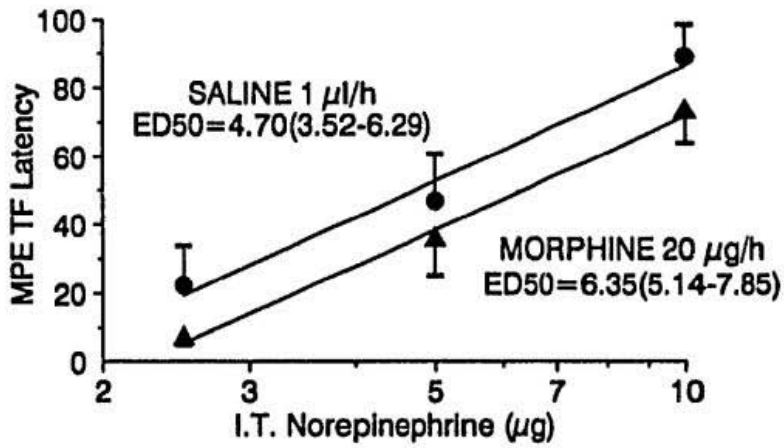

Figure 10B. NE dose-response curves (expressed as MPE) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or morphine (20 µg·h^{-1}; ▲) for 6 days. NE was tested on days 1-3 post-infusion with only one dose injected daily. Data are expressed as the mean ± SEM of 7-14 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

TABLE II. ED_{50}, 95% Confidence Interval (CI) and ED_{50} Ratio for Intrathecal Norepinephrine Following the Continuous Intrathecal Infusion of Saline or Morphine

TREATMENT (dose)	ED_{50} (95% CI)[a]	ED_{50} Ratio[b]
SALINE (1µl·h^{-1})	4.33 µg (3.10-6.05)	
MORPHINE (5 µg·h^{-1})	4.84 µg (3.77-6.22)	1.12
SALINE (1 µl·h^{-1})	3.72 µg (2.54-5.44)	
MORPHINE (10 µg·h^{-1})	5.60 µg (4.63-6.77)	1.51
SALINE (1µl·h^{-1})	4.70 µg (3.52-6.29)	
MORPHINE (20 µg·h^{-1})	6.35 µg (5.14-7.85)	1.35

a ED_{50} and 95% CI calculated using MPE data

b ED_{50} ratio is defined as ED_{50} in opioid-infused rats·ED_{50} in saline-infused rats^{-1}

No significant differences ($P>0.05$)

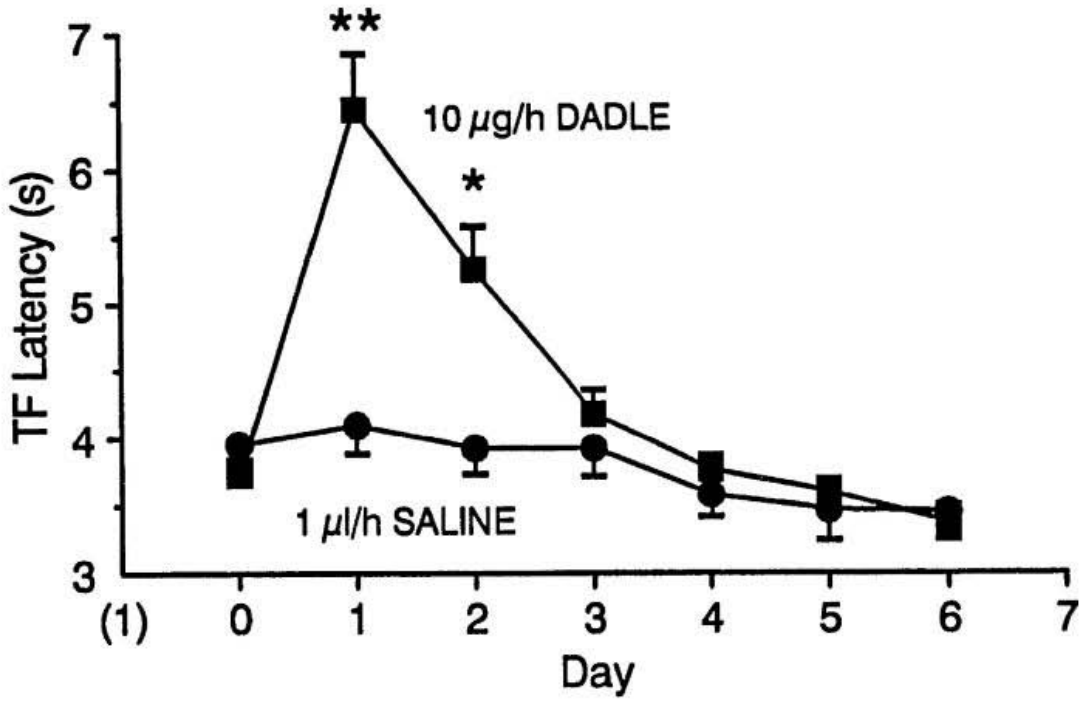

Figure 11. The time-course of tail-flick latency during the continuous i.t. infusion of DADLE (10 μg·h^{-1}) or saline (1 μl·h^{-1}). Data are expressed as the mean ± SEM of 7-21 rats and the asterisks denote a significant difference between the DADLE and saline groups (Student's t-test or Mann-Whitney two sample test, **P<0.005; *P<0.05).

3.2.2 Behavioral Effects with DADLE

All animals exhibited normal grooming and feeding behaviour throughout the i.t. infusion period; both DADLE- and saline-treated rats had comparable increases in body weight on days 1-6 (Figure 12). Forty percent of DADLE-infused rats (8 of 20) exhibited stiff tails on day 1 of infusion. When the pumps were removed at the end of the infusion period and the i.t. catheters were flushed with saline, 65% of DADLE-infused rats (40 of 61), but no saline-infused rats, displayed a cataleptic condition for up to 1 h. These animals had muscular rigidity and could be easily manipulated into an upright "sitting" position where they remained stationary until moved by the observer. There was no startle response and no effort to escape. DADLE-infused rats also exhibited a significant decrease in body weight as compared to control in the immediate post-infusion period (Figure 12), indicative of opioid-withdrawal. Collectively, these data are consistent with the continuous spinal infusion of DADLE and indicate that the decline in antinociceptive activity was not due to a problem with the drug delivery system (i.t. catheter and/or osmotic pump).

3.2.3 Recovery from DADLE Tolerance

The time-course of recovery from DADLE tolerance is shown in Figure 13. The DADLE test dose (3-μg i.t.) significantly increased tail-flick latency in saline-infused animals on days 1-4 post-infusion, yielding a mean peak effect of 9.5 ± 0.4 s (upper solid horizontal and adjacent dashed lines). In DADLE-tolerant rats, the

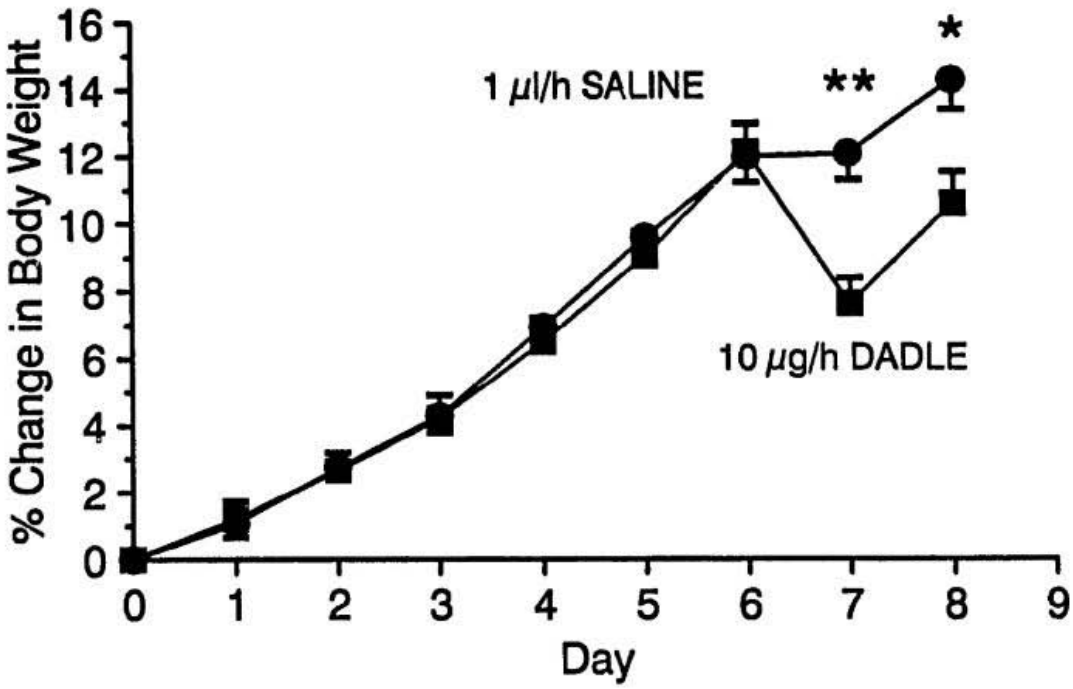

Figure 12. The change in body weight during (days 1-6) and after (days 7-8) the continuous i.t. infusion of DADLE (10 µg·h^{-1}) or saline (1 µl·h^{-1}). Each point represents the mean±SEM of 7-21 rats and the asterisks indicate a significant difference between the saline and DADLE groups (unpaired Student's t-test or Mann-Whitney two-sample test, where applicable; **P<0.005; *P<0.05).

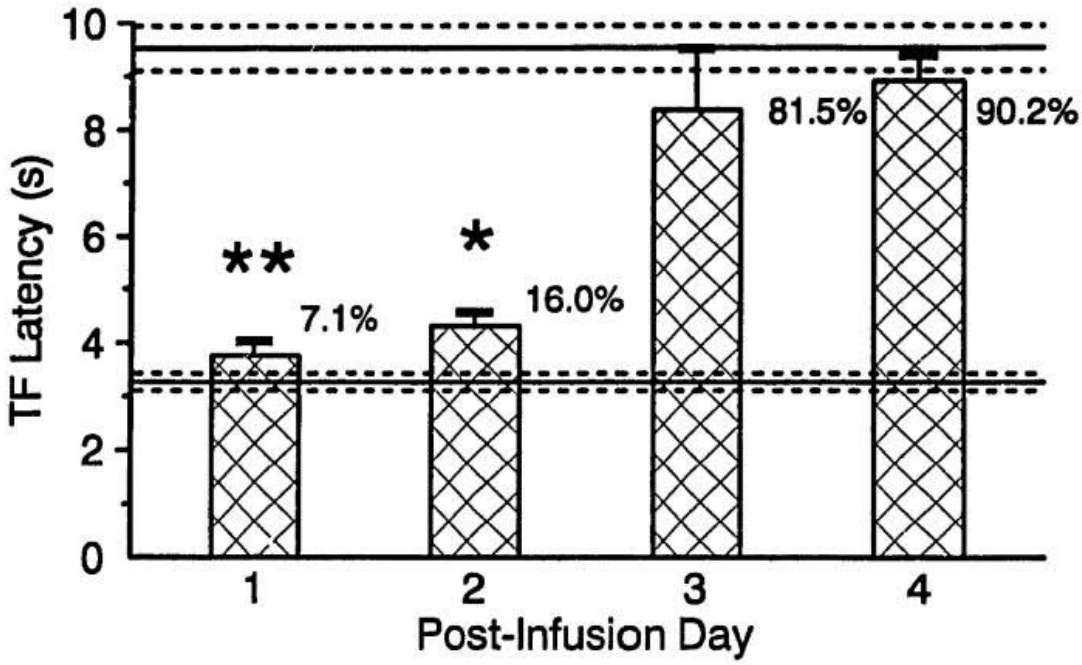

Figure 13. The time course of recovery from DADLE tolerance following the continuous i.t. infusion of DADLE (10 μg·h^{-1}) for 6 days. Histograms indicate the maximum tail-flick response to a test dose of DADLE (3 μg i.t.), injected on consecutive days after the infusion was discontinued. Separate groups of rats were used on each post-infusion day and the results represent the mean ± SEM of 4-5 rats, except on day 4 where n = 3. The results are also expressed as a percent of the DADLE effect in saline-infused rats. The upper solid horizontal line and adjacent dashed lines represent the maximum tail-flick latency ± SEM produced by the DADLE test dose in saline-infused rats (n=13). The lower solid line and adjacent dashed lines indicate the baseline response ± SEM before injection of the test dose. Asterisks denote a significant difference from saline-infused animals (unpaired Student's t-test or Mann-Whitney two sample test, where applicable,**P<0.0001; *P<0.005). Significant recovery from tolerance was observed on day 3 post-infusion.

same test dose had little antinociceptive activity on days 1 and 2 post-infusion. Peak tail-flick latency was only 7.1% and 16.0%, respectively, of that induced by DADLE in the saline-infused group. By day 3 post-infusion, this effect increased to 81.5% (8.4 ± 1.1 s) and recovery of antinociceptive activity was virtually complete (90.2%) by day 4 post-infusion. Accordingly, cross-tolerance experiments to DADLE were conducted on days 1-2 post-infusion.

3.2.4 Cross-Tolerance Studies between DADLE and NE

AUC analysis of the time-course profiles for individual test doses of i.t. NE (see Figure 14) revealed a significant reduction in antinociceptive activity in rats previously infused with DADLE (10 $\mu g \cdot h^{-1}$) as compared to saline (Table III). This attenuated response was confirmed by the rightward displacement of the NE dose-response curve in DADLE-tolerant rats (Figure 14) and the corresponding increase in the ED_{50} of NE (ED_{50} ratio was 2.54; Table IV). The dose-response curves did not differ from parallelism ($P>0.05$). Thus, rats made tolerant to DADLE using continuous i.t. infusion for 6 days displayed significant cross-tolerance to i.t. NE in the post-infusion period. For completeness, NE dose-response curves were analyzed as both the absolute latency (s) and as MPE. There was no difference in the overall result using either method of calculation.

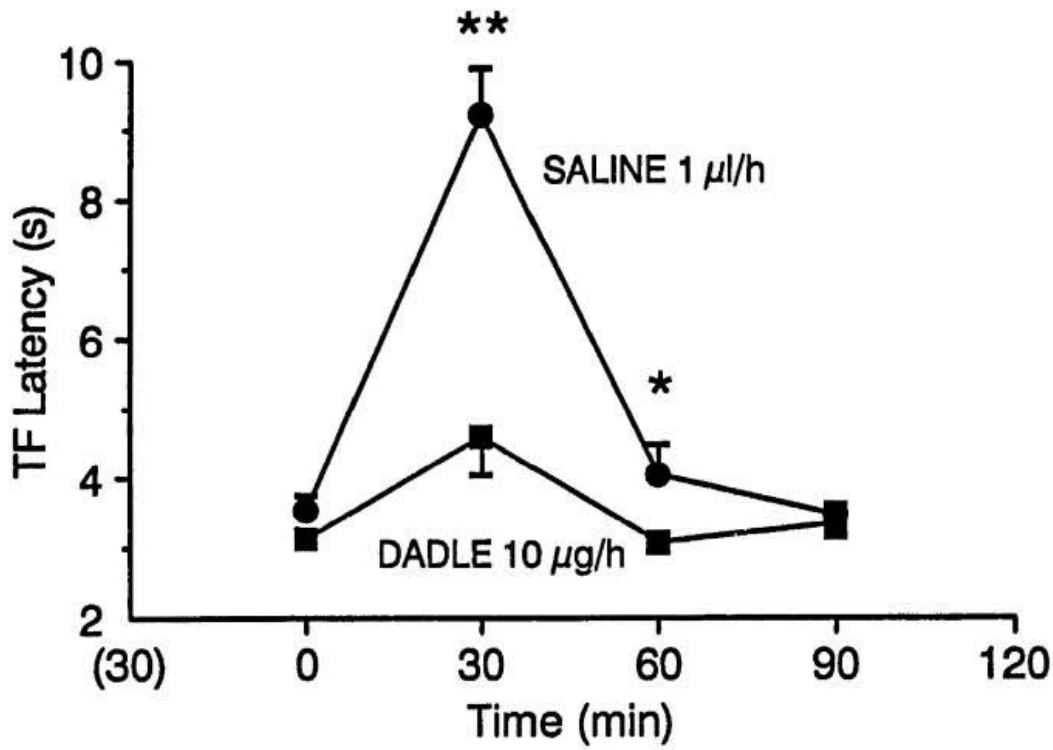

Figure 14. The time course of tail-flick latency following the acute i.t. injection of NE (5.0 µg) to rats previously infused with DADLE (10 µg·h^{-1}; ■) or saline (1 µl·h^{-1}; ●). Data are expressed as the mean ± SEM of 7 saline- and 12 DADLE-infused rats. Asterisks indicate a significant difference between the two groups (unpaired Student's t-test or Mann-Whitney two-sample test, where applicable; ** P<0.0001; *P<0.05).

TABLE III. Summary of the Differences in the Area Under the Tail-Flick Latency versus Time Curve (AUC) Following the Intrathecal Injection of Norepinephrine (NE) in Rats Previously Infused with DADLE or Saline for 6 Days

NE DOSE (μg)	TREATMENT (dose)	AUC (s·min)
2.5	SALINE (1 μl·h^{-1})	377 ± 29
	DADLE (10 μg·h^{-1})	302 ± 6*
5.0	SALINE (1 μl·h^{-1})	503 ± 27
	DADLE (10 μg·h^{-1})	327 ± 17†
10	SALINE (1 μl·h^{-1})	617 ± 36
	DADLE (10 μg·h^{-1})	434 ± 24**

* P<0.05; ** P<0.005; † P<0.0001

TABLE IV. ED_{50}, 95% Confidence Interval (CI) and ED_{50} Ratio for Intrathecal Norepinephrine Following the Continuous Intrathecal Infusion of Saline or DADLE.

TREATMENT (dose)	ED_{50} (95% CI)	ED_{50} Ratio†
SALINE (1μl·h^{-1})	2.64 μg (1.56-4.48)	
DADLE (10 μg·h^{-1})	6.70 μg (5.05-8.88)	2.54*

† ED_{50} ratio is defined as ED_{50} in opioid-infused rats·ED_{50} in saline-infused rats^{-1}

* Statistically significant shift

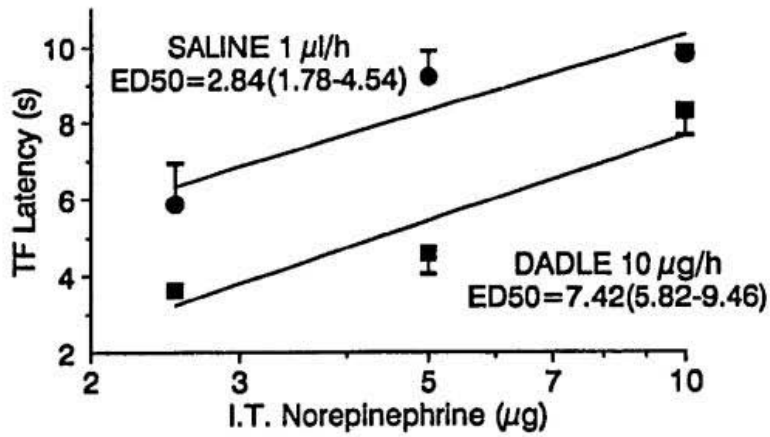

Figure 15A. NE dose-response curves (expressed as peak tail-flick latency in seconds) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or DADLE (10 µg·h^{-1}; ■) for 6 days. NE was tested on days 1-2 post-infusion and rats received only one dose daily. Data are expressed as the mean ± SEM of 6-13 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

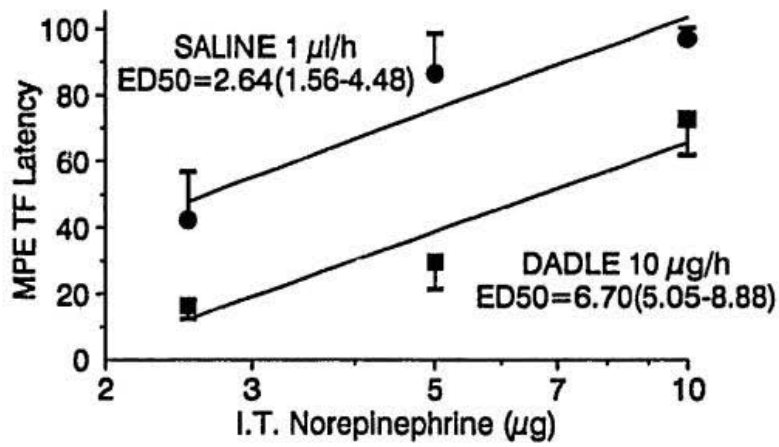

Figure 15B. NE dose-response curves (expressed as the maximum percent effect) determined after the continuous i.t. infusion of saline (1 µl·h^{-1}; ●) or DADLE (10 µg·h^{-1}; ■) for 6 days. NE was tested on days 1-2 post-infusion and rats received only one dose daily. Data are expressed as the mean ± SEM of 6-13 rats. The corresponding ED_{50} (95% CI) of i.t. NE, expressed in µg, is indicated on the figure.

3.2.5 Cross-Tolerance Studies between DADLE and DX

The time course of tail-flick latency following the i.t. injection of DX (0.1 μg) to rats previously infused with saline (1 $\mu l \cdot h^{-1}$) or DADLE (10 $\mu g \cdot h^{-1}$) for 6 days is illustrated in Figure 16. Peak antinociception was observed 15 min after injection and there was no significant difference between the two groups at any time point. Similar time-course experiments were conducted with 0.2, 0.3 and 0.5 μg of DX (data not shown). AUC analysis of the time-course data revealed no significant difference between saline- and DADLE-infused rats for each i.t. dose of DX (Table V). No sedation was observed with any of the doses of i.t. DX.

TABLE V. Summary of the Differences in the Area Under the Tail-Flick Latency versus Time Curve (AUC) Following the Intrathecal Injection of Dexmedetomidine (DX) in Rats Previously Infused with DADLE or Saline for 6 Days

DX DOSE (μg)	TREATMENT (dose)	AUC (s·min)
0.1	SALINE (1 $\mu l \cdot h^{-1}$)	268 ± 7
	DADLE (10 $\mu g \cdot h^{-1}$)	259 ± 16
0.2	SALINE (1 $\mu l \cdot h^{-1}$)	255 ± 12
	DADLE (10 $\mu g \cdot h^{-1}$)	265 ± 23
0.3	SALINE (1 $\mu l \cdot h^{-1}$)	290 ± 7
	DADLE (10 $\mu g \cdot h^{-1}$)	326 ± 18
0.5	SALINE (1 $\mu l \cdot h^{-1}$)	370 ± 28
	DADLE (10 $\mu g \cdot h^{-1}$)	366 ± 34

No significance ($P>0.05$)

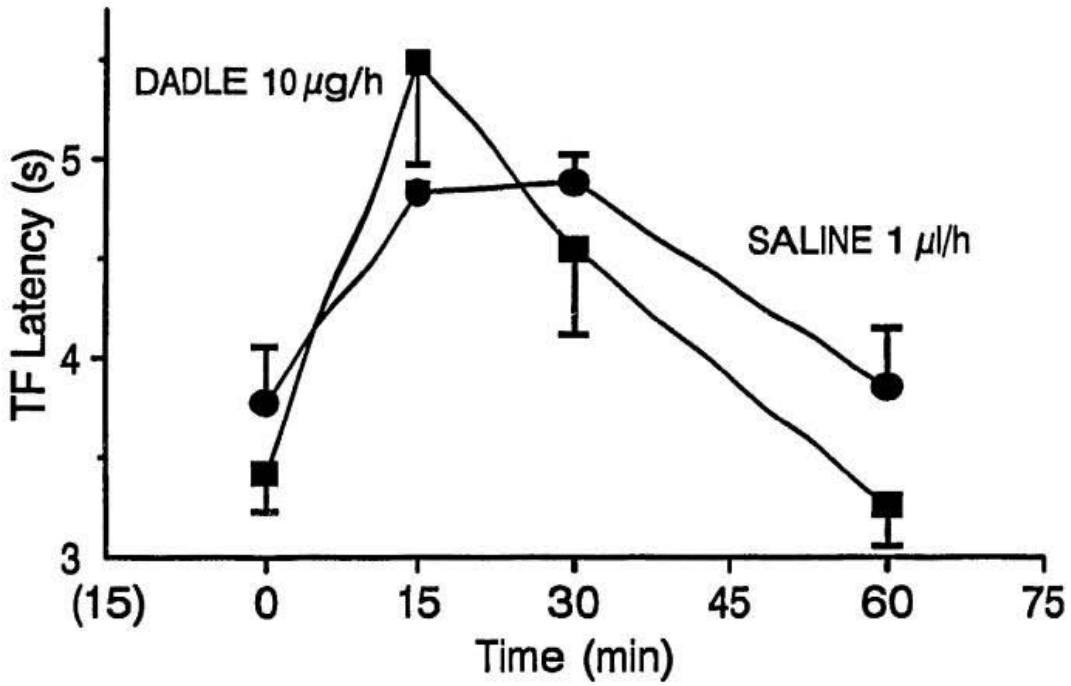

Figure 16. The time course of tail-flick latency following the acute i.t. injection of DX (0.1 µg) to rats previously infused with DADLE (10 µg·h^{-1}; ■) or saline (1 µl·h^{-1}; ●). Data are expressed as the mean ± SEM of 4 saline- and 5 DADLE-infused rats. There were no significant differences between the two groups (unpaired Student's t-test or Mann-Whitney two-sample test, where applicable).

Peak tail-flick latencies (15 min) were used to construct the DX dose-response curves (Figure 17). The ED_{50} and 95% CI for DX in DADLE- and saline-infused animals was 0.24 µg (0.17-0.36) and 0.38 µg (0.29-0.49), respectively (Table VI). For comparative purposes, dose-response curves were also constructed using the MPE (Figure 17B). The corresponding ED_{50} and 95%CI was 0.25 µg (0.16-0.38) in DADLE-tolerant rats and 0.38 µg (0.29-0.50) in saline-controls (Table VI). The slopes of the linear portion of the DX dose-response curves for both treatment groups were parallel. Thus, there was no evidence of cross-tolerance between DADLE and DX.

TABLE VI. ED_{50}, 95% Confidence Interval (CI) and ED_{50} Ratio for Intrathecal Dexmedetomidine Following the Continuous Intrathecal Infusion of Saline or DADLE

TREATMENT (dose)	ED_{50} (95% CI)	ED_{50} Ratio†
	ABSOLUTE LATENCY (s)	
SALINE (1 µl·h^{-1})	0.38 µg (0.29-0.49)	
DADLE (10 µg·h^{-1})	0.24 µg (0.17-0.36)	0.63
	MAXIMUM PERCENT EFFECT	
SALINE (1 µl·h^{-1})	0.38 µg (0.29-0.50)	
DADLE (10 µg·h^{-1})	0.25 µg (0.16-0.38)	0.66

† ED_{50} ratio is defined as ED_{50} in opioid-infused rats·ED_{50} in saline-infused rats^{-1}

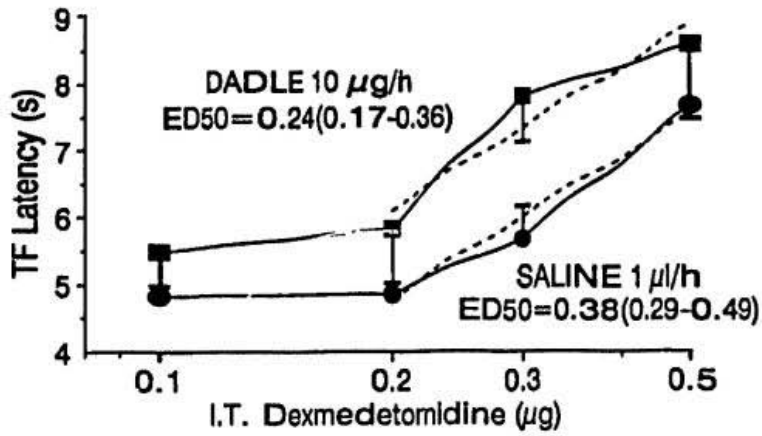

Figure 17A. DX dose-response curves (expressed as peak tail-flick latency in seconds) determined after the continuous i.t. infusion of saline (1 μl·h^{-1}; ●) or DADLE (10 μg·h^{-1}; ■) for 6 days. DX was tested on days 1-2 post-infusion and separate groups of rats were used for each dose. Data are expressed as the mean ± SEM of 3-6 rats and the dashed lines represent regression lines used to calculate the ED_{50} and 95% CI (expressed in μg as shown).

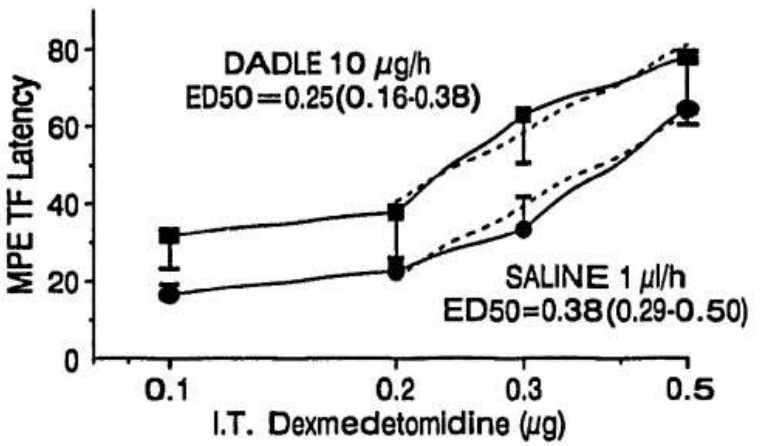

Figure 17B. DX dose-response curves (expressed as the maximum percent effect) determined after the continuous i.t. infusion of saline (1 μl·h^{-1}; ●) or DADLE (10 μg·h^{-1}; ■) for 6 days. DX was tested on days 1-2 post-infusion and separate groups of rats were used for each dose. Data are expressed as the mean ± SEM of 3-6 rats and the dashed lines represent regression lines used to calculate the ED_{50} and 95% CI (expressed in μg as shown).

3.3 Antagonist Experiments

3.3.1 Differential Antagonism of Morphine (20 μg·h^{-1})- and DADLE (10 μg·h^{-1})-Induced Antinociception by Naloxone and Naltrindole

To assess the relative selectivity of DADLE and morphine for μ- and δ-opioid receptors, respectively, during continuous i.t infusion, naltrindole (1.0 mg·kg^{-1}) or naloxone (1.0 mg·kg^{-1}) was injected i.p. at the time of maximum antinociception (day 1 of infusion). Prior to naloxone, maximum tail-flick latency during morphine-infusion was 9.0 s (dashed line in Figure 18A; defined as 100%). After naloxone, the morphine effect decreased to 46% and remained significantly antagonized from 15-60 min (**P<0.01,*P<0.05). The same dose of naloxone had a small but non-significant effect on DADLE-induced antinociception (P>0.05). The maximum tail-flick latency during DADLE infusion before naloxone administration was 6.2 s (100% effect). Neither naloxone nor naltrindole (1.0 mg·kg^{-1} i.p.) had any effect on baseline tail-flick latency in rats continuously infused with i.t saline (data not shown).

The effect of the δ-selective antagonist, naltrindole, on DADLE- and morphine-induced antinociception was determined in a separate experiment. The maximum increase in tail-flick latency during DADLE infusion (6.4 s; 100% effect) was significantly antagonized by i.p. naltrindole from 45-60 minutes (Figure 18B). In contrast, naltrindole had no significant effect in rats receiving continuous i.t. morphine (peak tail-flick latency = 8.7 s). These data suggest that DADLE retained its δ-receptor selectivity in the early period of continuous i.t. infusion; an observation

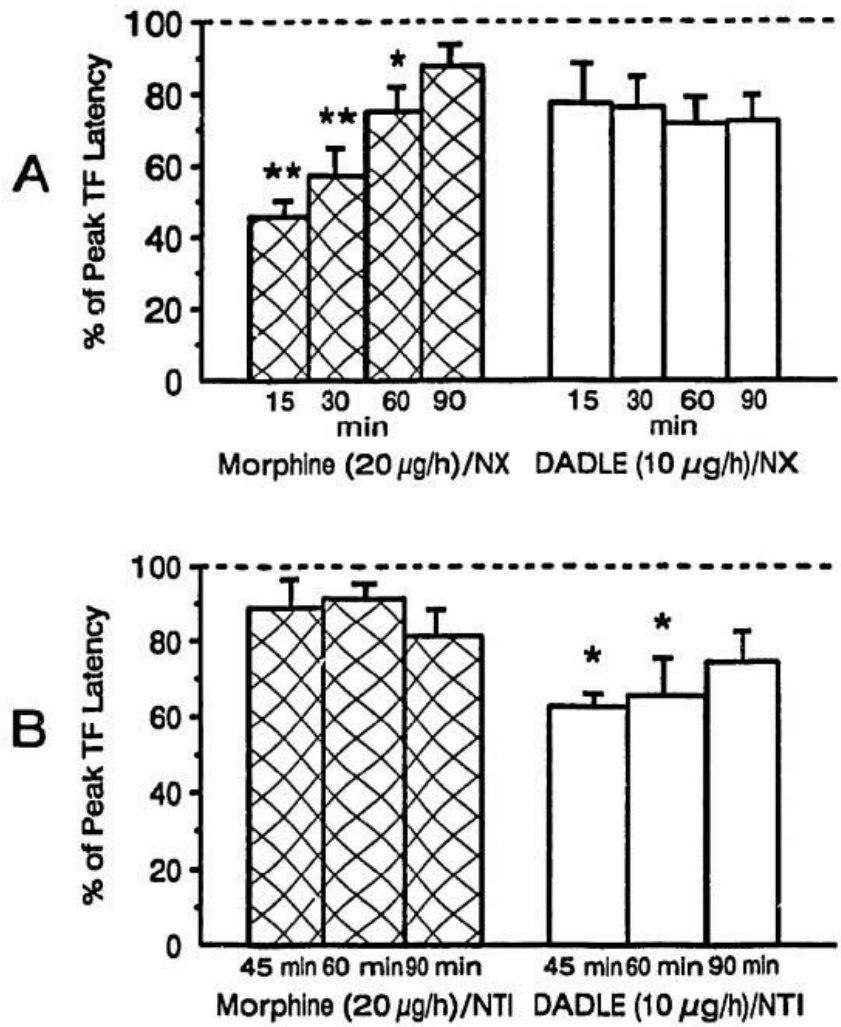

Figure 18. The effect of naloxone (A; NX) or naltrindole (B; NTI) on tail-flick latency during the continuous i.t. infusion of morphine (20 μg·h^{-1}) and DADLE (10 μg·h^{-1}). Naloxone and naltrindole (1.0 mg·kg^{-1} i.p.) were injected on day 1 of infusion. Data are expressed as the mean ± SEM of 5 rats. Asterisks denote a significant difference from peak antinociception (before the antagonist) as determined by repeated measures ANOVA followed by Newman-Keuls test (**P<0.01; *P<0.05).

that is consistent with the differential cross-tolerance results of this study.

3.3.2 *Effect of Wyeth 27127 and Prazosin on Dexmedetomidine-Induced Antinociception*

To confirm the selectivity of high dose DX for α_2-adrenoceptors, and thus its utility as a probe agonist in the cross-tolerance experiments, three separate groups of naive rats were injected with DX (0.5 µg i.t.) and the tail-flick latency determined 15 min later. Rats were then injected i.t. with either WY 27127 (10 µg; α_2-selective antagonist), prazosin (10 µg; α_1-selective antagonist), or vehicle (DMSO; 5 µl). The antinociceptive effect of DX was markedly antagonized from 30-60 min by WY 27127 (Figure 19). In contrast, rats treated with prazosin showed no attenuation of the antinociceptive effect of DX; the time course was identical to receiving DX + DMSO (Figure 19), DX + saline or DX alone. For the purposes of clarity, the latter groups were omitted from the graph. AUC analysis of the time course data for WY 27127-treated animals was also significantly different from both the DMSO- and prazosin-treated groups. These data confirm the α_2-selectivity of i.t. DX at the highest dose used in the cross-tolerance experiments (0.5 µg).

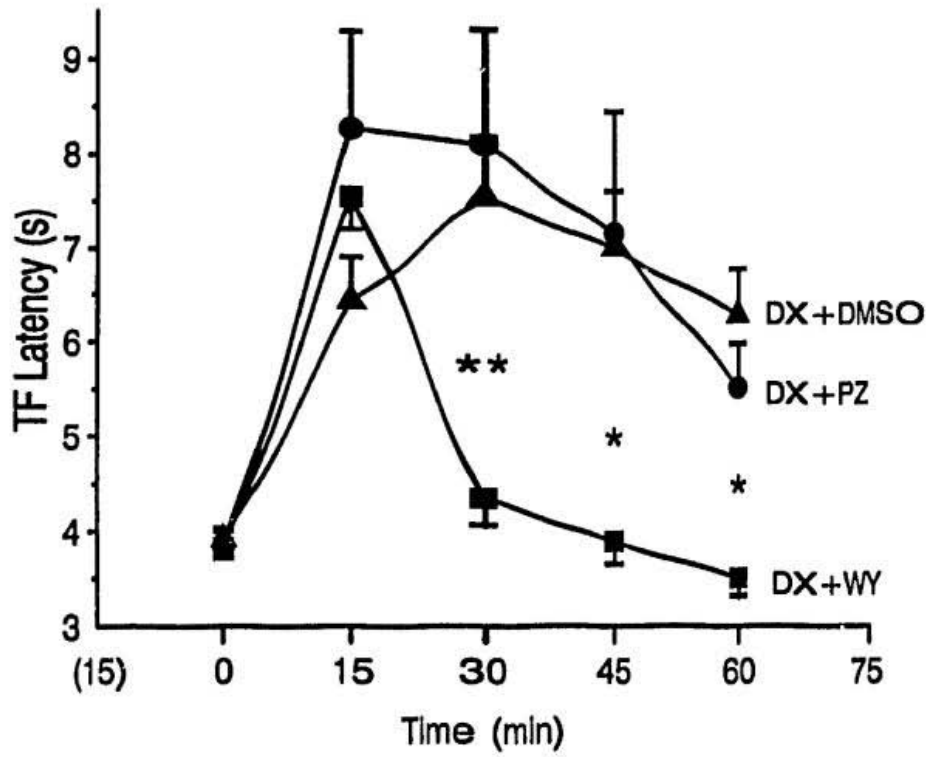

Figure 19. The effect of i.t. prazosin (PZ; ●) , Wyeth 27127 (WY;■) or DMSO (▲) on dexmedetomidine (DX)-induced antinociception in three separate groups of naive rats. Dexmedetomidine (0.5 µg i.t.) was injected at time 0. Immediately after the 15-min time point, prazosin (10 µg), Wyeth 27127 (10 µg) or DMSO was injected i.t. to one of the groups. Data, expressed as the mean ± SEM of 4-9 rats, were analyzed using one-way ANOVA followed by a Newman-Keuls test. ** Indicates a significant difference of Wyeth 27127-treated from both DMSO- and prazosin-treated animals ($P<0.01$). * Indicates a significant difference between Wyeth 27127- and DMSO-treated rats ($P<0.01$); and between Wyeth 27127- and prazosin-treated rats ($P<0.05$).

4.0 DISCUSSION

Endogenous opioid and noradrenergic systems are well recognized for their ability to *independently* modulate nociceptive transmission in the dorsal horn of the spinal cord (see reviews by Cousins and Mather, 1984; Yaksh and Noueihed, 1985; Yaksh, 1985; Maze and Tranquilli, 1991). Nevertheless, pharmacological evidence, including the blockade of spinal α-adrenergic antinociception by opioid antagonists (Tung and Yaksh, 1982; Loomis *et al.*, 1987a; Yang *et al.*, 1994) and the observation of cross-tolerance between i.t. α-agonists and opioid agonists (Sherman *et al.*, 1988; Stevens *et al.*, 1988; Kalso *et al.*, 1993; Paul and Tran, 1995), suggest that spinal noradrenergic modulation includes an endogenous opioid-dependent mechanism.

The results of the present research provide further support for this hypothesis, and in particular, the model illustrated in Figure 1. Thus, the non-selective α-agonist, NE, exhibited cross-tolerance to DADLE, but not morphine, in the rat as assessed by dose-response analysis. In contrast, the highly selective α_2-agonist, DX, exhibited no cross-tolerance to DADLE using the same experimental paradigm. These data are also consistent with the results of acute experiments investigating the antinociceptive interaction between spinal α_1- and α_2-adrenoceptors that preceded this work (Loomis *et al.*, 1992a; 1992b; 1993).

4.1 *Relationship of the Present Work to Acute Studies of an α_1- and α_2-Adrenoceptor Interaction*

A threshold dose of MX (10 µg i.t.), producing <5% MPE in the tail-flick test and 0% in the paw pressure test, when combined with i.t. DX significantly shifted the DX dose response curve to the left. A combination of otherwise inactive doses of MX+DX, effecting near maximal activity in the tail-flick test and intermediate activity in the paw pressure test, was significantly blocked by naloxone (30 µg i.t.), ICI 174,864 (75 µg/kg i.p.), or antiserum to Met-enkephalin (10 µl i.t.)(Loomis *et al.*, 1992b). Moreover, pretreatment with the neutral endopeptidase inhibitor SCH 32615 (75 µg i.t.), but not vehicle, further potentiated the MX+DX interaction (additional leftward shift in the MX+DX dose response curve). The potency shift with this combination exceeded the small effect of SCH 32615 on DX alone. These results indicate that the acute synergistic interaction between i.t. DX and MX involves a local enkephalin-dependent process mediated by spinal δ-receptors; a mechanism supported by the well established interaction between exogenous opioids and α_2-agonists in the rat spinal cord. The blockade of this supra-additive interaction by either prazosin (10 µg i.t.) or WY 27127 (0.5 µg i.t.) indicated that the respective antinociceptive effects of MX and DX were mediated by distinct α-adrenoceptor subtypes.

4.2 Continuous Opioid Infusion and Tolerance

In the present study, dose-dependent antinociception was observed with continuous i.t. morphine (5, 10 and 20 $\mu g \cdot h^{-1}$) in the tail-flick test (mean peak tail flick latency and AUC analysis of the time course data). The time course data are in agreement with previous studies using osmotic mini-pumps for spinal drug delivery (Milne *et al.*, 1985; Russell *et al.*, 1987; Stevens *et al.*, 1988; Stevens and Yaksh, 1989a, 1989b, 1989c). Unlike the morphine experiments, only one infusion dose of i.t. DADLE (10 $\mu g \cdot h^{-1}$) was used. In preliminary experiments, a lower infusion dose (3 $\mu g \cdot h^{-1}$) yielding significant antinociception was tested. However, these animals rapidly recovered from tolerance (overnight) making cross-tolerance studies impractical. Solubility limitations prevented a higher infusion dose of i.t. DADLE from being used.

Continuous i.t. opioid infusion for 6 days induced significant tolerance to the spinal antinociceptive effect of morphine and DADLE. Tolerance was confirmed by: a) the absence of antinociceptive activity of morphine or DADLE probe doses in opioid-, but not saline-infused rats during the immediate post-infusion period; b) the rightward (6-fold) shift in the morphine dose-response curve in morphine- as compared to saline-infused rats; and c) the reversibility of this process upon removal of the opioid (see below). These data indicate that the decline in tail-flick latency during opioid infusion was the result of progressive pharmacodynamic changes to

the continuous exposure of opioid receptors to their respective agonists (e.g. uncoupling of μ- or δ-receptors from the effector mechanisms normally mediating their antinociceptive effects). It was not due to pump or catheter failure, repeated behavioral testing, degradation of the opioid (Stevens and Yaksh, 1989b) or pharmacokinetic factors (Yaksh, 1991).

The integrity of the drug delivery system was also confirmed by the dose-dependent reduction in body weight in opioid-, but not saline-treated animals during the immediate post-infusion period. This effect, characteristic of opioid withdrawal (Wei *et al.*, 1973; Suzuki *et al.*, 1988; Cridland *et al.*, 1991) and reflecting a decrease in food and/or water consumption during the first 24 h, is consistent with the continuous delivery of opioid agonists via the mini-osmotic pump/catheter system. Opioid-infused rats did not exhibit any signs of withdrawal when probe doses of opioid- or α-agonists were tested, indicating that this abstinence effect had a rapid onset and short duration.

4.3 Recovery from Opioid Tolerance

Significant recovery of antinociceptive activity did not occur until 4 days after the discontinuation of i.t. morphine infusion (5 $\mu g \cdot h^{-1}$). This time course provided a 3-day window in which to conduct cross-tolerance experiments without the complication of concurrent recovery in opioid sensitivity. In the interests of time and efficiency, recovery was determined using only the lowest infusion dose of

morphine. Since the magnitude of spinal opioid tolerance is dependent on the i.t. infusion dose (Stevens and Yaksh, 1989c), the time course of recovery from 10 and 20 $\mu g \cdot h^{-1}$ morphine should not be less than 3 days. Accordingly, cross-tolerance experiments were limited to 3 post-infusion days in morphine-tolerant rats.

In vivo studies of the rate of offset of opioid tolerance have yielded variable results, depending on the opioid agonist, the route of drug administration and the behavioral measure of opioid activity. Using a stereotypical pattern of automatic running activity ("running fit") in mice, Goldstein and Sheehan (1969) estimated the time of recovery from levorphanol tolerance (induced with 20 $mg \cdot kg^{-1}$ i.p. every 8 h) to be equal to rate of onset of tolerance (approximately 48 h). Although not quantitatively measured, these investigators noted that, in some experiments, analgesic tolerance appeared to develop considerably more slowly than tolerance to the running fit. Cox *et al.* (1975) measured the antinociceptive response in rats (paw pressure withdrawal test) to a standard infusion of morphine (5 $mg \cdot kg \cdot h^{-1}$ i.v.) + cycloheximide (200 $mg \cdot kg \cdot h^{-1}$ i.v.) to prevent the further development of tolerance at various time intervals after cessation of repeated s.c. morphine injections. A biphasic pattern of recovery, consisting of an initial rapid phase (4 days duration) and second prolonged phase (mean half-time of 13 days) was reported; a comparable recovery pattern was also observed in mice. Tung *et al.* (1981) induced analgesic tolerance (hot-plate test) using once daily injections of i.t. morphine (45 µg) for 8 days. Seven days after the termination of chronic morphine, rats regained

85% of their initial responsiveness to i.t. morphine. Yaksh (1991) reported a 50% recovery of the i.t. morphine ED_{50} in the rat hot-plate test, nine days after terminating a continuous i.t. morphine infusion (20 nmol/h). In contrast, μ-receptor binding and antinociceptive activity of PL 017 were restored 3 days after the continuous i.t. infusion of PL 017 was discontinued in the rat (Nishino *et al.*, 1990). Although time course data provide no specific information about the molecular/cellular events underlying tolerance, the prolonged and sometimes biphasic recovery after chronic morphine treatment implies that multiple factors are involved.

Recent studies using continuous i.t. infusion of receptor selective agonists in rats indicate that opioid tolerance *in vivo* is based on pharmacodynamic rather than pharmacokinetic factors nor is it the result of learned behaviour (e.g. associative tolerance). Thus, tolerance is observed only after the activation of spinal opioid receptors, the degree of opioid tolerance is proportional to both the log of the infusion dose and the fractional receptor occupancy of the agonist used, and there is minimal loss of response between agonists which interact with distinct receptors (e.g. minimal cross-tolerance between μ, δ, α_2 agonists) (Stevens and Yaksh, 1989a,b; 1992). These data are consistent with the down-regulation of a fraction of the spinal opioid receptor population and/or an uncoupling of opioid receptors from their corresponding effector system.

Evidence from studies using NG 108-15 and 7315c cells in culture (expressing δ and μ receptors, respectively) indicate that the induction of tolerance

in these cells involves two phases (see review by Johnson and Fleming, 1989). Acute changes, characterized by rapid onset and rapid recovery (e.g. min to hours) and initiated by high concentrations of agonist, include receptor desensitization (e.g. the functional uncoupling of μ and δ receptors from their associated G proteins [Law *et al.*, 1983; Puttfarcken *et al.*, 1988]) followed by internalization of uncoupled cell surface receptors into lysosomes where they are degraded (Law *et al.*, 1984). Receptor desensitization occurs prior to any change in the number of opioid binding sites. Long-term changes, characterized by slow onset and slow recovery (e.g. days to weeks), may reflect altered expression or repression of mRNA for specific proteins (e.g. receptors, G-proteins, ion channels, ion pumps, enzymes of 2nd messenger systems). For example, chronic morphine administration to the rat has been shown to decrease mRNA coding for POMC in the hypothalamus (Mocchetti *et al.*, 1989).

The relevance of these observations to the progressive loss of opioid activity in conscious behaving animals remains to be determined. As noted above, many characteristics of opioid tolerance *in vivo* can be explained by the functional uncoupling of opioid receptors from the G proteins linking them to their effector systems (Cox, 1991). Concurrent changes in cholecystokinin activity, an important physiological modulator of morphine analgesia, has also been implicated in the development of opioid tolerance (Xu *et al.*, 1992; Zhou *et al.*, 1992; Hoffmann and Wiesenfeld-Hallin, 1994). Interestingly, tolerance to opioids acting on spinal δ-

receptors is not altered by pharmacological manipulation of cholecystokinin neurons. Our data are consistent with the time-dependent recovery of functionally coupled μ- and δ-opioid receptors and/or from compensatory changes to cholecystokinin neurons in the case of μ-receptors.

Unlike morphine, significant recovery from DADLE tolerance was apparent 3 days after discontinuation of DADLE infusion (10 $\mu g \cdot h^{-1}$) and complete by day 4. On the basis of these data, cross-tolerance studies with NE or DX were carried out on days 1-2 post-infusion in all DADLE-tolerant rats. Our results are similar to those of Russell *et al.* (1987) who reported a slightly slower rate of recovery to the μ-agonist, PL017 (0.5 $\mu g \cdot h^{-1}$ i.t.) as compared to DADLE (2 $\mu g \cdot h^{-1}$ i.t.) in the rat. Complete recovery from PL017 and DADLE tolerance was observed on days 6 and 5, respectively. The modest difference in recovery between DADLE and morphine is probably related to the lower intrinsic activity of morphine (Stevens and Yaksh, 1989c) and/or differences in the selectivity of the two agonists for their respective opioid receptor subtypes (see review by Yaksh and Noueihed, 1985; Russell *et al.*, 1987).

4.4 Cross-Tolerance Between Spinal Opioids and α-Adrenergic Agonists

In an early study using continuous i.t. infusion of morphine (10 $\mu g \cdot h^{-1}$ for 5 days) followed by NE (15 $\mu g \cdot h^{-1}$ for 7 days), Milne and co-workers (1985) reported significant cross-tolerance in the rat tail-flick test. The absence of antinociceptive activity in morphine tolerant rats was not due to the oxidation of NE in the osmotic

mini-pump (as confirmed by the fluorometric analysis of NE taken from the pumps at the end of the experiment). However, only one dose of i.t. morphine and NE was tested. Since the extent of tolerance, and the development of cross-tolerance, is influenced by the dose of the tolerance-inducing agent (Stevens *et al.*, 1988), we used three different infusion doses of morphine, and three probe doses of NE (for quantitative dose-response analysis) in the present experiment.

Animals made tolerant to i.t. morphine using three infusion doses (5, 10 and 20 $\mu g \cdot h^{-1}$) exhibited no significant cross-tolerance to i.t. NE in the tail-flick test. While there was an apparent attenuation of low dose NE in rats treated with 10 $\mu g \cdot h^{-1}$ morphine, no such effect was observed in rats infused with a higher dose of morphine (20 $\mu g \cdot h^{-1}$) indicating that this trend was not pharmacologically significant. Previous studies reporting cross-tolerance between continuous i.t. morphine (0.76-7.6 $\mu g \cdot h^{-1}$) and test doses of ST-91 (Stevens *et al.*, 1988), and between morphine (10 $\mu g \cdot h^{-1}$) and i.t. oxymetazoline (29.7 µg) (Sherman *et al.*, 1988) in the rat hypothesized that re-distribution of morphine to the brain during i.t. infusion, and the consequent activation of descending noradrenergic fibers, was most likely responsible for the observed interaction.

The locus of action of drugs injected into the spinal subarachnoid space is an important consideration. Hydrophilic drugs like morphine undergo slow cephalad migration in the CSF while more lipid soluble drugs can be redistributed via the systemic circulation after uptake in the microvasculature of the spinal cord. The cell

bodies of noradrenergic neurons modulating nociceptive transmission in the spinal cord are located in pontine nuclei (LC, medial and lateral parabrachial nuclei, nucleus subcoeruleus, A5 and A7 nucleus) (Proudfit, 1988; Jones, 1991), and morphine, focally injected near these brainstem sites, evokes the release of NE from nerve terminals located in the spinal dorsal horn (see review by Yaksh, 1985). Thus, the rostral migration of morphine, and its attendant activation of bulbo-spinal noradrenergic fibers, can confound the interpretation of cross-tolerance studies in the spinal cord.

The absence of cross-tolerance between morphine and NE in the present study (using three different infusion doses), indicates that: 1) the locus of action of morphine, delivered by continuous i.t. infusion, and thus the site of morphine tolerance, was primarily in the spinal cord; 2) any rostral migration that did occur was insufficient to effect detectable α-adrenoceptor tolerance; and 3) spinal μ-receptors are unlikely to mediate the endogenous opioid component of spinal noradrenergic antinociception in the rat. A predominant spinal site of action is consistent with the results of a previous study investigating the activation of brainstem μ-receptors during continuous i.t. morphine infusion. Rats receiving twice daily injections of s.c. morphine or a continuous infusion of morphine (1 $\mu g \cdot h^{-1}$) in the brainstem subarachnoid space for four days exhibited pronounced rhythmic fictive swallowing (300 per minute) 30 min after i.v. naloxone (Bieger *et al.*, 1992), reflecting an alteration in the *brainstem* opioid receptors that modulate

buccopharyngeal and esophageal activity, chronically exposed to morphine. This was six times greater than the response evoked by naloxone in rats treated with continuous i.t. morphine (5 $\mu g \cdot h^{-1}$ for four days). These data indicate the limited effect of spinal morphine on relevant brainstem opioid receptors during continuous i.t. infusion, and are in agreement with a previous study (Loomis *et al.*, 1987b) demonstrating that: 1) the antinociceptive effect of i.p. morphine is unaffected by tolerance to spinal morphine (10 $\mu g \cdot h^{-1}$ for 7 days); and 2) the distribution of an i.t.-infused dye is almost entirely restricted to the spinal segments adjacent to the tip of the i.t. catheter. Nevertheless, any differences in the rostral migration of morphine between the present study and previous investigations using the same type of delivery system are not easily explained.

α-Agonists, including NE, are known to induce acute tolerance (tachyphylaxis). Depending upon the intrinsic activity and the frequency of administration of the probe drug, this phenomenon can influence the interpretation of cross-tolerance experiments. To minimize this potential problem, rats were allowed to recover for 24 h between probe doses of NE, and an escalating dose of NE was administered on subsequent post-infusion days (i.e. 2.5 µg NE on day 1, 5.0 µg NE on day 2, etc). This testing regimen was based on preliminary experiments indicating that the antinociceptive effect of NE in saline-infused rats (5 and 10 µg i.t. on days 2 and 3 post-infusion, respectively), was identical to that observed in naive rats, administered 3 days apart (data not shown). These results strongly

suggest that acute tolerance to NE was not a factor in this study.

Because of the rapid recovery from DADLE tolerance, cross-tolerance experiments were limited to days 1 and 2 post-infusion. To assess the problem of acute tolerance with daily i.t. injections of DX, probe doses were tested in saline-infused rats on consecutive post-infusion days. The degree of acute tolerance observed was sufficient to preclude the further use of this treatment schedule (data not shown). Instead, each rat received only one dose of DX during the 2-day "window".

If NE effects spinal antinociception, in part, through the α_1-adrenoceptor mediated release of enkephalin (Loomis *et al.*, 1993), then down-regulation of the spinal opioid receptors responsible for the inhibitory effect of enkephalin should attenuate this NE response. Indeed, the ED_{50} of i.t. NE was significantly reduced in rats made tolerant to the δ-agonist, DADLE as compared to saline-infused animals. It was not possible to test this hypothesis using selective α_1-agonists like methoxamine or phenylephrine in this experimental paradigm because of the exaggerated motor responses evoked by these drugs in the spinal cord. Such effects confound the intepretation of changes in behavioral responses to noxious stimulation (e.g. TF test) as previously described (Section 1.5.2). To our knowledge, this is the first study of cross-tolerance between DADLE and NE. Although the shift in the ED_{50} of NE was relatively small (2.54) in DADLE-tolerant rats, this was expected given the spectrum of NE activity at both α-receptor subtypes (Loomis *et*

al., 1993), the predominant role of α_2-adrenoceptors in spinal adrenergic antinociception, and evidence for the dissociation of α_2-adrenoceptors from endogenous opioid systems (Fleetwood-Walker *et al.*, 1985; Maze and Tranquilli, 1992).

Further support for the research hypothesis is indicated by the absence of cross-tolerance between DADLE and the highly selective α_2-agonist, DX. These results are similar to those of Kalso and co-workers (1993), who reported no cross-tolerance between spinal Tyr-D-Ser(otbu)-Gly-Phe-Leu-Thr (DSTBULET; δ-agonist) and i.t. DX in the rat as determined from electrophysiological recordings of dorsal horn nociceptive neurons. In these studies, rats were administered two daily injections of i.t. DX (5 μg) for 10 days or i.t. DSTBUTLET (50 μg) for 5 days before receiving the probe doses of DSTBUTLET or DX, respectively. These behavioral data provide further support for the dissociation of opioid- and α_2-mediated mechanisms of spinal antinociception, as reflected in our model (Figure 1). The attenuation of i.t. NE, but not DX, in rats treated identically with continuous i.t. DADLE cannot be explained by the redistribution of DADLE to noradrenergic cell bodies located in the brainstem. In such a case, both drugs would be expected to exhibit cross-tolerance to DADLE. Thus, there is no evidence in either of the cross-tolerance studies (morphine-NE or DADLE-NE/DX) to conclude that sites of action, other than those in the spinal cord, contributed significantly to observed results. In view of the absence of the cross-tolerance between i.t. morphine and NE, and

between DADLE and DX (as assessed using dose-response analysis), similar studies of i.t. morphine and DX were not undertaken.

High doses of i.t. DX (1-6 μg), (acute or continuously infused) are known to induce profound sedation in rats (Fisher *et al.*, 1990; Kalso *et al.*, 1991; Takano and Yaksh, 1992; Takano and Yaksh, 1993; unpublished results). This is a supra-spinal inhibitory effect that arises from drug redistribution into the systemic circulation (Savola *et al.*, 1986; Kalso *et al.*, 1991). It is important to note that no sedation was observed at any of the doses used in the present experiments (0.1-0.5 μg i.t.). Moreover, it is known that the antinociceptive effect (TF test) of i.t. DX in rats perists beyond the time course of sedation (Kalso *et al.*, 1991).

An interesting observation was the apparent potentiation of i.t. DX in DADLE-tolerant animals. There was an parallel, but statistically non-significant, leftward shift of the DX dose-response curve in DADLE- as compared to saline-infused animals. Such an observation has, to our knowledge, not been previously reported. This effect was unlikely to be due to the inadvertent delivery of residual DADLE solution during the i.t. injection of probe doses of DX (resulting in potentiation), since all catheters were thoroughly flushed with saline following the removal of the osmotic pump. Stress-induced antinociception, possibly related to the discontinuation of DADLE infusion, also seems unlikely as we observed no abnormal behaviour at the time of testing nor were the baseline tail-flick latencies any different compared to saline-infused rats.

One possible explanation is that the sudden withdrawal of DADLE infusion caused a "rebound" release of endogenous enkephalin. An increase in the extracellular concentration of enkephalin might be sufficient to potentiate the antinociceptive effect of DX, subsequently injected into the spinal subarachnoid space. We did not determine enkephalin concentration in the present study but there are reports of such changes in the brain (Simantov and Snyder, 1976) and spinal cord (Cridland *et al.*, 1991) of morphine-dependent rats, and the cerebrospinal fluid of humans (O'Brien *et al.*, 1988). Using nearly identical conditions to those of the present study (continuous i.t. morphine infusion - 5 $\mu g \cdot h^{-1}$ for 6 days), Cridland *et al.* (1991) reported a significant increase in met-enkephalin immunoreactivity in the rat lumbar and sacral spinal cord, 24 h after i.t. naloxone-precipitated withdrawal. These changes were localized to the spinal cord; the caudate-putamen and globus pallidus were unaffected by the naloxone challenge. Moreover, the i.t. infusion of morphine by itself (no naloxone) did not affect met-enkephalin immunoreactivity in the brain or spinal cord. The delayed appearance of this neurochemical change is in agreement with the time course of DX administration in the present study (i.e. for the first day of DX testing). To the extent that this mechanism is responsible for the increased activity of DX in DADLE-infused rats, our data suggest enkephalin levels may remain elevated for up to 48 h after the infusion is discontinued. It should be noted that a similar synergisitic interaction between DX and morphine would also be expected during opioid withdrawal, but this

was not investigated in the present study. The interaction between endogenous enkephalin and i.t. DX is in agreement with the proposed model of spinal adrenergic antinociception (Figure 1). In future experiments, it would be useful to determine the change in met-enkephalin immunoreactivity as well as mRNA for met-enkephalin in the spinal cord of naloxone-treated rats following the continuous i.t. infusion of DADLE.

4.5 Receptor Selectivity of Morphine, DADLE and Dexmedetomidine

The reliability of the cross-tolerance data depend on a number of important factors. These include: 1) the selectivity of morphine and DADLE for their respective opioid receptor subtypes during continuous i.t. infusion; and 2) the selectivity of DX as an agonist at α_2-adrenoceptors.

The modest but selective blockade of morphine by naloxone, and DADLE by naltrindole, on day 1 of continuous i.t. infusion is the only direct evidence of μ- and δ-receptor selectivity in the present study. DADLE has been reported to have modest activity at μ-receptors (Tseng, 1983; Russell *et al.*, 1987; Stevens and Yaksh, 1992). Indeed, a low dose of i.p. naloxone yielded a small but non-significant reduction in tail-flick latency during DADLE infusion. However, systemic naloxone is only 3-10 times more potent against μ-agonists such as morphine and sufentanil, than against DADLE (Tung and Yaksh, 1982; see review by Yaksh and Noueihed, 1985; Yaksh *et al.*, 1986). Significant and quantitatively greater blockade

of DADLE was achieved with the δ-selective antagonist, naltrindole. This is in agreement with previous *in vitro* results indicating the relative inhibitory potency of naltrindole to be over 100 times greater for DADLE than for morphine (Portoghese *et al.*, 1988). It is also consistent with the failure of naltrindole to affect morphine antinociception in the present study, although conflicting results with naltrindole have been reported *in vivo* (Tiseo and Yaksh, 1993). While the naloxone and naltrindole data are only relevant to day 1 of infusion, they do correspond to the time of maximum antinociception with morphine and DADLE. As discussed in section 4.4, the selectivity of these agonists in our experimental paradigm is also inferred from the outcome of the cross-tolerance experiments, and the different time-courses of recovery from opioid tolerance. Although [D-Pen^2, D-Pen^5]enkephalin (DPDPE) is more selective than DADLE at the δ-receptor (Clark *et al.*, 1986), the large amounts required for continuous i.t. infusion and the related cost of DPDPE made it impractical for use in this study.

To verify the α_2-selectivity of DX, we tested the effect of both prazosin and WY 27127 on DX (0.5 µg), reasoning that any activity at α_1-adrenoceptors would be most evident with the highest probe dose. Prazosin (10 µg i.t.) was without effect whereas the selective α_2-antagonist, WY 27127 (10 µg i.t.), completely reversed Dx-induced antinociception. It should be noted that the 10-µg dose of prazosin is well above the i.t. dose (2.5 µg) that blocked the tail-flick effect of the α_1-selective agonist, ST-587 (Loomis and Arunachalam, 1992) and equal to the ID_{50} of i.t.

prazosin (9.8 µg) in blocking i.t. NE in the tail flick test (unpublished results). These results indicate that the probe doses of DX used in the present study had no detectable activity at spinal α_1-adrenoceptors, consistent with the pronounced selectivity of DX for α_2-adrenoceptors (α_2-selectivity ratio of 1620 versus 220 for clonidine; Virtanen *et al*, 1988), and the results of our cross-tolerance experiments.

Prazosin is a classical α_1-selective antagonist which has been used extensively to characterize adrenergic binding sites and functional α-adrenoceptors. In recent years, multiple subtypes of α_1- and α_2-adrenoceptors have been identified through the molecular cloning of cDNA/genes encoding for these receptors (Lomasney *et al.*, 1991). Binding experiments using cells that selectively express specific α-adrenergic subtypes have shown that prazosin, in addition to being an α_1-antagonist, binds to specific subtypes of α_2-adrenoceptors (Bylund, 1988; Lomasney *et al.*, 1991). Moreover, studies on the spinal pharmacology of α_2-agonists, including the rank order of antagonist potency, suggest that at least two subtypes of the α_2-adrenoceptor (designated α_{2A} and α_{2nonA}) effect antinociception in the rat spinal cord. The α_{2A}-receptor, which is not blocked by prazosin, mediates the antinociceptive activity of agonists like DX and clonidine. The α_{2nonA}-receptor, which is blocked by *high* doses of prazosin, mediates the effect of ST-91 (Takano and Yaksh, 1992; Takano *et al.*, 1992). The failure of prazosin to block DX in the present study is consistent with the high doses of i.t. prazosin required to block α_{2nonA} receptors (ID_{50} = 38 µg; 95%CI = 22-65; ST-91 in rat hot-plate test), and the

>100 µg required to block DX or clonidine at α_{2A} receptors (Takano and Yaksh, 1992). These dose-response data strongly suggest that prazosin induced selective α_1-adrenoceptor blockade in the present study.

The antagonist profile of WY 27127 at different subtypes of the α_2-receptor has not been reported but, on the basis of other published data (Takano and Yaksh, 1992; Takano *et al.*, 1992, Millan, 1992), we believe that the effect of DX was mediated by α_{2A}-receptors. Future experiments will be required to clarify this issue.

4.6 Antinociceptive Interaction Between Spinal α_1- and α_2-Adrenoceptors

Clearly, the results of the present study provide no detailed mechanistic information about the coupling of α_1-adrenoceptors to the enkephalin-dependent process in spinal α-adrenergic antinociception. Based on the available information, we have proposed that the activation of α_1-adrenoceptors results in an increase in the extracellular concentration of enkephalin which then effects antinociception via spinal δ-opioid receptors located on relevant neurons of the nociceptive pathway. In the absence of release studies, it is not known if the activaton of α_1-adrenoceptors actually evokes release or merely facilitates the stimulus-evoked release of enkephalin. Future studies measuring the spinal release of this peptide will be required to answer this and related questions. Moreover, it is unclear if this is a direct excitatory effect on enkephalin neurons or whether this involves internuncial neurons. The role of other inhibitory inputs (e.g. GABA) in spinal α_1-

induced modulation also remains to be investigated. This possibility, and the underlying hypothesis that spinal α_1-adrenoceptors participate in spinal adrenergic antinociception are supported by the recent report that NE modulates nociceptive transmission in the substantia gelatinosa of the rat by facilitating GABA and glycine inhibitory post-synaptic potentials through α_1-adrenoceptors (Baba *et al.*, 1995).

An interaction between monoamine receptor subtypes is not unique in the central nervous system; an analagous situation has been shown to occur between dopamine receptor subtypes. Using animal models of Parkinson's Disease, concurrent activation of D_1- and D_2-receptors in the striatum and substantia nigra produces an enhancement of motor skills and alleviation of the Parkinsonian symptoms (Robertson, 1992a, 1992b). A similar effect on Fos expression in the striatum following administration of a combination of D_1- and D_2-selective agonists has provided additional support for an this interaction within the dopamine system (Paul *et al.*, 1992).

4.7 Significance of the Results

The results of this thesis provide additional evidence for an antinociceptive interaction between opioid and α-adrenergic systems in the spinal cord of the rat, effected by the concurrent activation of α_1- and α_2-adrenoceptors (Figure 1). These results are important in our basic understanding of α-adrenergic pain modulation, and its relationship to spinal opioids. They indicate that in the rat, α_1-adrenoceptors

could play an important role in spinal antinociception by potentiating the inhibitory effects mediated by α_2-adrenoceptors. These data provide a pharmacological explanation for the often conflicting results of cross-tolerance studies between i.t. opioid and α-adrenergic agonists (Post *et al.*, 1988; Solomon and Gebhart, 1988; Ossipov *et al.*, 1989), and the sensitivity of i.t. α-agonists to opioid antagonists (see reviews by Yaksh, 1985 and Maze and Tranquilli, 1991). Based on our model, an opioid component in spinal α-adrenergic modulation would be evident when spinal α_1-adrenoceptors, located on or linked to enkephalin-containing neurons, were intentionally or inadvertently activated by the test agonist. Thus, the α_1/α_2 selectivity ratio and the dose of the α-agonist would be critical factors in the outcome of such studies. It is noteworthy that previous studies of opioid and α-adrenergic interactions have generally used α-agonists with modest selectivity for α_2-adrenoceptors (e.g. ST-91 and clonidine; see Virtanen *et al.*, 1988).

To the extent that a comparable interaction occurs in human spinal cord (a question yet to be investigated), these results also have significant implications in the rational selection of α-agonists as spinal analgesics, and thus the drug regimen used in the clinical management of pain. The functional interaction between spinal α_1- and α_2-adrenoceptors shown in this and previous studies suggest that a low dose combination of α_1- and α_2-agonists could optimize adrenergic spinal analgesia. Currently, analgesic doses of spinal clonidine produce adverse cardiovascular effects that often require pharmacological intervention. These arise from the

inhibition of sympathetic outflow following the interaction of clonidine with α_2-adrenoceptors on preganglionic sympathetic neurons. However, central α_1-adrenoceptors oppose the hypotensive and bradycardic actions of clonidine. Thus, the concurrent use of an α_1-agonist could potentially augment analgesia while attenuating the adverse cardiovascular effects of α_2-agonists. Such a combination may also represent a more rational approach to the treatment of chronic pain. The overall reduction in α-receptor stimulation achieved by using a low dose combination of α_1- and α_2-selective agonists may delay the development of analgesic tolerance.

In summary, the results of this study are consistent with a spinal enkephalin-dependent, δ-receptor mediated process in α-adrenergic antinociception. This opioid component appears to be selectively coupled to α_1-adrenoceptors, that may facilitate the stimulus-evoked release of enkephalin in the dorsal horn of the spinal cord.

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